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ANIMAL HEALTH PERSPECTIVES

Editors:

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VOLUME 2

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PREFACE

The well-being of animals is pretty much intercalated. It's impossible to ensure human health, food security and food safety, and welfare without considering animal health.

The need to enhance the collaboration within animal health workers, researchers and academicians has moved the editors to develop this publication. The book takes into account the major threats of animal health. It's a unique compilation of bacterial, viral, parasitic, vector-borne diseases and metabolic disorders of animal health significance. The book highlights the important diseases of livestock and pet animals. Concepts presented in the book could be a way forward in devising ways to improve food safety from farm to fork.

It is anticipated that this book would be of great use to a variety of readers. University students, graduates, practitioners, and animal healthcare providers would definitely find this book of great importance. The language of book has been intentionally kept easier for a non-technical person to grasp the concepts on importance of veterinary health from a global perspective. The editors wish to publish a series on the subject keeping in view the urgency to highlight these areas for awareness, research and development.

Editors

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CHAPTER 01

THE CORRELATION BETWEEN GUT MICROBIOTA AND BOVINE MASTITIS

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INTRODUCTION

Mastitis is a highly prevalent disease in dairy cows and humans, which is characterized by inflammation that occurs in the mammary gland. It is considered as one of the most threatening diseases in that mastitis reduces milk production to unprofitable levels, makes milk discarded, decreases conception, and culls the cows early and even death, which causes enormous economic losses (Hertl et al. 2010). Approximately 220 million dairy cattle are raised worldwide and about 10 million were in China in 2018. The incidence of clinical mastitis is approximately 30% and it not only causes substantial economics but also leads to public health threats including the potential to transmit pathogenic and/or antibiotic-resistant organisms to humans. Generally speaking, bovine mastitis is identified as a complex disease affected by many factors including artificial feeding conditions, management, udder hygiene, pathogen infection, and health status of cows.

It is believed that pathogenic microorganisms mainly cause cow mastitis. There are two types of mastitis, clinical and subclinical infections. Depending on the primary reservoir and mode of transmission, this pathology can be contagious or environmental. Compared with clinical mastitis, the incidence of subclinical mastitis in dairy cows is 20-40 times higher, and the duration of the disease is longer. It is also classified as environmental types or contact infectious types according to the transmission amplification of pathogens. Numerous pathogens are responsible for mastitis with the majority of infections caused by *staphylococci, streptococci,* and *enterobacteria*. The main contagious microorganisms are *Staphylococcus aureus* and *Streptococcus agalactiae*, which are the main source of the mammary gland of infected cows (Gomes et al. 2016). On the other hand, the pathogenic bacteria which come from the body surface and the environment the dairy cows live in also cause mastitis, including *Streptococcus mammary*, *Escherichia coli,* and *Klebsiella*. A study of 161 large farms in 21 provinces in China showed that the average incidence rate of mastitis was 3.3% per 100 dairy cattle per month. The isolation and identification results of pathogenic bacteria showed that *Escherichia coli* was the highest, about 14.4%, followed by *Klebsiella* (13.3%), Coagulase negative *staphylococcus* (11.3%), *Streptococcus agalactiae* (10.5%) and *Staphylococcus aureus* (10.2%). The isolation of *Streptococcus agalactiae* and *Streptococcus* mammalia were respectively about 2.8 and 2.1%, lower than expected. The proportion of Coagulase negative *staphylococcus, Escherichia coli,* and other enterobacteria isolated from northwest China was higher than that from northeast and south China. *Streptococcus agalactiae* and other *Streptococcus* were isolated more in the winter (October to March), and *Escherichia coli* and *Klebsiella* were higher in the summer (April to September).

For a long time, the major strategy of treating mastitis is antibiotic therapy, but the cure is not very successful, and the prevention and treatment measures mostly emphasize environmental disinfection and mammary gland antibacterial and anti-inflammatory treatment. The side effects come with the use of antibiotics cannot be neglected in the coming term such as drug resistance and antibiotics residues in milk. Thus, detailed knowledge of the mechanism and the cure of mastitis is urgently needed to safeguard dairy industry's economic benefits and the human health.

Diagnosis of Cow Mastitis

The symptoms of clinical mastitis include redness, fever, and swelling of breast tissue. The color of the milk appears yellow, gray or red. The milk itself presents muddy, flocky, and sometimes as thin as water. The diagnostic methods for subclinical mastitis are cell count (SCC) and California mastitis Test (CMT) (Ashraf et al. 2018).

SCC is a classic and the most common detection method, which is widely used as a measure of milk quality, and the relationship between SCC and breast infection becomes a research topic. Traditional SCC detection relies on laboratory microscopy and cell staining techniques (Harmon 1994; Schukken et al. 2003). With the advancement in technology, there are automated electro-cellular counting machines based on imaging technology and flow cytometry, such as DeLavalTM and FossomaticTM. A more practical test for estimating SCC in farm is the California Mastitis qualitative test (CMT). In this approach, the sodium dodecyl sulfate is used to dissolve cell membranes and precipitate DNA and proteins into observable gels. The Wisconsin Mastitis Test (WMT) is an improvement

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on CMT. It increases the accuracy of viscosity measurements. CMT is still a suitable screening method for the mastitis. In the test of subclinical mastitis samples at dry milk stage, CMT shows higher sensitivity and specificity(Schukken et al. 2003).

The gold standard for identification of mastitis pathogen is microculture. The results depend on the growth count, colony phenotype, drug sensitivity test, and biochemical test of the sample on the medium at certain temperature and time. Laboratory culture has requirements for environment, equipment and operators, and the operation is tedious and time-consuming. Commercial microbial media kits have been introduced to facilitate the use of field tests, such as AccumastTM, Virba, CHROMagar and Hardy diagnostics.

PCR and LOOP-mediated isothermal amplification (LAMP) using pathogen DNA as template can obtain high sensitivity and specificity in a short time. PCR tests often require specialized equipment and trained personnel so that they are usually performed in the laboratory. LAMP assay is an economical and efficient diagnostic method in low-resource environment because of its convenience in field application, relatively simple sample preparation, and low sensitivity to inhibitory substances in biological samples. LAMP can detect pathogens such as *Staphylococcus aureus* and *Streptococcus* in milk, and the results can be obtained within 1-2 hours with high accuracy (Ashraf et al. 2018; Griffioen et al. 2020).

Treatment of Cow Mastitis

The prevention and treatment of dairy cow mastitis is a difficult point in dairy industry. There is no perfect method to prevent and treat this disease to maintain a balance between cow health and food safety. At present, the main treatment strategy for mastitis is still the extensive use of antibiotics. Penicillin, streptomycin and gentamicin are used by injecting into the breast, and depending on the degree of sickness after infection, it can be injected intramuscular or intravenous. In addition, prepare before injection to ensure that the injection equipment is sterilized and clean and massage the breast after injection to ensure absorption. The extensive use of antibiotics in treatment has brought many negative effects, such as drug resistance and the residue of antibiotics and antibiotic resistance genes (Krömker et al. 2017; van den Borne et al. 2019). To solve this problem, more and more scientists are focusing on developing new treatment methods, especially natural compounds, traditional Chinese medicine, and probiotics to replace the use of antibiotics. Traditional Chinese medicine and natural compounds contain a variety of active ingredients, which has high efficiency, low toxicity, and detoxification, alleviating the swelling and pain, and not easy to cause drug resistance (Hu et al. 2019; Zhao et al. 2018). Astragaloside is a Chinese herbal medicine widely used in the treatment of mastitis. It has anti-inflammatory effects by inhibiting the infiltration of inflammatory cells in mammary tissue and reducing the expressions of TNF-A, IL-1β and IL-6 (McDougall et al. 2021).

Gut Microbiota and Mastitis

Studies of the relationship between intestinal flora and human diseases have been reported often and it is also a promising way to treat diseases of dairy cows in dairy farming. Gut microbiota are an essential part of animals and humans. Shaped by eons of co-evolution, host-bacterial associations have developed into prosperous relationships creating mechanisms for mutual benefits to both microbe and host. The symbiotic relationship between host and bacteria is maintained by a highly intricate and extensive ecosystem (Jami et al. 2013). The disturbance in microbe-host ecosystem contributes to intestinal disease metabolic disease and even infectious illnesses such as diabetes, non-alcoholic fatty liver disease, colitis, obesity, and other diseases (He et al. 2015)

There are large quantity kinds of bacteria carried by dairy cows and a considerable number of them are mainly distributed in the intestinal cavities connected with the outside world, such as oral cavity, respiratory tract, rumen and intestinal tract. The special physiological structure endows cows with the ability to eat cellulose that is difficult to be digested by other animals and to biotransform nutrients that humans cannot digest for use. The implementation of this important physiological process cannot be achieved without the assistance of a large number of symbiotic bacteria in dairy cows, which exchange material and energy closely with the host and transfer information to each other. Symbiotic bacteria are closely related to the nutrition and immunity of dairy cows. Abundant microbial communities in rumen and intestines of dairy cows not only help to digest cellulose, hemicellulose, lignin and other indigestible crude fibers so that plant fibers become energy sources for dairy cows' life, but also have the ability to decompose toxins produced in the host metabolism (Hu et al. 2019). As the natural habitat of microorganisms, gut contains a large number of microbial communities such as archaea, bacteria, eukaryotes and even viruses. Calves are first exposed to microorganisms in the cow's birth canal and the external environment after birth. These microorganisms form the initial structure in newborn animals within a few hours and influence the final composition of the intestinal flora. After weaning, the relative homeostasis of intestinal flora will change, the structure of intestinal flora will change, and the new homeostasis will be formed. The intestinal bacteria of dairy cows mainly belong to *Firmicutes, Bacteroidetes* and *Proteobacteria* are involved in the metabolism of most nutrients, including fiber, hemicellulose, starch, protein and lipid substances, and the production of nitric acid, lactic acid, amino acids, ammonia and fatty acids. Fungi in the digestive tract of dairy cows can degrade the plant crude fiber in the feed, and at the same time mutually symbiosis with the bacteria. Compared with the high-concentrate diet, the high-fiber diet is more conducive to the fungi in the body. Anaerobic fungi with high cellulose degradation efficiency have been used as feed additives in dairy cows. The number of archaea in the gastrointestinal tract is small, and the characteristic of the methanogens is significant, which is the most abundant and complex. Methane is one of the final products of rumen microbial ecosystem after fermentation of plant substances, mainly produced by methanogens. Some researchers believe that methane contributes to global warming and climate change, so ruminant livestock is responsible for the greenhouse effect to some extent (Hu et al. 2019, Hu et al. 2020).

There are many kinds of symbiotic microorganisms in the digestive tract of dairy cows, and their composition and structure are very complex. Traditional microbial culture methods have limitations in studying the complex microflora in the intestinal tract. The application of metagenomics technology addresses the issue that the diversity of intestinal microorganisms cannot be accurately analyzed due to the inability to obtain non-cultured microorganisms.

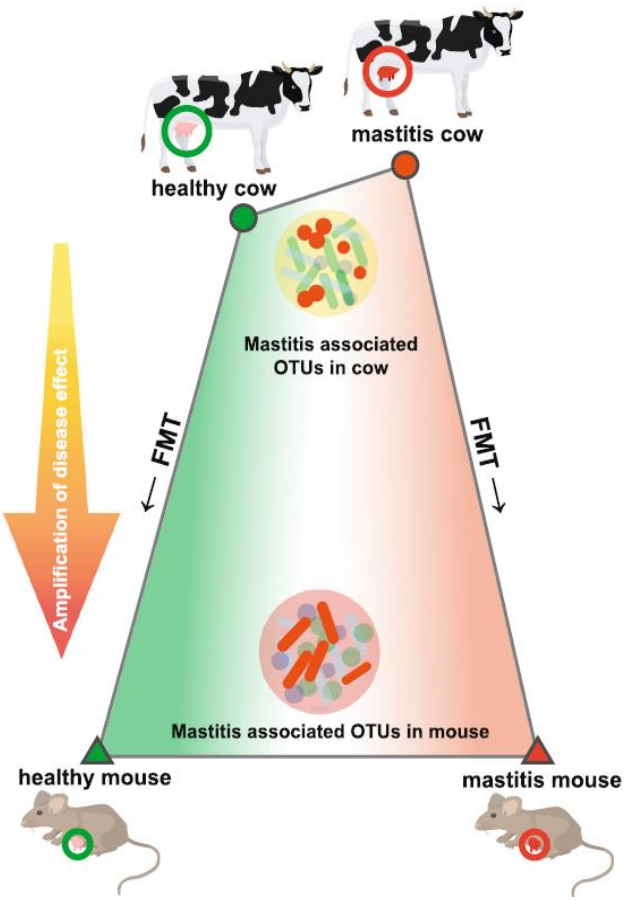

Fig. 1: Fecal microbiota transplantation (FMT) from cows with mastitis to germ-free mice leads to mastitis symptom (Ma et al. 2018).

With the rapid development in sequencing technology, the application of various omics technologies and the collaborative development of systems biology, bioinformatics and other disciplines have jointly promoted the research on the interaction mechanism between the intestinal flora and the host. After the comparison of microbial genomes attached to rumen epithelial cells and rumen contents, it was found that *Firmicutes* was the dominant bacterial community in the epithelial cells and *Bacteroides* was dominant in the contents (Li et al. 2012). Sequencing analysis of the full-length 16sRNA gene in the gastrointestinal tract of cattle showed that *Firmicutes* was the dominant bacterial community, followed by *Bacteroides* and *Proteobacteria* (Petri et al. 2013).

Research studies found that samples collected from various parts of the gastrointestinal tract of healthy cattle, such as the anterior stomach, small intestine, and large intestine showed no significant difference in the microbial community, most of which belonged to *Firmicutes* and *Bacteroides*. After comparison of the microbial diversity in rumen and intestinal tract, it was found that although the microbial community composition was in the same digestive tract, there were still differences, which might be related to the different physiological functions of each part (de Oliveira et al. 2013). Gut microbiota has a large number of microorganisms higher than the total host cells. It not only participates in energy metabolism of the body, but also plays an important role in the regulatory mechanism of host immune system. Intestinal flora structure is very complex, there are still some bacteria posing a threat to health. Disturbance in intestinal flora not only causes the digestive tract inflammation disease, irritable bowel syndrome, colon cancer, obesity, diabetes and other common diseases, but also leads to some systemic diseases, such as hardening of the Peripheral Arteriosclerotic Disease (PAD), nonalcoholic liver damage, depression, Alzheimer's disease, breast cancer, and immune system diseases such as food allergy, etc. (Xie et al. 2018; Li et al. 2019). The abuse of antibiotics and the change of diet destroy the normal structure of intestinal flora and break the balance, causing intestinal stress syndrome, inflammatory bowel disease, constipation, cardiovascular disease, nervous system disease, obesity, metabolic syndrome, allergic diseases, and autoimmune diseases which are difficult to be treated (Groen et al. 2017). Healthy intestinal flora has a direct protective effect, and it enhances the barrier's protective function of intestinal epithelial cells. The gut microbiota can stimulate the immune system, improve the immunity, produce bactericidal substances, and inhibit the colonization of pathogenic bacteria. Intestinal microflora can stimulate intestinal mucosa and significantly increase the content of T Helper 17 cells. The *Bacteroides* can increase the number of CD4+ T cells (Atarashi et al. 2011; Wang et al. 2019).

Recent research shows that there is a close connection between gut microbiota and mastitis. How the gut microbiota in gastrointestinal tract get connection with inflammatory disease in udder is a scientific issue which deserves investigation. It was believed for a long time that the internal environment of mammary gland is germ-free, but with the development in technology, and the use of more sensitive molecular methods for microbial identification, the statement that the healthy udder is germ-free has been challenged (Oikonomou et al. 2012). In most situations, the incidence rate of mastitis in high-producing dairy cows is much higher than that of dairy cows with a normal yield, and the disease happens more frequently during the lactation period. Dairy industry is committed to getting more dairy products and dairy cows are fed concentrated feeding stuff which alters the composition and quantity of the gut microbiota in rumen and gastrointestinal tract.

Lipopolysaccharide (LPS), one of the main elements of the cell walls of gram-negative bacteria, is an important factor that induces inflammation. In many cases, LPS metabolized by gut microbiota is closely related to mastitis (Wang et al. 2017). A large number of gram-negative bacteria are present in the gut and can lead to LPS accumulation.

High-concentrate (HC) diet was overused in cows' lactation period. HC can reduce the pH in ruminal and therefore leads to subacute ruminal acidosis (SARA). SARA is often associated with laminitis, liver abscess and other diseases, which are usually related to the massive release of LPS in the rumen and the transfer of LPS to various organs. Similarly, mastitis is often associated with SARA (Zebeli et al. 2012). The incidence of SARA in dairy cows is up to 18-40% in early lactation due to the increase of high-concentrate diet, while the incidence of mastitis in dairy cows is up to 30-45% at this time (Hu et al. 2019). The disturbance of rumen microflora caused LPS release in SARA cows which may be related to mastitis (Guo et al. 2017). Rumen-derived LPS in gram-negative bacteria cross the rumen epithelium, translocate from the digestive tract into the interior circulation and pass through the whole body. Systemic inflammatory response happens when LPS from gastrointestinal tract enter into the bloodstream. Moreover, these LPS from rumen in bloodstream increase the levels of

inflammatory cytokines TNF-α, IL-1β, and IL-6 in peripheral blood (Hu et al. 2019). At this time, the persistent feeding of an HC diet to dairy cows accelerates the increase in inflammatory cytokines. Liver is the organ which cleans the circulating LPS. The chronically high levels of LPS in the blood afflux to liver further injure the hepatocytes and inhibit liver function. Under the circumstances, LPS in the blood and rumen ulteriorly injure the rumen epithelium which serves as a biological barrier of innate immunity.

Rumen epithelium's barrier function is dependent on the multicellular structure, which includes the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale, as well as on the tight junctions that exist in the stratum granulosum (Graham et al. 2005). LPS and high acidity in the rumen injure the rumen epithelium and lead to dysfunction. When the structural integrity of the rumen epithelium is damaged, the epithelium barrier has an increased permeability, and LPS and pathogenic bacteria in the rumen may translocate across the epithelium barrier, leading to a systemic inflammatory response (Liu et al. 2013).

The most direct evidence of gut microbiota-mastitis correlation is that mice fed with high-fat diet can show increased serum LPS and result in mammary gland inflammation (Subbaramaiah et al. 2011). Other studies showed that dysbiosis of intestinal microbiota can lead to mastitis. Fecal microbiota transplantation (FMT) from cows with mastitis to germ-free healthy mice resulted in mastitis symptoms in mammary gland as well as inflammations in a wide range of tissues including serum, spleen, and colon in the mice (Ma et al. 2018). Similarly, cows fed with HC diet show increased levels of LPS and inflammatory genes such as IL-1β, IL-2, IL-22, CCL19 and so on. In addition, the levels of TNF-α, IL-1β, IL-6, NF-κB and TLR4 pro-inflammatory cytokines in the mammary gland increased (Zhang et al. 2016).

HC diet (or changes of feeding) leads to disturbance in rumen microbiota. Then, the dysbiosis or changes of rumen microbiota make the LPS levels increase significantly, and LPS injure the rumen Epithelium and recede its barrier function so that the permeability of the rumen epithelium barrier increased which allows LPS to pass through epithelium barrier and enter circulation system. When LPS go into organs via the blood, liver works to remove these LPS until the excessive LPS exhaust liver function and promotes the entry of LPS into the circulatory system and organs, leading to chronic low-grade inflammation in the whole body. In return, the chronic low-grade inflammation enhances susceptibility to mastitis and ultimately LPS enter mammary gland, leading to inflammation.

LPS and Blood-milk Barrier

Blood-milk barrier is one of host physiological barriers which include gut barrier, blood-brain barrier, blood-testis barrier, and the blood-milk barrier. Mammary epitheliums constitute the main structure of blood-milk barrier, which is the main defensive line of mammary gland that controls the passing of water molecules, ions, and bacteria. The junctions between mammary epithelial cells are mainly composed of tight junctions, adherent junctions, and gap junctions. Tight junction protein is the main connection between adjacent blood-milk barrier and endothelial cells, constituting the main components of the blood-milk barrier. Tight junction is constituted of Occludin, Claudins, ZOs and JAMs proteins.

Research studies show that tight junction proteins' functions can be reduced by LPS which allow more LPS to enter the blood and open a vicious circle. In most situations, mastitis occurring in animals alter the occludins, resulting in structural defect to the tight junctions (Chen et al. 2011). It is significant evidence of mastitis as well as a reliable indicator of dairy quality that somatic cell numbers (SCCs) increase as well as the contents of inflammatory cytokines TNF-α, IL-1β, IL-6 and SAA, corresponding to a reduction in the quantity and quality of dairy products. The SCCs' major component are neutrophils (PMNs) which form an important part of the body's natural immune system, responding quickly against pathogen infection. During the cows' lactation, the number of PMNs in milk is low, when pathogens intrude mammary tissue through papillary tube, macrophages in mammary gland play phagocytosis and release inflammatory cytokines and chemokines to induce PMNs in peripheral blood to cross the blood-milk barrier and enter mammary tissue to fight against pathogens.

When mammary gland gets infected, the PMNs pass through the blood-milk barrier constituted of vascular endothelium and mammary epithelial cells into mammary acini, playing an important role in determining the severity and prognosis of mastitis. The process of PMNs' crossing from the bloodstream to milk is under the control of the blood-milk barrier. It lowers the threshold of PMNs crossing the barrier and the dysfunction of the blood-milk barrier often means an increase in permeability. It explains the reason why plentiful PMNs enter the mammary acini when the mammary gland was irritated by pathogens. After entering mammary tissue, PMNs are in contact with pathogenic bacteria, which are recognized and phagocytized under the corresponding receptors' transduction, and finally the microorganisms are degraded by respiratory burst and enzymes. PMNs have a protective effect to mammary gland tissue. On the other hand, excessive inflammatory cytokines and reactive oxygen cause serious damage to mammary gland tissue and increase the inflammation degree and susceptibility of mammary gland tissue.

Probiotics and Mastitis

Probiotics have made great progress in the prevention and treatment of cow mastitis or mastitis in other animals or even human by re-equilibrating the microbiota. Probiotics are defined as live microorganisms that confer health benefits on the host and maintain balance of enteric microorganism. Various strains of probiotics have been isolated from milk or other sources. In medical applications, the probiotics have been used to treat mastitis by breastfeeding. Clinical research studies show that the incidence of mastitis in women who received *Lactobacillus salivatius* PS2 in approximately week 30 (25%) was lower than that in the control group (57%) (Fernandez et al. 2016). *Lactococcus lactis* DPC 3147 is a food-grade microorganism with broad spectrum antibacterial activity against mastitis caused by *Streptococcus* and *Staphylococcus aureus*. *Lactococcus lactis* treatment has a good result similar to antibiotics in the treatment of clinical mastitis. It can promote immune regulation by injecting lactic acid bacteria in the mammary gland of healthy cows (Twomey et al. 2000). Infusion of probiotics lactic acid bacteria to cow mammary gland causes recruitment of lymphocytes and neutrophils and increases the expression of immune-related genes. Some probiotics with the ability to inhibit the growth of mastitis pathogens by secreting bacteriocin were isolated from milk. 165 strains of lactic acid

bacteria were isolated from bovine papillary duct, among which 10 strains could inhibit *staphylococcus aureus*, *Escherichia coli* and *Streptococcus*, which can cause mastitis, and reduce the colonization of these pathogens on mammary epithelial cells. *Lactobacillus casei* BL23 inhibited the invasion of *S. aureus* into mammary epithelial cells by inhibiting adhesion and cohesiveness (Bouchard et al. 2015).

Probiotics can pass through the intestinal epithelial, enter into the circulatory system by swallowing function of dendritic cells, and displace to the mammary gland tissue. It is believed that gut bacteria may be derived from milk at first, and impact on flora of mammary gland after the formation of intestinal flora (Jost et al. 2013). *L. casei* can reduce the amount of *Staphylococcus aureus* colonizing in the breast by 80%, and have significant therapeutic effect on mastitis (Bouchard et al. 2013). In one study, *L. salivarius* and *L. gasseri* isolated from breast milk were taken orally to treat lactating women with mastitis caused by *Staphylococcus* infection, and the experimental results showed that the treatment effect was significant (Arroyo et al. 2010). Probiotics directly reach the mammary gland through endocytosis to inhibit the reproduction of pathogenic bacteria, and improve the host immunity.

Many subsequent studies have proven that probiotics play a protective role in mastitis through their metabolites, which is called short-chain fatty acids (SCFAs), that are produced by bacterial fermentation of dietary fiber in the gut or rumen, having an anti-inflammatory property. It is reported that SCFAs have been shown to inhibit the production of pro-inflammatory cytokines and to decrease the pathological injury in mammary gland induced by LPS. SCFAs are carboxylic acids defined with the presence of an aliphatic tail of 2 to 6 carbons, including butyrate, acetate and propionate, produced by gut microbiota's fermentation of dietary fiber. SCFAs are important components of milk, and for ruminants, it is the major source of energy (70%). SCFAs paly many roles such as cell differentiation, proliferation, motility, apoptosis, and immune regulation (Spichak et al. 2021). Acetic acid, propionic acid and butyric acid get the highest proportion in short-chain fatty acids, accounting for more than 95% of the total content of short-chain fatty acids. There is around 50% acetate, 27% propionate, and 23% butyrate in cows ruminal fluid (Xu et al. 2015). SCFAs participate in the modulation of metabolic, neuroregulation, inflammatory response, and immunological functions and G protein–coupled cell surface receptors (GPCR: GPR41, GPR43, and GPR109a) are activated. GPCRs are present in epithelial cells, neutrophils, and macrophages in the immune system. Butyrate and propionate inhibit LPS-induced TNF-α and NOS expression and NF-κB activation, inhibiting HDACs in neutrophils by activating GPCR.

Conclusion

Gut microbiota is closely associated with development of mastitis. Subacute rumen acidosis (SARA) occurred in 18 to 40% of dairy cows due to the addition of high concentrate feed in early lactation. Clinical data also showed that the incidence of mastitis was highest in early lactation (35 to 40%). The pH of rumen fluid was significantly reduced when cows were fed with high-concentrate diet and induced SARA. The high acid vinegar of rumen microenvironment resulted in rumen epithelial injury. The rumen microbial were disordered while a large number of gram-negative bacteria died, and released LPS into the blood continuously, resulting in systemic low-grade inflammatory reaction in the body. When dairy cows are exposed to lactation stress, LPS in the circulation system cross the blood-milk barrier and displace into the mammary gland and accumulate to injury the mammary tissue, leading to mastitis ultimately. On the other hand, gut-derived LPS entry into the mammary gland leads to more neutrophils to enter the mammary gland, and release more inflammatory cytokines, aggravating the inflammatory response of the mammary gland. According to the pathological mechanism that rumen flora disturbance leads to subclinical endotoxemia and causes systemic low-grade inflammation in the body, it is concluded that there is correlation between gut microbiota and bovine mastitis.

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CHAPTER 02

BETA-LACTAM ANTIBIOTICS RESISTANCE IN *S. AUREUS*: MECHANISMS AND RESISTANCE MODULATION STRATEGIES

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INTRODUCTION

Staphylococcus (S.) aureus is considered a bacterium of utmost importance regarding both animal and human health concerns. The genus consists of 81 species and subspecies in which the majority of the species are mammalian commensals or opportunistic pathogens that establish in habitats like skin, nares, and various mucosal membranes of the target host (Haag et al. 2019). Several species are having significant medical and veterinary importance e.g., *S. aureus* is considered the most pervasive and significant pathogen on the human and animal side. It involves a wide range of conditions in human and animal health ranging from mild infections to lethal disease manifestations which can lead to life-threatening conditions (Leonard and Markey 2008). The pathogenic properties like phagocytic evasion, biofilm formation, and the development of antibiotic avoidance, shield the bacteria from degradation and evolve to be a pathogen of worry around the globe (Monistero et al. 2018).

Antibiotic resistance, being an emerging global public health concern, has become one of the biggest challenges of the 21st century (Roca et al. 2015). The emergence of antibiotic resistance and the spread of antimicrobial-resistant pathogens can be anticipated by a proper understanding of evolutionary events responsible for the development of antimicrobial resistance. *S. aureus* exhibits resistance to antibiotics by *mecA* and *blaZ* gene responsible for encoding penicillin-binding protein 2a (PBP2a) (Aires-de-Sousa 2017). As beta-lactams are the most commonly used antibiotics in field conditions so it is necessary to understand the molecular mechanisms of resistance to these antibiotics (Alves et al. 2020). This mechanistic knowledge, along with structural information will help plan control strategies to reduce the clinical problem caused by MRSA. Continued and multidimensional efforts by the Antimicrobial Stewardship Program are urgently needed to promote the rational use of antimicrobials, infection prevention, and containment of antimicrobial resistance (Rice 2012).

Staphylococcus aureus: A prevalent pathogen of human and veterinary significance

S. aureus is considered a contagious pathogen that can be transferred between animals and humans posing a serious public health threat. Besides research on *staphylococcal* pathogenesis in humans, *S. aureus* is considered a major cause of infection and sickness in a variety of animal species, resulting in important public health and agricultural consequences (Peton and Le Loir 2014). The resistive action of *S. aureus* against antibiotics facilitates the establishment of persistent and recurring infections and made the treatment strategies ineffective. The dearth of remedies against this pathogen along with the lavish use of beta-lactam antibiotics has given rise to resistance against *S. aureus* strains (Gao et al. 2012). Methicillin-resistant *Staphylococcus aureus* (MRSA) strain is an important drug-tolerant microorganism with higher genetic capabilities having the characteristic of evading host immune response (Brady et al. 2011). Studies have proved MRSA is a zoonotic bacteria that can transfer from infected bovine milk and the environment to the people who are taking care of animals but also those who are consuming milk and milk products, animal handlers, and veterinarians are also at risk to get MRSA infection (Juhász-Kaszanyitzky et al. 2007).

In humans, MRSA has long been thought to be a healthcare-associated pathogen, with known risk factors for nosocomial infection including protracted antibiotic treatment, surgical intervention, delayed recovery, treatment in an ICU, and close contact with other MRSA-infected or colonized persons (Devriese et al. 2005). In *S. aureus* infection, the frontal nares can be thought of as a natural ecological niche in infected persons. In nasal carriers, *S. aureus* also inhabits the skin occasionally and the transmission is thought to occur mostly via hands. In humans, the first report on MRSA was published in the United Kingdom in 1961, followed by reports in Europe in 1965, Australia in 1966, the United States in 1968, and Asia in the 1970s. However, recent studies have also shown that MRSA from livestock sources which are known as livestock-associated MRSA (LA MRSA) can also colonize associated occupational workers in the vicinity of animals (Rinsky et al. 2013).

In animals, *S. aureus* can be a cause of wide range of infections which may include skin and soft tissue infections, joints, bones, implant infections, pneumonia, septicemia, and mastitis. It may produce systemic disease in animals, but it is considered a major cause of bovine mastitis (Holmes and Zadoks 2011). Even the first isolation of MRSA from animals was found in the milk of mastitic cows (Devriese and Hommez 1975). Mastitis

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in bovines caused by *S. aureus* is the most prevalent, accounting for 30-40% of all mastitis cases and 80% of subclinical bovine mastitis. The ever-increasing prevalence of antimicrobial-resistant strains of *S. aureus* proved a significant constraint in developing a dairy industry globally (Fey and Olson 2010). Infected udders and teat skin are the principal reservoirs in a dairy herd. Infected cows can shed bacteria through their milk, and MRSA mostly spread from udder to udder during milking due to contaminated milking machines, farmer's hands, or contaminated bedding (Sakwinska et al. 2011).

The rise in the global incidence of virulent and multidrug-resistant *S. aureus* strains poses a new threat to the dairy business, necessitating more concern to address the problem (Cuny et al. 2015). Hence, the current chapter discusses the evolution of resistance mechanisms in *S. aureus* with the main context of the beta-lactam resistance development over time. Moreover, different therapeutic regimes and resistance modulation strategies for beta-lactam resistant *S. aureus* using herbal, nanoparticles, and non-antibiotics in combination will be reviewed.

Evolution of antimicrobial resistance mechanisms in *S. aureus*

Antibiotics are widely employed in both human and veterinary medicine, as well as in other agricultural operations. Over the last several decades, greater industrial usage of these medications increased the number and kind of drug-resistant bacteria, resulting in increased public health issues in terms of morbidity, mortality, and treatment costs. The trend of resistance, which began in hospitals and subsequently expanded to the general population and livestock, is now well-established and repeats itself with each new wave of antimicrobial resistance (Chambers 2001).

In the laboratory, bacterial mutants may be produced spontaneously at a rate of 10^6 to 10^8 per cell. Therefore, when first antibiotic resistance appeared in natural populations, it was presumed that selection and mutation had developed these resistant species (Bitrus et al. 2018). However, once extra-chromosomal DNA elements and horizontal genes transfer (HGT) was discovered, gene mutation was gradually limited to a secondary role in the development of antibiotic-resistant bacteria. It is now known that the acquisition of distinct genetic 'accessory' elements such as transposable genetic elements, plasmids, and genomic islands drives *staphylococci* resistance to various antimicrobial drugs. These elements include pre-formed antimicrobial resistance genes and are passed down through generations of bacteria by horizontal gene transfer across related strains and even between different genera and species.

In *S. aureus*, the classical microbial HGT pathways including transduction, transformation, and conjugation have been established. Although, there is a fourth mechanism that is responsible for the more rapid transfer of plasmid DNA is known as "phage-mediated conjugation". However, it's unknown how much each of these pathways contributes to the spread of antibiotic resistance genes in the environment. Furthermore, the availability of restriction-modification systems containing enzymes that may destroy unmodified DNA might impact the acceptability of certain *S. aureus* strains as HGT receivers (Waldron and Lindsay 2006).

Through the horizontal transfer of mobile genetic elements, this pathogen may rapidly gain and lose virulence features and resistance from other members of the genus *Staphylococci*, which is responsible for its worldwide growth and stability (Waldron and Lindsay 2006; Bloemendaal et al. 2010; Bitrus et al. 2018). According to whole-genome sequencing studies, the *S. aureus* genome has two parts: 1) Stable core genome which consists of 75-80% of the total genome 2) Less stable mobile genetic elements (MGE) that include plasmids, pathogenicity islands, Staphylococcus cassette chromosomes, transposons, insertion sequence, and bacteriophage (Lowy 2003; Holden et al. 2004). With the help of a horizontal transfer, lineage-specific MGEs in *S. aureus* easily integrate, recombine, and move in and out of the genome (Lindsay 2014). They encode a diverse set of virulence and resistance genes, as well as immune evasion genes, allowing MRSA to adapt and form new, pathogenic, and highly resistant clones (Bitrus et al. 2018). Methicillin was the first semisynthetic penicillinase-resistant penicillin, being introduced in 1961. The acquisition of the *mec*A gene, which encodes the penicillin-binding protein 2a (PBP2a), a slightly different PBP with a poor affinity for beta-lactam antibiotics was the cause of such a novel resistance. The *blaZ* gene, which codes for beta-lactamase enzymes, is involved in penicillin resistance (Deurenberg et al. 2007). Beta-lactamase is an extracellular enzyme that is produced in response to beta lactam antibiotics. It decreases penicillin therapeutic action by hydrolyzing the beta lactam ring (Lowy 2003).

Beta-lactam resistance: Mechanisms and impact

The development of resistance by *S. aureus* against various antibiotics has led to the fact that even minor infections and injuries can prove fatal for the life of host (Miragaia 2018). The resistance development in pathogens can be better understood by keeping methicillin-resistant *S. aureus* as a model because of the gradual development of resistance against methicillin in *S. aureus* and the abundance of published literature on MRSA, a global health hazard of public and animal origin (Lindsay 2013; Otto 2013).

The most important antibiotics that have become resistant to *S. aureus* infections belong to the beta-lactam group. Beta-lactam antibiotics including penicillin are the most commonly used in human and veterinary practice (Lowy 2003). These antibiotics are highly efficacious drugs with the least toxicity issues which is the reason for their abundant usage (Shahid et al. 2009). These antibiotics are bactericidal and inhibit the synthesis of the cell walls in gram-positive bacteria like *S. aureus* (Rice 2012). The synthesis of the *S. aureus* cell wall is mainly comprised of transglycosylation, in which the glycan strand elongation occurs, and transpeptidation in which cross-linking occurs between stem peptides of different glycan strands (Macheboeuf et al. 2006). The resultant major structural constituent of the cell wall, peptidoglycan, comprises glycan strands that are composed of N-acetylglucosamine and N-acetylmuramic acid disaccharides linked by peptide cross-links between N-acetylmuramic acid moieties on adjacent strands (Holden et al. 2004). Both reactions i.e. transglycosylation and transpeptidation included in the polymerization of peptidoglycan are mainly mediated by a protein named, Penicillin-binding protein (PBP) (Giesbrecht et al. 1998). Beta-lactam antibiotics are known to target PBP which is an essential constituent to synthesize peptidoglycan of the cell wall. The inactivation of penicillin-binding protein after attachment with beta-lactams causes the inhibition of peptidoglycan synthesis by blocking transpeptidation which will ultimately stop the cell

wall synthesis and cell growth usually resulting in cell death (Tang et al. 2014).

Beta-lactam resistance associated with beta-lactamase enzyme production

The resistance against beta-lactam antibiotics has emerged over time which is just because of continuous exposure of *Staphylococci* to these antibiotics in different forms. The tremendous use of these antibiotics as food additives or supplements in the feed of farm animals, the routine clinical use of this antibiotic group in dealing with the bacterial infections of animals and humans, and the co-existence of *Staphylococci* with penicillin producing fungi in the soil are considered major driving forces for continuous exposure of the bacteria with the beta-lactam antibiotics and ultimately the emergence of beta-lactam resistance in *Staphylococci* (Westh et al. 2004; Castanon 2007). The beta-lactam resistance in *S. aureus* can be possibly due to three different mechanisms. The first mechanism is the use of the beta-lactamase enzyme to hydrolyze the beta-lactam ring of these antibiotics to make them ineffective. The other possible mechanisms include the decreased reach of beta-lactams to penicillin-binding proteins or the development of intrinsic resistance both in beta-lactamase resistant and sensitive *S. aureus* by reducing the binding affinity of these antibiotics with PBPs (Rice 2012).

The *blaZ* gene typically resides on a large transposon on a plasmid and is responsible for the production of the beta-lactamase enzyme (Lowy 2003). These enzymes are extracellular and are of four different types A, B, C, and D. These enzymes are synthesized by *S. aureus* on exposure to penicillin or other beta-lactam antibiotics that are opposite to the beta-lactamases produced by gram-negative bacteria which produce this enzyme continuously (Lyon and Skurray 1987). The production of this enzyme under the influence of the *blaZ* gene results in hydrolysis of beta-lactam ring making the beta-lactam antibiotics ineffective against *S. aureus* (Rice 2012). The *blaZ* gene is controlled by two adjacent regulatory genes including repressor *BlaI* and antirepressor *BlaR1* genes (Lowy 2003). The current studies revealed that the cleavage of regulatory proteins BlaR1 followed by the cleavage of *BlaI* on exposure of bacteria to beta-lactam antibiotics is necessary for the production of the β-lactamase enzyme. In presence of beta-lactams, BlaR1, a transmembrane sensor-transducer, cleaves itself (Gregory et al. 1997) and is thought to cleave the repressor *BlaI* either directly or indirectly and permits the blaZ gene to synthesize beta-lactamase enzyme (Fig 1) (Lowy 2003). This enzyme has higher efficacy to hydrolyze the beta-lactam ring of ampicillin and penicillin-G while the efficacy against semisynthetic penicillins like oxacillin and methicillin is poor (Lyon and Skurray 1987). The beta-lactamase associated beta-lactam resistance has emerged in over 90% of *S. aureus* isolates of human origin (Lowy 2003).

Beta-lactam resistance associated with the formation of PBP2a protein

To lessen the health hazards caused by beta-lactamase producing *S. aureus*, semi-synthetic penicillin named methicillin was used effectively but in the same year, in 1961, the resistance against methicillin was documented in *S. aureus* isolates. It was hypothesized that continuous exposure of bacteria to methicillin has resulted in the development of resistance (Leonard and Markey 2008). The methicillin resistance is acquired by the acquisition of the *mecA* gene by *S. aureus* (Chambers 2001) which is responsible for the production of PBP2a (Penicillin-binding protein 2a) (Hartman and Tomasz 1984; Song et al. 1987). The gene is a part of a large mobile genetic element named *Staphylococcal* chromosomal cassette *mec* (*SCCmec*) (Itou et al. 2000). Upon exposure to beta-lactam antibiotics, just like the regulation of blaZ gene, the response of *mecA* gene is also regulated by two regulatory genes, mecI and mecR1. In the presence of beta-lactam antibiotics, mecR1 induces synthesis and inactivates the MecI that will allow the PBP2a synthesis (Lowy 2003). Unlike the PBP, the active sites of protein PBP2a prevent binding of all beta-lactams and thus exhibit low affinity for these antibiotics. This protein will allow the transpeptidation process to proceed and help bacteria to survive even in high concentrations of beta-lactam antibiotics (Lowy 2003). MRSA responds to antibacterial agents in a much poorer way compared to methicillin-sensitive *S. aureus* (Hurley et al. 2003).

Current therapeutic regimes used against *S. aureus* infections

Multidrug-resistant strains are becoming more common, and they are becoming a primary source of illness and mortality. As a result, creative approaches to combat multidrug-resistant organisms are critical. Endolysins, which are produced from bacteriophages and other peptidoglycan hydrolyses with the capacity to rupture cell walls, might be used as an alternative to antibiotics. These lytic enzymes have a high level of host specificity and might replace or complement antibiotics in the treatment of infections caused by Gram-positive drug-resistant bacterial pathogens like methicillin-resistant *S. aureus* (MRSA). *LysK* is a well-studied endolysin that has an action against a variety of *staphylococcal* species (Ajuebor et al. 2016; Hosseini et al. 2016; Schmelcher and Loessner 2016).

Initially, penicillin was considered efficacious for treating *S. aureus* infections; however, penicillin resistance is commonly prevalent in most countries of the world (Levy and Bonnie 2004). Other-lactam antibiotics, such as penicillins, cephalosporins, carbapenems, and monobactams, have also become resistant to methicillin-resistant *S. aureus* strains (Rayner and Munckhof 2005). In the United States before the development of CA MRSA infection, infections caused by *S. aureus* were regularly cured with oral antistaphylococcal penicillins, like dicloxacillin, or cephalosporins, such as cefadroxil or cephalexin (Stevens et al. 2005).

Daptomycin is a new lipoglycopeptide antibiotic that is rapidly effective against MRSA infections (Straus and Hancock 2006). It was recently approved for the treatment of bacteremia and right-sided endocarditis caused by *S. aureus* including MRSA after a study proved that daptomycin was less effective as compared to vancomycin for this treatment. The strains of MRSA with heteroresistance towards vancomycin may also exhibit daptomycin heteroresistance even if they have never been exposed to daptomycin (Pillai et al. 2007). Although the resistance against daptomycin has been reported in some clinics, the higher concentrations of this antibiotic are still efficacious toward *S. aureus* (Bayer 2013).

Quinolones are a group of broad-spectrum antibiotics assumed to give promising results in treating the hospital-acquired infections caused by already resistant strains to some older antibacterial agents (Wafi Siala 2014). In hospitals,

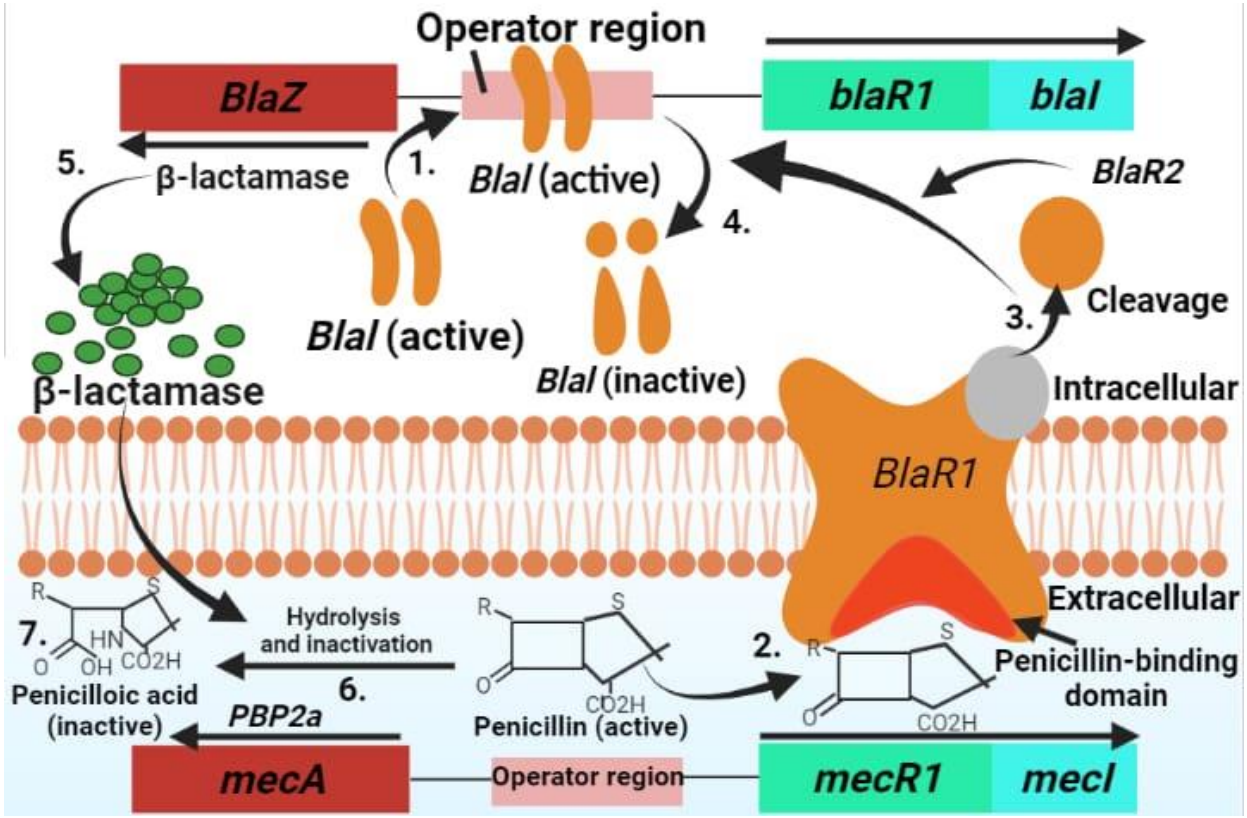

Figure 1. Mechanism of Beta-lactam resistance in *S. aureus*

fluoroquinolones can be used as an effective antimicrobial against MRSA infections (Haas et al. 2009). However, resistance to fluoroquinolones has also been detected in *S. aureus* isolates (Redgrave et al. 2014), and this antibiotics class is linked with a higher risk of MRSA colonization due to which its use is prohibited (Tacconelli et al. 2008).

At the moment, trimethoprim-sulfamethoxazole (TMP-SMX) and clindamycin are the most often used antimicrobial medicines for the outpatient treatment of CA MRSA infections in the United States while in other parts of the world combination of rifampin and fusidic acid is commonly used. TMP-SMX appears to be the agent of choice for adult therapy in the US, although clindamycin is also chosen by many pediatricians (Robert et al. 2007). From the results of *in-vitro* evidence-based recent study, the use of TMP-SMX was considered in favor to treat MRSA infections and it exhibited increased bactericidal activity than the other drugs like linezolid, clindamycin, rifampicin, and minocycline (Kaka et al. 2006).

Despite the lack of evidence that a combination medication is beneficial, some physicians have used a combination of rifampin with TMP-SMX or long-acting tetracycline regimens. During therapy, rifampin is usually used with fusidic acid to avoid mutual resistance. In clinical studies, linezolid is the only orally accessible antibiotic that is effective against MRSA infection. Pediatricians also commonly prescribe clindamycin to treat skin and soft-tissue infections, particularly CA-MRSA infections. This drug has also the potential to minimize MRSA's production of Panton-Valentine leukocidin and other virulence factors (Moellering 2008).

Based on clinical trials, Linezolid, an antibiotic belonging to the oxazolidinone group is effective in the treatment of MRSA infections and more effective than vancomycin in treating complicated skin infections due to MRSA (Weigelt et al. 2004). Linezolid has a bioavailability of 100% and penetrates deep into the epithelial lining of the lungs as well as diseased skin and soft tissues in diabetic individuals (Leach et al. 2011; Liu et al. 2011; Rodvold and Mcconeghy 2014). Although linezolid resistance in MRSA has been identified it has not yet become a severe clinical problem (Tsiodras et al. 2001). It may also be particularly useful for serious skin infections like necrotizing fasciitis due to its ability to inhibit the production of toxins (Stevens et al. 2007). The continuous medication with linezolid includes reversible myelosuppression (including anemia, leucopenia, thrombocytopenia, and pancytopenia), therefore complete blood counts should be examined weekly in those patients who are receiving linezolid for more than 2 weeks. With prolonged therapy (>28 days) sometimes adverse events are also observed that include lactic acidosis, optic neuropathy, and peripheral neuropathy (Rodvold and Mcconeghy 2014).

Based on clinical controlled trials, US Food and Drug Administration (FDA) has approved the following 5 anti-infective agents like daptomycin, tigecycline, linezolid, ceftaroline, and telavancin for the treatment of MRSA infections (Nguyen and Graber 2009; Stryjewski and Corey 2009). Vancomycin is considered the gold standard parenteral therapy for treating complicated MRSA infections. However, decreased efficacy due to rising resistance among MRSA strains has tainted that benchmark (Sakoulas and Moellering 2008; Tenover and Moellering 2007). Over the last decade, daptomycin and linezolid have recognized a significant role as first-line treatment in specific patients. Other older and newer agents have been limited in their usage due to a lack of clinical effectiveness data, medication product availability, and safety concerns. The present pipeline of research drugs used for the treatment of MRSA infections seems promising, and numerous new alternatives to overcome antibacterial resistance must be developed.

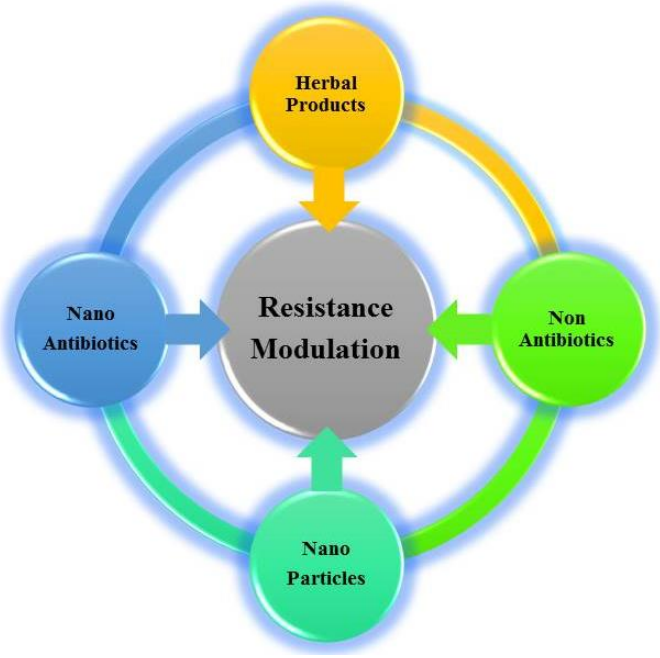

Figure 2: Resistance modulation strategies to combat antimicrobial resistance

Resistance Modulation Strategies for Beta-lactam Resistant *S. aureus*

Use of Nanoparticles

Various chemicals and drugs other than antibiotics can be used to modulate resistance produced by microorganisms (Fig. 2) (Wei et al. 2004). Several organoselenium compounds have been produced and demonstrated *in-vitro* for their remarkable antibacterial properties, including 2,4,6-tri-paramethoxyphenylselenopyrylium chloride, 9-para-chlorophenyloctahydr oselenoxanthene, and perhydroselenoxanthene, which are particularly effective against *S. aureus* (Küçükbay et al. 2003; Pietka-Ottlik et al. 2008; Tran and Webster, 2011). Antibiotics (Katva et al. 2018; Mazur et al. 2020), hydrogen peroxide (Alkawareek et al. 2019), and some of the other metallic nanoparticles (copper & tungsten carbide), all have synergistic effects with AgNPs, according to several investigations (Katva et al. 2018; Aqib et al. 2022; Ijaz et al. 2022). Moreover, the free hematoporphyrin and silver nanoparticles show minimal photodynamic action against methicillin-resistant *S. aureus* (MRSA). The antibacterial activity of hematoporphyrin is significantly increased when it is integrated into a silver-silica shell (Ahmadov et al. 2016; Bankier et al. 2019).

In addition, some inorganic nanoparticles also exhibited great capability in the treatment of *S. aureus* infections. Because of its ability to stimulate bone regeneration, the β-tricalcium phosphate nanoparticles have undergone remarkable progress in the treatment of *S. aureus* osteomyelitis (Chou et al. 2014; Uskoković and Desai 2014). The antibacterial action against intracellular *S. aureus* was significantly higher when niosomes loaded with ciprofloxacin were phagocytosed rather than free ciprofloxacin (Akbari et al. 2015).

The smart novel nano systems having simulated invasion mechanisms for *S. aureus* which is particularly targeted at the intracellular *S. aureus*, the resistant *S. aureus*, and *S. aureus* with biofilm must be developed in the future by studying the modification mechanisms of nanoparticles at the cellular, molecular, and animal levels to achieve satisfactory results. To increase the therapeutic effects of *S. aureus* infectious disease, there is a need of producing smart nanoparticles aimed at the invasion process of *S. aureus*. Later, the erythrocyte membrane-coated nanogel system was discovered based on the characteristics of *S. aureus* cell membrane penetration (Zhou et al. 2018). When bacteria recognize certain receptors on the erythrocyte membrane and subsequently penetrate the RBC-nanogel by producing β-toxin and δ-toxin, the loaded antimicrobial chemicals can make use of the RBC-nanogel to kill *S. aureus*.

Nanoparticles with reduced toxicity have recently become a popular option and an important component of nanotechnology in treatment trials. Garlic-derived phytochemically reduced NPs had improved bactericidal activity against multiple drug-resistant *S. aureus*at higher doses (0.5, 1.0 mg/50ul). However, green manufactured NiO-NPs are potential activists in the fight against drug resistance as well as an environmentally friendly catalytic agent that might be used on a large scale (Haider et al. 2020).

Inorganic minerals such as Ag, Cu, Au, ZnO, CuO, and TiO2 have been shown to have substantial antibacterial efficiency; among these nanoparticles, ZnO is a promising contender due to its simplicity of synthesis, environmentally compatible, and cost-effectiveness. Therefore, green generated ZnO-NPs derived from *Zingiber officinale* root extracts could be a possible antibiotic replacement in advanced medicine and could help to resolve the global issue of drug resistance against pathogenic bacterial diseases as well (Haider et al. 2020).

Use of Nano antibiotics

Antibacterial nanomaterials on their own, or nanomaterials that enhance the efficacy and safety of administering antibiotics (Li et al. 2008; Abeylath and Turos 2008) are referred to as "Nano antibiotics," and their ability to overcome infections both *in vitro* and *in vivo* has been explored and demonstrated. In contrast to many antimicrobial drugs currently being used in the clinics, antimicrobial NPs may not have any immediate or acute adverse effects, and their long-term toxicity is unknown (Kim et al. 2007). The most important aspect is that the antimicrobial NPs target many biological pathways prevalent in a wide range of bacteria; therefore, developing resistance to NPs' antimicrobial activity would necessitate numerous concurrent changes. Furthermore, some NPs can endure extreme circumstances, such as sterilization at high temperatures, which makes the use of conventional antibiotics unsuccessful. There are several advantages of using nanoparticles to administer antibiotics: 1) regulated and rather uniform dispersion in the target tissue, 2) better solubility, 3) release in a controlled and sustained manner, 4) enhanced patient compliance, 5) minimal adverse reactions, and 6) improved cellular internalization in the target tissue (Sosnik et al. 2010; Mansour et al. 2009; Santos-Magalhães and Mosqueira 2010).

Antibacterial NPs include naturally occurring antibacterial chemicals, metals and metal oxides, some carbon-based nanomaterials, and surfactant-based nano emulsions. According to a recent study, naturally occurring bacteria do not acquire antimicrobial resistance to metal nanoparticles (Kędziora et al. 2020). The antimicrobial processes of nanomaterials include 1) the photocatalytic production of

reactive oxygen species (ROS) that cause damage to the cellular and viral constituents, 2) disruption of bacterial cell wall, 3) inhibition of enzyme activity and synthesis of DNA, and 4) the disruption of the energy transduction (Rabea et al. 2003; Kim et al. 2007; Huang et al. 2008; Li et al. 2008). The metallic NPs (such as cadmium sulfide [CdS], gold [Au], and silver [Ag]) have recently been synthesized intracellularly or extracellularly using microbial cells or enzymes as a new biological and environment-friendly production of NPs (Saravanan and Nanda 2010; AbdelRahim et al. 2017).

Use of Herbal Products

Due to the high expense of effective antimicrobials in undeveloped countries, a considerable part of the population relies on medicinal plants to cure infectious diseases. Several research studies have explored the antibacterial activities of herbal plants against MDR infections in recent years, as people have become more aware of the therapeutic potential of plants and herbal components. This pattern can be found throughout the world with no exception in developed countries. Research using extracts of plants in Thai traditional medicine indicated a significant action against MRSA (*Garcinia mangostana, Quercus infectoria*) (Voravuthikunchai and Kitpipit 2005) and Australia (*Lepidosper maviscidum, Amyema quandong, Eremophila alternifolia, Eremophila duttonii*) (Palombo and Semple 2002).

Use of non-antibiotics

Antibacterial activity has been discovered against a wide range of bacteria in compounds from numerous drug families, including anti-inflammatory, antihistamines, antihypertensive, tranquilizers, and antispasmodic drugs, as a result of systematic screening of licensed non-antibiotic compounds (Mazumdar et al. 2009). Diclofenac sodium improves the efficacy of streptomycin against *E. coli*, *S. aureus*, as well as *Mycobacterium* spp., and that of gentamicin against *L. monocytogenes*. Antibacterial activity has been demonstrated in several phenothiazine-derived drugs, synergistic to antibiotics currently used in clinics, against a broad range of bacteria, which includes Gram-positive and Gram-negative bacteria, as well as mycobacteria (Kristiansen et al. 2007; Amaral and Viveiros 2012). In various clinical isolates, the antipsychotic drug phenothiazine thioridazine has proven to enhance resistance to oxacillin in MRSA (Klitgaard et al. 2008; Bonde et al. 2011) and dicloxacillin (Poulsen et al. 2013). Oxacillin-induced transcription of mecA and expression of PBP2a, in addition to transcription of some more genes that belong to the regulon of VraSR, are suppressed in the presence of thioridazine (Klitgaard et al. 2008).

The use of combinations of antibiotic–adjuvant has several advantages over the use of novel antibiotics, including a lower risk of developing resistance (Balaji et al. 2009; Nanda and Saravanan 2009). There are various approaches including modern developments in the conventional adjuvants like beta-lactamase and efflux pump inhibitors; novel approaches like targeting bacterial signaling pathways to interfere with bacterial response to antibiotics; and the use of high-output screening of already approved drugs to find out the drugs with unexpected adjuvant activity. The lack of introduction of modern antibiotic classes, the considerable issue of resistance acquirement to medicinal methods that depend solely on bacteriostatic/bactericidal activity, and the clinical efficacy of antibiotic/adjuvant combinations like Augmentin render the adjuvant approach a very appealing method for developing novel and effective therapeutic regimes for MDR bacterial infections (Bonde et al. 2011).

Conclusion

Antimicrobial resistance has emerged as a greater public health challenge. Beta-lactam antibiotics are widely used against *S. aureus* infections in animals and humans. With time, *S. aureus* has developed resistance to penicillin, cephalosporin, tetracyclines, chloramphenicol, methicillin, sulfonamides, and vancomycin. So, there is a need for alternative treatment strategies to combat antimicrobial resistance caused by this emerging pathogen. So, there is an urgent need for treatment options for patients infected with resistant *S. aureus*. More research is needed focusing on clinical outcomes and identifying the dynamics that promote resistance, high-risk strains, and the genetic basis of resistance.

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CHAPTER 03

BIOCHEMICAL MECHANISMS OF DRUG RESISTANCE AGAINST BACTERIA AND FUNGI IN ANIMALS

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INTRODUCTION

Antimicrobial resistance is acquired by a microorganism when it proliferates in the presence of an escalated concentration of antimicrobial drug on comparing with phylogenetically related strains. Antimicrobial resistance is a relative terminology that can only be measured after juxtaposition with one or more microbial strains under an analogous set of conditions. Improper and burgeoning dosages of antimicrobial drugs have substantially enhanced the susceptibilities of drug resistance. To elevate weight and growth rate in veterinary animals like herds or flocks, an optimized dosage of antibiotics is provided to them which also compensates for the unhygienic conditions in the crowded farms. It ultimately leads to an increase in the excessive collation of antibiotics in the environment and accretion of antibiotic resistance in the microorganism on their contact with the antibiotic drugs inside animal bodies (Kumar et al. 2020). Livestock animals greatly contribute to this resistive outspread. Antibiotic dissipation is highest in China (23%) followed by US (13%), Brazil (9%), and India (3%) (Kakkar et al. 2017). A survey in India reported the Vancomycin-resistant strains of *Staphylococcus aureus* from cow and goat milk. Furthermore, isolates derived from poultry were examined and found resistive to Streptomycin (75%), erythromycin (57%) along with more than 40 % combinative resistance against Kanamycin, tobramycin, rifampicin, and ampicillin (Bhattacharyya et al. 2016). Extended-spectrum β-lactamase (ESBL) constituting pathogens instigate a worldwide expansion of resistance against highly competitive third-generation antibiotics like cephalosporins.

ESBL-producing *E. coli* strains have been isolated from livestock, poultry feces or remnants, and animal feed in developed countries of the world (Allen et al. 2014). This case was first reported in the US when salmonella and *E. coli* strains possessing CTX-M extended-spectrum lactamase ($bla_{CTX\text{-}M}$) were examined inside cattle in Ohio. These strains stimulated the inhibitory action against the antimicrobial activity of cephalosporin (Wittum et al. 2010).

Susceptibility and Resistance are directly associated with the Minimum Inhibitory Concentration (MIC). This minimum concentration of antibiotics is responsible for the inhibition of microbial growth. Bacterial susceptibility to the antibiotic or drug is correlated with the range of average MICs. Intrinsic resistance can be acquired with this MIC value in the resistive part of the range.

General Mechanisms of Resistance against Antimicrobial Drugs

In addition to the intrinsic resistance, gram-positive and gram-negative bacteria have acquired intrinsic resistance in several modifying and mechanistic pathways. In animals, antimicrobials have been practiced for medicinal and prophylaxis purposes. But, excessive incorporation of these antimicrobial systems has stimulated a competent group of microbes for inhibiting the effect of added antimicrobials and producing resistance against them. Many animals like pets, as well as domestic animals (chicken, dogs, cats, horses, cows, and pigs), can harbor these antibiotic-resistant bacteria and transmit them from animals to humans and vice versa through different Horizontal and Mobile genetic transfer of genes, plasmids, transposons, and Integrons, enhancing the dissemination of the Multidrug-Resistant Genes (MDR) to multiple class of antibiotic in one step or event (El Salabi et al.2013). The Horizontal Gene Transfer mechanism is accountable for the acquisition of foreign DNA and transmission of antimicrobial resistance (Briceno et al. 2011) and (Verraes et al. 2013). The mechanisms of antimicrobial resistance have been briefly summarized here. A detailed description of mechanisms has been delineated in this chapter.

Strategies for Antimicrobial Resistance

- Enzymatic modification.
- Efflux pumping and reduction in cell permeability.
- Ribosomal modification or Target modification.
- Ribosomal protection.
- Inhibition of Protein formation.
- Modification of metabolic pathways.

Mechanistic Resistance against Antibiotics Polymyxin

Polymyxins are produced naturally by gram-positive bacterium *Bacillus polymyxa* using non-ribosomal protein synthetase enzymes. These antibiotic drugs exhibit prodigious efficacy against a wide range of bacteria (gram-negative) through the electrostatic attractions between the cationic peptide chain of Polymyxins and negatively charged phosphate groups in the innermost anchoring lipid A component of the bacterial outer membrane (OM) which is formulated with lipopolysaccharides (LPS) chains.

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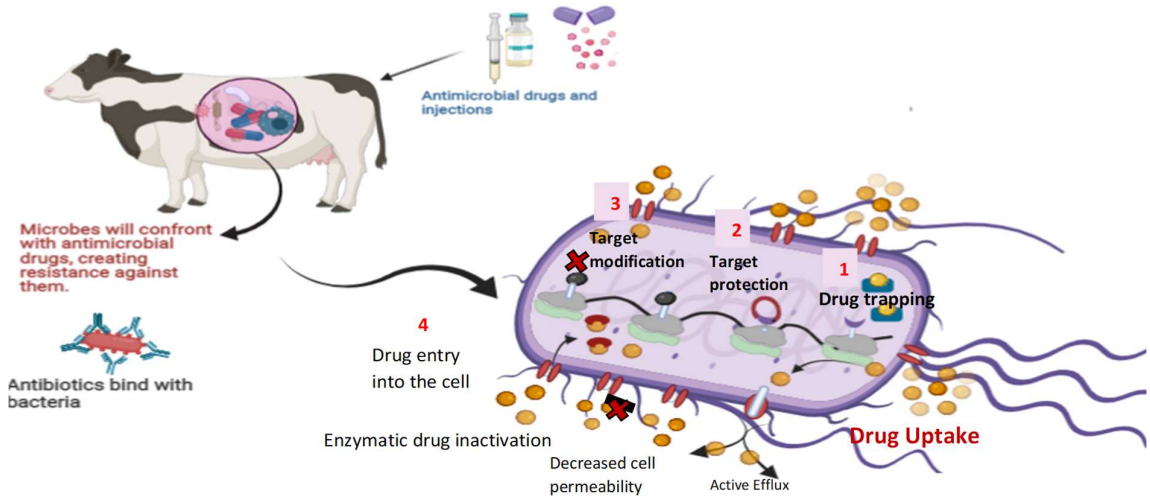

Fig. 1: With the continuous dosage and collation of antimicrobials and other injections, competent microbes inside the animals adapt several mechanisms to inhibit the functioning of antimicrobials. The drug enters the cell from protein channels mentioned as **1, 2, 3**, and **4**. The drug is trapped by another substrate to reduce the binding of antimicrobial with the target site. The targeted site is blocked or shielded to inhibit antimicrobial attachment. Target is modified or altered so antimicrobial will not recognize and bind with the specific site. The drug is enzymatically inactivated by phosphorylation. The drug is released out of the cell through active pumps (ABC). The permeability of the cell is declined to terminate the entry of antimicrobials.

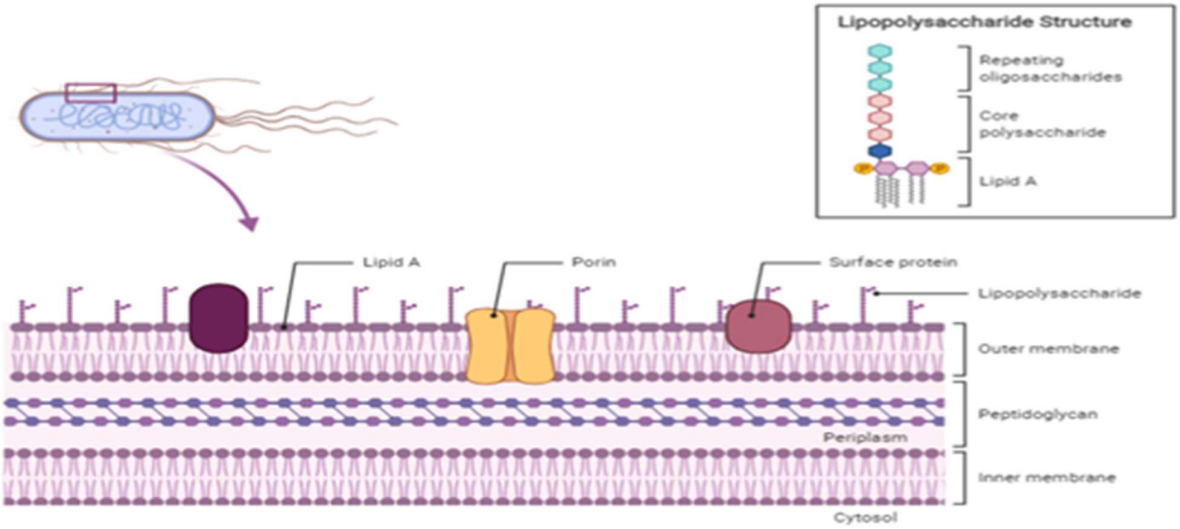

Fig. 2: (a). It stipulates the membrane structure of gram-negative bacteria along with the protein channels. (b) . It indicates the segmental division of lipopolysaccharide.

Table 1: Working modes of various classes of antibiotics have been mentioned below.

Mechanism of Antibiotic Action	Antibiotic Family
Disruption of bacterial outer membrane	Polymyxin, colistin, Lipopeptides, and Deptomycin
Inhibition of cell wall formulation	Penicillin, Cephalosporin, Monobactams, Carbapenems and Glycopeptides.
Inhibition of DNA synthesis	Deptomycin, Fluoroquinolones
Inhibition of protein synthesis	Aminoglycosides, Streptogramins, Deptomycin, Ketolides, Macrolides, Lincosamides, and Tetracyclines.
Inhibition of Folic acid synthesis	Trimethoprim and Sulfonamides.
Inhibition of RNA synthesis	Rifampin and other Metronidazoles.

(a). Operational Activity of Polymyxin and bacterial resistance against the drug: (b). Operational Activity of Macrolides accompanied by the Mechanism of Bacterial Resistance against it.

Operational Activity of Polymyxin

The asymmetric and semi-permeable outer membrane (OM) of gram-negative bacteria is responsible for the intake of essential entities like nutrients and the release of toxic moieties outside the cell. The bacterial OM is surrounded by a leaflet structure of lipopolysaccharide (LPS). The LPS is constituted by the three domains. The most distinct compartment is an anchoring hydrophobic chain of lipid A (endotoxin) which constitutes the tight packaging with fatty

acyl chains and serves as a membrane stabilizer, a central portion or core of oligosaccharide, and the outermost compartment of a distal polysaccharide (O-antigen) (Yu et al. 2015).

The bactericidal activity of polymyxin is conciliated by the electrostatic interaction with Lipid A, which anchors the drug in the bacterial membrane, disrupting the permeability of the cell. The negative charge of lipid A is due to the presence of phosphate ions (PO_4^{3-}) which are stabilized by the electrostatic interaction with divalent cations like calcium (Ca^{2+}) and magnesium (Mg^{2+}),γ-amines on the positively charged Dab residues (di-amino butyric acid) undergo protonation which creates the binding targeted sites of polymyxin drug with the Lipid A. These cationic peptide chains of polymyxin antibiotic exhibit greater affinity to the phosphate ions as compared to these divalent cations followed by the incorporation of N-terminal fatty acid chain D-Phe6-L-Leu7 (polymyxin B) or D-Leu6-L-Leu7 (polymyxin E) and can invent the curvature on the membrane surface, facilitating the formulation of destabilized areas on the outer membrane, accompanied by the penetration into the periplasm which leads to the increased Permeability of membrane, non-regulated release of divalent Ca^{2+} and Mg^{2+} ions, instability of cell integrity and eventually the death of bacterial cell (Falagas et al. 2010; Yu et al. 2015; Jian and Roger 2019).

In addition to this membrane lysis mechanism, an innovative survey has elicited that polymyxin can expeditiously instigate cell death via the aggregation of hydroxyl radical (•OH). In these bactericidal antibiotics, reactive oxygen species (ROS) like superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and •OH can enhance oxidative stress within the cell. It has been postulated that polymyxin can potentially induce superoxide O^{2-}, once they enter and cross the OM and then IM. These superoxides are transfigured to the H_2O_2 by superoxide dismutase enzymes. This newly synthesized H_2O_2 will be responsible for the oxidation of ferrous ion (Fe^{2+}) into ferric ion (Fe^{3+}) along with the biosynthesis of free hydroxyl radicals (•OH). This is characterized as the Fenton reaction. Excessively produced concentrations of these free radicals can stimulate the mutilation of DNA, lipids, proteins, and the ultimate cell death via oxidative stress (Yeom et al. 2010; Yu et al. 2015). This methodology has been operated in the species like *pseudomonas aeruginosa* and *Pseudomonas putida*. But many species like *Escherichia coli* have been reported to be resistant against these fatal effects of antibiotics by the cell-permeable chelators which hinder the Fenton reaction accompanied with the suppression of oxidative stress (Yeom et al. 2010).

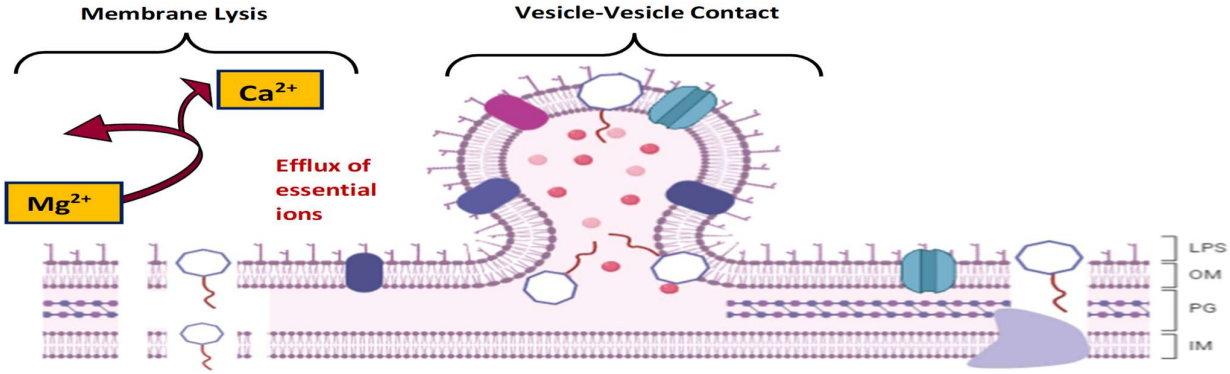

Fig. 3: The cationic Polymyxin targets lipid A, contriving a curvature at the outer membrane, then penetrating into the periplasm of the gram-negative bacteria, disturbing the permeability of the cell membrane, causing the efflux of Ca^{2+} and Mg^{2+} ions. (a). Polymyxin instigates the lysis of the membrane, devastating the integrity of the bacterial cell. (b). the drug also penetrates the inner and outer leaflets of phospholipids. This phenomenon is referred to as vesicle-vesicle contact, resulting in an osmotic imbalance and ultimate cell lysis.

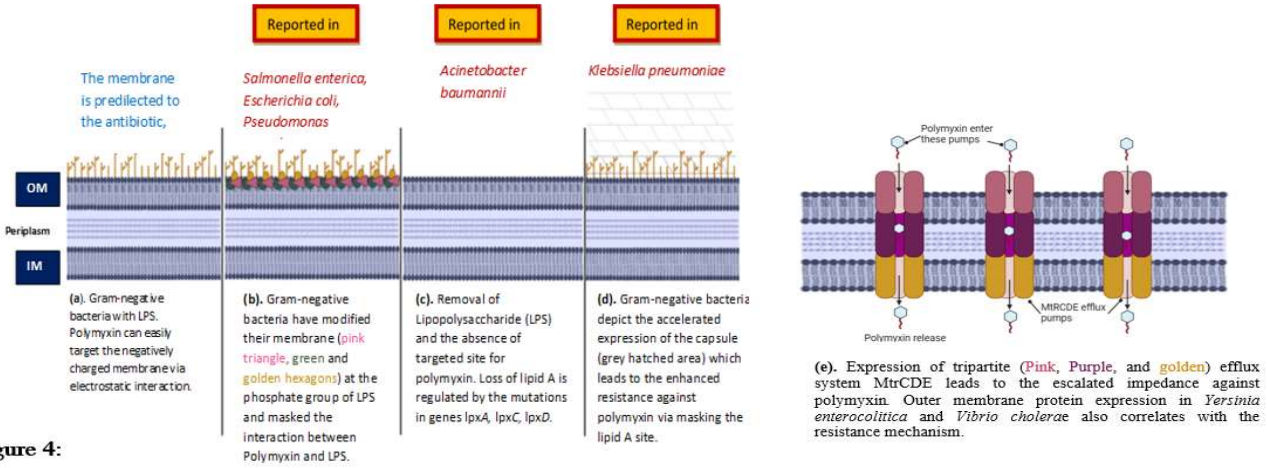

Figure 4:

Bacterial Resistance against the Polymyxin Drug

Various bacterial species like *Proteus spp, Serratia spp, and Burkholderia spp* have been found as intrinsically resistive to this drug. However, this resistance has been burgeoned after the development of acquired resistive mechanisms in *Klebsiella pneumoniae, Pseudomonas aeruginosa, Yersinia spp, Escherichia coli, Salmonella enterica, Vibrio cholerae, Yersinia enterocolitica*, Among the aforementioned categories, the highest observed mechanism is the modification of lipopolysaccharide at the phosphate group of lipid A by the addition of 4-amino-L-arabinose (L-Ara4N), phospho ethanolamine (PEtn), and galactosamine (GalN). These alterations mitigate the negative

Modification of LPS via 4-amino-L-Ara4N

The L-Ara4N, being the most pervasive modification, has been explicated in detail. The biosynthesis of L-Ara4N has been mediated by the genes of polymyxin resistance operon arn (formerly known as pmr). This operon constitutes of genes pmr HFIJKLM. The multi-steps involved in the biochemical formulation, conversion, and addition of L-Ara4N to the lipid A have been represented above in **Figure 5**.

The biochemical formulation and addition of L-Arabinose are acquired by the synchronized activity of enzymes PmrE, PmrH, PmrF, PmrI, PmrJ, PmrK, PmrL, and PmrM. This process proceeds in the cytoplasm with the conversion of UDP-glucose into UDP-glucuronic acid by PmrE/Ugd dehydrogenase enzyme which is accompanied by the oxidative decarboxylation via PmrI/ArnA and converts into and *Acinetobacter baumannii* (Olaitan et al. 2014). These bacterial species acquire mcr-1 genes which assist in resistive maneuver. The efficacy of polymyxin can be curtailed by adopting the following features:

I. Modification of LPS.
II. Loss or elimination of LPS and lipid A.
III. Plasmid Mediated Efflux Response.
IV. Capsule Expression.

UDP-4-keto pyranose. The enzymatic activity of PmrH/ArnB then converts UDP-4-keto pyranose into UDP-beta-L-arabinose which is further formylated by PmrI/ArnA to synthesize UDP-beta-L-Ara4FN to the Undecaprenyl phosphate carrier (Undp) which is present in the inner membrane of the bacterial cell where ultimate deformylation is carried out by the activity of PmrJ/ArnD and the formerly synthesized compound is flipped across the inner membrane into the periplasm by the consolidated activity of PmrL/ArnE with PmrM/ArnF charge characterized by the phosphate groups which leads to the reduction of anionic domains or drugs-binding sites. All these events exotically dwindle the affinity between the bacterial outer membrane and polymyxin (Nang et al. 2021).

PmrJ/ArnD and the formerly synthesized compound is flipped across the inner membrane into the periplasm by the consolidated activity of PmrL/ArnE with PmrM/ArnF. Eventually, L-ara4N is transferred to lipid A by the biochemical activity of the glycosyl transferase enzyme. **Figure 5.** This modification of lipid A by L-arabinose mitigates the predisposition of drug-binding sites and contributes to bacterial survival. Biosynthesis of the addition

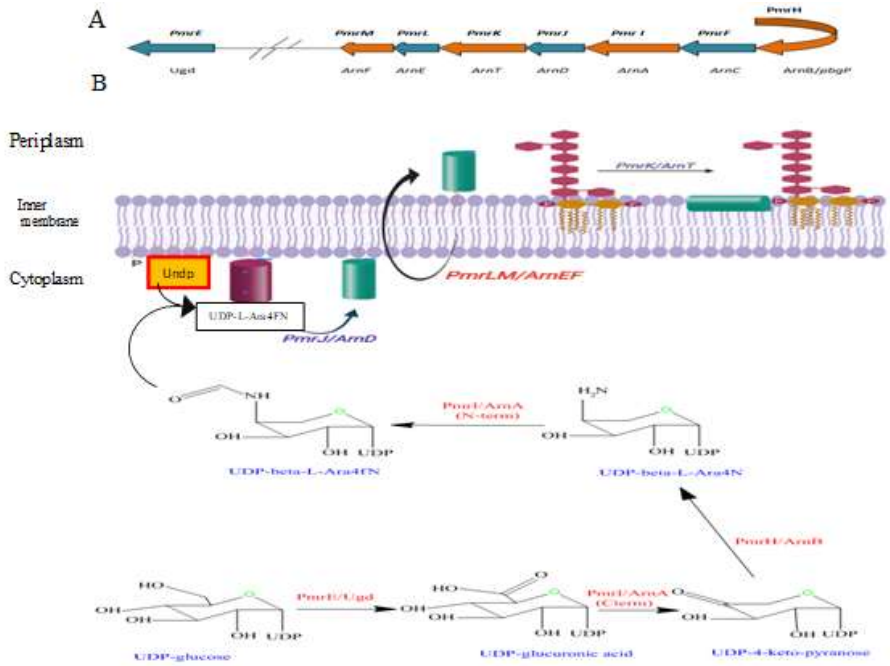

Figure. 5: This pictorial diagram predicts the conversion, addition, and synthesis of L-Ara4N on the lipid A membrane. Each step is initiated in the presence of a specific catalyst. The mechanistic pathway is triggered with the conversion of UDP-glucose into UDP-gluconic acid by the catalytic activity of PmrE/Ugd enzymes. The UDP-gluconic acid undergoes the multistep conversions and is transformed into UDP-4-Keto-pyranose via PmrI/ArnA dehydrogenase in the process known as oxidative decarboxylation which is then formylated into UDP-beta-L-arabinose and UDP-beta-L-Ara4FN through the enhanced catalytic activities of enzymes. ThePmrH/ArnB and PmrI/ArnA respectively. This newly synthesized product is transferred to the Undecaprenyl phosphate (UnDP) carrier through PmrF/ArnC. After deformylation of UDPL-Ara4FN (purple cylinder) via PmrJ/ArnD, it is flipped across the inner membrane by the synergistic activity of PmrL/ArnE and PmrM/ArnF. The L-Ara4N segment (zinc cylinder) of this molecule is translocated to the lipid A of Lipopolysaccharide by the catalytic activity of PmrK/ArnT: **UDP** = Uridine diphosphate: **Undp** = Undecaprenyl phosphate.

of L-Ara4N varies in terms of gene expressions among different species (Jian and Roger 2019). In addition to this enzymatically controlled phenomenon, another two-component regulatory system PhoP/PhoQ also contributes to the modification of lipid A in many bacterial species like *p.aeruginosa*. This regulating system is mediated in the presence of high iron concentration (Fe^{3+} ions and reduced pH) and low magnesium concentration (Mg^{2+}) or calcium (Ca^{2+}) ions (Falagas et al. 2010).

Resistance through the Expression of Capsule

Capsulated species of gram-negative bacteria were observed to depict more resistance against polymyxin drugs. While a capsular mutants were found to be more predilcated towards the polymyxin and its attachment at the bacterial surface when juxtaposed with the capsulated bacterial mutants (Jian and Roger 2019). Thus, the capsular expression has incremented the resistivity against the targeted drug in *Klebsiellapneumoniae*. This mechanism has been established on the principle of electrostatic interference between the anionic capsule and the polymyxin drug. These bacterial species can easily shed their anionic or negatively charged capsular polysaccharides (CPs) and cohere with the positive peptide chains of polymyxin (Olaitan et al. 2014). This crucial step neutralizes the positive charge of polymyxin drug, and it abates the susceptibility of the Lipopolysaccharide layer to bind with the drug via electrostatic correspondence (Yu et al. 2015).

Multidrug Efflux System (MDES)

Multidrug resistance has been accomplished by the mediated expression of efflux pumps in gram-positive and gram-negative pathogens colonizing within the animals. MexAB-OprM, AcrAB, and NorM have progressively contributed to the mechanism of bacterial resistance. These pumping systems transport polymyxin in and out of the cell. These pumping systems have been operated inside the bacterial species mentioned below.

Loss of Lipopolysaccharide (LPS)

A few bacterial species have adapted the complete elimination of the lipopolysaccharide layer which has delineated the escalated degree of antibacterial resistance. But this strategy also exerts a negative impact on the fitness of bacterial cells because the lipopolysaccharide chain is the utmost requirement for the stabilization of the outer membrane of bacteria. Only the *Acinetobacter baumannii* strains have been reported to exhibit this unusual mode of impedance. Analysis of strains in the mouse infection model, the loss of lipid A and the alteration of lipid A has curtailed the competitive index of *A. baumannii* to 0.09 and 0.35 sequentially (Johnson et al. 2017).

Macrolides antibiotics have been extracted from *Streptomyces spp.* through the synergistic activity of polyketide synthase. Chemical representation of macrolides manifests them as 12-18 membered lactone rings attached with 1 to 3 various hexose components like desosamine.

Macrolides, Lincosamides and Streptogramins (MLS)

Macrolides, lincosamides, and streptogramins exhibit the same mode of spectral activity. In the early era, macrolides and related drugs were manipulated for the medication of respiratory tract and infection in skin tissues but further next-generation advancement expanded the usage of macrolides (Zhanel et al. 2001). This activity has been extended to the intracellular bacteria or other pathogens like (*Chlamydia trachomatis, Treponema pallidum, Mycoplasma pneumoniae,* and *Rickettsia*) and gram-positive bacteria (*staphylococcus aureus* and *Streptococcus pneumoniae, Streptococcus pyogenes,* and *bacilli* species). Gram-negative bacteria are intrinsically resistant to these MLS antibiotics except *Helicobacter, Campylobacter, Legionella, Bordetella pertussis, Chlamydia species,*

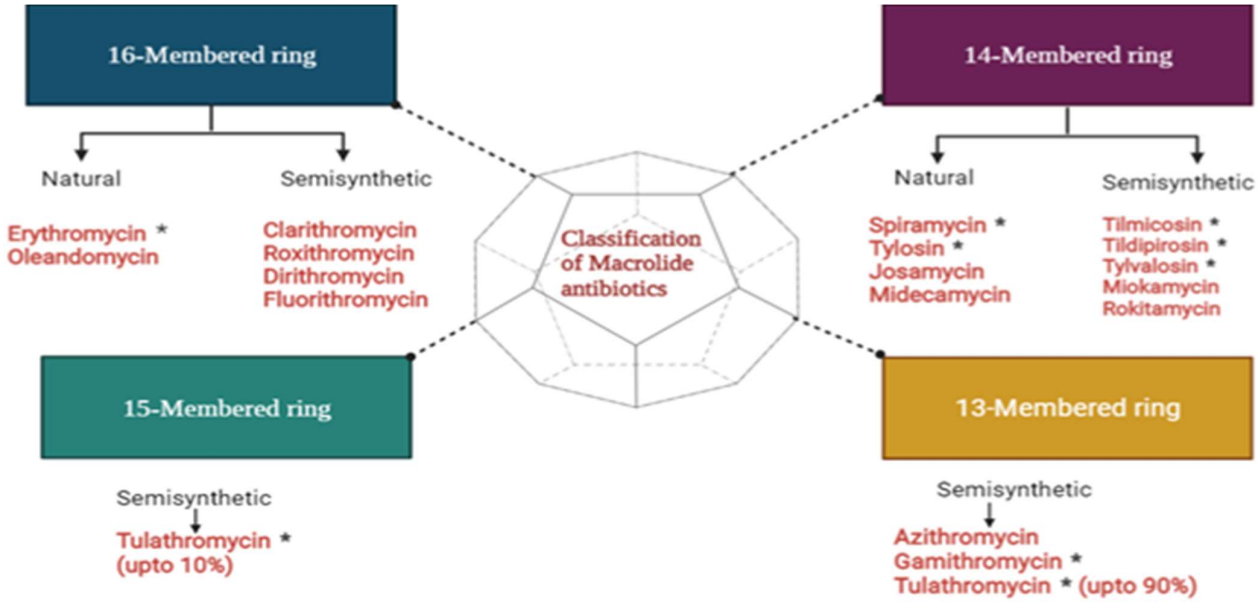

Fig. 6: Divisional model of Macrolides according to the different membered lactone –rings. Antibiotics mentioned with (*) sign are authorized for veterinary practices.

Table 2: Various Efflux systems have been mentioned in different Antibiotic classes.

Multidrug Efflux system	Bacterial species with the corresponding pumping system
MexAB-OprM	*Pseudomonas aeruginosa*
AcrAB	*K. pneumoniae, E. coli*
NorM	*Burkholderia veitnamien*
MtrCDE	*Neisseria meningitidis*

(Yu et al. 2015; Jian and Roger 2019).

Table 3: Administrative quantities, inhibitory activities, and injective time periods of various classes of antibiotics have been assayed in dogs, cats, ruminants (cattle, sheep, goat, and buffaloes), horses and swine (Giguère and Prescott 2013).

Species	Antibiotics	Dosages (mg/kg)	Defensive activity against the Selected bacterial species.	Intervals (hours)
Dogs and cats	Tylosin	10-20 (dogs), 5-10 (cats)	Heals abscesses, wound infections, tonsillitis, trachea bronchitis, and pneumonia caused by pathogens like *staphylococci, streptococci*, anaerobes, and *Mycoplasma*.	12
	Clarithromycin	5-10	Cures gastric ulcers associated with *Helicobacter* spp. in dogs	12
	Azithromycin	5(cats), 10(dogs)	Eliminates *Babesia gibsoni* from continuously infected dogs, effective against *Chlamydophila felis* infections in cats,	24
Ruminants	Tylosin	4-10	Treats pneumonia associated with *Mycoplasma bovis* and otitis media and intern in calves, foot rot, metritis, and mastitis caused by Gram-positive cocci.	24
	Erythromycin	1.1-2.2	Exhibits inhibitory activity against *H. somni, A. pyogenes*, and anaerobic bacteria	24
	Gamithromycin	6	Effective against bovine respiratory disease associated with *M. haemolytica*, *P. multocida*, *Mycoplasma bovis*, or *H. somni* in beef and non-lactating dairy cattle.	Single dose
Horses	Erythromycin	25	Cures infections caused by *Lawsonia intracellularis, Rhodococcus equi* pneumonia in foals, staphylococcal and streptococcal infections in horses.	6-8
	Clarithromycin	7.5	Assists in treatment of *Rhodococcus equi* infections in foals	12
	Azithromycin	10	Assists in treatment of *Rhodococcus equi* infections in foals.	24- 48
Swine	Tylosin	9	Inactivates *Pasteurella multocida,* reduced the frequency of *M. hyopneumoniae* lesions, *P. multocida* and *A. pyogenes* pneumonia	12-24
	Tildipirosin	4	Aids in the treatment of respiratory disease associated with *A. pleuropneumoniae*, *P. multocida*, *Bordetella bronchiseptica*, and *H. Parasuis*.	Single dose
	Tulathromycin	2.5	Active against swine respiratory disease caused by *A. pleuropneumoniae*, *P. multocida*, *B. bronchiseptica*, *H. parasuis*, or *Mycoplasma hyopneumoniae*.	Single dose

and *Haemophilus influenza* (Leclercq et al. 2002). Macrolides are characterized by a lactone ring. Erythromycin-derived macrolides constitute 15-membered clarithromycin, roxithromycin, azithromycin and dirithromycin. The pharmacokinetic (PK) profile has been enhanced due to the structural modification of erythromycin but cross-resistance has been observed in these antibiotics. Therefore, better spectral activity has been expressed by 16- membered macrolides (Spiramycin, Midecamycin, Josamycin, and Miocamycin) and veterinary medicine (Tylosin) (Felmingham et al. 1991).

Manipulation of MLS antibiotics in Animals

For veterinary purposes, various macrolides against bacterial infections and syndromes have been administered in specific concentrations and intervals of time. These regulations have been elucidated in the Table-2.

Operational Activity of Macrolides

This intrigued class of antibiotics exerts its antibacterial effect in bacterial strains via inhibiting protein synthesis and ribosomal inhibition of the microbes. Macrolide binds to23rRNA of 50S bacterial ribosome only at a related site (v-domain) to avoid the interferences of other antibiotics like chloramphenicol on this site. This binding was further declared when the binding of macrolide Erythromycin was acknowledged via the Fragment reaction studies and dimethyl sulfate and kethozol probing (Moazed et al.1987). Macrolides annihilate bacterial growth by targeting ribosomes and inhibiting the phenomenon of protein synthesis. After incorporating into the bacterial cell, this antibiotic increments the affinity and coheres with the peptide exit tunnel of the larger ribosomes which is directly adjoined with the peptidyl transferase. Thereby, this devastating antibiotic obstructs the exit channel of protein and prevents the elongation of the polypeptide chain. Depending upon the dual antibacterial nature (bacteriostatic and bactericidal), macrolides can terminate the protein formulation and thus the growth of bacterial cells (Svetlov et al. 2017). Other antibiotics like Lincosamide as well as streptogramins also exhibit the analogous mode of attack on the bacterial surface (Matzov et al. 2017). The hydrophobic and hydrophilic-faced lactone rings bind to the ribosomal subunit with their hydrophobic end accompanied by the specific hydrogen bondings of desosamine or mycaminose entities with nucleotides A2058 and A2059 of the host cell, thus, escalating the interaction of macrolides with the bacterial ribosomes (Hansen et al. 2002). Another 18-membered ring macrolide has the potency to interfere with the protein synthesis via inhibiting RNA polymerase rather than binding to the ribosomal subunit (Artsimovitch et al. 2012).

Mechanism of Resistance against Macrolides

Miscellaneous mechanistic pathways have been operated by the bacteria to antagonize the effect of the antibacterial drug. Bacteria can

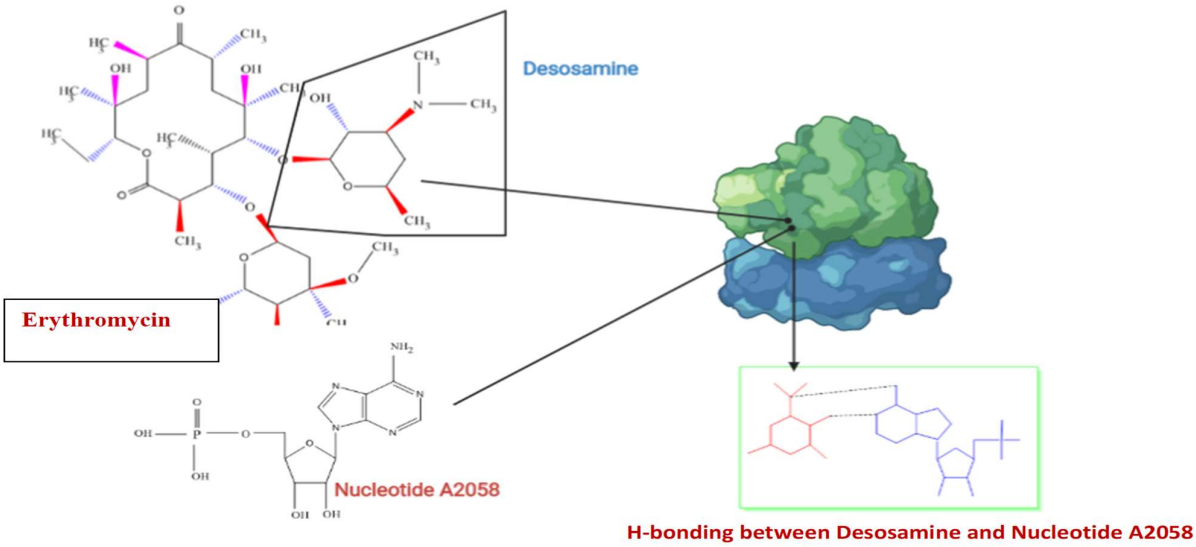

Fig. 7: (Macrolide) Erythromycin interacts with 23S subunit of RNA nucleotide A2058. After internalization of macrolide with the binding site on the 50S subunit, a Hydrogen bond is formulated between the 2-OH group of desosamine and Nitrogen (N_1) in A2058.

Table 4: Efflux pumping system marked with (*) and subfamilies provide substantial resistance against macrolides (Gomes et al. 2016).

Efflux pumping system	Symbols/ Ideogram	Bacterial strains
Major facilitator Superfamily	MFS* subfamily Mef	Mef(A) in *Streptococcus (pneumonia, pyogenes, agalactiae)*
ATP-binding cassette	ABC* subfamily Msr	Msr(A) in *Staphylococcus xylosus* and *S. epidermidis, S.aureus*
Multidrug and toxic compound extrusion	MATE	
Resistance nodulation division	RND	
Small multidrug resistance	SMR	

I. Diminish the Concentration of Macrolides inside the cell.
II. Modify or amend the target (Ribosome).
III. Modify the Macrolides.
IV. Shielding or Protecting the Ribosome.

Diminish the Intracellular Concentration of Macrolides

Various bacterial species are competent enough to elude the working of macrolide via decreasing their concentration inside the cells. This mechanism is acquired through the optimized activity of efflux pumps encoded on the plasmid or chromosomes (Ambrose et al. 2005).
Multicomponent Mef efflux system belongs to the MFS family and transports the antibiotic to the exterior of the cell via the secondary active transport rather than the direct utilization of ATP. Mef (A) and Mef (B) are the effectively operated determinants against macrolide. Mef (A) is resistive against macrolides, with minimum inhibitory concentration MIC of erythromycin, clarithromycin, and azithromycin against the bacterial strains as mentioned in **Table: 4**. On the contrary, Msr related to the ABC family utilize ATP as an energy source for active transport. It encrypts a protein with the 2-ATP binding compartment of ABC transporters. Both aforementioned efflux systems proved to be efficient against14 and 15- membered ring macrolides like erythromycin and ketolides (telithromycin). Streptogramins and clindamycin are not resistive to bacterial strains as they don't induce Msr (A) genes (Leclercq et al. 2002; Li et al.2009).

Modification of Ribosome

Various categories of alteration at the ribosome also contribute to macrolide resistance. These resistive modifications include methylation of ribosomal subunits, mutations at ribosomal RNA and proteins. The Emr family of enzyme methyltransferase involves the prepotent methylation of 23S subunit of rRNA at the N6 position of nucleotide A2058,(see figure-7) which formulates unique chemical bonding with the saccharides entities (hexoses) at the C5 position of macrolactone rings of the drug, accompanied by the hydrogen bonding in the drug structure (Poehlsgaard et al. 2005). Monomethylation demonstrates a low resistive activity in contrast to dimethylation. The phenomenon of dimethylation encounters the prodigious resistance to all MLS_b antibiotic drugs (see figure-8) against *Mycobacterium avium, Helicobacter pylori, Treponema pallidum, Propionibacterium,* and *Streptococcus pneumoniae* (exhibiting 4rrn genes). Ketolides (telithromycin) have been reported to be resistant against gram-negative and gram-positive bacterial strains.

Modification of Macrolides

In this resistive pathway, enzyme-catalyzed modification of antibiotics is adapted to mitigate the level of interaction and binding of antibiotics to the target site (50S ribosomal subunit) inside the bacterial cell. This intrigued class of bacterial enzymes has been diversified into various subclasses.i.e. Macrolide phosphotransferases (MPHs),

Macrolide esterases (Eres), Macrolide glycosyltransferases. MPH(A) and (B) genes induced phosphorylation against 14, 15 and 14, 16 membered ring macrolides respectively(Fyfe et al. 2016).This enzymatic alteration was observed from the clinical analysis of Escherichia coli strains which inhibited the action of Oleandomycin in 1988. Later, it was confirmed that an enzyme in those was responsible for the phosphorylation of the hydroxyl group at the 2nd position of C5 linked desosamine entity of Oleandomycin. Due to the phosphorylation reaction, the enzyme was named "Macrolide 2'-phosphotransferase". Later, this mechanism was also declared in another macrolide Erythromycin. In the 1980s, a substantial level of resistance was observed in Erythromycin but not in Lincosamide and Streptogramins. It was due to another enzyme known as Esterase (Ere) (Barthelemy et al. 1984). A detailed mechanism of this reaction has not been explicated in this chapter.

Glycosyltransferase enzyme has been reported for the "Antibiotic Resistance and Immunity" in the Host cell (Fyfe et al. 2016). For an instance, *Streptomyces spp*, from which macrolide originated, operate this resistive mechanism for instigating the Self-resistance against the antimicrobial generated by itself (Bolam et al. 2007).

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Antifungal Drugs

Fungal cell membranes have distinctive ergosterol, sterol, which replaces cholesterol present in mammalian cell membranes. An antifungal agent is a medicinal elixir that selectively exterminates fungal pathogens from a host with minimum toxicity to the host.

Three commonly used classes of antifungal drugs are:
1. Flucytosine (5-FC).
2. Ergosterol Synthesis Inhibitors.
3. Polyenes.

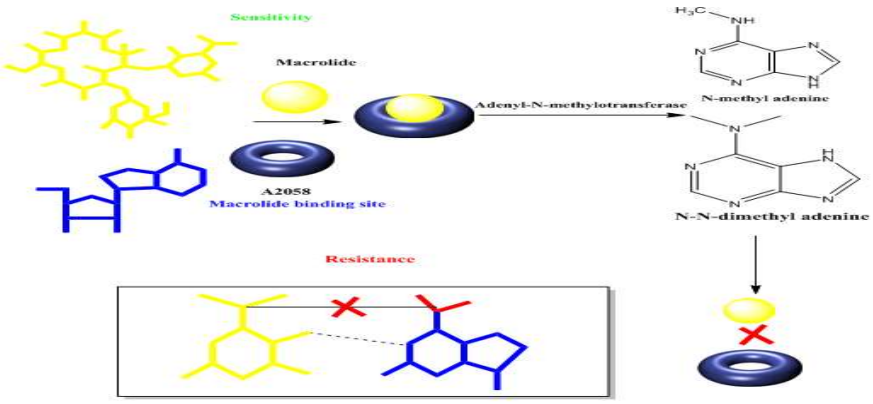


Fig. 8: It explicates the modification of antibiotic sites and stimulates resistance to Macrolide, Lincosamide, and streptogramins (MLS). After the action of Adenyl-N-methyltransferase enzymes, adenine is methylated and reduces the binding of erythromycin to the 50S targeted site of the ribosome.

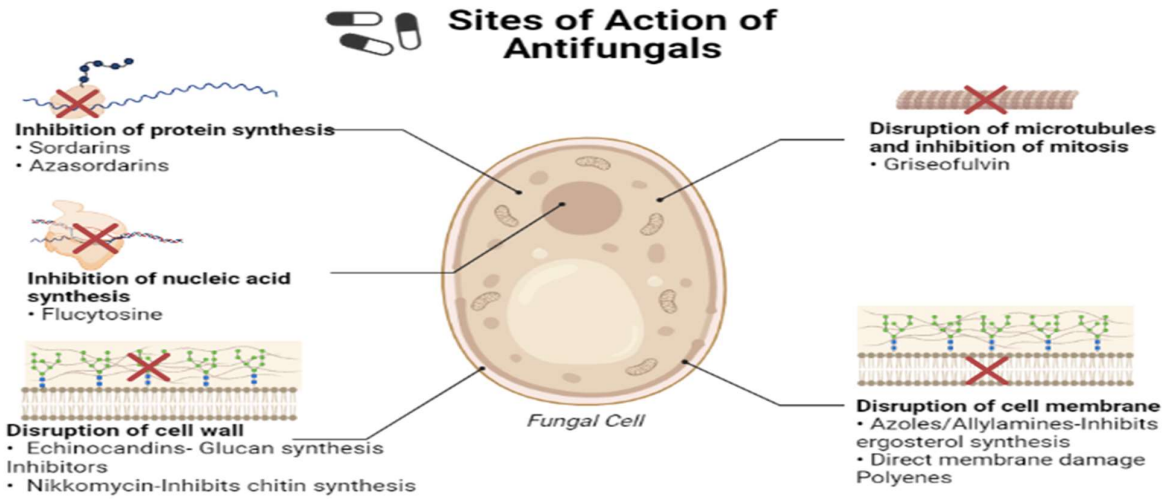
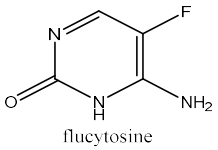


Fig. 9: Simplified and Schematic presentation of the mode of action of Antifungal Drugs.

Flucytosine

Flucytosine (5-FC) is an anti-metabolite initially synthesized in 1957 while looking for new antineoplastic agents. Although it lacked anticancer capabilities, it quickly became clear that it was a powerful antifungal agent. Flucytosine is a pyrimidine analogue that is water-soluble and related to the chemotherapy drug 5-fluorouracil (5-FU). It has a substantially restricted range of action than amphotericin B. Ascomycete and basidiomycete yeasts, as well as several hyaline and melanized filamentous fungi, are all susceptible to 5-FC (Vermes et al2000). The drug is especially crucial for treating fungal infections in body regions where other antifungal drugs have limited penetration, such as inflammatory infections of the urinary system, brain and eyes, or heart valves (Chandra et al. 2017).



flucytosine

Mode of Action of 5-Flucytosine

Flucytosine (5-FC) has a unique method of action among antifungal drugs in that it targets DNA, RNA, and protein synthesis. 5-FC is a drug precursor, which means that it must be metabolized through the pyrimidine salvage route to be activated, where it serves as a disruptive substrate, producing poisonous nucleotides and disrupting DNA and protein synthesis. Among antifungal agents, the mediated activity of 5-Flucytosine is unique, as it targets protein, DNA, and RNA synthesis.

Activation of Flucytosine is carried out by metabolization through the pyrimidine salvage route, where it functions as a disruptive substrate, producing poisonous nucleotides and disrupting the formation of DNA and protein (Figure 9). Membrane permeases actively transfer them into the fungal cell (cytosine permease encoded by the gene (FCY2) and other encoded by FCY21 and FCY22), flucytosine (5-FC) is transformed via Fluorouracil (5-FU) to 5-Fluorourdine monophosphate (5-FUMP) under the action of cytosine deaminase enzymes, encoded by the FCY1 gene and Uracil Phosphoribosyl-transferase (UPRT) is encoded by the gene FUR1, respectively. Two specific kinases phosphorylate the 5-FUMP, resulting in 5-fluoro-UTP, which is integrated into the RNA.

FUMP, resulting in 5-fluoro-UTP, which is integrated into the RNA. 5-FUMP is also converted to 5-fluoro-2′-deoxyuridylate, which suppresses the activity of thymidylate synthase and consequently DNA synthesis by reducing the nucleotide pool available. The cytosine deaminase enzyme is absent in mammalian cells. As a result, 5-FC is not transformed to 5-FU, and these cells are not immediately exposed to 5-FC's harmful effects (Lestrade et al. 2019).

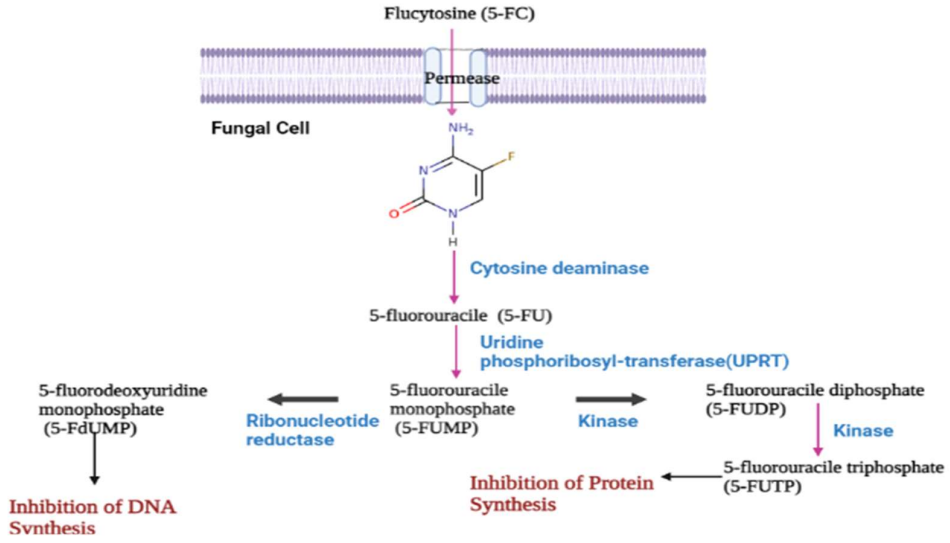


Fig. 10: Schematic modeling of Flucytosine (5-FC) activity. The enzyme 1: cytosine permease transports (5-FC) into the cell. The enzyme 2: cytosine deaminase deaminates 5-FC to 5 fluorouracil (5-FU). The enzyme 3: uridine monophosphate pyrophosphorylase converts 5FU to 5-fluorouridine monophosphate (FUMP), then to 5-fluorouridine diphosphate (FUDP), and finally to 5-fluorouridine triphosphate (FUTP). FUTP is integrated into RNA, causing tRNA amino acylation to change and protein synthesis to be inhibited. Furthermore, the enzyme 3: uridine monophosphate pyrophosphorylase converts 5FU to 5-fluorodeoxyuridine monophosphate (FdUMP). FdUMP prevents DNA production by preventing thymidylate from being incorporated into DNA.

Table 5: Different antibiotic Modification systems have been mentioned in different bacterial strains.

Modification system	Bacterial strain	Antibiotic System
EreA2	*Vibrio cholerae, Pseudomonas spp, Salmonella Indiana, Klebsiella pneumoniae, E. coli, Klebsiella oxytoca*	
EreC	*Klebsiella pneumoniae*	Active against Erythromycin
EreD	duck pathogen *Riemerella anatipestifer*	

Resistance Mechanism to Flucytosine

Flucytosine is analogous to pyrimidine that prevents the synthesis of DNA and RNA in cells. *Candida spp., Candida neoformans,* and certain molds are all susceptible to this chemical. Primary flucytosine resistance is still uncommon in *Candida* and *Cryptococcus* isolates (less than 2%). However, secondary resistance to flucytosine can develop in these yeasts, necessitating its use only in conjunction with other antifungal drugs, primarily amphotericin B or fluconazole (Hospenthal et al.1998).

Flucytosine resistance is stimulated by mutations in the purine-cytosine permease enzyme (encoded by the FCy2 gene), which is accountable for the drug's absorption into the cell. Alterations in the cytosine deaminase enzyme (encoded by the FCy1 gene) leads to the conversion of 5-fluorouracil to 5-fluorouridine monophosphate. Resistance to this antibiotic is triggered by changes in the uracil phosphoribosyl-transferase enzyme (encoded by the FUR1 gene), which converts 5-fluorouracil to 5-fluorouridine monophosphate. The majority of these pathways have been connected to resistance in *Candida albicans* resistance (Espinel-Ingroff2008).On the other hand inactivation of the *Candidalusitaniate* FCy2 gene, led to flucytosine cross-resistance.

Laboratory mutants of *Candida glabrata* were produced by exposing a wild-type isolate to 5-FC in separate research. Two of these mutants were selected for further study of the molecular underpinnings of 5-fluorouracil (5-FU) resistance based on their susceptibility to the drug. A missense mutation in the gene coding for cytosine deaminase enzyme accompanied with the reduction in the expression of the gene coding for uridine monophosphate pyrophosphorylase was found in one mutant that was resistant to both drugs. The other mutant with lower susceptibility to 5-FC and 5-FU had overexpression of the thymidylate synthase and cytosine permease, which was linked to a missense mutation in the last gene. This investigation revealed that, in addition to mutations in the FUR1 gene, which are the generally observed cause of resistance to 5-FC, alternative pathways exist in *C. glabrata* (Vandeputte et al. 2011).

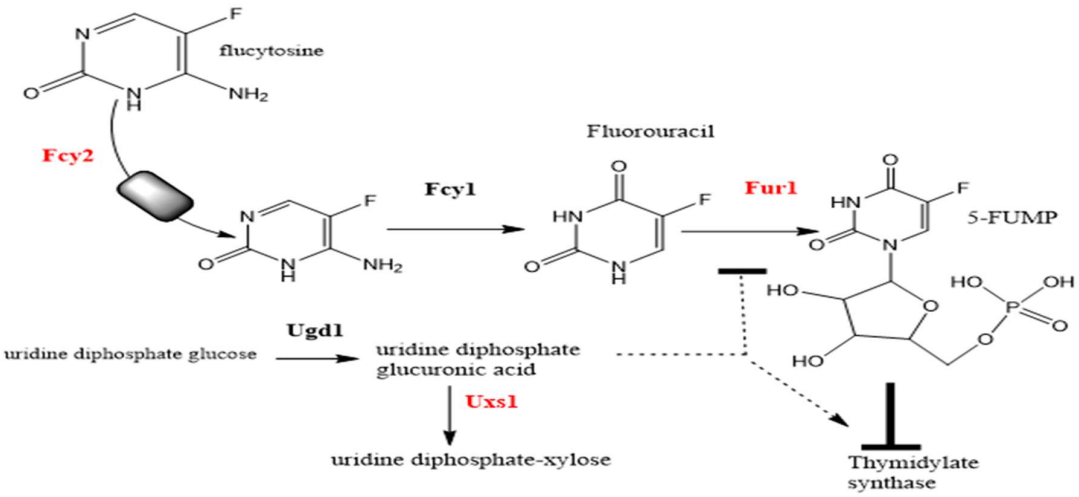


Fig. 11: Inhibition model of 5-flucytosine/5-fluorouracil toxicity by uxs1 mutation: uxs1 mutations can initiate resistance to both 5FC and 5FU through a variety of pathways. A mutation in uxs1 results in a buildup of UDP-glucuronic acid, a product of Ugd1, which either prevents the synthesis of hazardous fluoride compounds or restores inhibition of their targets, such as thymidylate synthase. For those proteins where mutations were discovered in this study, the names are highlighted in red.

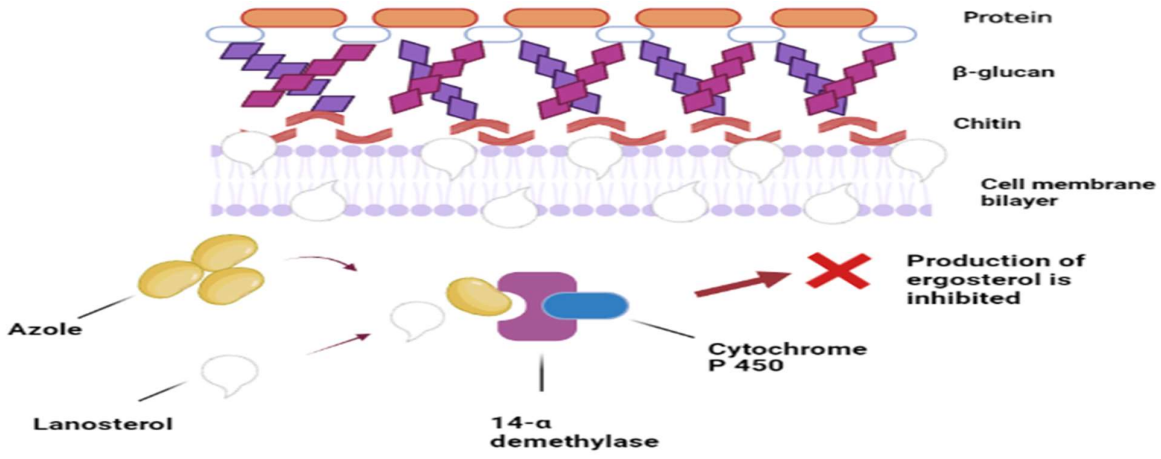
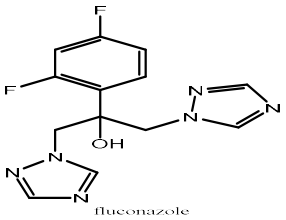


Fig. 12: The mechanism of action of antifungal drugs from the azole group. Inhibition of the production of ergosterol.



Azoles

Azole antifungals prevent the conversion of lanosterol to ergosterol, a key component of the fungal cell membrane. To treat chronic mycoses, they can be taken orally. The first azole based oral drug, ketoconazole has succeeded more effective and less toxic derivatives voriconazole, posaconazole, isavucazole and itraconazole (Campoy et al.2017).

Mode of Action

The azoles work by inhibiting 14α-lanosterol demethylase, which converts lanosterol to ergosterol in the cell membrane and is encoded by the ERG11 gene (Figure 3). The active site of this enzyme comprises an iron protoporphyrin unit. The ergosterol biosynthetic route is blocked when azoles bind to iron (Sanglard et al. 2015; Shukla et al. 2016). The accumulation of 14α-methyl sterols occurs when ergosterol synthesis is interrupted, altering the action of the enzymes, membrane stability, and permeability linked to it (Cowen et al. 2015).

Resistance Mechanism to Azole

Resistance to azole antifungal drugs has been explored in *Candida spp.* species and can be divided into categories:

I. Imports of drugs are being reduced.
II. Changes in drug processing within the cell.
III. Changes in the target enzyme.
IV. Changes in the ergosterol biosynthesis pathway's other enzymes.
V. Modifications in Efflux pumps.

I. Reduction of Drug Import

The first line of defense against drug resistance is a defect in drug import. To prevent medications from entering the cells, cells might change the composition of their membrane. *C. albicans* resistance is influenced by changes in the sterol and phospholipid components of the cytoplasmic membrane.

II. Changes in Drug Processing within the Cell

For the veteran-medically important fungi, little research on drug alteration or degradation within a resistant cell has been done. Azoles are not metabolized by *Candida albicans*.
In *Streptococcus cerevisiae*, plasmid complementation of the ERG3 mutation implies that the ERG3 mutation can generate azole resistance on its own.

Modifications in Efflux Pumps

Drug resistance is known to be caused by two types of efflux pumps found in eukaryotic cells:
(I) Adenosine triphosphate (ATP)-ABCT (binding cassette transporters) family.
(II) Major facilitator family (MF).

III. Changes in the Target Enzyme

Drug resistance has a common mechanism. It is the ergosterol biosynthesis pathway for azole-based antifungal medicines. ERG1, ERG2, ERG3, ERG4, ERG5, ERG6, ERG7, ERG11/ERG16, ERG24, ERG25, ERGX, and ERGY are the genes involved in the ergosterol biosynthesis pathway (Demuyser et al.2019). In *C. albicans*, ERG1 to ERG12, ERG20, ERG24, and ERG25 have been discovered. The ERG11 gene produces lanosterol demethylase, because of a point mutation in ERG11 caused by the substitution of a lysine for arginine at amino acid.

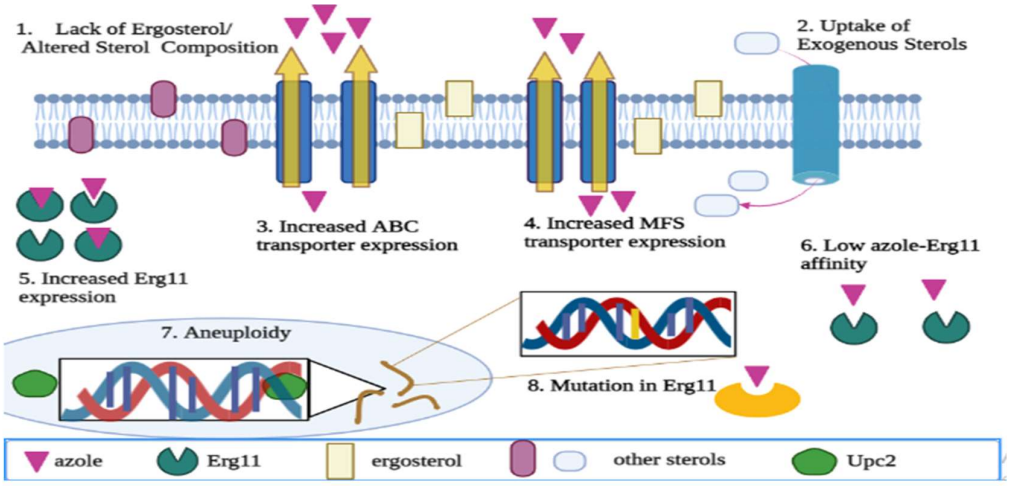


Fig. 13: Mechanism of azole resistance in Candida species. (**1**) Production of alternative sterols due to Erg3 inactivation. (**2**) Uptake of exogenous sterols. (**3**) Increased production of efflux pumps of type ATP-binding cassette and (**4**) major facilitator family reduces intracellular levels of azoles. (**5**) Increased expression of Erg11 can overcome the activity of azole drugs. (**6**) Low affinity of azole binding to Erg11 may reduce the azole's potential to inhibit the proton. (**7**) Genetic adaptability to azole exposure may be aided by aneuploidy. (**8**) Erg11 mutations can cause proteins to have a lower affinity for fluconazole binding.

IV. Changes in the Ergosterol Biosynthesis Pathway's other Enzymes

Another resistive mechanistic pathway includes the alteration in the targeted enzymes and other supporting enzymes present in the biochemical pathway. Modifications in the ergosterol pathway are potent to cause resistance not only to the drug (to which the cells are exposed) but also moreover the related drugs.

Prevention and Control of Antifungal Resistance

Antifungal resistance prevention and suppression strategies have yet to be developed; however, the following antibacterial resistance prevention and suppression strategies should be implemented.

- Cautious usage of antifungal drugs.
- Right dose, with a focus on avoiding a low antifungal dosage.
- Treatment with existing drug combinations.
- Use surveillance studies to figure out how common antifungal medication resistance is.

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CHAPTER 04

DRUG RESISTANCE OF GRAM-NEGATIVE PATHOGENS IN LARGE ANIMALS WITH SPECIAL REFERENCE TO PAKISTAN

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INTRODUCTION

Non-hazardous bacteria are useful microbes for animals and humans, which help in disease prevention and food digestion (Stark 2010). However, pathogenic bacteria are important disease-causing entities. Of these, endotoxin producing bacteria (gram negative bacteria) are important pathogens in large animals, causing systemic infections. The importance of these pathogens has been augmented after the emergence of antimicrobial resistance, which even can be transmitted between humans and animals (Idrees et al. 2011). Gram negative bacteria can cause intestinal and extra-intestinal infections in animals. Treatment of infections caused by resistant pathogens is costly because treating individuals with alternative drugs is always more expensive than conventional treatment (Burroughs et al. 2003). In addition, no new antibiotics have been developed specifically for multi-drug resistant gram-negative bacteria (Giske et al. 2008). Therefore, the clinical and economic impact of resistant gram negative bacteria is greatly worrisome. Knowing the sources and level of antimicrobial resistance in gram negative bacteria are of dire need.

Gram Negative Bacteria

Bacteria, a well-known type of micro-organisms, are differentiated traditionally based on the retention of crystal violet stain in a Gram staining method. Gram negative bacteria, owing to lower quantity of peptidoglycans in the cell wall, do not retain the crystal violet and appear pink/red under microscope. This property of bacteria is associated with the amount of peptidoglycans in their cell wall.

Antibiotic Resistance

Since the advent of first antibiotic 'penicillin' by Alexander Fleming, number of new antibiotics introduced, continued to show ineffectiveness against microbial pathogens. This phenomenon is known as antimicrobial resistance (AMR). Drug resistance has come forth as global challenge in the recent decades and it is anticipated to be one of leading causes of death in 2050 and emerging global public health concern that endangers efforts to achieve the 2030 Sustainable Development Goals (SDG). Overuse and misuse of antimicrobials in people and animals, often without professional oversight, contribute to AMR development. Antimicrobials are commonly misused to treat viral infections in humans and for growth promotion in food animals. Resistant microbes can be found everywhere, in humans, animals, our food and the environment. They are capable of spreading between animals and humans, including through food animal products. Impact of AMR is expected to be quite extensive in developing countries. Pakistan is the first country of the Eastern Mediterranean Regional Office (EMRO) of the WHO to establish the early implementation of the WHO Global Antimicrobial Resistance Surveillance System (GLASS). Furthermore, in line with the five strategic objectives of the WHO Global Action Plan (GAP) for AMR, Pakistan also developed the National Action Plan on AMR for the AMR containment through one health approach. As per recommendations given in NAP for AMR, Pakistan also has developed "national surveillance strategy for antimicrobial resistance in healthy food animals (AHC 2021) and "national surveillance strategy for antimicrobial resistance in bacterial isolates from sick food animals" (AHC 2022). Similar strategies are essential for knowing the status of antimicrobial resistance in different types of bacterial pathogens followed by developing antimicrobial stewardship program in animal health sector. Gram negative bacteria are important public health concern, as their outer membrane protects them from many antibiotics, including penicillin.

AMR in Large Animals as one Health Issue

According to one health concept, health of humans is closely related with the health of animals and environment. Resistance is transmitted from animals to humans and vice versa. Due to emergence of zoonotic pathogens, AMR has become an important one health issue. Similarly, resistance in commensals including E. coli, Salmonella spp., Campylobacter and Enterococcus spp. in large animals has also public health importance. These bacterial species have become resistant to even highest priority critically important antimicrobials and reserved antibiotics.

Mechanisms of Acquiring the AMR

There are certain mechanisms of acquiring resistance by gram negative bacteria. The bacteria can modify the antibiotic molecules by either chemical / enzymatic alterations or

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destruction of antibiotic molecules by β-lactamases. The permeability of antibiotics is decreased by changes in qualitative functioning or number of pores. This decreased permeability results into limited influx.

(i) Decreased permeability resulting limited influx of the antibiotic substances

(ii) Increased extrusion of substances by efflux pumps.

(iii) Modification of the target site and genetic mutations as well as enzymatic alteration of the target site also help bacteria to acquire resistance.

Pathogens and Resistance

Escherichia coli

Escherichia coli has substantial importance for the antimicrobial resistance studies because this resistance can gauge the levels of resistant bacterial phenotypes in the environment (Anjum et al. 2021). Therefore, E. coli is considered for the monitoring of AMR in surveillance programs of developed nations including WHO GLASS, WHO tricycle, EARS-Net and DENMAP. Similarly, commensal E. coli is chosen as AMR sentinel, as it provides valuable data and constitutes a reservoir of resistance genes, which can spread horizontally to zoonotic and other bacteria (EFSA and ECDC 2019). With the passage of time, the magnitude of resistance is being increased. Two decades earlier, E. coli isolated from cattle were resistant (<5%) to all the tested antibiotics including ampicillin (3%); chloramphenicol, cephalothin, and nalidixic acid (2.3% each); cefoxitin (1.5%); and ciprofloxacin, trimethoprim-sulfamethoxazole, and amoxicillin-clavulanic acid (0.1% each) (Schroeder et al. 2002). Lower resistance pattern was also observed at Switzerland in 2003 where none of the E. coli isolates from cattle showed resistance to amoxycillin-clavulanic acid, cephalothin, cefoperazone, enrofloxacin and Polymyxin B while chloramphenicol was resistant to 10% isolates and ampicillin, streptomycin and sulfonamide were >20% resistant (Lanz et al. 2003). As the human illness due to E. coli is linked with meat source (Schroeder et al. 2002) so the increasing level of resistant E. coli in humans and animals are analogous. In addition to showing 9% resistance to ampicillin and 66% resistance to streptomycin by bovine E. coli isolates in 2004, 68% of the isolates had multi-drug resistance (MDR) (Wilkerson et al. 2004). E. coli can acquire the co-resistance due to use of other types of antibiotics in animals, as the selection of chloramphenicol resistant E. coli is associated with the administration of dihydrostreptomycin and trimethoprim to the animals (Harada et al. 2006) and it is assumed that irrespective of the antibiotics use in calves, there is co-selection of tetracycline resistance due to unknown mutation (Walk et al. 2007). The increased resistance in E. coli commensals isolated from cattle is also attributed to earlier Salmonella infection and similarly the higher MDR was seen in isolates from such animals (DeFrancesco et al. 2004). Apart from systemic and enteric infections, E. coli also causes mastitis (predominantly sub-clinical) in the ruminants and has shown a high resistance to highest priority critically important, high priority critically important and highly important antimicrobials, and producing even fluoroquinolone resistance and extended-spectrum β-lactamase (Su et al. 2016). There is another fact that E. coli isolates from large animals were comparatively higher resistant to tested antibiotics than isolated from humans (Wilkerson et al. 2004; Mora et al. 2005). Although animals are considered as source of resistant E. coli to humans (Tanih et al. 2015), but there is another thought that milk can't be the source of transmission (Botrel et al. 2010).

Salmonella

Salmonella is one the major foodborne pathogens in humans, worldwide. According to United States Centers for Disease Control and Prevention (CDC), United States Department of Agriculture (USDA), University of Minnesota, and the National Institute of Health, Salmonella is 3rd most important zoonosis in Pakistan (CDC 2017). Multi-drug resistant Salmonellosis is endemic in humans in Pakistan, with the varied prevalence of Salmonella (Butt et al. 2005; Mirza and Khan 2008), Salmonella meleagridis, Salmonella montevideo, and Salmonella typhimurium are frequently recovered from faeces of cattle. Humans can acquire the infection from ruminants due to households' contamination or utilizing as food source. Similarly, humans can acquire the resistant bacteria from animals and Salmonella may acquire resistance during treatment with antibiotics for any other disease, due to its potential selective pressure. Salmonella had shown variable resistance to most of the antibiotics including cephalosporins over the longer periods, which is thought to be attributed to occurrence/emergence of different serotypes in an area (Davis et al. 1999; Frye et al. 2007; Hong et al. 2016). This is why, the resistance pattern appears uneven in surveillance programs for Salmonella. Like E. coli, Salmonella species show the higher phenotypic resistance in animals than humans (Davis et al. 1999). The effective choices against Salmonella infections include chloramphenicol and fluoroquinolones (Anderson 1968; Piddock 2002). Salmonella species isolated from cattle are highly resistant to ampicillin, streptomycin, chloramphenicol and tetracycline (Davis et al. 1999; Poppe et al. 2006; Marrero-Ortiz et al. 2012). All isolates of Salmonella from meat in Pakistan showed resistance to ampicillin and evidence of resistance evolution in Salmonella spp. are found in livestock. Before 2000, Salmonella from bovine species were highly susceptible to the antimicrobials; now the situation is opposite i.e., high resistance including to the extended spectrum cephalosporins.

Campylobacter

The commensal species of Campylobacter in bovines are C. jejuni and C. coli which induce diarrhea. However, C. fetus (previously known as Vibrio fetus) causes venereal disease, Campylobacteriosis in cattle causing infertility. C. fetus can be transmitted through male during coitus and have even been isolated from seminal vesicle of the bull (García et al. 2021). Unlike other commensals, isolation of Campylobacter spp. is difficult due to its anaerobic and other requirements. Unlike Salmonella and *E. coli*, the critically important antibiotics are different for Campylobacter. Resistant Campylobacter can be transmitted to humans from livestock species (Noreen et al. 2020). Moreover, its zoonotic potential makes it more important. Fluroquinolones and macrolides including erythromycin, clarithromycin, or azithromycin are considered the drugs of choice against Campylobacter infections but high resistance against these drugs has become public health concern. Additionally, Campylobacter coli and C. jejuni have developed high resistance for tetracyclines, beta-lactams, aminoglycosides, and lincosamides as well (Hull et al., 2021).

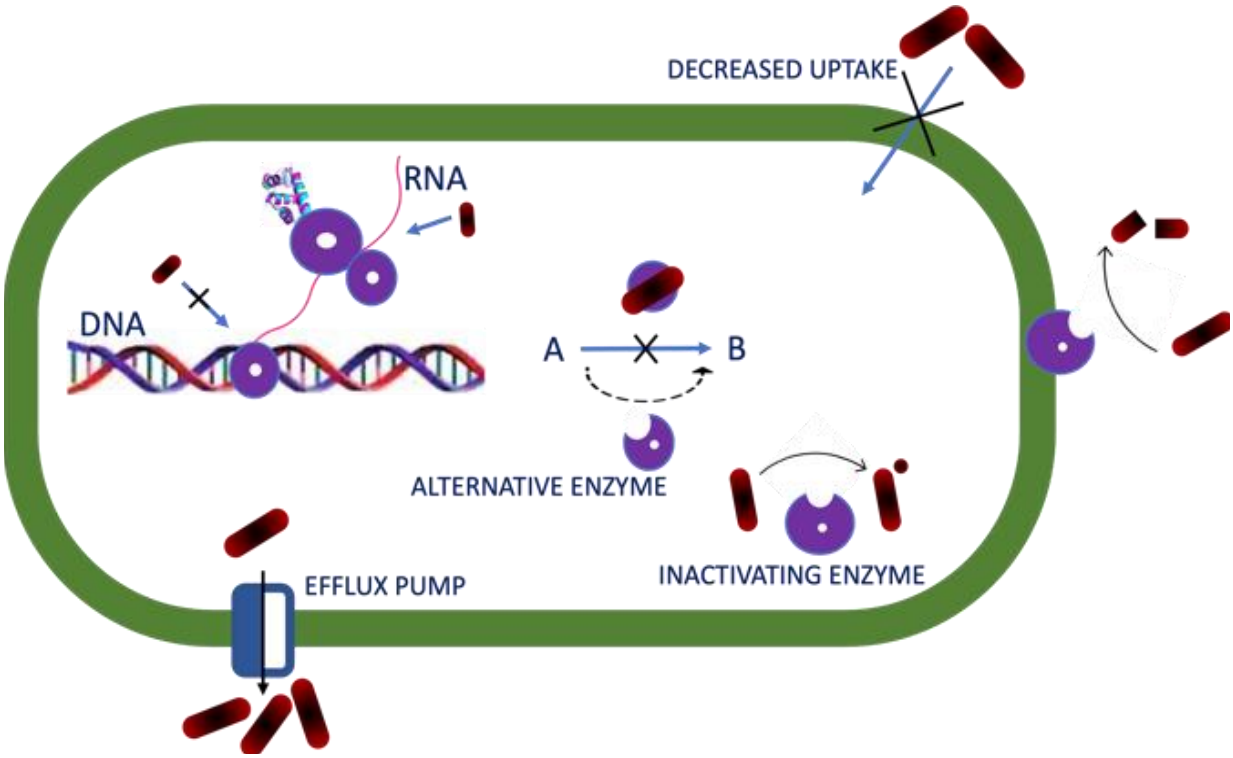

Fig. 1: Mechanisms of acquiring resistance by gram negative bacteria.

The resistant is possibly transmitted from animals to humans. Campylobacter has emerged as resistant to florfenicol and linezolid (Zhao et al. 2019).

Helicobacter

Helicobacter pylori is a spiral gram-negative bacillus that infects mostly the gastric lining of different animals. Most of the infections caused by Helicobacter are silent, causing ulcers related to the stomach and intestine, thus contributing to significant levels of morbidity and mortality and also can cause lymphoma and adenocarcinoma. Helicobacter also play role in thrombocytopenic purpura and anemia related to iron and B12 deficiency. Transmission routes of Helicobacter species are not confirmed, but there is evidence of fecal-oral and gastro-oral transmission. AMR of Helicobacter is an emerging issue related to its treatment. Helicobacter has shown high resistance against tetracycline and metronidazole, which results in treatment failure. Growth in antimicrobial resistance against these drugs is due to an increasing rate of treated patients and the growing usage of antibiotics. Other antibiotic drugs like clarithromycin, furazolidone and amoxicillin are better choices to treat Helicobacter infections (Bahrami et al. 2011). Different antibiotic drugs show resistance to several species of Helicobacter. Metronidazole is an antimicrobial having nitro-reductases as an active part. In case of Helicobacter, process of resistance is more complex. Factors like decrease in antibiotic uptake, increase in penetration of the drug through the bacterial cell wall and reduced action of nitro reductases are contributing to the non-activation of metronidazole. Levofloxacin, fluoroquinolones are also used as treatment therapy to control Helicobacter infection. This drug hinders DNA gyrase and causes disturbance in the synthesis of DNA. Mutation in A or B subunits of DNA gyrase are contributing to causing resistance by Helicobacter against this drug. Clarithromycin is a bacteriostatic drug used for the treatment of different Helicobacter species. Different point mutations in the 23S rRNA gene decrease the ability of ribosomes for the antibiotic. In this way, Helicobacter becomes resistant (Alba et al. 2017). Helicobacter pylori is a vital pathogen responsible for increasing antimicrobial resistance and causing a serious threat related to health. Resistance can be single, multiple, or hetero drug. Mechanisms involved in AMR are the mutational transformation of chromosomes and disturbance in the cellular activities of drugs. Growing AMR rates result in a considerable decrease in the efficacy of treatment related to different species of Helicobacter, globally (Tshibangu and Yamaoka 2021). Tetracycline-linked resistance is attained through protecting ribosomal proteins. These proteins enhance the resistance by minimizing the efficiency of ribosomes for this drug or by escaping the antibiotic from the ribosome in bound form. Activation of enzymes of tetracycline and point mutations linked to genes having 16 rRNA, also play role in causing resistance. Due to area-wise variation in incidences of antimicrobial resistance and clinical status of decreased treatment protocols, there is dire need to check the proportion of antimicrobial resistance of local drugs. This action helps in the selection of suitable antibiotic solutions and in changing the views of consultants about AMR, before ultimate treatment. These strategic protocols help in the reduction of treatment failures and the eradication of waves of increasing treatment resistance related to different Helicobacter species, in different populations of the world (Thung et al. 2016).

Proteus

Proteus mirabilis is a motile bacillus of Enterobacteriaceae family, normal inhabitants of intestines. However, six species of Proteus including P. vulgaris, P. cibarius and P. terrae are most commonly identified from clinicals samples related to different infections. These are opportunistic pathogen of vital

importance and present in soil, water and also in mammalian intestinal tract (Li et al. 2021). It acts as major cause of urinary tract infections in different animals. Biofilm production by P. mirabilis has become an emerging issue of much concern. Bacteria isolated from animals having diarrhea showed high resistance to ampicillin and doxycycline (Ghazay et al. 2019). Biofilm producing bacteria (Proteus), with higher rate of genes expression, are significantly more resistant to cefoparazone, piperacillin, and imipenem than non-producers (Schaffer and Pearson 2015). Biofilm production is linked with expression of certain genes like rsmA, hmpA, ureC, atfA and pmfA, showing resistance to kanamycin, tetracycline, doxycycline and cephalothin with almost 3/4th of the MDR and XDR isolates (Alabi et al. 2017). MDR in isolates of P. mirabilis is a growing health concern now a days and also has a serious threat for animals and humans. P. mirabilis isolated from intestines of large animals carry resistant genes of blaOXA-1, blaNDM-1, and blaTEM-1 (Kang et al. 2021). Proteus has shown relation with Salmonella regarding antibiotic resistance, identified based on Salmonellae genomic island 1 (SGI1) (Wang et al. 2019). Resistance related to cephalosporins in Proteus species is caused by extended-spectrum β-lactamases (ESBLs) and plasmid related cephalosporinases (Bonnin et al. 2020)

Moraxella

Morexella catarrhalis is diplococcus pathogen for infections related to respiratory tract. It causes sinusitis, pneumonia, otitis media and bronchitis (Goldstein et al. 2009), while Moraxella bovis is cause of infectious keratoconjunctivitis in bovines. Although there is low mortality, but morbidity is high with production losses. Antibiotics 'oxytetracycline and tylosin' are widely used for the treating keratoconjunctivitis but tulathromycin has been recognised as rational single dose treatment option (Lane et al. 2006). Other susceptible antibiotic options include ampicillin, ceftiofur, tilmicosin, tylosin, erythromycin oxytetracycline, and gentamicin. Amoxicillin and clavulanate remained drug of choice against Morexella related infections and involving all strains of it (Hsu et al. 2012). A slight decrease has been reported in MICs of cefotaxime and amoxicillin and a significant decrease in minimum inhibitory concentration of drug clarithromycin for mutants acrA, acrB, and oprM as compared to O35E which is a wild type of strain (Spaniol et al. 2015). Polymyxin drug is last approach against different infections caused by gram negative pathogens which are carbapenem resistant.

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CHAPTER 05

MULTIDRUG RESISTANCE IN CLOSTRIDIA

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INTRODUCTION

Anaerobic bacteria including Genus Clostridium have been showing an increasing trend in resistance patterns in recent years against routinely used antimicrobial agents. There are many resistance mechanisms, which have been evolved either intrinsically or acquired from the external environment by bacteria to overcome these agents (Munita and Arias 2016). The most notable being the production of the enzyme β-lactamase (Kuriyama et al. 2000). Other types of resistances include decreased drug accumulation (either through decreased membrane permeability or more active efflux of antibiotic), drug inactivation or modification (enzyme production), alteration of drug binding and/or target sites (alteration of binding proteins), and alteration in metabolic pathways (Li et al. 2016; Munita and Arias 2016; Brook 2017). Many drugs have become ineffective, especially against infections caused by *C. difficile*, *C. perfringens,* and some other species. Meanwhile, there are still some drugs having 80-100% sensitivity against most clostridia which include chloramphenicol, metronidazole, or even ceftriaxone and penicillin in combination with other drugs (Brook 2017). This chapter discusses mechanisms of resistance development including resistance rates reported by various studies against important clostridial species including C. *difficile*, *C. perfringens*, *C. tetani*, *C. sordellii,* and *C. chauvoei*.

Resistance against β-lactams

Beta-lactams are among the most recommended antibiotics which are routinely used. They inhibit bacterial cell wall synthesis and possess four-membered core-lactam ring. Based on adjoining structures, beta-lactams have four groups i.e., penicillin group, cephalosporin group, monobactams, and carbapenems (Petri 2011).
Resistance against beta-lactams most commonly occurred via the enzyme, beta-lactamase. There are four classes of these enzymes namely A, B, C, and D. A, C and D are serine hydrolases while B class is metallohydrolase (Majiduddin et al. 2002). Class D lactamases have been found in gram positive bacteria which also include the one conserved in *C. difficile* species (Toth et al. 2016).

Clostridioides (Clostridium) difficile

The genetic basis of resistance in *C. difficile* included the production of inducible lactamase (Toth et al. 2016). BlaCDD is the gene responsible to produce this enzyme, CD0457 encoding putative membrane-protein *bla X* is usually co-transcribed with *bla CDD*. *bla X* and *bla CDD* confer resistance against ampicillin and have a signal sequence associated with the cell membrane (Zhang and Shen 2017; Armenteros et al. 2019). Bla operon regulated by BlaR1 exhibits dose mediated expression in lactams. However, the resistance may vary depending upon the geographical situation and is thought to be directly related to the clinical use of cefoxitin. Beta-lactams are relatively ineffective against many clostridial species.
A study reported from Texas in swine production groups has shown all 131 isolates resistant to cefoxitin, imipenem, and ciprofloxacin whereas susceptibility was noticed for amoxicillin/clavulanic acid, piperacillin/tazobactam and vancomycin. Isolates were having intermediate resistance to ampicillin (Norman et al. 2009). In another study, an antimicrobial resistance pattern was studied using 523 *C. difficile* isolates, in an integrated population comprising humans and swine. Swine isolates were those collected from farrows, nurseries, breeding-place, and other production places, while isolates of human origin were obtained from workers at swine raising places and non-workers also. The majority of the strains were resistant to ciprofloxacin and cefoxitin while all of them were susceptible to amoxicillin/clavulanic acid, and vancomycin. The non-significant association was found among these studied groups indicating that transmission is unlikely to occur in an integrated population as proven by the results (Norman et al. 2014). Different studies reported susceptibility to co-amoxicillin using ≥16/8mg/L. Out of 2803 isolates, only 4 were

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found resistant; WPR was 0% having low heterogeneity. However, subgroup analysis was not carried out. The piperacillin/tazobactam susceptibility was studied applying breakpoint (≥128/4mg/L). Out of 3041 isolates, 8 were found resistant; the WPR was 0 % and further subgroups were not compared. The meropenem susceptibility was investigated in 17 studies at breakpoint (≥16mg/L). From total 275 *C. difficile* isolates, 20 were found resistant. No significant difference was found in data from 1992-2014 and the data from 2015-2019 (Sholeh et al. 2020a).

Clostridium perfringens

The most commonly used antibiotics in humans and animals belong to a β-lactam class of antibiotics (Price et al. 2019). Due to this prolonged usage, resistance against β-lactams has been found most frequently in bacteria, which has posed a difficulty in treating and overcoming these bacterial infections. Most of these bacteria produce beta-lactamase enzyme, which hydrolyzes the beta-lactam ring present in these compounds, thereby making them ineffective (Bush and Bradford 2016). The situation is worrisome not only for aerobes but also in case of anaerobes like *C. perfringens*, due to the existence of *bla 2* gene which has been reported in some of the studies from clinical settings (Mishra et al. 2016). This *bla2* gene was initially reported in Firmicutes, and bacteria like *C. perfringens* have acquired it from the external environment and incorporated it into their genomes.

Usually, penicillin G has been used as a drug of choice and most Clostridium strains have also been found susceptible to penicillins until recently. The exceptions include *C. ramosum*, *C. innocuum,* and *C. clostridioforme*. Clinically management of infections caused by anaerobes had been good with penicillin G. Semisynthetic penicillins are less effective as compared to penicillin, ampicillin, and amoxicillin. However, due to the production of BLs by anaerobes, these drugs have limited use. The first generation cephalosporins have activity similar to penicillin G, however, they have been found effective against *C. perfringens*. Additionally, the biofilm formation by *C. perfringens* might protect the cells from atmospheric oxygen and higher concentrations of the antibiotic penicillin. The resistance mediated by biofilm formation in *C. perfringens* has been found against penicillins, virginiamycin, lincomycin, tylosin, and even salinomycin, monensin and narasin (Charlebois et al. 2017). In a study carried out to find the antimicrobial susceptibility of *C. perfringens* against different antibiotics, the resistance rate was 26% against ampicillin, 3.7% (chloramphenicol), 15.2% (Ciprofloxacin), 6% (clindamycin), 32.9% (erythromycin), 45.4% (gentamicin), 0% (metronidazole), 52.5% (nalidixic acid), 10% (vancomycin), 21.8% (penicillin), 32.1% (trimethoprim-sulfamethoxazole), 19.3% (amoxicillin), 38% (imipenem) and 2.5% against ceftriaxone respectively. The resistance rate to cloxacillin was 100%, to cephalexin was 0%, to oxacillin 45.6%, to cephalothin 8.6%, bacitracin 89.1%, and colistin 40% respectively. Overall high dosage of penicillin is an effective therapy against soft tissue infections caused by *C. perfringens* in humans. Penicillin resistant *C. perfringens* strains are rare, as no strain was found resistant to it, in studies carried out in Brazil (Silva et al. 2009), Canada (Leal et al. 2008), and New Zealand (Roberts et al. 2006). In Iran, however, the resistance rate reported was higher, i.e., 21.8%. This difference might be due to the difference in the type of samples used. It has been known that *C. perfringens* is a normal flora of the GIT of animals and humans and may lead to foodborne diseases. These microorganisms also can transfer resistance genes via mobile elements to other gut microbiota (Hosseinzadeh et al. 2018).

Resistance against Cephalosporins

Anaerobes have evolved three major mechanisms conferring resistance to them against beta-lactam antibiotics. Firstly, inactivating enzymes i.e., β-lactamases including penicillinases and cephalosporinases (Kuriyama et al. 2000); secondly, low-affinity penicillin binding proteins (PBPs); thirdly, decreased permeability via alterations in porin channels. Cephalosporinases are quite often belonging to subgroup 2e and may inhibit BL inhibitors like clavulanic acid, tazobactam, sulbactam). Cephalosporins have either a class or specific beta-lactamase enzymes, which can inactivate them (Bui and Preuss 2021). The aerobic anaerobic i.e., polymicrobial infections usually require metronidazole in addition to beta-lactam, cephalosporin, or fluoroquinolones for treating anaerobes as BLs are becoming ineffective against anaerobes. There has been an emerging trend in the resistance of anaerobes against penicillins, cephalosporins, clindamycin, and fluoroquinolones.

Anaerobic microorganisms can be tested for enzyme beta-lactamase by using chromogenic cephalosporin test like nitrocefin disks (Papanicolas et al. 2014). Many anaerobes possess cephalosporinase enzyme, which is the reason for limited efficacy of cephalosporins against anaerobes. First generation cephalosporins have activity much like penicillin G against anaerobes. The second-generation drugs like cefoxitin have efficacy against anaerobes however this efficacy varies geographically and also these drugs a relatively less effective against Clostridia with the exception of *C. perfringens*. Third generation drugs have also raised concerns regarding antimicrobial resistance and are of limited use against clostridia.

Clostridioides (Clostridium) difficile

C. difficile is usually evaluated routinely for antibiotics, having an association with CDI. Cephalosporins and clindamycin are considered high risk agents for CDIs. Cephalosporin has been known to be resistant to *C. difficile*; even studies have reported its overgrowth following CFs therapy. The mechanism of resistance to these drugs is not known in-depth and they are termed as constitutively resistant to cephalosporin (Spigaglia 2016). The resistance may be strain dependent also and it is known that antibiotic degrading enzymes and modification of the target site are mainly involved in making these drugs ineffective.

C. difficile resistance pattern against β-lactam antibiotics has been found variable in different studies. Against cephalosporins resistance rates are 14.3%(ceftriaxone), 3.5% (cefoperazone), 10.5% (Cefepime) which are low as compared to 76% against ceftazidime and 95% for cefotaxime. Recent studies have shown that lactamase enzyme in *C. difficile* imparts resistance to penicillins, cephalosporins and monobactam class of lactams (Banawas 2018). Ceftriaxone susceptibility was also reported in various studies. From 3476 isolates used in various studies, 1289 were having resistance. The percentage was 37.1 at breakpoint ≥64mg/L. WPR for this drug was 47% with substantial heterogeneity.

Resistance against Chloramphenicol

Anaerobes were not found to have resistance against CH, although few clinical studies have reported treatment failure.

Lack of resistance might be due to its infrequent use clinically. In the USA, this drug has been used rarely. Resistance against chloramphenicol is rare however some strains have MICs clustered nearby breakpoints. This resistance is because of the drug inactivation due to nitroreduction and/or acetyltransferase. It is a bacteriostatic agent having good susceptibility against anaerobes, MICs of this drug is usually clustered near the susceptibility range. Some reports do show treatment failure using chloramphenicol but on the other hand, this drug has been in use for the last 65 years against anaerobic infections.

Clostridioides (Clostridium) difficile

Multidrug resistance has been observed in *C. difficile* isolates obtained from animal sources including chloramphenicol. In a study on swine population, the strains were found resistant to clindamycin, intermediate susceptible to ampicillin, and susceptible to chloramphenicol and tetracycline (Norman et al. 2009). In another study carried out on an integrated population of humans and swine, the human isolates were obtained from wastewater collected from workers and non-workers. All the strains of *C. difficile* isolated from human subjects were susceptible to chloramphenicol (Norman et al. 2014). In *C. difficile* resistance against chloramphenicol has been mediated by catD gene encoding CAT enzyme , present on mobilizable transposans Tn4453a and Tn4453b, having structural and functional relatability with *C. perfringens* transposan Tn4451 (Lyras et al. 1998).

Chloramphenicol antibiotics are still recommended against *C. difficile* infections. In Iran, a total of ten studies have reported resistance patterns of *C. difficile* against various antibiotics. The fixed effect model was used for studying some antibiotics including Chloramphenicol. A resistance pattern against this drug was observed in 6.2% isolates. In Europe resistance reported was 3.7%. Data have indicated that Chloramphenicol can still be recommended for CDI.

Clostridium perfringens

Previously isolates of *C. perfringens* from swine have shown multidrug resistance against clindamycin, erythromycin, and tetracycline, but these isolates were susceptible to chloramphenicol. On the other hand, isolates identified from African (Cote d'Ivoire) cooked beef have shown resistance to chloramphenicol including some other drugs (Kouassi et al. 2014). In another study multidrug resistance to chloramphenicol along with some other drugs was found in 5% isolates of *C. perfringens* from animal origin (Mau-Inchaustegui and Rodriguez-Cavallini 2011). It is notable that that commonly reported resistance determinants to date have been found associated with bacitracins, MLS, tetracyclines, and chloramphenicol drugs.

Resistance against CH has been mediated via acetyltransferase enzymes encoding cat(P) and cat(Q) genes. Cat(Q) gene has been shown to have variation from *C. perfringens* cat(P) gene. Moreover, cat(Q) monomer has 53% sequence conservation with cat(P) and 39-53% with other cat proteins at amino acid level. Phylogenetic analysis has shown that cat(Q) is closer to Cat proteins from *S. aureus* and *C. coli* like cat monomers from Clostridial species. In the case of *C. perfringens*, mobilizable transposans including Tn4451 and Tn4452 have been identified (Adams et al. 2002) conferring resistance against chloramphenicol antibiotic, but these genetic elements are not conjugative, which is taken care by co-resident elements thereby facilitating transfer to the cells. Tn4451 mobilizable transposan has elicited transposition dependent upon unusual resolvase enzyme. Conventionally transposition has been dependent upon transposase or integrase enzymes (Adams et al. 2002). PIP401,53kb plasmid has been the first conjugative plasmid identified from *C. perfringens* which imparts resistance against chloramphenicol as well as tetracycline. It was obtained from human isolate of *C. perfringens* type A strain (CP590).

Resistance against Macrolide–Lincosamide–Streptogramin (MLS_B)

Clostridioides (Clostridium) difficile

C. difficile has acquired resistance against clindamycin, erythromycin. *C. difficile* isolates became resistant to MLS_B family i.e., Macrolide-lincosamide-streptogramin B, through ribosomal methylation process. Erythromycin ribosomal methylase B (ermB) has been the most widespread resistance gene detected in *C. difficile* isolates (Schmidt et al. 2007; Spigaglia et al. 2005). The erm class B is usually present on mobile genetic elements, best known of them is Tn 5398, a 9.6 kb mobilizable transposan sequence (Farrow et al. 2001). This element has two copies of ermB and is known to be transferable *in vitro* from *C. difficile* to *S. aureus* and/or *B. subtilis*. Transfer of Tn5398 from donor to recipient is carried out by other conjugative transposans responsible for Integration and/or excision in the donor genome as Tn5398 does not encode gene for producing recombinase enzyme (Mullany et al. 2015). Integration process into recipient may occur through homologous recombination or site-specific recombinase enzyme of the recipient cell. It is also recently known that a portion of genome having Tn5398 integrate in to the recipient cell through homologous recombination (Wasels et al. 2015b). Resistance against erythromycin, clindamycin or erythromycin alone has also been reported in erm negative *C. difficile* strains. Some of these strains have alterations in 23RrDNA/ribosomal proteins (L4 or L22). However, these alterations also exist in the susceptible isolates which exclude their role in imparting resistance (Spigaglia et al. 2011). It is further noticed that resistant erm-negative strains when treated using 2 pump inhibitors (reserpine and carbonyl cyanide m-chlorophenyl hydrazone–CCCP), didn't reduce MICs, thereby indicating non-involvement of efflux mechanisms in mediating resistance (Spigaglia et al. 2011). In this perspective, in the absence of *erm* genes, other determinants might have role in *C. difficile* resistance to MLS_B. The other determinants including cfrB and/or cfrC, encoding 23SrRNA methyltransferase impart resistance against phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (PhLOPSA) have also been detected in several *C. difficile* strains (Candela et al. 2017). The cfr gene denominated as Tn6218 is a non-conjugative element, which is also associated to Tn916. A study from Japan reported more than 96% resistance in *C. difficile* isolate against lincosamide and macrolides (Senoh et al. 2015). Prevalence data from Iran shows 61.5% of isolates are resistant to erythromycin (Khademi and Sahebkar 2019).

Clostridium perfringens

Cross resistance has been observed in macrolide, lincosamide, and streptogramin B groups. MLS_B antibiotics inhibit protein

synthesis in gram positive as well as gram negative bacteria and resistance against these antibiotics is because of methylation of 23SrRNA encoding *erm* genes, thus preventing MLS_B antibiotics binding with ribosomes. The resistance mechanism in anaerobes against MLS_B antibiotics has been conferred by five genes including *erm*(Q), *erm*(B), *erm*(C), *erm*(F) and *erm*(G). However, not even a single gene has been identified in these bacteria that encode MLS resistant efflux proteins and/or inactivating enzymes (Roberts 2003).

Mef(A) gene encoding efflux pump associated resistance to macrolides alone (while lincosamides and streptogramin B were still effective) was first identified in *S. pneumoniae*. This gene was later on identified in *C. perfringens*. The sample source was soil, sewage and water across 14 US states (Soge et al. 2009). Besides other antibiotic resistance genes, the study also reported *erm*(Q) and *erm*(B) highlighting that environmental *C. perfringens* isolates might act as reservoir for these resistance genes.

Macrolides have medium to good in vitro activity against most anaerobes, but they cause little toxicity also. The most effective macrolide against gram positive oral anaerobic microflora is Clarithromycin. Similarly, erythromycin is effective to some extent against severe soft tissue infections caused by anaerobes, providing good adequate cleaning and drainage of the infected tissue. Erythromycin resistance has been observed in 32.9% of samples in a study from Iran (Khademi and Sahebkar 2019). A study on three *C. perfringens* genomes revealed *erm*(Q) gene that encodes resistance to MLS_B. The *erm* gene i.e., *erm*(Q) has been first detected in *C. perfringens*.

Resistance against Clindamycin

Clindamycin, a broad-spectrum antibiotic has proven clinical effectivity against anaerobes as shown in the previous research/clinical trials. It has been used against dental infections in people allergic to penicillin as well as against aspiration pneumonia. Clindamycin hydrochloride has rapid complete absorption from GIT and also reaches all body tissues including sputum, saliva, soft tissues, respiratory tissue, prostate, semen as well as bones and joints**.** Resistance is conferred by macrolide-lincosamide-streptogarmin (MLS) type 23 S methylase, usually encoded by one of the *erm* gene expressed in higher amounts. This antibiotic is not recommended nowadays against intraabdominal infections. Several species of clostridia have become resistant to this drug. 20% of *C. ramosum* isolates have been found resistant to clindamycin (Goldstein and Citron 2011).

Clostridioides (Clostridium) difficile

C. difficile has shown variable susceptibility against clindamycin. Resistance has been on the increasing side in treating skin and soft tissue infections. *C. difficile* isolates from equine origin identified over a period of seven months have shown high levels of resistance to clindamycin. A Spanish study reporting 144 isolates of porcine origin reported a higher incidence of RT078 (94.4%) having multidrug resistance in 49.3% of isolates tested (Peláez et al. 2013). This study reported more than 27.8% resistance to clindamycin. Similarly, Italian isolates of *C. difficile* sampled from swine and dogs have shown 10 and 6 ribotypes from both species respectively (Spigaglia et al. 2015). The major strain found was RT078 (50% in swine isolates), whereas non-toxigenic canine strain RT010 was found in 64% isolates. 15% resistance was reported against clindamycin in pigs and 51% was found for isolates of canine origin (Spigaglia et al. 2015). The isolates belonging to ribotype 027FQR had 75 -100% resistance against clindamycin. A detailed study from 15 regions of Italy has shown MDR in more than 59% of isolates and 100% resistance in PCR ribotypes 365/607 & 018. MDR pattern in this species is often linked to resistance against clindamycin (Spigaglia 2016).

A meta-analysis of the data included 64 studies reporting around 20,000 *C. difficile* isolates; 6685 (34%) were found resistant to clindamycin. The WPR to clindamycin was 59% having no significant difference on the basis of time of study when it was conducted (Sholeh et al. 2020a). Significant differences were found when continent wise data was compared. The highest resistance was found in isolates from Asia followed by South America. When compared on the basis of the quality of studies set using different parameters, the high, moderate, and low quality data reported 63%, 57%, and 17% respectively (Sholeh et al. 2020a). The method used for antimicrobial susceptibility testing was also having significant differences among them.

Clostridium perfringens

Studies carried out on samples of porcine origin of *C. perfringens* have reported resistance against erythromycin, tetracycline as well as clindamycin. Some recent studies also reported 28% resistance against *C. perfringens* isolates of porcine origin reported from Canada. The *C. perfringens* isolates from bovine origin have also reported reduced susceptibility to clindamycin. 5% of *C. perfringens* isolates of animal origin have shown resistance clindamycin in other studies (Mau-Inchaustegui and Rodriguez-Cavallini 2011).

Resistance against Metronidazole

Metronidazole usually gives well anaerobic coverage. Nitroimidazoles are effective against anaerobes as intracellular reduction of the drugs into active antibacterial metabolites takes place anaerobically. However, these are genotoxic and therefore not used in food animals in various countries.

Resistance against metronidazole is attributable to nitroimidazole reductase (nim) enzyme, which converts 4-,5-nitroimidazole into 4-,5-aminoimidazole and avoid toxic radical formation required for antimicrobial effect of drug. Nim has been found in aerobic as well as anaerobic bacteria. Nim genes are usually located on mobilizable plasmids and can lead to 5-Ni drugs ineffectiveness. However, resistance sometimes exists even in nim negative strains due to the sub-MIC concentrations of metronidazole.

Clostridioides (Clostridium) difficile

C. difficile infections have been treated commonly using oral metronidazole, fidaxomicin etc. (Spigaglia 2016). However, this microorganism possesses many resistant mechanisms, like, metabolic pathway changes, biofilm formation, *ermB* gene (Resistance against MLS_B). *C. difficile* ribotype 027 has been shown to have unusually high resistance against metronidazole drugs (Peng et al. 2017). Although this type of resistance has been quite uncommon. Recent studies on ribotypes 027 and 010 have shown the resistance to metronidazole a complex process. Alterations in metabolic pathways involve

nitroreductases activity, iron uptake, DNA repair as well as biofilm formation are thought to play a vital role (Chong et al. 2014). It is predicted that biofilm matrix alters the physiological state of bacteria thereby acting as a protective barrier as well as imparting more resistance against antibiotics (Vuotto et al. 2016).

A study carried out in Western Australia from 2007 to 2009 found 23% of diarrheal horse isolates susceptible to metronidazole (Thean et al. 2011). A cross sectional study including diarrheal and non-diarrheal foals reported 7 samples positive for *C. difficile* A/B toxin (from diarrheal foals), having susceptibility to metronidazole (Silva et al. 2013). Resistance against metronidazole was however observed in horses having an acute gastrointestinal disease (Magdesian et al. 2006). Studies involving human patients have been reporting high resistance against metronidazole. There has also been an increase in geometric mean of MICs i.e., for RT027 (1.1–1.42mg/L), RT001/072, RT106 and RT356 (0.6 mg/L), RT010 (1.5mg/L) and other RTs (0.13–0.4mg/L) (Freeman et al. 2015). Metronidazole susceptibility to 19645 isolates of *C. difficile* was investigated in more than 100 studies. EUCAST breakpoint 2mg/L was taken as standard in 32 studies reporting 5900 isolates. About 190 were reported to be resistant; WPR to metronidazole was 1%. When the data were compared based on the difference in time range; there was an increase in resistance during time period 2015-2019 as compared to 1992 to 2014. Highest resistance was seen in isolates of Asian origin. The CLSI breakpoint (32mg/L) when applied, 129 out of 13207 isolates were found resistant. There was non-significant association when different parameters were compared like time period and geographical location (Sholeh et al. 2020a).

Clostridium perfringens

Resistance against metronidazole has rarely been reported in *C. perfringens* isolates obtained from humans and/or animals. A study from Sweden included 50 *C. perfringens* isolates obtained from acute diarrheal dogs reported 54% of isolates had decreased susceptibility to metronidazole, having MIC 4mg/L (Gobeli et al. 2012). Another study from Costa Rica reported multiple resistance to several drugs including metronidazole in 5% of *C. perfringens* isolates obtained from animal sources (Mau-Inchaustegui and Rodriguez-Cavallini 2011). Most of these isolates possessed intermediate susceptibility to the drug, metronidazole (57% susceptible; MIC:16mg/L). Similarly, an isolate obtained from a dog has reported a metronidazole resistant strain of *C. perfringens* (Marks and Kather 2003).

Resistance against Tetracyclines

The second most commonly used broad spectrum antibiotic after β-lactams is tetracycline, which nowadays has limited use against anaerobic infections because of the rapid development of resistance against it. Tetracycline analogs including minocycline and doxycycline also have limited use owing to the significant resistance and therefore require susceptibility testing before use to confirm effectiveness. Tigecycline has been found active against anaerobic gram positive bacteria (Frampton and Curran 2005).

Clostridioides (Clostridium) difficile

In *C. difficile*, resistance to tetracycline is mediated by tet genes including *tet(M)*, *tet(P)*, *tet(K)*, *tet(L)*, *tet(W)* and *tet(X)*. The most commonly widespread is tet(M), which is usually carried by conjugative Tn916 like elements (Spigaglia 2016). Both tet(M) and tet(W) have been identified in human and animal isolates of *C. difficile* (Fry et al. 2012). The latest detected tet(X) genes or mutations in the existing *tetM* and *tetW* classes might increase the resistance to tigecycline (He et al. 2019).

This transposan family is responsible for conferring antibiotic resistance to pathogens not only against tetracycline but also other classes of antibiotics. The commonly well-known element in this family is a 21kb Tn5397 which has *in vitro* capability of transfer between *C. difficile* and *B.subtilis*/*E.faecali* (Jasni et al. 2010). Tn5397 element having *tndX* genes encodes for serine recombinase enzyme inserts DNA predicted filamentation processes induced by cAMP (Fic) domain (Wang et al. 2006). Group II intron and a variable excision/insertion module distinguish Tn5397 from Tn916. Tn916 containing xisTn and intTn, encodes excisionase and tyrosine integrase enzyme inserts at multiple regions into the genome of *C. difficile* and carries tetM alleles (Mullany et al. 2012). Around 31 studies indicating the susceptibility of 4861 *C. difficile* isolates to tetracycline have reported 886 isolates under the resistant category (breakpoint 16mg/L). The weighted pooled resistance was 20% having substantial heterogeneity. The continental categorization was having significant differences while there was non-significant difference in two different points of time in the same region. The resistance patterns against tetracycline were 34%, 26% and 16% in Oceania, Asia, and Europe respectively. Categorization on the basis of quality of articles gave resistance rates as 22%, 16% and 40% for high, moderate and low quality data (Sholeh et al. 2020b).

C. difficile isolates from swine kept in the US have shown resistance against tetracycline indicating the presence of tet(M) gene in 97% of isolates, tet(W) in 32%, and a subset of 31%, having both of these genes. However, these 31% isolates showed different MIC values within the "resistance" category (Fry et al. 2012). Besides tetM, various tet genes also existed in *C. difficile*. The presence of both *tetM* and *tetW* has been found in human as well as animal origin isolates (Fry et al. 2012). Tn6164, a 106kb element was reported in M120 *C. difficile* strain. This element contains parts from different bacteria like *S. pneumoniae* etc also predicted to confer resistance to tetracycline etc. Since this M120 strain is susceptible to tetracycline class, Tn6164 is not seemed to be involved in resistance. However, it has been found to enhance virulence of this strain, which results in mortality in more patients as compared to the people infected with strains not having this element.

Clostridium perfringens

Bacterial resistance to tetracycline came from one or more of the 36 tet genes which follow any one of the three resistance mechanisms (Sheykhsaran et al. 2019). *tetA*(P) and *tetB*(P) were the first identified two functional overlapping resistance genes in *C. perfringens* R-plasmid pCW3. The reduced susceptibility has also been reported in poultry to *tet(M)* gene, in addition to tet(P) genes. Other studies also reported tet(Q), tet(K), tet(L), tet(O), and tet(W) (Gholamiandehkordi et al. 2009). The tet P determinant commonly associated with conjugative as well as non-conjugative plasmids has been present only in Clostridium spp. and has demonstrated the capability to spread in the whole clostridium genus (Vidor et al. 2019). TetA(P) has been found to be linked to all tetracycline resistant strains. Most isolates

resistant to tetracycline also possess tetB(P) or tet(M) genes. TetB(P) has been shown to be associated with low level resistance and did not disturb MIC of isolates already possessing tetA(P) gene (Johansson et al. 2004).

A study on 124 resistant strains isolated from dogs revealed 96% isolates carrying tetA(P) and 41% isolates having tetA(P) and *tetB*(P) genes. *tet(M)* or alone *tetB*(P) was not observed in these isolates (Kather et al. 2006). Tetracycline resistance commonly observed in *C. perfringens* has been associated with antibiotics used in animal feed. A study conducted on 81 tetracycline resistant strains reported all strains carrying the tetA(P) gene with 43 strains having *tetB*(P) on the tet(P) operon. The other 32 strains were having chromosomally encoded tet(M) gene along with tetA(P) gene. The tetracycline resistance has also been reported worldwide in *C. perfringens* isolates identified from poultry. Previously studies have reported resistance to oxytetracycline (MIC>1mg/L) in samples from countries i-e, Sweden (76%), Denmark (10%), Norway (29%), and Belgium (66%) against *C. perfringens* isolates (Johansson et al. 2004; Martel et al. 2004). The isolates from Canada and Korea have also shown high resistance patterns against tetracycline (Park et al. 2015).

Resistance against Fluoroquinolones

Clostridioides (Clostridium) difficile

There are concerns over the use of fluoroquinolones in treating anaerobic infections because of the rapid increase in merging resistance against bacteroides and anaerobic cocci, as well as the impact of these on increasing infections of *C. difficile*. Anaerobes are having natural resistance against older fluoroquinolones. In *C. difficile* resistance against FQs is mainly because of the alterations in quinolone resistance determining region i-e, QRDR of either GyrA or GyrB. This gyrase subunits i.e., GyrA or GyrB may have several amino acid substitutions. However, the most common substitution in FQs resistant *C. difficile* strains was found to be Thr82Ile in GyrA (Spigaglia et al. 2011; Kuwata et al. 2015). This substitution however did not affect *C. difficile* strains *in vitro*, indicating that it can be sustained even in situations, where antibiotic selective pressure is not present at the population level (Wasels et al. 2015a). Repeated exposure to moxifloxacin and levofloxacin also gave rise to mutant resistant strains of *C. difficile* (Spigaglia et al. 2009). As the drug concentration in the intestine of humans is not inhibitory at earlier treatment stages, therefore there are chances that mutation may be acquired in the bacteria against FQs. Ciprofloxacin, a second generation FQ was observed to have 0-1% susceptibility against *C. difficile* strains (Kuwata et al. 2015). Resistance in fourth generation antibiotics like moxifloxacin and gatifloxacin was observed against 36% and 68% *C. difficile* strains respectively (Freeman et al. 2015; Kullin et al. 2017).

The *C. difficile* hypervirulent 027 PCR ribotype has shown frequent alterations in GyrA and/or GyrB subunits, imparting resistance against fluoroquinolones. In a study conducted in Virginia USA on 3118 isolates, resistance against fluoroquinolone and MDR 027 ribotype was frequently seen and reported in 32% of *C. difficile* isolates (Carman et al. 2018). Another study carried out in Japan has shown susceptibility in *C. difficile* isolates against metronidazole and vancomycin, however more than 96% of ribotypes 018 and 369 were having resistance against fluoroquinolones, lincosamides and macrolides (Senoh et al. 2015). Moxifloxacin is not recommended for CDI treatment however resistance against this drug is an important marker for spread of *C. difficile* in health care setups (Dingle et al. 2017). When ciprofloxacin and moxifloxacin were used as representative fluoroquinolone drugs against *C. difficile* isolates, the former showed highest resistance (95% WPR), and the latter was having 32% (CLSI standard) and 49% (EUCAST standard) resistance (Sholeh et al. 2020a).

The usage of antibiotics including clindamycin and cephalosporins along with fluoroquinolones, ampicillin, and amoxicillin are associated with a high risk of CDI. The resistance rate may vary in different places. In Iran, *C. difficile* were found to have high resistance i.e., 69.5% against ciprofloxacin, 93.4% levofloxacin, 92.9% against nalidixic acid, and 67.9% against moxifloxacin. High resistance against moxifloxacin were also found in *C. difficile* strains from countries including China, Korea, and Germany reporting 61.8%, 62.6%, 68% resistance respectively, and 100% resistance against isolates from the Czech Republic and Poland. Low resistance rates were seen in Brazil (8%), France (8%), Hungary (41.2%), Israel (4.7%), Japan (0%), New Zealand (0%), Sweden (15%), Spain (43%), and United States (36%) (Banawas 2018). in addition over usage of fluoroquinolones has been found associated with hypervirulent 027/BI/NAP1 *C. difficile* strain (Peng et al. 2017).

Clostridium perfringens

Anaerobes are no more susceptible against first generation FQs. The newer class of quinolones, however, has significant activity against anaerobes like *C. perfringens*. Low susceptibility quinolones include levofloxacin, ofloxacin, ciprofloxacin, enoxacin, pefloxacin, fleroxacin, and lomefloxacin. gatifloxacin, grepafloxacin. Moxifloxacin, Sparfloxacin, and Trovafloxacin have intermediate anti-anaerobic activity. Trovafloxacin has restricted use as it is hepatotoxic. Highly susceptible drugs include clinafloxacin and sitafloxacin, as they show the highest *in vitro* activity against anaerobes (Stein and Goldstein 2006). FDA has approved moxifloxacin usage and it has been successfully used against the skin and mixed intraabdominal infections caused by anaerobes including *C. perfringens*.

Resistance against Aminoglycosides

Anaerobic bacteria have a natural resistance to aminoglycosides, owing to their requirement of oxygen for their movement to the cytoplasm of the cell.

Clostridioides (Clostridium) difficile

Bacitracin antibiotic has BcrA,-B, and –C; an ATP-dependent ABC type efflux system responsible for its non-effectiveness against *C. difficile* isolates. Resistance against kasugamycin is mainly because of KsgA gene producing dimethyl transferase enzyme (Duffin and Seifert 2009).

Clostridium perfringens

Aminoglycosides aren't able to reach the target site in the bacterial cell. In a cell free environment streptomycin and gentamicin can bind and stop protein synthesis in *C. perfringens* ribosomes. Uptake of these drugs is either energy dependent

or energy independent. When energy dependent, it will be available from O_2 or N_2 dependent electron transport system. However strict anaerobes lack this system and don't have the capability to import these drugs (Ricci and Piddock 2003). These drugs, therefore, don't accumulate inside *C. perfringens.* Trimethoprim-sulfamethoxazole also is ineffective against anaerobes.

Antibiotic Resistance in other Species of Clostridium

Clostridium tetani

Clinical isolates of *C. tetani* have been found susceptible to penicillin, although most studies have targeted antitoxin and vaccine developments against this bacterium. Penicillin has been considered the standard treatment (Campbell et al. 2009) but the efficiency of penicillin depends on its efficiency to reach the infection site effectively. In most studies all strains were found sensitive to penicillin, highest MIC was found 0.25ug having zone of 29mm using 10ug discs commercially available (Campbell et al. 2009). A study from Pakistan also reported all *C. tetani* isolates were sensitive to penicillin. However, another report from Canada has reported alive *C. tetani* in wounds treated for two weeks using high penicillin doses. Some patients have prolonged recovery time which lasted for 16 days in a study using penicillin as a treatment option.

A study on 45 clinical *C. tetani* isolates reported none of the strains were resistant to penicillin. In this way unlike other clostridial species e.g., *C. tetani* has not been found to acquire resistance against commonly used antimicrobial drugs (Sebaihia et al. 2006). The results, therefore, highlighted the fact that penicillin can still be used for treating tetanus along with additional therapeutic agents. The inefficiency to treat with penicillin is sometimes due to the reason that the appropriate therapeutic dose administered intravenously could not be able to reach the infected tissue in required amount.

Metronidazole has nowadays been considered as the first line of treatment against *C. tetani* infections as an alternative to penicillin. Other drugs effective against tetanus includes cephalosporin, chloramphenicol, clindamycin, macrolides, and tetracycline (Sebaihia et al. 2006). Metronidazole is an alternative drug against CT after penicillin has the highest MIC of 1.0ug having zone of 26mm using 5ug discs. A study on clinical *C. tetani* isolates reported none of the strain resistant to Metronidazole (Campbell et al. 2009). *C. tetani* has been found resistant against erythromycin in studies.

Clostridium chauvoei

The virulent *C. chauvoei* strain gives potential insights into the genome of this microbe and revealed its replication in infected tissues of the host and the role of various virulence genes during that process. The chromosomal region of this microorganism has resistance genes conferring resistance against antibiotics. Nevertheless, *C. chauvoei* has been found sensitive to many antibiotics. The MICs for JF4335 strain using CLSI standard on Mueller Hinton broth were 2ug/ml for Cephalotin, 0.5 ug/ml for Clindamycin, 0.25ug/ml for Enrofloxacin, 0.25ug/ml for Erythromycin, <0.012 ug/ml for Penicillin, 1ug/ml each for Vancomycin and Tetracycline and <4ug/ml for chloramphenicol (Frey and Falquet 2015). These MICs have given clear indication of failure of treatment of blackleg using antibiotics which lead to rapid death of animals. The strain JF4533 has also been found to have a gene for resistance against penicillin and an elongation factor G type gene for tetracycline resistance. The Vancomycin B type (vanW) gene has also been found to be present along with other resistance genes. Besides these, there are multi-antimicrobial extrusion proteins that confer resistance against antibiotics. While all these genes have been present in JF4533, they are either not expressed or are producing non-functional proteins doing functions other than exporting antimicrobial agents (Frey and Falquet 2015).

Table 1: Antibiotic activity against *C. difficile*

Resistant	Intermediate	Susceptible
Beta lactam	Ampicillin	Metronidazole
Tetracycline	Moxifloxacin	Vancomycin
Lincosamide	Rifampicin	Fidaxomicine
Microlides	Gatifloxacin	Chloramphenicol
Fluoroquinolones	Clindamycin	Cefoperazone
Ciprofloxacin		Ceftriaxone
Cephalosporin		Cefepime
Erythromycin		
Ceftazidime		
Ceftaxime		
Aminoglycosides		

Table2: Antibiotic activity against *C. perfringens*

Resistant	Intermediate	Susceptible
Beta lactam	Semi-synthetic Penicillin	Penicillin
Cloxacillin	Cephalosporin	Ampicillin
Oxacillin	Sparfloxacin	Amoxicillin
Cephalothin	Grepafloxacin	Metronidazole
Bacitracin		Trovafloxacin
Colistin		Gatifloxacin
Tetracycline, Doxycycline		Moxifloxacin
Viirginiamycin		Chloramphenicol
Macrolides		Vancomycin
Lincosamide		Ceftriaxone
Ciproflaxacin ,Ofloxacin		
Levofloxacin, Fleroxacin		
Pfloxacin, Enoxacin,		
Lomefloxacin		
Clindamycin		
Erythromycin		
Aminoglycosides		

Table 3: Antibiotic activity against *C. tetani*

Resistant	Intermediate	Susceptible
Co-Trimoxazole	Macrolides	Penicillin
Erythromycin	Clindamycin	Metronidazole
Ofloxacin		Chloramphenicol
		Tetracycline
		Cephalosporin
		Cefaperazone

Table 4: Antibiotic activity against *C. chauvoei.*

Resistant	Intermediate	Susceptible
Lincomycin	Neomycin	Chloramphenicol
Metronidazole	Kanamycin	Tetracycline
Bacitracin	Ampicillin	Baquiloprin/ Sulphadimidine
		Erythromycin
		Gentamicin
		Sulphonamides
		Penicillin

Table 5: Antibiotic activity against *C. sordellii*

Resistant	Intermediate	Susceptible
Aminoglycosides	Clindamycin	Benzyl Penicillin
Streptomycin	Lincomycin	Ampicillin
Kanamycin		Carbenicillin
Neomycin		Co amoxiclav
Tobramycin		Cefoxitin
Gentamicin		Erythromycin
Tetracycline		Metronidazole
Oxytetracycline		

The susceptibility of different drugs to *C. chauvoei* has been 100% against chloramphenicol, 93.7% for baquiloprim/ sulphadimidine and tetracycline, 93.5% for erythromycin, 87.5% for gentamicin, 87.7% for sulphonamides and 75% for penicillin. The less susceptible antibiotics included 55.2% for neomycin, 43.7% for kanamycin, and 62.5% for ampicillin. The species showed resistance against bacitracin, metronidazole, and lincomycin (Rais et al. 2016). Another study reported sensitivity against penicillin, oxytetracycline and chlortetracycline. Moreover, it is important to administer the drug both locally and systemically during the early stages of disease onset.

Clostridium. sordellii

C. sordellii cause severe infections which lead to death in a very short duration. The only way is to have antibiotic therapy at the earliest. A study has shown *C. sordellii* is sensitive to B-lactams including ampicillin, benzyl penicillin, carbenicillin, cefoxatin and coamoxiclav and resistant against cephalothin (Sasaki et al. 2001). Another study using 12 isolates for susceptibility testing has reported all isolates susceptible to Erythromycin and metronidazole except one which has been found resistant. Resistance has also been observed against aminoglycosides i-e, gentamycin, kanamycin, neomycin, streptomycin, and tobramycin (Nakamura et al. 1986). Nakamura and other colleagues in 1986 have shown a *C. sordellii* isolate with high MIC for vancomycin. Clindamycin and lincomycin also behaved differently in different studies. Perhaps one reason might be the difference in the methods used for susceptibility testing. Brazier et al., have used the disc diffusion method for sensitivity testing and found that 50% of the tested isolates were resistant to clindamycin. The other studies carried out on a panel of 12 and 24 isolates have used the agar dilution method and have found no resistance against clindamycin. Similarly, Nakamura has reported complete sensitivity to lincomycin against all 24 isolates of *C. sordellii* (Dornbusch et al. 1975; Nakamura et al. 1986). The other two studies however reported resistant strains. The resistance pattern has also been seen in case of tetracycline antibiotic. Similarly, 100% sensitivity to doxycycline has also been found in a study.

Isolates of *C. sordellii* obtained from malignant edema in cattle were also tested for susceptibility against oxytetracycline. A high resistance pattern was observed in all three confirmed *C. sordellii* isolates. Molecular analysis revealed tetracycline resistance genes namely tetA(P) and *tetB*(P) previously reported only in *C. perfringens* and consist of tetracycline resistance determinant TetP, arranged in a distinctive 17bp pattern and linked transcriptionally. The *tetA*(P) is 46kDa tetracycline efflux protein that causes active efflux of the tetracycline drug from the prokaryotic cell (Bannam and Rood 1999). The *tetB*(P) is 72.5kDa ribosomal protection protein, which dissociates tetracycline from the target and binds to bacterial ribosomes. *tetP* in *C. perfringens* is found on pCW3 (47kb) or other conjugative plasmids (Bannam et al. 2011). There is a lot more to know about the location of tetA(P) and *tetB*(P) genes found in *C. sordellii* isolates.

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CHAPTER 06

CLOSTRIDIAL DISEASES HEALTH PERSPECTIVE IN FARM ANIMALS

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INTRODUCTION

In nature, Clostridium (C.) is a genus of widely distributed commensal or soil-borne bacteria as well as often being a part of the enteric flora of many animals and humans. We tend to pay more attention to pathogenic clostridium species as *C. botulinum, C. chauvoei, C. haemolyticum, C. novyi, C. perfringens, C. septicum*, and *C. tetani*, rather than the commensal members of this genus. Clostridium species are distinguished from other bacteria by their anaerobicity and the presence of heat-resistant endospores. Predisposing conditions are always needed for clostridial infections to occur, i.e., a deep wound or a traumatic injury that compromises the skin or intestinal barrier, or the alteration of the gastrointestinal microbiota because of a change in feed or the treatment of antimicrobial agents. When these conditions are met, the bacteria usually produce toxins which are primarily responsible for the pathogenesis of the diseases caused by the bacteria. Because the bacteria's toxins act quickly, treating diseases caused by them is very challenging. We can classify clostridial diseases and infections into three major categories: neurotoxic diseases, histotoxic diseases, and enteric diseases. Animals that are afflicted with clostridial diseases typically die suddenly, with no apparent symptoms. Bacteria present in animal' intestine can multiply, causing the bacteria to spread throughout the carcass. In addition to putrefaction of carcasses caused by growth of clostridial organisms' postmortem, other pathologies can be affected. So, carcasses must be examined early to make a definitive diagnosis and tested specifically for bacteria and their toxins, with histopathology also crucial. As well as morphological and biochemical characteristics, antigenic specificity of toxins and surface antigens are used to distinguish between the various pathogenic and related species. In order to prevent these diseases, vaccination is the best method for protection.

The current chapter reviews a number of histotoxic and neurotoxic clostridial diseases as well as enterotoxaemia's that have been reported in farm animals. Moreover, it includes information concerning their etiology, epidemiology, and the mechanisms underlying their pathology. Furthermore, it covers treatment, control, and prevention strategies.

Clostridial Histotoxic Infections

Blackleg

Overview

'Blackleg,' or clostridial myositis is a highly fatal and febrile bacterial disease that affects cattle as well as sheep, goats, and infrequently horses. Blackleg breaks out in either the shoulder or the hindquarter then triggers black, edematous, emphysematous, or crepitating swelling of the heavy body muscles, and severe toxaemia and myonecrosis of the skeletal and/or cardiac muscles (Abreu et al. 2018).

Etiology and Epidemiology

An infection occurs when a rod-shaped Gram-positive, spore-forming bacterium called *C. chauvoei* gets into the body. Despite environmental variations and disinfectants, the spores remain viable for many years**.** According to current information, the predominant route of infection in sheep is believed to be penetrating skin or mucosal injuries, but the primary entrance point in cattle is unknown. However, ingestion of contaminated feed or eruption of teeth might allow entry through the alimentary mucosa (Radostits 2007). There is a positive correlation between annual rainfall and incidence of blackleg during warm, wet months. In spite of the lack of understanding of this correlation, it is hypothesized that rain may aid in spores' allocation. Moreover, water saturation supports anaerobiosis coupled with enhanced pasture growth, thus encouraging pastured cattle to consume more feed (Useh et al. 2006). While sheep of any age can potentially be infected, fast-growing cattle under two years of age that are on a great amount of nourishment and are rapidly growing in particular are most susceptible (Radostits 2007; Snider and Stern 2011; Cooper et al. 2016).

Pathogenesis

As previously stated, *C. chauvoei* spores are prevalent in soils, and the disease usually manifests in injured sheep, whereas in cattle, the spores multiply after ingestion before crossing the intestinal mucosa. Spore-intake causes spores to multiply and enter the general circulation, where they are distributed in a variety of tissues, including skeletal muscles (Quinn et al. 2011; Frey and Falquet 2015; Cooper and Valentine 2016).

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Blackleg has been categorized as an "endogenous" clostridial infection since the pathogen seems to be set up in tissue in a dormant state prior to disease happens (Cooper and Valentine 2016).

An anaerobic environment for germination and multiplication is created by local muscle damage ahead of low oxygen tension, which triggers the dormant spores to germinate. As soon as *C. chauvoei* reverts to its vegetative form, it generates a number of toxins, including oxygen-stable and oxygen-labile hemolysins, DNase, hyaluronidase, and neuramidase, causing substantial necrotizing myositis locally and a fatal toxemia systemically (Abreu et al. 2017; Pires et al. 2017).

Clinical Findings

The majority of blackleg cases are acute or sub-acute, though cases can also occur chronically. When acute cases are found, animals are dead without clinical signs, but for some of the cases where clinical signs are evident, such as depression, lethargy, anorexia, and refusal to move, clinical signs are followed by circulatory collapse and death (Groseth et al. 2011; Snider and Stern 2011). Observations of the animal before death usually reveal severe lameness, as well as swelling of the upper part of the leg. At first, the swelling is painless and hot to the touch with discolored skin covering, then quickly it come to be cold and painless, with edema and emphysema being felt and the skin appears dry and cracked (Irisk 2007). When blackleg lesions are present in the limb musculature of sheep, there is a stiff gait, and the sheep experiences lameness in one limb or more along with is reluctant to move. Subcutaneous edema, however, is scarce and gaseous crepitation cannot be felt before death (Radostits 2007). The skin may appear discoloured above and beyond, although there is no skin necrosis or gangrene (Coetzer et al. 1994). Clinically, the syndrome in horses is not well characterized, yet it has been associated with edema of pectoral muscle, stiff gait, and incoordination (Radostits 2007).

Gross and Microscopic Lesions

The carcass of a blackleg-dead animal is often found lying sideways with the affected hind limb sticking out. In a few hours, bloating, putrefaction, and blood-stained froth is exuded from the nostrils and anus (Songer 2004). Acute hemorrhagic inflammation is evident in internal organs such as the lungs, heart, stomach, and intestines. Moreover, local lymph nodes are emphysematous and swollen as a result of acute lymphadenitis. Besides, there is also blood-tinged fluid in the serous cavities and the internal organs display degenerative changes. Swelling is often observed externally in superficial skeletal muscles, as well as stretched and dark skin overlying the affected muscles. Under the skin of affected areas, crepitation can be palpated since the sero-sanguineous fluid, which is accompanied by gas bubbles, usually expands the subcutaneous tissues and fasciae in affected areas (Hogarth 2000). On cutting sections of the affected muscles, dark red to black discolorations are observed, a sweet odor, similar to rancid butter can be smelled along with edema at the periphery of the lesions can be noticed. The center of the lesions is usually dry and friable, with numerous tiny cavities filled with gas bubbles.

Microscopically, there are swollen, hypereosinophilic, vacuolated, fragmented muscle fibers; they have lost cross striations; gas bubbles separate the fascia from the muscular fibers, and hypercontraction bands are often seen. Infiltrated neutrophils may infiltrate fragmented myofibers; however, macrophages, plasma cells, and lymphocytes are gradually replacing the neutrophils as the lesions progress. likewise, Gram-positive bacteria occur singly or in small irregular clumps could be observed. Hemorrhage and proteinaceous edema expand the interstititium, whereas the interstitial vessels contain fibrin thrombi besides arterioles and arteries show fibrinoid necrosis, with fibrin thrombi in the interstitial vessels and fibrinous necrosis in arteries and arterioles along with intramural neutrophil infiltration (Abreu et al. 2018).

Treatment, Prevention and Control

Unless the animal is morbid, penicillin and surgical debridement of the lesion, including fasciotomy, are recommended. Unfortunately, the extensive nature of the lesions, however, limits the recovery rate. In the presence of high fever and a toxemic condition, antiallergic and antipyretic as well as large doses of crystalline penicillin (44,000 IU/Kg BW) should be given intravenously, followed by a longer-acting preparation. Blackleg antiserum, unless very high doses are given, is unlikely to be of much benefit in treatment (Radostits et al. 1994; Constable et al. 2017). In order to prevent the spread of infection, the corpse of an infected animal must be buried deeply.

In order to control blackleg, Bacterins vaccines prepared from *C. chauvoei* cultures that were formalin-inactivated are generally used for vaccination against blackleg. Most of the vaccines can be found in a polyvalent formulation with *C. novyi, C. septicum*, and *C. sordellii*. A vaccination schedule of two vaccinations given four weeks apart followed by a booster vaccination at the end of the first year is generally recommended on farms where the disease is enzootic (Butler 1998; Tolera et al. 2019).

Malignant Edema

Overview

Known also as gas gangrene, malignant edema is a type of necrotizing clostridial infection of horses, sheep, and cattle that is characterized by a short duration, fever, and the presence of hot, painful swelling in the vicinity of the site of infection (Cebra and Cebra 2012; Silva et al. 2016).

Etiology and Epidemiology

Several Clostridium species have been isolated from injuries associated with malignant edema in animals, involving *C. septicum*, *C. chauvoei*, *C. perfringens* and *C. sordellii* (Peek et al. 2003; Silva et al. 2016). Oftentimes, mixed infections occur. Additionally, *C. sordellii* has been linked to malignant edema in cattle, as well as swollen heads in sheep. Infection is caused by gram-positive bacteria that enter the body through skin wounds or mucosal wounds. Like *C. chauvoie*, these organisms generate spores that are resistant to heat, alcohol and acid pH leading to endurance of the infection for extended times in a local area but germinate when subjected to an anoxic environment with the proper nutrients and temperatures. Gas gangrene's clostridial agents, including their spores, are ubiquitous. Animals' intestinal contents, organic matter, and soil containing high levels of humidity are the major sources of

these clostridial agents. Thus, seasonally flooded soils are more likely than dry soils to contain these microorganisms**.** The disease can be sporadic and harm animals of all ages and species in response to injections, while outbreaks in sheep can occur following some management practices such as vaccinations, umbilical cord contamination in newborns, shearing and docking or after lambing. When it comes to cattle, following parturition, lacerations of the vulva are often associated with the precipitating cause, whereas in equine, intramuscular inoculation of medications, usually associated with colic therapy, is the most common cause (Constable et al. 2017).

Pathogenesis

The disease set off when wounds are contaminated with the spores or with the vegetative forms of one or more clostridial species, germination, vegetation, and production of toxins then occur; hemolytic and necrotizing toxins, in particular (alpha toxins), are most threatening. The histotoxic clostridia produce toxins that act locally, causing tissue necrosis, which offers an ideal condition for the continued multiplication of these microorganisms and higher concentrations of toxins, which are ultimately released into the body's circulation, causing toxaemia, shock, and death (Morris et al. 2002; Costa et al. 2007; Popoff and Bouvet 2009; Aronoff 2013; Silva et al. 2016; Sunagawa and Sugitani 2017).

Clinical Findings

Most animals show similar clinical signs, including depression, tachycardia, muscle tremors, and hyperthermia within about six to 48 hours after infection. Usually, when a limb is affected, the animal lame, reluctant to move, and eventually falls to its death within 48 hours. A reddish-brown fluid is released from the vulva within 2-3 days when an infection occurs at parturition. In some cases, swelling extends to the pelvis and perineum. Among young male rams, fighting injuries can cause the development of a clinical condition referred to as "big head." This is a specific type of gas gangrene, distinguished by an edematous swelling of the head, face, and neck that is not gaseous or hemorrhagic (Choi et al. 2003; Costa et al. 2007; Odani et al. 2009; Farias et al. 2014; Silva et al. 2016)

Gross and Microscopic Lesions

In most animals, gas gangrene results in comparable gross changes, with diffuse hemorrhagic and gelatinous subcutaneous edema and emphysema, irrespective of the clostridial species. A soft, doughy swelling is produced at the site of infection accompanied by local erythema, severe pain on palpation, then the swelling becomes more edematous, but less painful. A diffuse edema involving the perineum and perivaginal area of cattle often occurs following postpartum gas gangrene, which, in some cases, extends to nearby muscles. Among sheep and cows, an externally visible line of demarcation dividing affected and unaffected tissue along with severe edema and bleeding of the subcutaneous tissue distinguishes gangrenous myositis. In an edematous or hemorrhagic area, gram-positive bacilli with central, subterminal, or sometimes terminal spores are usually observed microscopically. There may also be infiltrated inflammatory exudate, mainly neutrophil-based, as well as vasculitis, and thrombosis, which may extend to the fasciae and muscle tissue, in addition to necrosis of adjacent skeletal muscle similar to that found in cases of blackleg. An unpleasant, putrid smell is frequently present with infections caused by *C. perfringens* and *C. sordellii*. Edema fluid is generally blood-stained and contains gas bubbles, apart from in *C. novyi* infections where it is gelatinous, clear, and without gas bubbles. It is not uncommon for subcutaneous hemorrhages and serosanguineous fluid to accumulate in body cavities. A ram's "swelled head" can involve the entire neck and head, as well as the pleura, the chest, and the lungs (Cooper and Valentine 2016; Constable et al. 2017).

Treatment, Prevention and Control

Locally, penicillin inoculation immediately into and across the edge of the lesions are often suggested with irrigation with hydrogen peroxide and iodine solution. In order to control the toxemia on a systemic level, high dosages of procaine penicillin G, repeated at 4-6 hours intervals along with antitoxin drugs, fluid therapy, and anti-inflammatory drugs should be given as soon as possible in the course of the illness (Constable et al. 2017). Vaccination of the animals, strict hygienic measures at lambing, shearing, castration, docking, navel treatment with antiseptics (acridine dyes, betadine lotion, etc.), as well as avoidance of soil or fecal contamination of wounds are all part of a proven approach to disease prevention. When gas gangrene is endemic in an area, animals aged 4 to 6 months or younger should be vaccinated with bacterin toxoid twice, with a gap of four weeks between each vaccination (Boyd et al. 1972; Barnes et al. 1975; Lewis 2011; Parish et al. 2019; Oliveria Jounior et al. 2020).

Bovine Bacillary Hemoglobinuria

Overview

Bacillary hemoglobinuria (BHU; red water disease) is an acute, toxemic, and often lethal disease of cattle, less frequently noticed in sheep, and it has been notified rarely in horses and pigs. It is marked by intravascular hemolysis, sudden onset of hemoglobinuria, hepatic infarction, and death within one to two days (Oliver and Staempfli 1999; Radostits 2000; Shinozuka 2011).

Etiology and Epidemiology

The strict anaerobic *C. haemolyticum* (*C. novyi* type D) is a Gram-positive, motile, and sporulated rod (Navarro and Uzal 2016) causes BHU, and its primary virulence factor is beta toxin, a phospholipase C (Oliver and Staempfli 1999; Radostits 2000; Shinozuka 2011). Under anaerobic conditions, it produces a necrotic and hemolytic beta toxin causing damage to the hepatocytes as well as capillaries endothelium.

Bacillary hemoglobinuria is usually sporadic, but it can be endemic in areas with a high prevalence of fascioliasis. Flooding, drainage, polluted hay from infected areas, or carrier animals spread the disease from infected areas to noninfected ones. Conditions of dry weather may affect animal movement patterns, which may result in animals converging on areas of pastures that have liver fluke infestations, such as those located around small ponds, drains, dams, and swamps, thus being more prone to exposure to metacercariae. The disease is more common in animals over the age of one year, in good nutritional condition and are often introduced to infected areas recently.

Pathogenesis

C. haemolyticum spores are soil-borne, they can withstand to exposure to environmental conditions and remain viable for several years (Jasmin 1947). Usually, the disease is caused by ingesting contaminated materials and after multiplying in the intestinal tract, bacteria enter the bloodstream, phagocytized by Kupffer cells, and then remain latent in the liver until hepatic damage renders the environment anaerobic (Van Kampen and Kennedy 1969). In addition to causing liver necrosis, migrating flukes also cause anaerobic conditions in the liver, which allow it to germinate, multiply, and produce toxins such as phospholipase C (hemolytic beta toxin), causing hepatocellular necrosis and intravascular hemolysis (Cullen and Stalker 2016; Navarro and Uzal 2016). As a result of beta toxin action, the arachidonic acid cascade is activated, thromboxane and leukotrienes are produced, platelets aggregate, and capillary permeability increases. Infarcts with large anemic area are typical of the disease both because of hepatocyte necrosis and thrombus formation due to endothelial disruption. Among the main clinical features of hemolysis are hemoglobinuria and jaundice, as well as severe hypoxia that leads to hemoglobinuric nephrosis (Navarro and Uzal 2016).

Clinical Findings

Clinically, it may present as either an acute form lasting 10 to 12 hours, or a subacute form lasting 3 to 4 days. Both forms are characterized by hemoglobinuria, jaundice, and high fever (40 to 41°C) that decreases rapidly at the time of death (Oliver and Staempfli 1999). A marked reduction in hemoglobin concentration, hematocrit, and red blood cell count is associated with hematological changes, followed by leukocytosis. Furthermore, symptoms include decreased rumination, lactation, defecation of dark brown feces, an increased heartbeat, severe dyspnea, and edema of the brisket (Van Kampen et al. 1969; Navarro 2017).

Gross and Microscopic Lesions

Macroscopically, it is characterized by rapid rigor mortis, soiled perineum with bloodstained urine and feces, subcutaneous gelatinous edema, and severe petechial or diffuse hemorrhages are common features. However, pathognomonic features of BHU are a large, usually single area of necrosis delimited by a hyperemic rim, typically seen at the liver's diaphragmatic surface. Microscopic examination of hepatic lesion reveals focally extensive coagulative necrosis with a rim of inflammatory cells mainly neutrophils, and fewer lymphocytes and plasma cells surround the necrotic tissue with large numbers of Gram-positive rods, mostly along the inner margin of the leukocytic rim in sinusoids of necrotic areas. A homogeneous or globular acidophilic substance occupies the glomerular spaces and tubular lumen, giving the kidneys a mottled appearance (speckled red or brown by hemoglobin). Furthermore, the tubule lumens contain varying levels of eosinophilic granules and protein casts as a result of epithelial degeneration and necrosis. Therefore, the urine in the bladder appears deep red colored (Ahourai et al. 1990; Hussein et al. 2013; Navarro 2017).

Treatment, Prevention and Control

If available, special treatment is immediate administration of antitoxic serum with Procaine penicillin G, plus supportive treatment such as blood transfusions, parenteral fluids, and mineral supplements containing iron, copper, and cobalt. Treatment must be in the early stages, unfortunately, there is not enough time to initiate treatment, and even when it is attempted, the success rate is low (Crowe et al. 1989; Oliver and Staempfli 1999; Shinozuka 2011). During an outbreak, it may be more useful in preventing the disease in animals that haven't been vaccinated. Considering the disease's association with liver flukes, reducing parasitic burdens and restricting access to swampy and poorly drained pastures have been very effective in reducing BHU incidences (Kahn and line 2005). Burning or deep burial is also suggested for disposing of animals who have died of BHU.

Black Disease

Overview

Infectious necrotic hepatitis (INH) is a bacterial infection, more commonly known as black disease in livestock, that occurs most often in sheep, infrequently in cattle, and is caused by a cytotoxin of *C. novyi* type B. It produces focal areas of coagulative necrosis in the liver (Cullen and Stalker 2016).

Etiology and Epidemiology

C. novyi type B, the etiologic agent of infectious necrotic hepatitis, a soilborne organism found in the intestinal microbiota and in herbivores' livers. It is also capable of infecting wounds by resting on the surface of the skin or lying dormant in muscle. Infection occurs via fecal-oral route and spread from farm to farm by transport of contaminated soil during flooding or by infected wild animals and birds. Adult animals are more susceptible to INH, while young animals are not affected as often. In many countries, outbreaks take place during the summer or autumn months and are correlated with liver damage caused by fascioliasis, and typically end within a few weeks after a frost since the encysted metacercaria is destroyed by the freezing

Pathogenesis

During infection, *C. novyi* passes through intestinal walls, settles in the liver, and remains dormant until local anaerobic conditions, such as those created by migrating flukes, which then promote *C. novyi* to multiply and produce alpha-toxin, which is necrotic and causes further liver necrosis and widespread damage to the blood vessels. In some cases, nervous signs can occur as a result of this general vascular disturbance or from the effect of specific neurotoxin.

Clinical Findings

Clinical signs are usually non-specific, such as weakness, restlessness, drowsiness, anorexia, hyperthermia, tachypnea, tachycardia, and recumbency are among the common symptoms. Affected sheep can die within a few hours of disease onset without any clinical signs being present. Cows show the same signs as sheep, but the course is more prolonged, the

feces are semifluid, feces are semi-fluid sometimes mixed with blood.
Peritonitis, severe progressive toxemia, depression, reluctance to walk, pain on palpation of the abdomen, frequent straining, and recumbency are the common symptoms displayed by horses with INH. They may also have variable degrees of jaundice, tachypnea, hematuria, and, rarely, neurological signs such as ataxia and head tilt. Jaundice is unexpectedly seen in horses but not in ruminants. This may be because horses are more susceptible to the action of C. novyi beta toxin (Smith 2015; Whitfield et al. 2015; Navarro and Uzal 2016).

Gross and Microscopic Lesions

A characteristic feature of this disease is hemorrhagic subcutaneous edema and congestion in ventral regions of the carcass that result in a very dark appearance, earning the name "black disease". A large amount of straw-colored fluid or blood-tinged fluid has been observed to accumulate in the pericardial, pleural, and peritoneal cavities as well as petechial hemorrhages on the epicardium and endocardium. The liver is swollen, gray-brown, and shows characteristic yellow necrotic areas approximately one to two cm in diameter that are bordered by red areas of hyperemia. On microscopic examination, the liver lesion appears as an eosinophilic inflamed fluke tract encircled by coagulation necrosis, dilated and congested vessels, and neutrophil infiltration. At the margin of the lesion, large, gram-positive bacteria are found just inside a zone of neutrophil infiltration (Nyaoke et al. 2017).

Treatment, Prevention and Control

Besides supportive care, procaine penicillin is given in high doses. The produced toxin may, however, make antibiotics ineffective (Cebra and Cebra 2002; Smith 2015). Vaccination is repeated every four to six weeks on affected farms, followed by annual vaccination. In outbreaks, vaccination with alum-precipitated toxoids is highly effective. By controlling the liver fluke and destroying the snails in streams and marshes by using molluscicides, this disease can be controlled (Hjerpe 1990).

Clostridial Neurotoxic Infections

Tetanus

Introduction

Tetanus, otherwise known as Lock Jaw, gets its name from the Greek word 'tetanos,' meaning 'contract'. Rather than a transmissible disease, it is a neurologic condition that has a worldwide distribution in humans and animals and results from the intoxication of the nervous system with the exotoxin of *C. tetani* and is characterized by persistent spasmodic contractions of the entire body musculature without impairment of consciousness (Bleck 1991; Hassel 2013).

Etiology and Epidemiology

As a result of wound contamination with soil-borne, sporulating, anaerobic bacterium infection by *C. tetani*, clinical disease often arises from tetanus neurotoxin (Bleck 1991; Hassel 2013; Popoff 2020). All farm animals are susceptible to tetanus throughout the world. The susceptibility of animal species to tetanus, however, varies considerably between them. The most susceptible species are horses, guinea pigs, monkeys, sheep, mice, goats, and humans but less sensitive species are cats and dogs, and birds are relatively invulnerable (Aslani et al. 1998; Wernery et al. 2004; Driemeier et al. 2007; Popoff 2020). A deep puncture wound, including those in the hoof, is commonly employed as the portal of entry in horses. Further, after castration in young pigs, as well after shearing, docking, vaccinations, or injection of pharmaceuticals, particularly anthelmintic drugs, among lambs. Additionally, insanitary conditions at parturition can cause tetanus to develop in the newborn (Linnenbrink and Macmichael 2006; Smith and Sherman 2009; Kumar Das et al. 2011; Pugh and Baird 2012).

Pathogenesis

When the oxygen tension in the local tissue decreases, the tetanus bacilli proliferate and produce tetanolysin and tetanospasmin. Whenever tetanolysin triggers local necrosis, surrounding tissues are invaded and tetanospasmin is brought into the bloodstream, bound to motor endplates, transported retrogradely by intra-axonal transport, inhibiting the release of neurotransmitters such as glycine and g-amino butyric acid, thereby hindering the inhibitory spinal interneuron activity. Inhibitory neurotransmitters act to inhibit the actions of excitatory nerve impulses from upper motor neurons. If normal inhibitory mechanisms cannot inhibit these impulses, generalized muscle spasms, and as a result, death by asphyxiation occurs (Wernery et al. 2004; Lotfollahzadeh et al. 2018).

Clinical Findings

An infection usually incubated for 3-4 weeks but may persist for several months afterward. At the beginning, muscle stiffness is accompanied by muscle tremor and the affected animal may continue to eat and drink, but tetany soon prevents mastication. Muscular tetany increases with the progression of the disease, which causes the animal to assume a "saw horse" posture. An unsteady, straddling gait caused by stiff hind limbs could also be noticed.
In all except sheep, quick movement of the third eyelid across the cornea before slow retraction is one of the initial and most reliable indications. Among young cattle, bloat is an initial sign but not always harsh and usually associated with frequent powerful rumen contractions. Infected horses initially display signs of colic and muscle stiffness in the lips, nostrils, ears, jaw (lockjaw), and tail. Death occurs as the entire musculature is affected. Horses and cattle usually die from a fatal illness within 5-10 days, but sheep usually die at about the third or fourth day.

Gross and Microscopic Lesions

The entry wound is the only visible change in animals dying of tetanus. Microscopically, there is tygrolysis of the C.N.S. neurons and a tabby-cat appearance caused by fatty changes in the myocardium

Treatment, Prevention and Control

For treating infections, penicillin G or metronidazole are the

best antibiotics, but they will not affect an existing disease. Although tetanus does not have a specific treatment, Anti-tetanus neurotoxin antibodies block free tetanus neurotoxin from entering neurons, but anti-toxin antibodies can't control toxin that has been taken up into neuronal cells. Once the infection site is found, a wound must be aggressively cleaned and debrided, but only after antitoxin is administered, as debridement, irrigation with hydrogen peroxide, and the use of local penicillin may facilitate toxin absorption. To avoid injury if convulsions occur, keep the affected animals calm and provide them with a calm, dark room with non-slip flooring. The injection of 1500 IU tetanus antitoxin can be effective for short-term prophylaxis. Furthermore, inactivated toxoid requires two doses 3-6 weeks apart. A protective antibody titer can be obtained within 14 days of the second injection and lasts for at least a year and up to 5 years (Acke et al. 2004; Sprott 2008).

Botulism

Overview

Botulism is a neuromuscular disorder that causes profound generalized flaccid paralysis in most of animals, and is often regarded as a zoonotic disease since foodborne botulism can end up infecting humans.

Etiology and Epidemiology

The disease is caused by one of seven neuroparalytic toxin subtypes (A to G) produced by *C. botulinum* (Galey et al. 2000; Ettinger and Feldman 2004; Bohnel and Gessler 2010), spore-forming anaerobe. Mammals, birds, and fish are typically affected; horses are mainly vulnerable to type B toxins; cattle and sheep are typically affected by types C and D. Under environmental circumstance, spores can survive for over 30 years, nonetheless if moist and warm conditions occur, the spores will germinate and revive vegetative cells that release a stable highly lethal toxin that blocks the release of acetylcholine at the neuromuscular junction, causing flaccid paralysis. Throughout drought times when feed is scant, phosphorus intake is minimal, and carrion is ample, silage-associated botulism outbreaks are most likely to occur. The majority of botulism cases are caused by ingestion of preformed botulinum toxins; additionally, toxins in feed can be caused by direct growth of *C. botulinum* in feeds (forage botulism) or contamination of feeds with toxin-containing carrion (carrion-associated botulism), while other less common ways to get botulism are through a wound or toxins produced by the growth and infection of the alimentary tract (toxicoinfectious botulism) (Rings 2004; Braun et al. 2005; Radostits 2007).

Pathogenesis

Soil and animals' gastrointestinal tracts can harbor *C. botulinum* spores. Under anaerobic or alkaline conditions, intestinal or wound-borne botulinum toxins are absorbed by the circulatory system and reach peripheral cholinergic terminals, and peripheral ganglia, causing functional paralysis without causing any pathological changes (Aoki et al. 2010). A toxin's heavy chain binds to receptors and translocates inside cells; the toxin's light chain blocks the release of acetylcholine at the neuromuscular junction as a result. The animal dies of respiratory failure after developing flaccid paralysis of the diaphragm (Lobato et al. 2013).

Clinical Findings

It can take from 18 hours to 17 days for botulism to develop. Acute cases can result in sudden death, whereas chronic ones aren't. When a cow becomes botulism-infected, it has difficulty moving, digestive problems, weakness in the hind limbs and sternal recumbency; while in horses, there is paralysis of muscles in the limbs, mandibles, larynx, pharynx, eyelids, tongues, and tails, usually end also with sternal recumbency. As the affected animals lie in sternal recumbency, they rest their heads on the ground or on their flanks, similar to a cow with parturient paresis while sheep hold their heads tipped to one side or move up and down while walking (limber neck). Furthermore, dysphagia and tongue weakness can also be seen in these sheep. Sometimes, the tongue will become paralyzed and hang from the mouth, incapable to grind or swallow, and will drool saliva (ACMSF 2006; Sherein 2013; Alemu and Ayele 2018).

Gross and Microscopic Lesions

The occurrence of suspicious feedstuffs in the stomach or forestomach can be a signal even if no specific changes are observed during necropsy. Jejunal hemorrhages can occur infrequently, especially in cattle. Horses with botulism types A and C have been reported to have edema of the nuchal ligament, which may result from weak neck muscles making it difficult for the horses to keep their heads up (Chao et al. 2004; Alemu and Ayele 2018).

Treatment, Prevention and Control

Advanced stages of botulism usually do not respond to treatment, and euthanasia is often recommended. In affected mammalian species, antitoxins can be given, but this approach must be applied before the toxins interact with the neuromuscular junction. Specific or polyvalent antiserums can be highly effective when given early in the course along with high quality fluid physiotherapy, enteral or parenteral nourishment, and mechanically ventilated if needed with inhaled oxygen nasally. It is important to dispose of carcasses hygienically in order to avoid further contamination of pastures. When herds are infested, vaccination can be effective (Beran 1994; Fitzpatrick and Katherine 2006; Nusair et al. 2009; Desta et al. 2016).

Clostridial Infections of Gastrointestinal System

Braxy

Overview

"Braxy" is another name for hemorrhagic abomasitis (gastritis) that occurs in sheep as well as other ruminants, which often results in rapid mortality with no or few symptoms (Cebra and Cebra 2012).

Etiology and Epidemiology

A gram-positive soil-borne bacillus called *C. septicum* causes

this infection, which is occasionally linked to other gastrointestinal infections as well as wound infection. During winter when grasses are of poorer quality, sheep may change their grazing behavior, resulting in increased consumption of woody forages and soil that holds *C. septicum* spores. As a result, this may damage the abomasal mucosa, thereby providing an entry point for *C. septicum* that may result in fetal toxemia in yearlings and weaner sheep.

Clinical Findings

After onset of disease, animals suffer from fever reaching 42°C followed by recumbency and death within 12–36 hours. Also, there may be sudden onset of symptoms, such as complete anorexia, depression, and bloating and distension of the abdomen. When an animal is comatose, there may be a bloody discharge from its nose

Gross and Microscopic Lesions

Macroscopically, abomasal wall is markedly edematous, thickened, congested, and hemorrhagic with blood-tinged fluid within the abdomen and abomasum (Maria et al. 2009). Upon microscopic examination, there is extensive necrosis, ulceration, edema, and congestion of the mucosa and submucosa. Besides, in the lamina propria and submucosa, there is heavy neutrophilic infiltration. Similarly, the small intestine is hemorrhagic, swollen, edematous, and necrotic.

Treatment, Prevention and Control

In most cases, antibiotics such as penicillin G cannot cure this disease because of its rapid nature. Disease at flock level, however, is usually sporadic, and can be prevented with clostridial bacterin toxoid vaccines administered to animals at risk on a routine basis.

Enterotoxaemia with type A *Clostridium perfringens*

Traditional associations with yellow lamb disease are strains of *C. perfringens* type A that release high-level of alpha toxin. Anecdotal evidence suggests a few cases occurred in South America, but the condition has been discovered mainly in the United States and Europe. As a result of the hemolytic lecithinase (phospholipase) alpha toxin, yellow lamb disease is associated with hemolysis and jaundice and characterized by depression, anemia, icterus, and hemoglobinuria, with sudden death occurring in rare cases. No specific findings can be identified during necropsy in animals with yellow lamb disease. In addition to generalized icterus and hydropericardium in the heart, red urine in the bladder, and an enlarged, pale and friable spleen are the most commonly defined gross findings. Upon microscopic examination of the liver, hepatocellular necrosis has been noted in the mid-zone central to the mid-zone areas and bile stasis has been observed in the bile canaliculi. The kidneys appear microscopically to contain multiple hemoglobin casts in the tubular lumens. In addition, the kidney's proximal and distal convoluted tubules are filled with eosinophilic, multifocal, and granular intracytoplasmic hyaline droplets. As of now, vaccines are not currently able to prevent yellow lamb disease; however, there is a chance of protection from vaccines meant for different types of *C. perfringens*.

Enterotoxaemia with type B *Clostridium perfringens*

Infections with *C. perfringens* type B have been reported in the Middle East, Europe, and South Africa. Usually, it infects lambs up to the second week of their life, calves of the comparable age, and foals in their earliest few days of life, causing lamb dysentery. Unlike cattle or horses, lambs suffer from an extremely acute disease, which often results in death without warning. There is usually watery bloody diarrhea, reluctance to suckle, and abdominal pain normally accompanying the illness. An older lamb may be suffering from an extremely chronic form of the disease, exemplified by depression, conditional loss, and reluctance to suckle. Opisthotonus, blindness, and absence of coordination are occasionally seen as neurological signs. It is characterized by deep-seated necrohemorrhagic or ulcerative enteritis with deeply penetrating, irregular, well-defined, mucosal ulceration bordered by a rim of hyperemia, with a fibrinous pseudomembrane overlying the peritonitis. Hyperemic intestinal mucosa and coagulative necrotic yellow necrotic areas may reach the muscularis as well as serosa and are usually non-sporulated, large, Gram-positive rods in the lumen. Blood usually stains the intestinal contents because of thrombosis of mucosal blood vessels. Occasionally, animals stay alive for a few days without hemorrhaging in more chronic cases. Possibly there will be a little blood or serous-stained fluid in the peritoneal cavity. In the epicardium and endocardium, there are petechiae and ecchymoses, as well as hydropericadium. Vaccination of the dam just before parturition, which lasts approximately 4 to 6 weeks, is an essential measure due to the high mortality rate among animals at very early ages. Afterward, a double vaccination should be given four to six weeks apart, followed by an annual booster.

Enterotoxaemia with type C *Clostridium perfringens*

Struck is a *C. perfringens* infection of type C, which causes hemorrhagic inflammation in the intestine with ulcerations, ascites, and peritonitis in adult feedlot ruminant early in the spring and winter. Associated with *C. perfringens* type C, enterotoxic hemorrhagic enteritis can occur in piglets within the first eight hours of life, causing the entire litter to die whereas calves, lambs, and foals may show no symptoms or suffer from bloody diarrhea during their first few days. During necropsy, hemorrhagic or necrotizing enteritis, frequently with gas inside the lumen as well as within the walls, may be found. Alpha and beta exotoxins are produced by *C. perfringens* type C, and neonates are predisposed to beta toxin since colostrum contains trypsin inhibitors. Consequently, excessive milk consumption in a protease-deficient digestive system may allow clostridial organisms to rapidly thrive, attach to villus tips, lyse the cells and produce beta toxin causing necrosis to enterocytes, which then extends downward leading to hemorrhage, necrosis, and edema of the lamina propria, and finally death due to diarrhea or secondary bacteremia and toxemia. As a result of the severity of the disease, treatment is usually ineffective, but if attempted, specific hyperimmune sera and oral antibiotics may be used. Vaccinating pregnant dams during the last third of pregnancy, first with two shots one month apart, then annually thereafter, is the best way to control the disease. In livestock born to unvaccinated mothers, antisera should be administered immediately after birth when outbreaks occur.

Enterotoxaemia with type D *Clostridium perfringens*

Overview

"Overeating disease" or pulpy kidney infection, produced by *C. perfringens* type D toxins, is a serious infectious globally distributed condition of sheep, goats, and calves. Grain overload (starch) and sudden dietary changes may contribute to the disease that affects the small intestine (Quinn et al. 2002). Historically, focal symmetrical encephalomalacia (FSE) was used to refer to the diseases that are one form of type D enterotoxemia of sheep, and sometimes it is yet used today when discussing these forms.

Etiology and Epidemiology

In small intestine enterotoxemia, *C. perfringens* type D produces a variety of toxins, among them the Epsilon toxin, which is the 3rd powerful clostridial toxin following botulinum and tetanus toxin, and it causes vascular damage and neuropathy characteristic of this condition (Niilo 1980; Jemal et al. 2016). Typically, lambs are the most susceptible, calves and goats also commonly contract the disease, horses are less vulnerable while adult cattle, deer, and domesticated camels, are rare cases. Between the ages of 3 and 10 weeks in lambs, enterotoxemia is most common, and the same risks apply to calves between the ages of 1 and 4 months. Foraging on lush, fast-growing grass or young cereal crops, intense grain nutrition, and abrupt dietary changes are among the conditions that favor the disease (Uzal et al. 1994). Under these conditions, it has been called 'overeating disease'. Following heavy rains, the disease often manifest within 5-14 days of a flock being introduced to lush pastures.

Pathogenesis

Despite large numbers of *C. perfringens* type D ingested, many are obliterated in the rumen and abomasum. However, a few make it to the duodenum alive, where they multiply and produce an epsilon prototoxin that is triggered by trypsin/chymotrypsin to produce an epsilon toxin. A number of conditions must be met before the disease can begin, such as excessive amounts of starch rich nutrient passing into the duodenum after sheep eat too much grain or are abruptly switched from a largely roughage-based ration to one that is mostly grain-based; furthermore, slowing of alimentary tract movement that allows excess toxin accumulation. Microvascular endothelial impairment caused by Epsilon toxin of *C. perfringens* type D results in boosted vascular permeability and harsh vasogenic edema. A part of the toxin may enter the brain after the blood-brain barrier breaks down, damaging neurons directly. As well, increased intracranial pressure and significant neurologic impairment are the consequences of generalized edema in the brain.

Clinical Findings

Lambs are frequently found dead within 2 to 12 hours without any previous signs of illness. Close observation of a flock may reveal symptoms such as yawning, dullness, depression, and loss of interest in feeding. Those who survive exhibit green, pasty diarrhea, staggering, recumbency, opisthotonos, frothing at the mouth and severe clonic convulsions. Most adult sheep will survive up to 24 hours, during which they may show staggering and knuckling, salivation, convulsions, muscle tremor, rapid, shallow and irregular breathing, and bloat in the terminal stages.

The symptoms exhibited in cattle are comparable to those in sheep and are primarily neurological in nature. Acute cases show bellowing, mania, and persistent convulsions, whereas peracute cases die without any signs of illness. Cases that are subacute tend to be calm, docile and go blind for 2 to 3 days, then recover (Blood and Henderson 1974; Jemal et al. 2016).

Gross and Microscopic Lesions

A typical examination of the dead animal shows fecal dirtiness of the perineum besides quick decay of the carcass, the presence of a clear, straw-colored pericardial and thoracic fluid that clots as soon as exposed to air; petechial hemorrhages are observed in the cardiac epicardial layer, endocardial layer; and there are patches of congestion in the abomasal and intestinal mucosa. In freshly examined specimens, the "pulpy kidney" lesion may not be noticeable. It is not considered to be a useful investigative finding (Itodo et al. 1986; Blackwell et al. 1992; Radostits et al. 2007; Jemal et al. 2016). On microscopic examination of the brain tissue, encephalomalacia is evident with necrotic and degenerated neuronal and neuroglial cells, spongy neuropil, axonal swelling, increased gitter cells and lymphoplasmacytic infiltration together with vascular changes including hypertrophic endothelium, hyalinized arterial walls, lymphoplasmacytic perivascular cuffing, capillary hemorrhage.

Treatment, Prevention and Control

Sheep are too acutely ill for effective treatment to be effective. It may be possible to treat goats with antitoxin plus sulfadimidine if the course lasts longer. As soon as an outbreak begins, you can administer antitoxin to all sheep, which will provide protective levels of circulating antitoxin for 21-29 days. When ewes are vaccinated twice a month, with the last vaccination occurring around one month before lambing, they will develop good passive immunity in young lambs and have positive levels of protective antibodies by the time they are eight weeks old. When lambs are between four to ten weeks old, they need to be vaccinated with toxoid and then again, one month later. While waiting for vaccination immunity to develop, decreasing food intake is an effective means of short-term control measure (Pawaiya et al. 2020).

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CHAPTER 07

PATHOGENESIS AND PREVENTION OF PORCINE CONTAGIOUS PLEUROPNEUMONIA

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INTRODUCTION

Porcine infectious pleuropneumonia has brought significant economic losses to the pig industry, so it has been listed as one of the important diseases endangering the pig industry in the world. After the pigs are infected with APP, they can be divided into acute and chronic diseases according to their clinical course. It mainly exists in the lungs and tonsils of diseased pigs, and pigs are the only host. All breeds and day-old pigs can be infected with the disease. Three to five months old pigs are susceptible to the disease, and adult pigs often show recessive process. The disease can occur all year round, such as winter and spring cold season or climate change season. The disease can be easily induced by high density of pigs, poor ventilation of piggery, poor sanitary conditions, or by transport, mixing, long-distance transportation, weather mutation and other stress (Blackall et al. 2002). The disease spread mainly at the respiratory level but can also spread by direct contact. Infected pigs will spread APP into the air when they cough or exhale and infect other healthy pigs through air droplets. In the acute stage, pig secretions contain a lot of pathogens, which can be carried by pig farm staff through clothes, shoes or tools.

The disease usually occurs in sporadic or small-scale outbreaks. In the acute outbreak stage, the disease can spread by "jump" in fat pig herds, with high incidence in large-scale intensive pig farms. The morbidity and mortality were different in different pig farms and different strains. The morbidity and mortality were higher in pigs with the first disease and tended to moderate after a period of time. The morbidity was generally 8.5-100% and the mortality was about 0.4-100%. Acute pleuropneumonia is characterized of dyspnea, frequent standing or sitting posture, foam-like secretions on the nose and mouth, blue-purple skin on the ears, nose and extremities, temperature up to 41.5℃, and high mortality (Rossic et al. 2013). Cellulosic necrotizing tinea plantar pneumonia is a typical symptom of chronic pleuropneumonia. Pigs with chronic pleuropneumonia may experience slow growth, but the mortality rate is low.

Characteristics of *Actinobacteria pleuropneumoniae*

Actinobacteria pleuropneumoniae (APP) is typically a rod shape, with capsule or slender small bacteria, polymorphism. The bacteria in the material can be bipolar color, facultatively anaerobic, with no spore formation and no motility. APP currently has a total of 15 serotypes, which can be divided into biological type I and biological type II based on the growth dependence on NAD (Nicotinamide Adenine Dinucleotide, nicotinamide adenine dinucleotide, also known as v factor). Except for serotypes 13 and 14 belonging to organism II with NAD-independent growth, the remaining 12 serotypes were NAD-dependent organism I and all associated with porcine pathogenesis.

Biological Characteristics

The main pathogenic factors of APP include Apx exotoxin, ApxI, Apx, capsular polysaccharide, lipopolysaccharide, outer membrane proteins, and transiron-binding proteins.

Exotoxin

At present, there are four kinds of Apx exotoxins found in APP: ApxI, ApxII, ApxIII and ApxIV, which all belong to the family of cell pore-forming proteins, with leukocytes to toxicity and hemolysis, which can damage the host defense mechanism and cause damage to host cells and tissues. Apx high concentration of exotoxin can perforate the cell membrane of phagocytes and other cells, which can effectively escape the relevant defense mechanisms and cause cell swelling and death due to altered osmotic pressure.

The major virulence factor of APP pathogenesis is its extracellular exotoxin-Apx toxin. Apx is a substance that acts on alveolar macrophages and suppresses the phagocytic activity of alveolar macrophages, which is thought to be one of the major causes of infection and severe damage to the lung tissue. Apx toxins, include ApxI, ApxII, ApxIII and ApxIV (Bode et al. 2003). ApxI can cause the disease to develop typical lung lesions, which is also the strongest virulence factor causing infectious pleural pneumonia in pigs. Apx is secreted by all serotype APP, and the toxin has so weak virulence that Apx expression is only induced by APP in animals, but not on any other medium. This is the only gene found in APP which can be expressed *in vivo*. Apx has strong specificity and does not cross-react in species genera closely associated with *Actinobacter pleuropneumoniae*. Once the pig is infected with APP, any serotype can respond to the expressed Apx. If

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biologically active Apx toxin, four genes CABD operon are required in certain order. C gene encodes toxin structural protein, A gene encodes toxin-activated protein, translational protein products of B gene and D gene produce transmembrane channels on the cell wall, responsible for the toxin from cell to cell (Qian et al. 2002).

Characteristics of ApxI

Apx I, expressed and secreted by serotypes 1,5,9,10 and 11, has strong hemolytic activity, and strong cytotoxic effects on alveolar macrophages and neutrophils, and is one of the potent protective antigens for APP (Meyns et al. 2007). The production, activation, and secretion activity of ApxI is regulated by an operon that includes the above four complete CABD genes: ApxIC, ApxIA, ApxIB and ApxID, a typical operon of RTX toxin, and the product of ApxI is responsible for secretion to extracellular (Nkando et al. 2012; Li et al. 2022). Serotypes I, 5a, 5b, 9, 10 and 11 are clinically APP outbreak serotypes with severe lung injury and high mortality, and strong hemolytic and cytotoxic ApxI because they have intact ApxI, while serotypes 2,4,6,7,8 and 12, although not producing ApxI, have a truncated ApxI retaining the original promoter whose expressed products have secretory functional (Nielsen R et al. 2000). It can be seen that ApxI toxin plays a dominant role in most serotypes. But the diagnostic method of ApxI is poorly specific and it is difficult to detect all serotypes of APP and it cross-reacts with other *Actinobacterium* (Liu et al. 2009). For example, the homology of ApxI with *Escherichia coli* HlyA and *Pasteurella multocida* was 60% and 44% (Wei et al. 2012) Therefore, the accuracy of the diagnosis and treatment of the disease is very difficult. In the early 20th century, the genetically engineered expression product of ApxI was applied to subunit vaccines, and many researchers studied its protective pyrogenicity, believing that the expression product is not only protected against isoforms, but also resistant to some extent. It has been shown that the expression of ApxI protein N-terminal by *E. coli* can basically achieve the immune effect of ApxI, laying a foundation for the study of subunit vaccine (Fang Xie et al. 2010.).

Characteristics of ApxⅣ

Apx was first identified in 1999 and was only expressed *in vivo*. In 1997, Anderson identified CM5, a sequence similar to the meningitis FrpA and FrpC genes, appearing downstream of the serum type 1 LacZ gene, suggesting the possibility of a fourth Apx toxin gene in the APP (Shin et al. 2011). A suspected fourth Apx toxin was identified by Sealler in 2014, both by gene sequencing and by gene expression, and it was named as ApxIV (Liu J et al., 2009.). Unlike ApxI, ApxII, and ApxIII, which have weak virulence and are not detected *in vitro* culture (Rossic et al. 2013), ApxIV is the only gene which was only expressed *in vivo* in APP. Because the operon structure is different from the first three toxins of Apx, the ORFI gene is immediately upstream of Apx A, but its expression products are functionally similar to Apx C and are also responsible for the activating of Apx (Nkandoi et al. 2012). The structure of the Apx has the similar characteristic of the RTX toxin (Bradford 2005.). Thus, Apx is secreted by all serotype APP, and no other strains in the Actinobacter genus secrete Apx, demonstrating that other *C. elegans* other than pleuropneumoniae lack of the Apx gene and have strong interspecies specificity (Frey et al. 2003.) but not by ApxI, ApxII, and ApxIII. Due to the specificity of Apx, the high specificity and conservation of the 442bP fragments are used as the target genes to detect APP. Domestic APP vaccines are mostly toxin-free inactivated seedlings, combined with Apx toxin expression characteristics only in the host, can distinguish between immune animals and wild virus infection (Shin M et al., 2011). Other researchers reported the establishment of the Apx-ELISA method, so that the Apx antibody cannot be detected in the APP carriers without infection symptoms (Rossic et al. 2013). This is also in line with the study showing that no serum cross-reactive was present between antibodies to the recombinant Apx A protein and other Apx toxins, and no Apx antibody was detected from both the other standard positive serum and whole APP inactivated seedlings, but Apx antibody was detected from the serum of animals infected with live APP (Bode et al. 2003; Li et al. 2022). The ELISA method established with recombinant Apx protein in Previous reports have suggested that APP could detect antibodies to Apx in animals, but not in animals immunized with inactivated seedlings (Christensen 1982). This indicates that Apx is not only species-specific and can only be used for the detection of APP infection, but also can be applied to differential diagnose of whole-inactivated vaccine or subunit vaccine from naturally infected pigs, so this protein is the best candidate antigen for the diagnosis of APP infection. However, Apx alone is difficult for the differential diagnosis of infectious pleural pneumonia in pigs. Apx antibodies can be detected in recovered pigs naturally infected with each serotype, and pigs recovered after infection can resist all toxins. Of course, Apx is also an important part of them. Some studies have shown that Apx can pass through thorns.

Persular Polysaccharide

Capsular polysaccharide (CP) is the main component of the extracellular membrane of the bacterial cell wall, with CP in all APP serotypes, mainly composed of sugars. Persular polysaccharide is one of the necessary virulence factors of porcine infectious *C. thyuropneumoniae*, and its virulence is determined by the type (Bradford 2005). It protects the bacterium from phagocytosis, and its specific antibodies can opsonize on Actinobacteria of *C. pleuropneumoniae*. The number and type of capsular polysaccharide and the secretion mechanism are directly related to APP pathogenicity, which shows that capsular polysaccharide is the factor affecting APP virulence.

Lipopolysaccharide

Lipopolysaccharide (LPS) is a surface antigen substance and a receptor for many phages, which is toxic to the host. As one of the virulence factors of Actinobacter, purified LPS can activate some blood clotting factors, which can induce blood agglutination and fibrinolysis, leading to tissue necrosis. Meanwhile, LPS acts together with the exotoxin to strengthen the toxic effect of Apx exotoxin on phagocytes, and LPS is also associated with APP adhesion to the trachea and pathological changes in the lungs. LPS is immunogenically weak. LPS is only resistant to disease and is not disease-resistant, so LPS only provides protection against APP attacks.

Exterior membrane Protein

External membrane protein is a very special but very important protein. Outer Membrane Proteins (OMP) is the main

structure of the outer membrane, is the direct permeability of cell membrane and efflux pump system, and plays an important role in ensuring material transport (Kamp et al. 2012). At the same time, OMP has stable structure of bacterial external membrane, which can adapt to the intracellular environment and resist tracellular sterilization and other important role, and is closely related to bacterial virulence. It can regulate phagocyte function, activate cellular actin production, and was first identified in the immunogenic and antigen-protective in the 1980s.The main antigen involved in immunity is 17 KD peptidoglycan-related lipoprotein, and 32 and 42KD proteins, which expressed an outer membrane protein PalA. It is immunogenic. But later studies showed that antibodies produced by this protein have negative effects on some serotype antibodies and therefore cannot be used as a vaccine component (Bagdasarian et al. 1998). It was believed that this may be one of the reasons for the unstable quality of some bacterial virulence.

Transgenic Iron-binding Protein

Transferring binding proteins (Tbp) is a transmembrane glycoprotein on the surface of most bacteria. However, iron is also a necessary material for bacterial growth. Under normal circumstances, all the iron ions required for cell metabolism have Tbp transport (Leiner et al. 1999). It has been shown that a part of bacteria in the Actinobacteria population have a mechanism to obtain iron. That is, transiron binding protein exists on its surface. When iron is absent, its receptor is expressed, thus helping bacteria to absorb iron from the animal body to meet the needs of bacterial growth and reproduction. The specific transiron binding protein located in the vitro membrane of APP bacteria is mainly composed of binding proteins A (TbpA) and B (TbpB). The bacteria can uptake iron ions, and Batles et al. confirmed that these two genes are virulence factors (Nielsen et al. 2000). In addition, Wang Fang used molecular biotechnology to amplify TbpB from five lines of APP, and cloned straight *E. coli*, providing technical help for the development of a new vaccine for infectious pleural pneumonia in pigs.

This bacterium is a facultative anaerobe. The optimum growth temperature is 37℃. It does not grow in the common medium, and V factor is needed to be added for the bacterium to grow. Under the condition of 10% CO2, myxoid colonies could be formed and cultured on chocolate AGAR for 24-48h to form opaque pale gray colonies with a diameter of length around 2mm. Two types of colonies can be formed. One is round, hard "waxy", and sticky. The other is a flat, soft, shining colony. Strains with pods can form rainbow colonies on AGAR plates. A-β-hemolytic ring is usually produced on AGAR plates of bovine or sheep blood. The hemolysin produced by *Staphylococcus aureus* had a synergistic effect with the β-toxin of *S. aureus*. That is, *S. aureus* could enhance the hemolysis of *S. aureus*, and CAMP reaction was positive.

The bacteria are not strong resistant to the outside world. It is sensitive to common disinfectants and temperature. General disinfectant can easily kill it, at 60℃ 5-20min. It usually survives for 7-10 days at 4℃. Pathogens that are not resistant to desiccation and are released into the environment are very weak, whereas pathogens in mucus and organic matter can survive for several days. It has certain resistance to crystal violet, bacitracin, lincomycin and spectacular mycin. It is more sensitive to tetracycline antibiotics such as oxytetracycline, penicillin, tylosinin, sulfadiazine, cephalosporin and other drugs.

Clinical Diagnosis and Symptoms

The natural infection incubation period of this disease is 1-2 days, and the clinical manifestations of pigs vary with the immune status, environment and management of pigs (Klausen et al. 2007). According to the length of the course of the disease, it is usually divided into four types: (1) the most acute, (2) acute, (3) subacute, and (4) chronic.

Regarding the most acute type, the following symptoms are specified. The body temperature of the pig rose to 41-42℃. The patient animals showed depression, loss of appetite, short-term diarrhea and vomiting. The patient animals also show cyanosis of skin of nose, ears, legs and sides, later severe dyspnea, mouth breathing, spasmodic cough, and sitting-dog position. There was a large amount of blood-colored foam discharge from the nose and mouth prior to death. Death usually occurs within 1-2 days. Some cases have asymptomatic and sudden death. The case fatality rate is up to 80-100%.

Regarding acute type, the following symptoms are specified. The sick pig spirit is listless, has appetite waste, temperature up to 40.5-41℃, cough, painful mouth and tongue, and abdominal breathing. Because of the different breeding and management conditions, the length of the disease is different, which can be subacute or chronic type.

Regarding subacute type and chronic type, the symptoms are no fever or mild fever, lack of energy, reduced feed intake, abnormal breathing, cough or intermittent cough, slow growth. The course of disease can last from a few days to two weeks (Christensen 1982). If the environment is good and there are no other complications, it can survive, but the disease has a certain impact on weight gain, and if other diseases are secondary, death is inevitable.

Pathological Changes

The Most Acute Type

Main pathological changes occur in respiratory tract, with bloody nose and outflow liquid, and tracheal and bronchial hemorrhagic secretions of foam samples. Pneumonia is more bilateral. The lesion area mainly includes the leaves, and part of the diaphragmatic leaves. There are clear boundaries between lesions and surrounding healthy tissue. There are lung congestion, hemorrhage, edema, a dark red or purple area, with the quality of the material to be solid. There is pink foam sample liquid when cut out (Fig. 1 & 2).

Acute Type

There is light red fluid in the chest, cellulose exudate on the lung and pleura surface, some adhesion of cases of lung and pleura, lung congestion, bleeding, and edema. The lung has solid texture, clear outline, purplish red lesions, obvious surrounding fibrosis and sections like liver sections. There is the trachea and a fibrinous exudate with blood-colored foam in the bronchus (Fig. 3).

Subacute or Chronic Type

The changes include lung serosal membrane and chest wall uneven thickening, a large number of small nodules protruding on the lung surface and internal. The lung surface has cellulose attached, lung serosal membrane and chest wall and pleura adhesion.

Fig. 1: Lung congestion.

Fig. 2: Bloody nasal discharge.

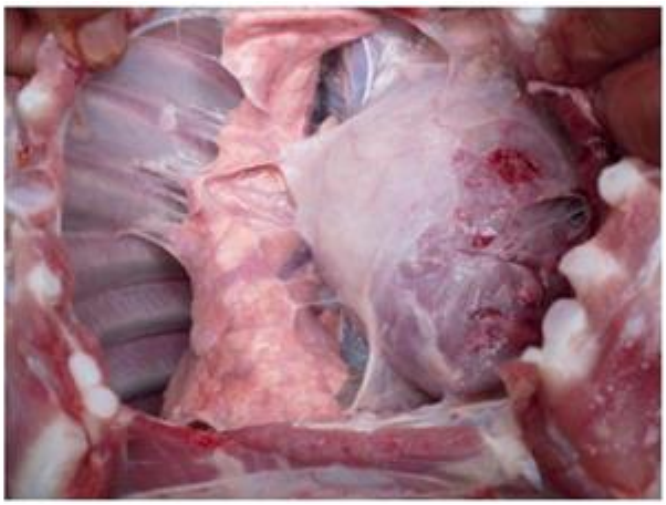

Fig. 3: The lungs are fused with the ribs.

Autopsy is difficult to separate. There are numerous purulent small nodules in the diaphragm surrounded by thick connective tissue. Arthritis, endocarditis, meningoencephalitis and other parts of the abscess can also be seen in varying degrees. This type of disease has a long course of the disease. Pigs have yellow-green spots on the pleura.

Laboratory Inspection

The diagnosis of the disease can be based on traditional methods of epidemiological investigation, clinical symptoms and pathological changes at autopsy, combined with laboratory diagnosis. Common laboratory diagnosis methods include the following five methods:

(1) Bacterial isolation and identification. Lung disease, heart, peritoneal effusion and nasal secretions of diseased and dead pigs were inoculated with lipid plate or chocolate plate and cultured overnight at 37°C. Waxy colonies were observed. *Actinobacillus pleuropneumoniae* inoculated in *Staphylococcus aureus* culture showed satellite appearance and typical β hemolysis. Combined with related biochemical examination, it was confirmed as pleuropneumoniae.

(2) Complement binding test uses the immune hemolysis mechanism as an indicator system to detect antigens or antibodies of another reaction system. In 1971, Nicolet established the complement binding test (CFT) method, which was improved by Lombin. In 1982, this method was internationally recognized as the standard method for APP detection. In 1990, Zhu Shisheng et al. made it convenient for the diagnosis of pleural pneumonia in pigs by freeze-drying multiple serotypes of antigens mixed with full-cell antigens in a certain proportion (Lara et al. 2008).

(3) Indirect hemagglutination test. It is to wrap antigens (or antibodies) on the surface of red blood cells to become sensitized carriers and then combine with corresponding antibodies (or antigens) to make red blood cells together and produce visible agglutination reaction. Mittal established indirect hemagglutination test (IHA) for APP detection and serological typing, which is fast and sensitive (Meyns et al. 2007). The method for APP detection can effectively distinguish serum type 4 from serum type 7.

(4) Agglutination test. Agglutination test (AA) is a simple and rapid method for APP detection. The direct antigen and corresponding antibody combine to agglutinate, which can be used for serotyping. The test includes tube agglutination, glass agglutination, co-agglutination, etc.

(5) Other diagnostic methods. Latex agglutination test (LAT) is a simple method. Immunodiffusion assay (IDT) is a classical immunological method with strong specificity (Liu et al. 2009).

Treatment

There are many causes of infectious porcine pleuropneumonia, so comprehensive prevention and treatment are appropriate. First of all, we should improve the level of feeding management, maintain piggery hygiene, good ventilation, regular disinfection, and achieve reasonable feeding density, in the process of feeding, and should also minimize various stress factors. Secondly, regular injection of vaccines, including inactivated vaccine, subunit vaccine and attenuated vaccine, can prevent APP infection to varying degrees, reduce the mortality rate of infected pigs and have certain protective effect, but it cannot reduce morbidity and chronic infection rate. Adhere to self-breeding, and try to reduce the introduction of diseases. In addition, standardized introduction procedures should be adopted. After the purchase of pigs from other places, some work should be conducted including isolation, and inspection, to ensure that no disease can enter the feed. For attenuated vaccine, although it has partial toxicity, which can easily cause disease and potential toxicity in inoculated animals, and the

accurate usage of attenuated vaccine cannot be controlled, attenuated vaccine has a good market prospect at present. Attenuated vaccine can be continuously screened to minimize virulence and have good immunity (Nielsen et al. 2000). Data show that attenuated vaccine nasal drip immunization can achieve good immune effect, and animals can also be inoculated by inhaling aerosol with APP through airtight fogging device, so as to achieve better infection effect (Huang et al. 2005). In addition, pigs of different age should be raised in different groups, and all pigs should be brought in, and all pigs should be brought out to reduce the probability of disease infection. At the same time, active drug prevention should be carried out to achieve better results as far as possible. Tallosin, cephalosporins and tetracycline antibiotics are more sensitive to *Actinobacillus pleuropneumoniae*, and appropriate drugs can be added to drinking water and feed for early prevention of pigs without disease, so as to effectively control the occurrence of the disease (Wei et al. 2012). Practice has proved that the use of such drugs has a certain degree of resistance, so in the process of use, it is necessary to frequently use different drugs, and do drug sensitivity test in advance, in order to conduct the prevention and control work as efficiently as possible.

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CHAPTER 08

BACTERIAL DISEASES OF FISH AND SHRIMPS/PRAWNS

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INTRODUCTION

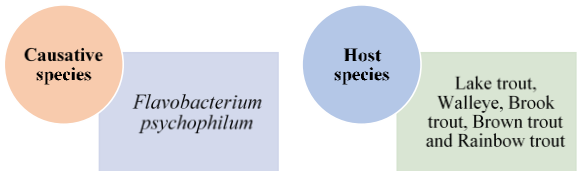
Because of the rising demand for edible fish, aquaculture is quickly becoming the world's fastest-growing food-producing sector. However, extensive food fish cultivation has resulted in outbreaks of a variety of bacterial diseases, causing annual economic losses to the aquaculture industry in the billions of dollars around the world. Feeding antibiotic-medicated feed to diseased fish is common practice, but it has resulted in antibiotic resistance in bacterial pathogens, requiring greater doses for effective management; a growing public concern. To treat bacterial infections in aquaculture, a number of vaccines have recently been produced.

Bacterial diseases in fish and shrimp/prawn

1. Peduncle disease (Flavobacteriosis)
2. Haemorrahgic Syndrome (Vibriosis)
3. Edwardsiella septicemia (Edwardsiellosis)
4. Columnaris (Flexibacteriosis)
5. Enteric Red mouth disease (Yersiniosis)
6. Motile Aeromonas Septicemia (Aeromoniasis)
7. Red spot disease (Pseudomoniasis)
8. Fish Tuberculosis (Mycobacteriosis)
9. Pop eye disease (Strptococcosis)
10. Necrotising hepatopancrease (NHP)
11. Furunculosis
12. Bacterial kidney disease
13. Acute hepatopancreatic necrosis
14. Luminous vibriosis
15. Photobacteriosis

Peduncle Disease (Flavobacteriosis)

Peduncle disease is a fish disease that is more common in cold-water fishes but has been observed in warm-water fishes as well (Bullock and Snieszko 1970).



When the water temperature is between 7 and 10 degrees Celsius, disease occurs. The incubation time is usually fewer than ten days.

Symptoms

Erosion of skin covering the yolk sac is a common symptom. Survivors have also exhibited spiral swimming and dorsal edema behind the skull. Eye lesions may lead to vision loss. Spinal compressions in the posterior, mid or anterior areas of the fish are the most prevalent abnormalities (Ostland et al. 1997). *F. psychrophilum* showed preference for muscle tissue and skin lesions with yellow margins may appear on the caudal peduncle area when feeding begins (Lumsden and Krumlauf 1996). Large numbers of bacterial diversity can be detected in the spleen, liver, air bladder, gut, pancreas, peritoneum and heart of seriously infected fish, indicating that the condition is septicemic (Evensen & Lorenzen 1996).

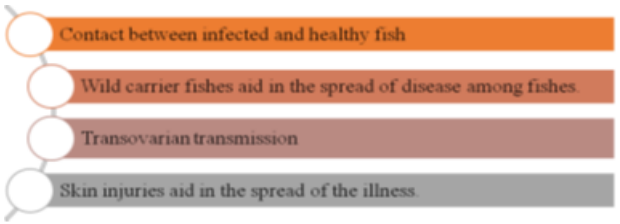
SYMPTOMS Peduncle DISEASE

Zoonoses

Flavobacterium psychrophilum and other *flavobacteria* are only known to infect fish and are not known to cause disease in humans.

Transmission

TRANSMISSION OF DISEASE



Factors cause disease

Factors responsible for this disease may include malnutrition, presence of toxic/harmful substance in water, harsh handling of fish and acidic water. Physiological imbalance of water makes fish more susceptible to infection. Overcrowding may increase the risk of the infection.

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Treatment

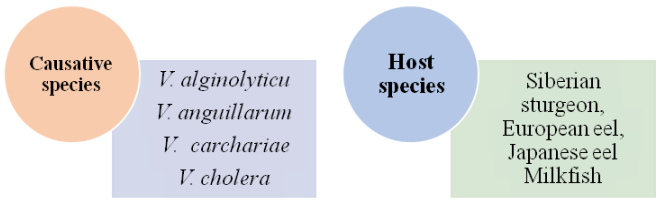
To minimize continuing shedding of the bacteria into the aquatic environment, oral antibiotics should be given in conjunction with 2 mg/liter potassium permanganate flushes and physical removal of most severely infected fish (Schachte 1983). Sulfisoxazole was helpful against *F. psychrophilum* in feeding fry when given as a prophylactic treatment at 88 mg /Kg /day for twenty-six days and as a therapeutic treatment at 220 mg /Kg/day for ten days (Amend 1970). Oxytetracycline, more effective than sulfonamides for control of bacteria cold water disease when given in feed at 75 mg/kg /day for ten days. Eggs can also be disinfected using 100 mg/litre hydrogen peroxide for 10 minutes or 200 mg/litre glutaraldehyde for 20 minutes (Cipriano and Holt 2005).

Control

Other important requirements that may assist to avoid and/or minimize the severity include proper management of fish culture conditions, operation and management of good standard for quality of water, and suitable sanitation methods. Equipment can be sterilized for 10 minutes in 1-2 percent formalin, 2 minutes in 0.5-1 percent chlorine, or 2 minutes in alkaline conditions corresponding to pH 13 (Rangdale et al. 1997).

Haemorrhagic Syndrome (Vibriosis)

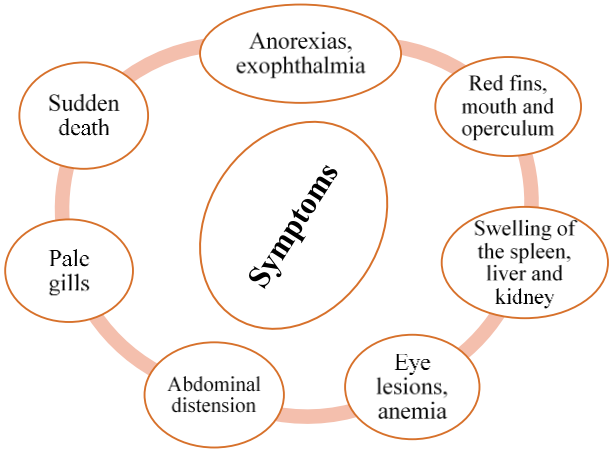
Vibriosis is among the most common fish diseases caused by the bacteria of the Vibrio genus. Septicemia, ascites, haematopiotic necrosis and dermal ulceration are all symptoms of this condition. Warmer temperatures are known to make Vibrio bacteria more harmful.

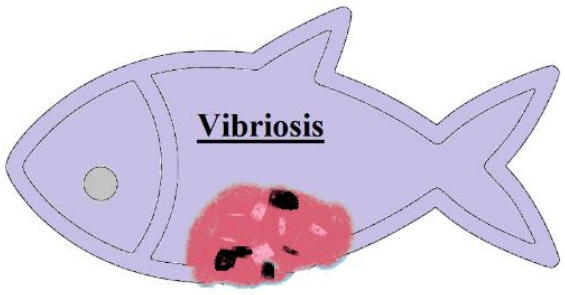


The incubation time could last up to three days. This is dependent on the pathogen's virulence and the fish's sensitivity (Aguirre-Guzmain et al. 2004).

Symptoms

There are many signs of Vibriosis that may be seen. These symptoms are not pathognomonic (specific to *Vibrio* bacteria) and may be caused by other diseases.





SYMPTOMS OF HAEMORRAHGIC SYNDROM DISEASE

Transmission

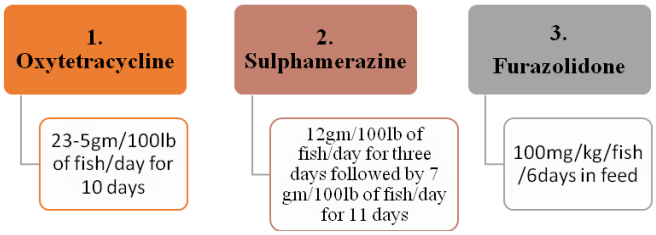
The infection can spread through the mouth and skin, as well as through external injuries. The bacterium was discovered in the gut, and it has the potential to infiltrate the host under any stress state. Transmission may also occur through contact between a sick and a healthy fish.

Zoonoses

Vibriosis is a zoonotic illness that can be transmitted to humans. Bacteria can enter the body through cuts and bruises on the skin, producing inflammation and infection in a specific area.

Treatment

If a fish has Vibriosis, it should be moved to quarantine tank. Antibiotics can be injected directly into the fish, incorporated into a diet, or administered through a water-based therapy.

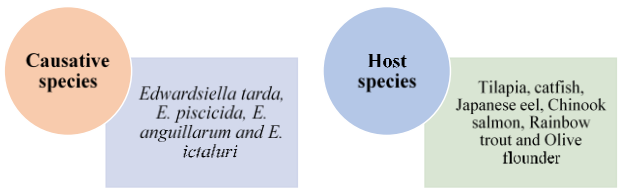


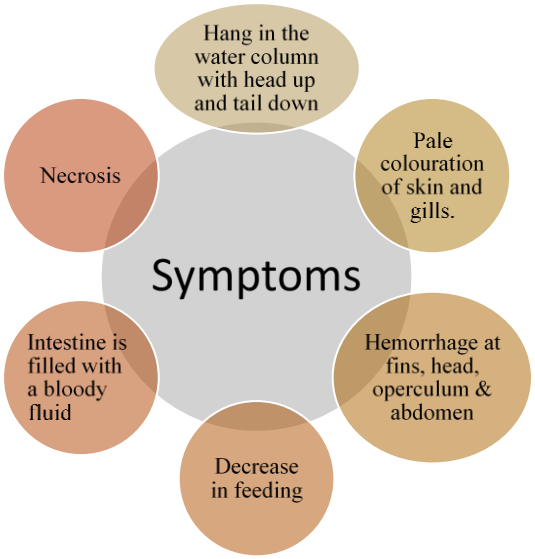
Control

Sources of stress must be minimized, and proper fish farm management, as well as the cleaning of fish eggs can help to prevent infection. If fresh fish is quarantined and sanitation is maintained, the spread of disease can be restricted. Healthy persons should not consume dry fish or infected fish viscera as a source of protein. Farm fish that have been affected should not be relocated to non-infested areas. Protection is offered when administered through intraperitoneal injection instead of immersion or oral administration (Cai et al. 2013).

Edwardsiella septicemia (Edwardsiellosis)

Edwardsiellosis is an acute, subacute, or chronic bacterial disease caused by Edwardsiella spp. in a variety of fish species. Septicaemia and abscess development are common symptoms (Park et al. 2012).





Symptoms of Edwardsiellosis

Transmission

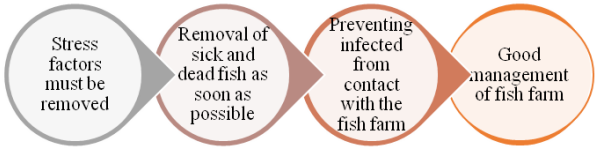
Consumption of infected fish or dead carcasses by the birds, aids in the spread of infection by picking up dead fish from one pond and flying to another, dumping diseased carcasses in a healthy pond. Contaminated nets and equipment have the potential to spread the disease from one pond to the next. Carriers aid in the spread of disease. Edwardsiella species can be found in aquatic invertebrates as well as humans. Any of those hosts could serve as a reservoir. The bacterium is normally present in the intestinal tract of fish, but when they are stressed, the bacterium has the capacity to penetrate the fish and cause infection (Meyer and Bullock 1973).

Zoonosis

In humans, *E. tarda* causes diarrhoea gastroenteritis, typhoid-like sickness, peritonitis with sepsis, and causes abscesses in the liver, among other things (Woo and Bruno 2011).

Treatment and control

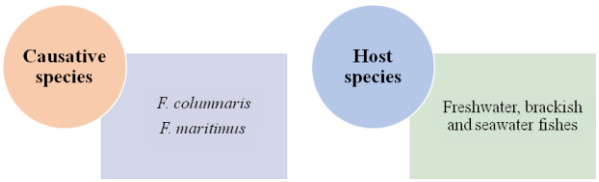
For ten days, **oxytetracycline** 50 mg/kg of body weight/day can be used to treat the condition (Hawke et al. 1981). The following procedures must be performed to control the infection:



CONTROL OF DISEASE

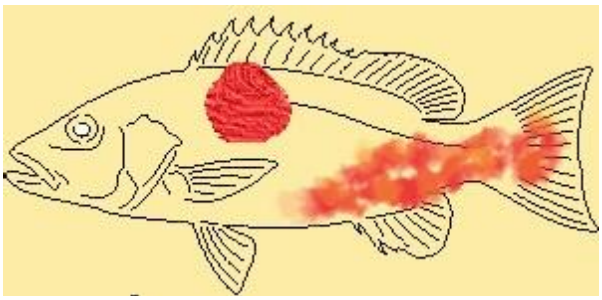
Columnaris (Flexibacteriosis)

Columnaris disease is a disease that affects salmonids and a variety of warm-water fish species. Columnaris disease has a variable incubation period, which is due to stress factors to which fish are exposed. Losses can occur after two to three days if the incubation period is less than 24 hours (Bernardet and Bowman 2006).



Symptoms

Small lesions appear as pale discoloration at the base of the pectoral or dorsal fins at first. These areas grow in size, eventually reaching 3-4 cm in diameter and covering 20-25 percent of the fish's total surface area. This could have a saddle-like appearance to it (saddle back disease). These areas have a slight lemon-yellow colour to their surface. The skin is completely eroded. At the lesion's advancing edges, a large number of bacteria are present. The necrosis causes the pectoral fins to completely disappear before spreading to the head. Necrosis appears in the form of yellow-orange areas that start at the gill's periphery and extend to the gill arch's base. As a result, the gill filament is completely destroyed. Appetite loss and sluggish swimming are also observed (Davis 1922).



Transmission

Flexibacter columnaris is a bacterium that can spread through water. When virulent strains are added to water, they infect the fish; less virulent strains only infect the fish when injected. Catostomids, cyprinids, and coregonids may act as infection reservoirs. Columnaris disease is spread by the stress of crowding fish, handling them, or maintaining them at or above marginal temperatures, and also the stress of injury.

Zoonosis

Flavobacterium columnare is not known to cause disease in humans.

Treatment

Copper sulphate can be used as a preventative in a 20-minute bath at 37 ppm (1:30,000) or as a 0.5 ppm addition to pond water. For an indefinite period, add 2 ppm potassium permanganate to pond water. Immunization against columnaris disease is available in two forms: oral and parenteral. Oxytetracycline (Terramycin) taken orally with food at a rate of 8 g per 100 kg fish per day for up to 10 days is particularly successful in both early and advanced outbreaks.

Nifurpirinol and nifurprazine can be added to water at concentration of 1 part per million (ppm) for 5 to 10 minutes or 0.05 to 0.1 part per million for an infinite period. These nitrofurans are administered orally with meal at a rate of 2 to

4 mg per kilogramme of fish for 3 to 5 days. For 24 hours, a bath of 1 ppm oxolinic acid is employed.

Sulfonamides, such as sulfamethazine and sulfamerazine, can be given to fish orally with feed at a dose of 10 to 20 mg/kg per day, however they are ineffective when compared to other medications (Suomalainen et al. 2009).

Control

Columnaris disease can be avoided by using water supplies that do not contain fish, as Flexibacter columnaris is shed into water by carrier fish. Injury, overcrowding, and unfavorably high temperatures are all factors that contribute to outbreaks and should be avoided (Suomalainen et al. 2009).

Enteric red mouth disease (ERM) (Yersiniosis)

It's a bacterial disease that affects salmonids and a few non-salmonid fish like goldfish and carp (FAO 2010). ERM infection took 5 to 10 days to incubate at 13 to 15°C in the lab. In all circumstances, the incubation time is based on the water temperature and fish susceptibility.

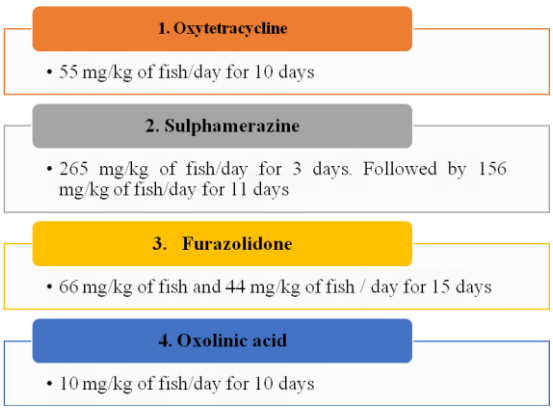
Symptoms

Subcutaneous haemorrhages induce the reddening of the throat and mouth. It's possible that the jaw and palate will erode. On the body surface, at the tips of the gills, base of the fins, and around the lateral line, haemorrhages develop. Skin darkening is also possible. Exophthalmia in one or both eyes causes partial or total blindness in fish. Some fish have a bloated abdomen, while others are malnourished (Tobback et al. 2007). The peritoneum is congested, and blood arteries are clogged. Petechial haemorrhages have been discovered in the liver, the swim bladder, the lateral muscles, and the adipose tissues. Swollen kidneys and spleen are possible. The digestive tract is clogged, irritated, and packed with bloody mucus, especially in the back (Manna et al. 2003).

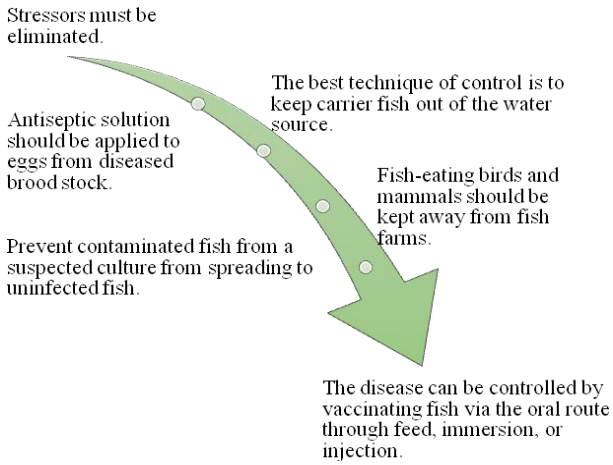
Transmission

Eating toxic food or water through the mouth is the route of transmission. Infection can also spread through wounds on the skin, scales, or gills. The bacterium may infiltrate the fishes if it is located in the alimentary system and is stressed. Carrier aids in the spread of infection from sick specimens to healthy individuals (Horne and Barnes 1999).

Treatment



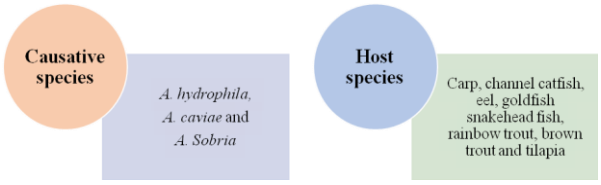
Control



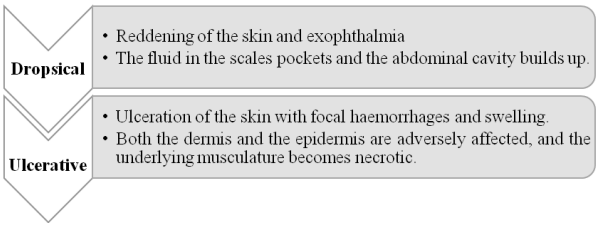
Control of disease

Motile Aeromonas Septicemia (MAS) (Aeromoniasis)

Ascitis, ulcer formation and exophthalmia characterize acute, subacute and chronic disease of freshwater fishes caused by motile *Aeromonas* bacteria (Chacon et al. 2002). It takes time from initial infection to the manifestation of disease symptoms is determined by the temperature of the surroundings.



Symptoms of disease



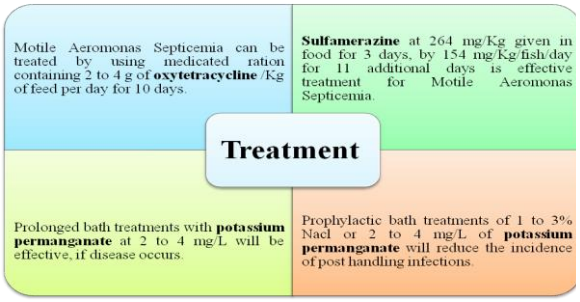
Zoonosis

Aeromonas hydrophila is a pathogen that affects amphibians, reptiles, and snakes, as well as cattle and humans (Khardori and Fainstein 1988).

Transmission

The causative agent is passed from person to person horizontally. It is widely distributed in pond water and sediments, and it can be transmitted through gastrointestinal discharge and external skin lesions. Infection produced by an epidemic can enter and spread amongst fish. Infection transmission is also aided by carriers (Griffin et al. 2013).

Treatment



TREATMENT OF DISEASE

Control

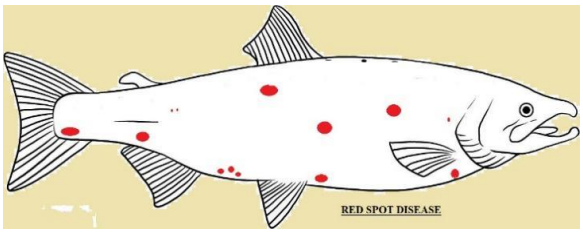
In order to prevent disease, ponds must be dried on a regular basis, kept clean, and disinfected. To prevent the spread of motile *Aeromonas septicemia* to fish hatcheries, disinfect new egg shipments with acriflavine (500-700 ppm active ingredient for 15 minutes) or Betadine (100-150 ppm active ingredient for 15 minutes) (Pridgeon et al. 2011b).

Red spot disease (Pseudomoniasis)

The majority of fish species are affected by a Pseudomonas species-caused acute septicemic bacterial disease (Haenen and Davidse 2001).

Symptoms

- Erythema can be found at the base of the fins, under the lower jaw, and around the anus.
- The peritoneum may develop small peticheal haemorrhages.
- The liver may appear pale and swollen.
- Kidneys can bae liquefying and soft.
- Fibrinous peritonitis and ascites are common in chronic cases (Tranzo et al. 2005).



SYMPTOMS OF RED SPOT DISEASE

Zoonosis

This is not a zoonotic species (Muroga and Sawada 1975).

Transmission

The bacterium enters the host through the mouth or by breaking or abrading the skin. Infection can also be spread through damaged gills. Carrier fishes may play a role in the disease's spread.

Treatment

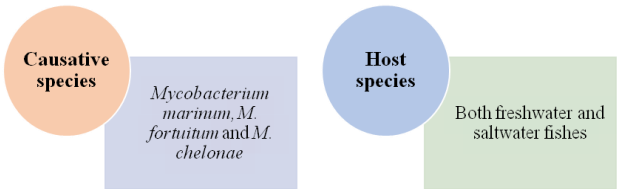
For 30 minutes, use chloride powder at a concentration of 5-10 mg/liter. It is necessary to eliminate the predisposing factors. A bacterial isolate is used in a drug sensitivity test to determine the drug's value for treatment.

Control

The fish farm is well-managed. External disinfection helps to keep the disease from spreading (Magi et al. 2009).

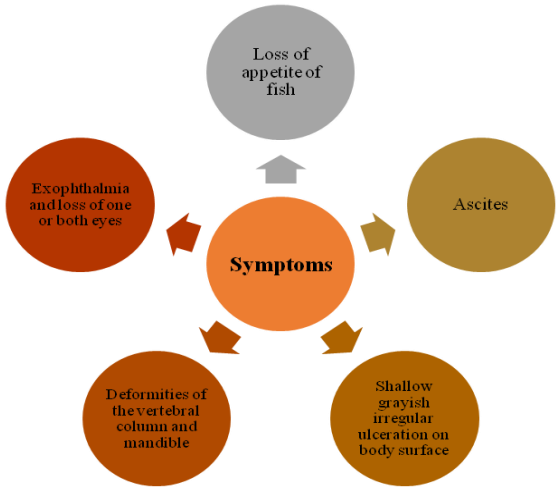
Fish tuberculosis (Mycobacteriosis)

Nontubercolous mycobacteria (NTM) cause fish tuberculosis, a chronic disease caused by common acid fast bacteria. The incubation time is highly variable, depending on susceptibility, temperature, and the severity of the exposure. The incubation period in salmon infected through the oral route can extend upto years. It may only last a few weeks or months in ornamental fish kept in overcrowded aquariums at higher temperatures. (Novotny et al. 2004).



Symptoms

The occurrence of a hallmark lesion of active tuberculosis with many bacteria in necrotic regions, is the most common lesion in late stages of infection in fish tuberculosis, which is identical to active human tuberculosis (Swaim et al. 2006).



Zoonosis

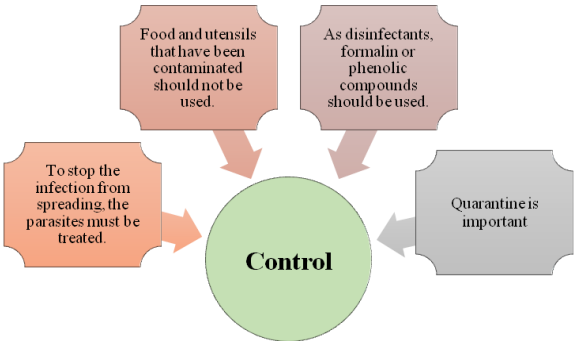
M. marinum is a zoonotic threat because it causes granulomatous lesions in the deep tissues and skin of human beings (Petrini 2006).

Transmission

The transmission of *M. marinum* across fishes is poorly understood. Oral infection occurs primarily through the eating infected dead fish, coming into contact with infected fish skin and gills (El Amrani et al. 2010).

Treatment and Control

To fully control mycobacteriosis, all affected stocks may need to be destroyed, and the holding tanks and plumbing may need to be disinfected (Roberts et al. 2001; Noga et al. 2011). In ornamental fish, the antibiotic Kanamycin mixed with food was effective in curing mycobacteriosis. The recommended dosage in food is 0.01 percent by weight. In aquaria, ethanol, lysol, and sodium chlorite have all been shown to be effective at killing *M. marinum*, while potassium peroxymonosulfate is ineffective (Mainous and Smith 2005). Furthermore, when the contact time is greater than 10 minutes, Sodium hypochlorite is a highly effective sterilizing agent (Anderson and Conroy 1970).

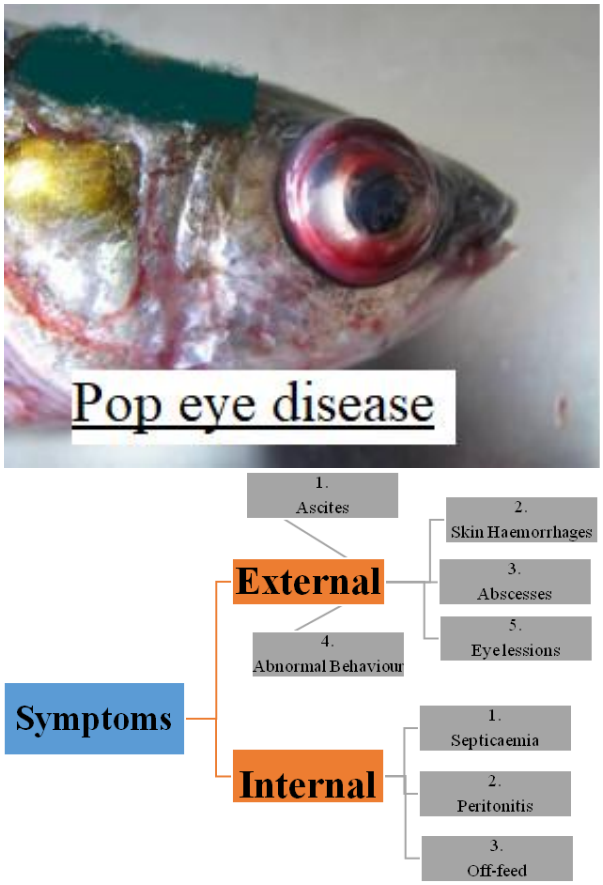


Pop eye disease (Streptococcosis)

Pop eye disease is a bacterial infection that affects warm-water fish in both salt and freshwater habitats, with the majority of cases occurring in tropical locations.

Symptoms

Exophthalmia, loss of orientation and erratic swimming, eye opacity, anorexia, stomach distention, hemorrhagic skin at the base of the fins or around the anus and darkening of skin are the most prevalent signs.



SYMPTOMS OF POP EYE DISEASE

Transmission

Intraperitoneal immersion, cohabitation and injections with infected fish, as well as gill and oral inoculation, have all been used to demonstrate *Streptococcus spp*. transmission in tilapia. Direct contact between diseased or dead fish and healthy fish in natural conditions, and indirect contact through the water in culture systems, appear to be the main pathways of disease transmission (Filho et al. 2009). *Streptococcus spp.* can be transferred horizontally in tilapia, according to several studies, yet spontaneous outbreaks of the disease on tilapia farms demonstrate that larvae and young fish under 20 g are not susceptible, meaning that the disease is not transmitted vertically (Geng et al. 2012).

Zoonosis

Streptococcus spp. is a bacterium that can infect both mammals and fish, including human being (Austin and Austin 2012).

Treatment

Antibiotics are only effective in treating a Streptococcus outbreak if they are administered early in the disease's progression. Because infected fish have a reduced appetite, oral antibiotics are ineffective. Immunostimulants such beta-glucans and nucleotides given to the meal have been demonstrated to help sick redtail black shark (ornamental fish) populations survive (Evans et al. 2004).

Control

Reduce feeding and stocking density, as well as the temperature of the water.

Necrotising hepatopancreatitis (NHP)

Necrotising hepatopancreatitis is caused by gram-negative bacteria, intracytoplasmic alphaproteobacterium that infects the hepatopancreas of prawns and is also known as NHP bacteria (NHPB) or rickettsial-like organism (RLO) (Gollas-Galvan et al. 2014).

Symptoms

Diseased animals may exhibit one or more of the symptoms listed below, however the pathogen may still be present even if no symptoms are present (Tang et al. 2017). At the farm, tank, or pond level, disease symptoms include:

- Lethargy
- Bacterial fouling
- Reduced growth rate
- Soft shell
- Flaccid body
- Black gills
- Empty intestinal tract
- Black streaks in hepatopancreas

Zoonosis

None

Transmission

H. penaei can be transmitted horizontally by cannibalism or polluted water. *H. penaei* has also been identified as a source of contamination in faeces thrown into the pond (Avila-Villa et al. 2012).

Treatment

The use of antibiotics such as oxytetracycline and florfenicol in medicated feeds every 8 hours for 10 days is probably the best treatment currently available, especially if infection with *H. penaei* illness is diagnosed early (Morales-Covarrubias 2019).

Control

For several weeks, avoid high water temperatures (more than 29-31 °C) and extreme salinities (greater than 20-38 ppt), which have been linked to the development of this epizootic sickness. Treatment of pond bottoms with hydrated lime (Ca $(OH)_2$) during pond preparation before stocking can help to prevent *H. penaei* infection. Many weeks of pond and water

distribution canal drying (by exposure to sunlight), calcium hypochlorite cleaning of fishing gear and other farm equipment, and thorough liming of ponds. It is possible to avoid infection by using specialized pathogen-free (SPF) broodstock.

Furunculosis

Furunculosis is a septicemic bacterial infection that mostly affects salmonid fish. It is found in goldfish and other cyprinids. The disease is named after the presence of "blisters" or furuncules on the surface of chronically afflicted salmonids. The incubation time for acute instances of furunculosis is 2-4 days. In chronic situations, especially at lower temperatures, the duration may be extended by several weeks.

Symptoms

In severely infected fingerlings, haemorrhages/ erosion of the pectoral fins at the base are prevalent. On the ventral surface, bloody or hemorrhagic vents and petechial haemorrhages are common. Typical "furuncules" or blisters on the skin in chronically infected adults may contain blood and amorphous yellow substance. This is uncommon in fingerling fishes because an acute infection often results in huge bacteria infection and gross lesions appear. The body cavity reveals a bloody fluid. Petechial hemorrhages in the body wall and viscera are fairly common.

Symptom of Furunculosis

Transmission

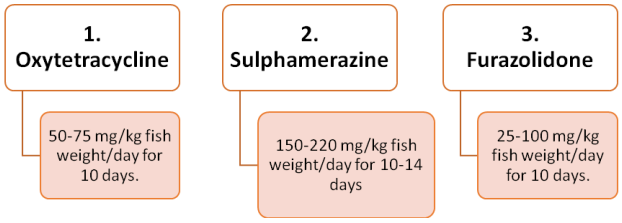
Contact with infected or carrier fish is the most common cause, although it can also happen when water is moved from raceway or pond to another. Infected equipment or clothing can potentially spread from one culture unit to the next unit. Fish-eating birds could possibly spread the disease by contacting diseased fish or droppings contaminated fish into an uninfected pond.

Zoonosis

No

Treatment

Oxolinic acid is given at a rate of 10 mg/kilogramme every day for ten days. Avoid vertical transfer; iodine is often used to clean the surface of fertilised eggs (passage of infection from parent to offspring).



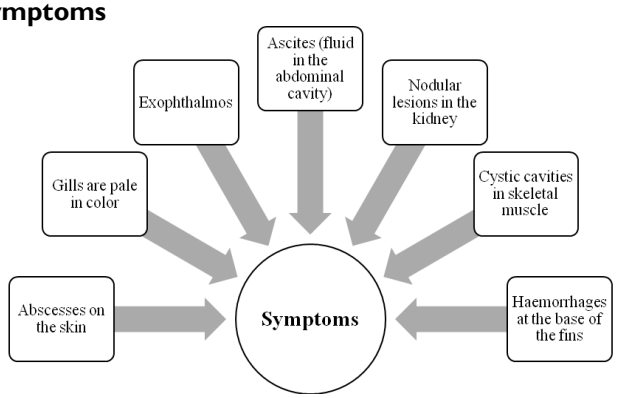
Control

Moving suspect or known carrier fish from hatchery to hatchery is considered useless. All sensitive species eggs should be treated for 10 minutes with organic iodine compounds at 100 ppm active iodine on water hardened eggs. Barriers should be placed in the hatchery to prevent the introduction of potential wild carrier fish. Disease-resistant fish strains should be employed as a disease-control strategy whenever possible. Only eggs from inspected and proven Furunclosis-free sources should be used if eggs from outside the hatchery system are necessary.

Bacterial kidney disease

Bacterial kidney disease occurs due to poor quality water. It occurs due to bacteria related to Salmonidae family, although it can also take an acute to subacute form. Dee Disease is another term used for bacterial kidney disease (BKD).

Symptoms



Transmission

Vertical transmission occurs through eggs or sperm, while horizontal transmission occurs through direct contact with diseased fish or water. Feeding raw, unpasteurized viscera of sick fish to other fish in early fish culture boosted the disease incidence in hatcheries. Another avenue for transmission is through infected male seminal fluids during conception.

Zoonosis

None

Treatment

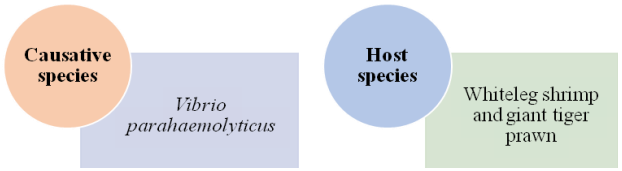
The disease is chronic in nature and microorganisms are present intracellularly. BKD is one of the most challenging fish disorders to cure with medications. Clindamycin, erythomycin, penicillin G, spiramycin, and lincomycin and cephradine are effective prophylactically but had limited therapeutic use. Lincomycin and rifampicin are effective prophylactically but had limited therapeutic use.

Control

Renibacterium salmoninarum can live in pond silt for up to 21 days, the fish farm is drained and disinfected before being replenished after 60 days. The imported fish must be accompanied by a certificate stating that it is disease-free. Disease-free fish eggs should be used. To avoid the spread of the virus, all equipment and tools must be disinfected. Prevent sick fish or fish eggs from moving from an infected environment to a free one. Vitamin C and iodine must be present in suitable amounts in fish diets. Avoid consuming contaminated food or water.

Acute hepatopancreatic necrosis disease (AHPND)

The infection with Vibrio causes AHPND, commonly known as early mortality syndrome (EMS).



Symptoms

- Pale to white hepatopancreas due to connective tissue capsule pigment loss
- Soft shell prawns
- Guts with sporadic or non-existent contents. Within the hepatopancreas, there are black (melanised) patches or streaks visible
- Massive and progressive degeneration of the proximal to distal tubules
- Sloughed cells have a massive secondary bacterial infection (Vibrio spp.)
- The hepatopancreas is completely destroyed

Transmission

AHPND has been transmitted in the experiment via immersion, feeding, and reverse gavage, replicating natural horizontal transmission via oral routes and cohabitation (Nunan et al. 2014; Dabu et al. 2017).

Zoonosis

Yes

Treatment

Not applicable

Control

Luminous Vibriosis

V. harveyi is the causative agent of luminous disease. *V. harveyi* is the main pathogen in both white and black tiger shrimp hatcheries.

Symptoms

- The larvae's internal tissues become densely packed with extremely motile bacteria, and the larvae become anorexic.
- Multiple melanized hemocytic nodules and extensive necrosis and bacterial invasion of the lymphoid organ.
- Lethargic swimming of shrimps at the end of the grow-out stage is one of the signs observed. Shrimps with the disease lost their escape instincts, develop a darkened colour, and are heavily fouled by epibionts. Pale and opaque fish with brown gills are less affected.
- Body reddening, expanded gill coverings, and minor uropod, pleopod, and periopod melanization.
- Except for a watery white liquid, the stomach and midgut are empty. On the lymphoid organ, there are little black dots.

Transmission

Transmission via oral routes

Zoonosis

Yes

Treatment

Chloramphenicol, sodium nifurstyrenate, and the nitrofurans (furazolidone, nitrofurazone, nitrofu-ran-toin, and prefuran) have all demonstrated to have low minimum inhibitory and bactericidal concentrations. Oxytetracycline (OTC) treated feeds have also been shown to be successful in preventing Vibriosis. OCT in a specific diet at 5 or 100 mg/kg body weight of shrimp per day for 4 to 6 days is proven to be effective.

Control

Female shrimps and their faeces should be separated from the eggs as soon as feasible after spawning. Before putting artemia nauplii into the hatchery, give them a good rinse. Enough chlorine should be added to the hatchery water.

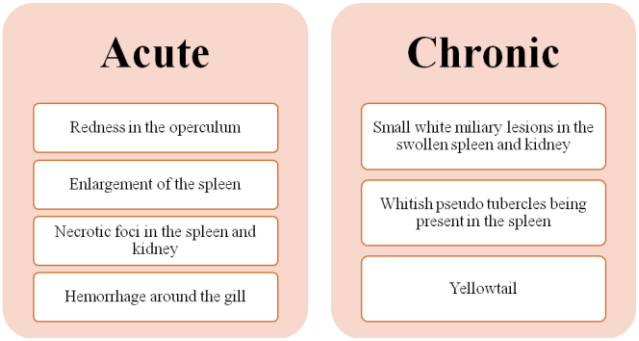
UV irradiation and filtration are used to treat the water. After each cycle of operation, thorough cleaning, disinfection with 200 ppm chlorinated water, and drying of the larval rearing tanks helps to regulate bacterial load and eradicate bacterial pathogens. In penaeid hatcheries, strict water quality control and cleanliness are strongly recommended for preventing luminous vibriosis.

Photobacteriosis (*Pseudo tuberculosis*)

White granulomatous lesions in the internal organs generate and characterize this chronic to subacute systemic infectious disease of marine and brackish water fishes.

Symptoms

The disease has two forms:



Transmission

During epizootics, horizontal transmission from fish to fish inside a culture unit is the most likely mechanism of spread.

Zoonosis

None

Treatment

The pathogen responds effectively to oxytetracycline, romet, oxolinic acid, ampicillin, amoxicillin, and florfenicol medicated meals if applied in a timely manner.

Control

Medicated feeds are rarely given early enough in the infection to be beneficial due to the quick onset of illness. Antibiotic use on Japanese fish farms has resulted in the isolation of resistant strains of the disease carrying numerous drug resistance R-plasmids. Vaccination is a logical method for future care due to the overall ineffectiveness of medicated feeds un battling the condition. Currently, commercial vaccines are in the research and development stage.

Summary

There are numerous bacterial aquaculture diseases around the world. Bacterial diseases and infections are common in fish, and they can cost a fish farmer a lot of money. Because some pathogens are contact-zoonotic, so, aqua farmers, field technicians and processors must practice good hygiene. It is critical to diagnose bacterial diseases correctly. Many diseases can be avoided with proper management, which includes the use of appropriate vaccines. If antibiotic treatment is required, an antibiogram should always be performed.

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CHAPTER 09

THEILERIOSIS: IMPACT ON ANIMAL HEALTH AND RECENT ADVANCES REGARDING ITS CONTROL

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INTRODUCTION

Theileriosis, Corridor disease (CD), or East Coast fever (ECF) is considered an important tick-borne disease of domestic cattle around the world. It is caused by phylum Apicomplexa member *Theileria* (*T.*) which circulates in bovines and causes economic losses to the livestock industry (Ota et al. 2009). Certain factors make the differentiation of *Theileria* with other Apicomplexa e.g. do not reside in the parasitophorous vacuole, sporozoites are free in host cell cytosol by dissolving the host cell membrane and convert into schizonts, non-motile and absence of well-developed apical complex like in Plasmodium and Toxoplasma (von Schubert et al. 2010). It is the main cause of high mortality, morbidity, main constrain in breeding development programs, and ultimately results in an economical loss (Moumouni et al. 2015; Lew-Tabor and Valle 2016; Kho et al. 2017; Hassan et al. 2018; Zeb et al. 2020). Economic losses caused by Theileriosis in Sub-Saharan Africa are 300 million USD and in India, it is 384.3 million USD per year (Vollmer 2009; Rajendran and Ray 2014; Mohamed et al. 2018).

Prevalence of Theileriosis

Tick genera involved in the transmission of *Theileria* sp. are *Hyalomma (H.), Haemaphysalis (Hae.)*, *Amblyomma (A.),* and *Rhipicephalus (R.)* described in Table 1 (Bishop et al., 2004; Gharbi and Darghouth 2014). Theileriosis is prevalent all over the world froinrope, Asia, the Middle East, and North Africa (Bilgic et al. 2010). The global prevalence of Theileriosis is mentioned in Table 2.

Theileria Species causing Infection in Animals

Species of *Theileria* (*T.*) that infect bovines are *T. annulata*, *T. orientalis*, *T. velifera*, *T. parva*, *T. mutans*, *T. taruortargi*, *T. sinesis,* and new species *T. yokoyama* which is closely related to *T. annulata* (Bishop et al. 2004; Cao et al. 2013; Anupama et al. 2015; Hasan et al. 2017; Ola-Fadunsin et al. 2017; Sivakumar et al. 2019; Niaz et al. 2021). Prevalence of *Theileria* species is also reported in disease-endemic areas of Pakistan and is mostly found in the bovine population where it is transmitted by *Hyalomma anatolicum* and its prevalence is reported by different researchers in different areas of the country **(**Durrani and Kamal 2008; Durrani et al. 2010; Khattak et al. 2012; Khan et al. 2013; Jabbar et al. 2015; Farooqi et al. 2017; Ali et al. 2019; Zeb et al. 2019; Zeb et al. 2020; Parveen et al. 2021). Disease caused by *T. annulate* is known as bovine theileriosis and *T. annulate* is known as the most pathogenic species having worldwide distribution (Khatoon et al. 2013; Anupama et al. 2015). *Theileria parva* is causing the economically most important East Coast fever in cattle, especially in Sub-Saharan Africa. Another form of the disease is corridor disease caused by buffalo-derived *T. parva* previously known as *T. lawrenci* (Lawrence et al. 2004). In African buffalos, *T. parva* is not associated with clinical disease development but these buffaloes play an important role in the epidemiology of the disease by acting as a natural reservoir host and infection source for ticks especially *R. appendiculatus* (Latif et al. 2019). In cattle buffalo-derived *T. parva* infection is clinically different as compared to East Coast fever e.g. disease course is shorter, parasitemia level is also lower, not transmissible from infected cattle, a low number of schizont-infected cells, self-limiting infection, no carrier state in cattle (Lawrence et al. 2004; Mbizeni et al. 2013; Mekata et al. 2018).

Life cycle and Morphology

Their shape varies from round to ovoid (Soulsby 1982). Both asexual and sexual life cycle stages are present i.e., merogony, sporogony and gametogony respectively and among these stages, sporozoites are transmitted to the host with the help of a tick vector. Gametogony is present in the midgut of the tick and sporogony is present in the salivary gland of ticks while merogony is present inside host cells. Merozoites are comma-shaped or signet ring-like and present inside red blood cells (Zaeemi et al. 2011).
Infection is transferred from the clinically infected hosts to the uninfected ticks. Infected tick takes gametocytes from their host during blood meal; gametes fuse to make zygotes which invade the tick salivary gland with the help of hemolymph. The size of ookinetes is large but their amount is lesser. The sporogony stage starts when ticks change into a next stage and feed on blood then they are transmitted to the host with the help of saliva and this is called transstadial transmission.

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Table 1: Distribution of *Theleria* sp. with their vector tick species

Theleria sp.	Tick vectors
T. annulate, T. ovis, T. lestoquardi, T. separate,	*H. detritum, H. lusitanicum, H. dromedarii, H. anatolicum*
T. parva, T. taurotragi	*R. appendiculatus, R. zambeziensis*
T. orientalis, T. sergenti, T. buffeli	*Haemaphysalis sp.*
T. mutans, T. velifera	*Amblyomma sp.*

Table 2: Global prevalence of Theileriosis

Species	Distribution	References
T. annulate	Pakistan, India, Iraq, Turkey, Egypt, Sudan, Portugal, China	Junlong et al. 2015; Gomes et al. 2016; Ozubek and Aktas, 2017; El-Dakhly et al. 2018; Mohammed-Ahmed et al. 2018; Selim et al. 2020; Ahmed et al. 2021; Ceylan et al. 2021; Zeb et al. 2022
T. luwenshuni	China, Pakistan, India	Li et al. 2013; Khan et al. 2020; Dhaygude et al. 2021
T. sinensis	China, Thailand, Russia	Altangerel et al 2011; Bursakov and Kovalchuk 2019; Agina et al. 2020c; Jia et al. 2020; Wang et al. 2021
T. orientalis	Russia, Australia, China, New Zealand, Italy, Portugal, Vietnam, Spain, India, Ethiopia, Malaysia, Hungary, Korea	Savini et al. 1998; García-Sanmartín et al. 2006; Eamens et al. 2013a, b; Bawm et al. 2014; Hornok et al. 2014; Gebrekidan et al. 2017; Jia et al. 2020; Ola-Fadunsin et al. 2020
T. parva	Uganda, Tanzania, Mozambique, Kenya	Oura et al. 2011; Kabi et al. 2014; Kerario et al. 2017
T. ovis	Palestine; Syria; Iraq, Turkey, China, Pakistan, Tunisia, Spain, Oman, Sudan, Italy, South Africa	Al-Fahdi et al. 2017; Hussein et al. 2017; Lee et al. 2018; Azmi et al. 2019; Remesar et al. 2019; Hassen and Meerkhan 2020; Rouatbi et al. 2020; Abid et al. 2021; Wang et al. 2021 Al-Renneker et al. 2013; Zhang et al. 2014
T. uilenbergi	China, Iraq	
T. lestoquardi	Ethiopia, Turkey, China, Pakistan, Iraq, Spain, Oman, Sudan, Poland, Syria, Italy	Nagore et al. 2004; Shayan et al. 2011; Iqbal et al. 2013; Renneker et al. 2013
T. separate	Ethiopia, South Africa	Gebrekidan et al. 2014; Berggoetz et al. 2014

Table 3: Suitability of various methods available for detection of theileriosis in livestock population (OIE 2014).

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection before movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
Microscopic examination	-	+++	-	+++	-	-
PCR	+	++	++	+++	+	-
Detection of immune response						
IFAT	+	+++	++	-	+++	-
ELISA	+	+	-	-	+	-

+++ = recommended method; ++ = suitable method; + = can be used; - = not suitable

Transovarian transmission is absent in *Theileria* sp (Mehlhorn and Schein 1984). Sporozoites convert into schizonts after entering the lymphoid cells of the host. Macrophages releases merozoites that infect surrounding RBCs and produce four daughter cells (Urquhart et al. 1996).

Sheep Theileriosis

Theileriosis in goats and sheep is caused by different species of *Theileria* viz; *T. lestoquardi, T. Ovis,* and *T. Separata,* and vectors of this protozoa are different species of *Amblyomma*, *Haemaphysalis,* and *Hyalomma* around the world (Uilenberg 2006). Among these *T. lestoquardi* is the most virulent as compared to others (Durrani et al. 2011).

In goats and sheep, theileriosis is diagnosed by fever, kidney and liver dysfunction, splenomegaly, and lymphadenopathy. When the animal is under stress then subclinical infections may change to clinical infections. A blood smear examination can also be used along with clinical signs for the diagnosis of Theileriosis. Blood serum can also be used to detect carrier animals but with low sensitivity and specificity. For confirmatory diagnosis of *Theileria* molecular techniques like PCR have been used (Aydin et al. 2015).

Cattle Theileriosis

Theileriosis is a disease of worldwide importance and causes a significant economic loss every year globally. This disease is spread by tick vectors and the causative agent is *T. annulate* which is vectored by ticks of the genus *Hyalomma*. Theileriosis is endemic and it poses a huge threat to exotic, crossbred, and local breeds.

Clinical Signs and Pathogensis

Clinical signs associated with theileriosis are nasal discharge, ocular discharge, high fever, anemia, jaundice, dyspnea, leucopenia, lymph node enlargement, emaciation, hematuria, petechial hemorrhages on conjunctival mucosa, diarrhea, and blood in feces, decrease milk production, abortion, stillbirth death due to asphyxia and in case of brain involvement; convulsions, profuse salivation, and head pressing (Qayyum et

al. 2010; Eamens et al. 2013a, b; Moumouni et al. 2015; Tretina et al. 2015).

Pathogenesis associated with Theileria infection are change in hematological factors i.e. complete blood count (CBC) change, reduction in packed cell volume (PCV) values, decrease in Hb and MCHC concentration, increase in MCV (Somu et al. 2017; Lawrence et al. 2018).

Leukopenia is also included in pathogenesis of theileriosis, but it depends upon the species of Theileria (Omer et al. 2002). Necropsy examination of theileriosis cases has demonstrated the p[resence of hemorrhages (petechial, ecchymoses) on GIT mucosa along that splenomegaly, lymphadenitis, emphysema and oedema are also reported (Urquhart et al. 1996).

Diagnosis

Different diagnostic methods are used for theileriosis having some merits and demerits, these methods are divided into 3 main categories i.e.

A. Conventional methods
B. Serological methods
C. Molecular methods

A. Conventional Methods

Conventionally blood smear examination is used for Theileriosis diagnosis along with lymph nodes biopsy, but this method is not sensitive and cannot be used in carrier animals (Bilgic et al. 2010; Hayati et al. 2020). Salivary gland staining by methyl green pyronin stain has been used to study the prevalence of *T. annuata* in *Hyalomma* (Haque et al. 2010). Giemsa staining is one of the most commonly used methods to detect schizonts in Lymphocytes and piroplasm in RBCs but it is less specific, especially in the case of low parasitemia (Shayan et al. 2008). Giemsa staining cannot differentiate between *T. annnulata* and other species which are mostly non-pathogenic and carrier animals may remain unnoticed. Almeria et al. (2001) researched to compare PCR with microscopic examination and found that pathogenic and non-pathogenic protozoa cannot be differentiated by microscopic examination while on the other hand, PCR is an effective tool for the detection of protozoa even the quantity of DNA is very low.

B. Serological Methods

Serological methods of examination of parasites depend upon the detection of antigen and antibodies relationship. Indirect fluorescent antibody (IFA) test and enzyme-linked immune sorbent assay (ELISA) are examples of serological methods used for the detection of the antibodies against *Babesia* and *Theleria* spp. (Burridge et al. 1974; Iseki et al. 2010). But they are unable to identify the specific species, due to the presence of polyclonal antibodies of the whole antigen. Moreover, it expresses the disadvantage of cross-reactivity (Burridge et al. 1974). However, these methods are more sensitive than Giemsa's staining. Serum antibodies produced against merozoite antigen are useful for detection purposes, but they may produce false positive and negative results on the base of cross-reactivity and may lead to weakening the host immunity (Ikadai et al. 2002). Moreover, recombinant proteins production may also lead to ELISA being used for the detection of *Theleria* sp, such as *T. annulata* and *T. parva* in cattle (Gubbles et al. 2000), however, its ability to differentiate between chronic and acute infection is not up to the mark.

C. Molecular Methods

Highly pathogenic and economically importantprotozoan parasite *Theleria* is present around the world and its detection is highly worthful; for their detection molecular techniques have been developed including the use of DNA probes and PCR (Allsopp et al. 1993; Collins et al. 2002). This technique has proved much more sensitive and specific than all the other techniques (conventional and serological) being used for the detection of *Theileria* species. Non-conventional methods used for the diagnosis of piroplasmosis (Basesiosis and Theileriosis) are polymerase chain reaction (PCR), quantitative PCR (qPCR), real-time PCR (rt-PCR), reverse line blot hybridization (RLB), and LAMP (loop-mediated isothermal amplification) nested PCR (Atlay et al. 2008; Liu et al. 2008). The vectorial capacity of ticks for theileriosis is very specific and seeking scientific attention for a long. Along with the development of disease in the host, protozoans also modulate themselves in vectors. *Theileria* is confined to the tick genus *Hyalomma* and first discovered *T. annulata* was found in *H. anatolicum* (Liebisch et al. 1978). The specificity and sensitivity of molecular methods are better as compared to conventional and serological methods (Sbaragano et al. 1999; Almeria et al. 2001) more specifically in low infectivity (Sbaragano et al. 1999). 18srRNA gene was extracted from the DNA of *H. marginatum* and amplified by PCR for confirmation of the presence of *T. annulate* (De Kok et al. 1993). Another species of *Hyalomma, H. anatolicum* have shown the presence of *T. lestoquardii* infesting the sheep and goat in 1988 and *T. annulata* in the blood of cattle and tick vector, and for detection of *T. Annulate,* the membrane surface protein gene was amplified and confirmed (Kirvar et al. 2000). *T. ovis* was found to be carried by *R. bursa* which was detected by PCR (Aktas et al. 2006). In Iran, *T. leastoquardi* and *T. ovis* were found in the *H. anatolicum* infesting the sheep, detected by amplifying the 18SrRNA and it has shown a higher prevalence of *T. leastoquardi* than *T. ovis* and they both have shown the clinical theileriosis in sheep (Namavari et al. 2011).

Restriction fragment length polymorphism is a modified type of PCR (RFLP-PCR) based on the restriction enzyme which has shown the simultaneous detection of ovine piroplasmosis in tick vectors (Karimi et al. 2012). Another study conducted in the North-west part of Iran revealed the *Babesia* and *Theileria* species infection in sheep and goat populations by screening vectors i.e. *R. Turanicus* and *H. anatolicum* (Abidgoudarzi 2013). Durrani et al. (2011) reported a higher prevalence of *T. lestoquardi (*66.5%) in *Hyalomma* than *T. ovis* (65.8%) in *Rhipicephalus* infesting the small ruminant in Pakistan. Various studies have been performed on the detection of theileriosis and reported the higher prevalence of the disease in endemic areas of Pakistan (Khattak et al. 2012; Farooqi et al. 2017; Zeb et al. 2019; Parveen et al. 2021).

Control of Theileriosis

The epidemiological investigations converge attention on the control of such deadly diseases. Demanded optimization of currently available control strategies and design of research-based novel authentic control strategies. The currently available *Theileria* control strategies are a managemental

augmentation of cattle barn, chemotherapeutic control, vector control, and immunological control (Mhadhbi et al. 2015; Gharbi et al. 2020). Each strategy is effective to some extent but has its limitations. Evidence about the resistance status of chemotherapeutics used against pathogen and vector are increasing. Barn up-gradation and immunological control should be prioritized as they are highly sustainable and in line with one health approach objective of the World Health Organization (WHO), Office International des Epizooties (OIE), and Food and Agriculture Organisation (FAO). These measures have the best turnover in terms of livestock productivity (Gharbi et al. 2011).

Immunological control of various infectious diseases is the most successful strategy and in the case of protozoal disease, a few agents are controlled through vaccination including theileriosis (Mcallister 2014). For immunological control of theileriosis, schizont-based cell culture vaccine was first performed in Israel during the 1970s. These cell culture vaccines have been produced in Morocco, Tunisia, and Sudan. The level of immune protection produced due to these cell lines depends on the attenuation level, infected cell culture dose, and heterology of the vaccine agent.

In the case of East coast fever schizont culture vaccine remain effective in comparison with tropical theileriosis and against them, sporozoite culture-based vaccine with infection treatment protocol (ITP) is in use (Nene and Morrison 2016). In the ITP, the sporozoite culture of *T. parva* is injected into the animal body in combination with treatment. Production of such vaccine is not cost-effective as a large number of animals are required for vaccine production and the use of highly expensive antibiotics is also demanded. Furthermore, the standardization, cryopreservation, and dissemination of such vaccines are highly difficult (Bastos et al. 2019). The availability of live attenuated organisms is limited to a few countries (Shayan and Rahbari 2005).

The ideology of vaccine production is the production and boost up of immunity against a pathogen by inducing the natural infection but without the production of clinical disease. Such immune responses will be long-lasting for the prevention of clinical infection, mortality following natural infection, and dispersal of such deadly pathogens among susceptible hosts. For effective immunization, various significant factors need to be considered including selection and purification of specific antigen along with appropriate adjuvant, immunization dosage, schedule, and delivery platform. Antigen selection depends on the immunogenic activity of antigen: epitope immunomodulation, production of neutralizing antibodies, and activation of T-cell response (cytotoxic). Adjuvant augments the immunomodulatory activity of antigen, prolongs the antigen persistence, acts as a co-stimulatory signal, induces local inflammatory process, and cytokines-based lymphocyte activation. The proinflammatory cytokines including IL-2 and IL-12 induce both innate as well as adaptive immune responses and promote T-lymphocyte proliferation. The addition of these cytokines (along with adjuvant) in subunit vaccines leads to immunopotentiator impacts (Preston et al. 1999). Vaccines are administered via various routes viz; subcutaneous, intramuscular, oral, intranasal, ocular, and in Ovo but the selection of these routes depends on the pathogen type, the tropism of the cell, and the infection stage. The needle-free and controlled release methods are still under research and in the developmental stage (Agina et al. 2020a).

In the case of *T. parva,* various antigens have been administered through different routes for the evaluation of immune-protective characteristics. The main hurdle in the production of subunit vaccines at the global level is the strain genetic complexities of the pathogen (Norling et al. 2015), polymorphic characteristics of MHC, pathogen biodiversity (Hemmink et al. 2018), and cellular immune response dominance (Morrison et al. 2015).

For the production of a new vaccine against theileriosis molecular and antigenic properties of Tp9 protein have been studied. Tp9 is expressed by both schizonts and sporozoites (Bastos et al. 2019). A signal peptide, tissue plasminogen activator, was replaced by aTp9 signal peptide to increase the production of Tp9 from mammalian cells. A significant quantity of interferons was produced from CD4+ T cells in response to Tp9 administration. Against Tp9 both cellular and humoral responses have been observed. Therefore, Tp9 can be used as a vaccine agent against East Coast Fever, and further studies are required in this regard (Bastos et al. 2019).

Production of mucosal antibodies and cellular (CD8+ T cell) immune response has been observed following the administration of schizont antigens (Tp1 to Tp12) (Morrison et al. 2015; Hemmink et al. 2018; Bastos et al. 2019) and sporozoite antigens p67 of *T. parva* (Dobbelaere et al. 1985), and sporozoite antigen (SPAG1) of *T. annulata* (Williamson et al. 1989). For clearance of protozoa from the host, the antigen-based specific immunological response is required. For searching for antigens as vaccine candidates, class I MHC restricted T-cell recognized antigens were tested. This antigen plays a pivotal role in the prevention of sporozoites' entry into host immune cells (Morrison et al. 2015).

The polymorphic immunodominant molecule (PIM): is a structurally complex protozoal protein, that has immunogenic attributes, is expressed by both schizont and sporozoite developmental stages and helps in the entry of the pathogen into immune cells (Philip et al. 2014). PIM is found to be rich in proline and glutamine and generate cellular and humoral immune responses but the sustainability of such immune responses in the long term is yet to be confirmed (Nene et al. 2016). Similar to PIM immunogenic proteins, *T. annulata* surface protein (TaSP) and *T. lestoquardi* surface protein (TlSP) are also found in respective species of *Theileria* (Schnittger et al. 2002; Agina et al. 2020b). These proteins can be used as subunit vaccine agents and these vaccines exhibit cross protectivity (Knight et al. 1996; Nene and Morrison 2016). The subunit vaccine against *T. orientlis* is ineffective and this might be due to pure isolate extraction difficulties and the involvement of various genotypes in a single infection (Ivanova et al. 2016).

The subunit vaccines are effective and a possible solution for them is the use of DNA-based vaccine technology. These DNA vaccines are effective in case of protection against intracellular organisms viz; mycobacteria, herpes simplex virus, and protozoa. DNA vaccines eliciting cytokines production are effective against theileriosis (Dong et al. 2017). DNA vaccine induces cellular immune response through the production of cytokines especially T-helper cell one cytokines (IFN, IL-12, TNF-α, IL21). These cytokines play a pivotal role in the induction of immune response against chronic infection (Villarreal et al. 2013). Few cytokines are also used as adjuvants along with DNA vaccines such as IL-21 which activates NK-cells and T-cells. The level of IFN-γ is increased which enhances the activity of NK-cells and T-cytotoxic lymphocytes that remove pathogen from the host.

The ineffectiveness of chemotherapeutic preventive measures for theileriosis demanded the immunological control strategies implementation. Cell culture of schizont and sporozoites stage of the pathogen are effective measures usedfor control but due to its higher dosage it can lead to disease beside that it is costly and requires specific storage/ transportation facilities. The subunit vaccines (p67, TaSP, SPAG, PIM, TISP) have been developed against theileriosis but remain unsuccessful due to strain diversity, genetic diversity, and MHC restriction variability in the host. DNA vaccines that induce cellular immune response are found effective in the prevention of theileriosis.

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CHAPTER 10

HANDLING OF OUTBREAKS OF ANTHRAX: FUTURE PERSPECTIVES AND CHALLENGES

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INTRODUCTION

Bacillus anthracis causes anthrax, a bacterial illness. It can appear on the skin, in respiratory tract, in intestinal tract, or any in one of four ways. Symptoms might appear anytime from 24 hours to more than two months after the infection has been transmitted (Janik et al. 2020). The incubation time of anthrax is unknown; however, it is thought to be between one and fourteen days. The illness is commonly per acute to acute in animals, with mortality occurring within one to three days (Nakanwagi et al. 2020). Blister usually of small size with swelling in the periphery appears on the skin, which commonly develops into a painless ulcer with a black core. High temperature, shortness of breath are symptoms of respiratory anthrax. The other type is characterized by bloody diarrhea, abdominal discomfort, nausea, and vomiting. It is usually associated with intestines (Bower et al. 2015).

History

Egypt and Mesopotamia are regarded to be the origins of anthrax. The "boils" on Pharaoh's livestock in a 1500BC papyrus may be the earliest known instances of anthrax. Many historians believe that this very disease triggered the plague of Egypt, which was depicted as illness affecting equines and livestock, during Moses' time, in the middle of ten outbreaks of Egypt. Anthrax was well in classical Greece, as proven by most of the important intellectuals of the time's historical texts (Breniquet 2014). A few, even believe that anthrax played a role in the collapse of Rome. The first clinical descriptions of cutaneous anthrax were given by Maret in 1752 and Fournier in 1769, according to the Centers for Disease Control (Piňosová et al. 2021). Robert Koch was the first scientist to discover *Bacillus anthracis*, the bacteria that causes anthrax (Braun et al. 2020).

Epidemiology

Although *B. anthracis* is found throughout the world, the incidence is unclear. It is rare in much of western Europe, Canada, the United States, and Australia, but widespread in southern and eastern Europe, numerous former Soviet Union nations in Central Asia, southern and central America, and Africa. Every year, at least 2,000 instances are reported worldwide (Sushma et al. 2021). Cases were reported occasionally several years in New Mexico. It has recently been identified in deer and livestock in areas near the Rio Grande in southeastern Texas. Many regions of the nation were "seeded" with anthrax spores during the large cattle drives of the 1800s, according to one concept. Even within endemic regions, anthrax strikes on a sporadic basis, with several years among outbreaks (Gillan et al. 2021). During natural circumstances, human disease (typically dermatological) is mostly induced through exposure to infectious animals or diseased animal products, such as hides or wool. Respiratory form of anthrax (wool sorter's sickness) has been reported in restricted production settings where high-volume handling of skins and wool occurred. The capacity of spore to cause disease via the breathing pathway is limited. Unreliable reporting makes estimating the real global incidence of human anthrax problematic (Finke et al. 2020).

Etiopathology

Bacillus (B.) anthracis is a spore-forming bacteria. Its morphology is rod shaped, diameter of 1-1.2 µm and a length of 3 - 5 µm. *B. anthracis* is an aerobic, non-fastidious bacterium that develops in a broad temperature range (12-45°C). The phenomenon of sporulation is aided by adverse circumstances such as distilled water, oxalate, O_2 and 2% NaCl which are all present at 25-30°C. It has the ability to produce long chains of bacteria cultivated in culture. It produces colonies those are several millimeters broad and whitish or cream-colored on agar media. The capsule produced by most *B. anthracis* strains gives colonies a slimy mucus-like look. Under aerobic or anaerobic circumstances, the bacteria may be grown in conventional nourishing media (Wood and Adrion 2019).
Endospore of this bacteria forms multiple layers in the cell membrane. It remains quiescent for years. It begins its development process when conditions become favorable. Development starts inside the rod-shaped structure. The position of endospore within the rod, the structure and form of the endospore, will tell if or not the endospore compels the rod body to come out (Janik et al. 2019). These are all traits

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that identify Bacillus species. It comprises of dipicolinic acid and very refractive. *B. cereus*, which is present in soil environments all over the globe, and *B. thuringiensis*, the pathogen for *Lepidoptera*, are genetically and phenotypically quite similar. The proliferative and spore states of the anthrax bacterium can both be detected. The illness is caused by the proliferative state, which is the reproducing form observed in affected animals (Villa et al. 2021). The proliferative state of such animals that died from anthrax is exposed to oxygen if the corpse is opened during necropsy, or by scavengers or decomposition. This permits spores to develop in the vegetative stage. The spores are resilient to certain chemicals and elements, and they can survive in the soil for almost 50 years. The bacteria return to the disease-causing vegetative stage when the spores gain entry in an animal, generally by feeding infected grass or by respiration (Jelinski et al. 2021).

Disease in Humans

Anthrax on the skin, commonly known as hide-illness, wool sorter's disease is referred to as cutaneous anthrax. It is the most frequent but least hazardous kind of anthrax. Erythema and edema may surround the lesion, although it typically feels like no pain area. Lymphadenopathy is a possibility, as are systemic symptoms including fever and headache (Hendricks et al. 2014). Anthrax associated with skin appears as a skin lesion that looks like a boil which develops into a black-centered ulcer (eschar). At the infection site, the black eschar generally manifests as big, non-painful decaying lesion which begins as an unpleasant, and a dark, irritating cutaneous sore or blister that generally appears as a dark patch like bread infected with *Rhizopus*. In general, cutaneous infections appear two to five days following spore penetration at the position where it is penetrated. Shivering with chills also present, although there are few additional symptoms. The bacilli persist within the skin sore in 90% of cases where it is evident of anthrax in human beings. Death is caused by the synthesis of two potent exotoxins and a fatal toxin (Akbayram et al. 2010).

Disease in Animals

The most prevalent type of anthrax in cattle is acute type, unexpected death is perhaps the most typical indication of anthrax. Fever, loss of rumination, excitation, mood disturbances, problematic breathing owing to fluid buildup in the lung tissue, uncontrolled movements, seizures, and death may occur after an epidemic has started (Hugh-Jones et al. 2002). The corpses get bloated with gases as they degrade quickly than in other situations. The stiffening, or rigor mortis, is not complete. Internal organ hemorrhages have been discovered during necropsy. Almost typically, the spleen is swollen. The sudden occurrence of anthrax-induced death, as well as the dark, fresh blood comes out of body orifices, can often be detected by veterinarians (Zohora et al. 2011).

Biological Warfare and Anthrax

Anthrax spores can be inhaled and cause illness and sickness. The spores of anthrax have been utilized in biological warfare. *B. anthracis* has become a preferred bioterrorism weapon, due to the capacity to create aerosols harboring anthrax spores. Several abilities may be capable of loading *B. anthracis* spores to weapons (Smith-Akin et al. 2007). Terrorists may devise techniques to disseminate spores through large-scale or localized assaults. Like a bioterrorism weapon, anthrax spores are intended to be sprayed in a cloud at a strategic position and inhaled by those who are being attacked. *B. anthracis* spores may be manufactured and kept dry, and they can last for decades in storage or after release (Hart et al. 2020).

During World War I in the early 1900s, the first purposeful use of anthrax as an act of hostility was documented. In 1916, Nordic rebels, armed with anthrax provided by the German General Staff, used it against the Imperial Russian Army in Finland, with unpredictable consequences (Farhatullah and Qureshi 2020). During the 1930s, in Manchuria, the Japanese Kwantung Army's Unit 731 tested anthrax as a biological weapon: most of this experimentation entailed the deliberate infection of war prisoners, hundreds among whom perished (Guillemin 2018). This domain has a significant history of operational bioterrorism research. For example, British bioweapons testing polluted Gruinard Island in Scotland with Vollum-14578 anthrax strain in 1942, establishing a ban region to be visited by any civilian before it was thoroughly cleaned by using different disinfectants in 1990. The Gruinard trials were designed to see how effective a cluster bomb of a "N-bomb" — It could be a contagious device made up of desiccated anthrax spores. Furthermore, five million "cows pies" were distributed (animal feed pellets coated with anthrax spores) were created and stockpiled (Balmer and Moon 2016). It was stacked at Porton Down for "Operation Vegetarian," which was to be used by Royal Air Force of British to conduct anti-livestock assaults against Germany. In 1944, anthrax-based bio weapons were to be unleashed on Germany. Palatable cattle cake and the bomb, on other hand, were not used; the cattle cakes were burned in 1945. United States joined the Biological Weapons Convention in 1972, weaponized anthrax existed in the US stockpile (Oliveira et al. 2020) In 1969, President Richard Nixon ordered the termination of US bio warfare projects, as well as the destruction of all existing bio warfare stocks (Eneh 2012) During war with separatists (1978–79), Rhodesian government employed anthrax on humans and animals. At Kantubek on Vozrozhdeniya Island, the Soviet Union developed and preserved 100 to 200 tons of anthrax spores, which were deserted around 1992 and annihilated by 2002 (Cross 2017).

Anthrax spores can be grown with only just few components of specialized technology and a basic understanding of microbiology from a first-year college course. To generate significant volumes of anthrax aerosol suited for bioterrorism, one need a lot of experience, training, and cutting-edge technology. In 2001 anthrax assaults occurred in North America, concentrated spores were utilized for bioterrorism by sending letters via mail carrying the pathogen. Letters were addressed a numbers of press outlets and also southern Dakota Democrats in the senate namely Vermont, Tom Daschle and Patrick Leahy. As a consequence, 22 people were diagnosed, with five of them dying. The attempts utilized only a few grams of material. The US Department of Justice declared in August 2008 about Bruce Ivins, a US government-employed top bio warfare investigator, was the culprit. Many anthrax conspiracies arose as a result of these incidents (Cole 2020).

Sverdlovsk Tragedy

Having signed the 1972's agreement to stop bioweapons production, the Soviet Union's leadership continued to operate

a biological warfare initiative with the participation of the manufacturing of *Bacillus* in the vast quantities after some time. In April of 1979, roughly one million residents of Sverdlovsk a city in Russia (now it is called Ekaterinburg), situated at a distance of 1370kms (850 miles) from Moscow, were exposed to anthrax leaked unintentionally from a biological weapon's factory nearby. A minimum of 94 persons became affected, with 68 of them succumbed to death. A person died four days after being discharged, ten additional victims died over the course of eight days during the climax of the deaths, and the final victim died six weeks after the first case was reported. Almost all the employees of pottery working at night time company directly all over the street from biology station (compound 19) got ill, and the majority of them deceased. Some NATO states believed that Soviets have created a sex-specific weapon since majority were males. Approximately 30 victims were saved thanks to extensive cleanup, immunizations, and medical assistance. Between 1979 and 1992, when Russian President Boris Yeltsin admitted the anthrax incident, the KGB engaged extensive hide and record destruction. To back up the hide theory, Soviet heath care and legal publications produced articles describing a livestock epidemic that resulted in gastrointestinal anthrax in humans in individuals who came into contact with the animals, and skin anthrax in those who interacted with organisms. The KGB took possession of all healthcare records. Aside from the medical concerns created by the epidemic, it spurred Western governments to become increasingly doubtful of a concealed Soviet bioweapons operation and to step up monitoring of suspicious areas. The US authorities' permission was given to examine the event in 1986, and it was determined that the transmission was caused by aerosol anthrax from a military equipment site. In 1999, Jeanne Guillemin stated that a joint Russian-American team probed the 1992 tragedy (Balmer 2021).

Outbreaks Handling and Diagnosis

- ✓ Anthrax should be suspected in any animal that dies suddenly and without warning (especially ruminants) (Avberšek et al. 2021).
- ✓ The diagnosis can be confirmed by aseptic conditions drawing deceased blood from a peripheral vein (e.g., the jugular vein or the ear vein) by using a suitable dye to analyze a sample of blood for the existence of the capsule. It can also be achieved by using Mc'Fadyean methylene blue stain or culturing the bacilli (McInnes 2015).
- ✓ For direct identification of *B. anthracis* in blood samples, a variety of approaches can be applied. To begin, Gram staining of specimens is one option. *Bacillus* spp. grow to be fairly large (3 to 4 μm long), may form lengthy chains, and stain Gram-positive (Markey 2013).

Laboratory Diagnosis of Anthrax

Before beginning antibiotic therapy, a sample should be collected. The anthrax type and clinical manifestations influence the sample selection. Pus, sputum, blood, CSF, stomach aspirate, and feces are all common specimens. A quick identification test for initial scrutiny for *B. anthracis* based on gross appearance of micro colonies has been published, as well as many nonselective and selective mediums for the detection and isolation of *B. anthracis*. Heat and alcohol shock can be used to separate *B. anthracis* from ambient materials before spreading on culture medium. Merely the spore-forming bacilli will endure the shock, assisting as an augmentation strategy (Kwon et al. 2018).

Confirmation Tests

Gram Staining

Gram-positive bacteria with distinctive purple color, massive, four-sided rods are discovered. Spores are seldom seen in clinical samples (Tekin et al. 2015).

McFadyean's Reaction Technique

Gurr's polychrome methylene blue dye can be used to illustrate polypeptide capsules for 30 seconds. Amorphous purple substance surrounds blue bacilli in the capsule. This is used to provide a preliminary diagnosis of anthrax in animals (Nagarajan et al. 2015).

Direct Immunofluorescence Test (direct-IF)

It uses fluorescent-tagged monoclonal antibodies to identify capsule and antigens which contain polysaccride. During bioterrorism epidemics, it is utilized to confirm the diagnosis (Maves and Berjohn 2020).

Ascoli's Thermos Precipitation Test

It is a test for ring precipitation identification. This test is primarily used when the material become rotten and the bacilli aren't likely to survive. Tissue samples are boiled and filtered after being crushed in saline. On a narrow capillary tube, this antigenic extract is placed over anthrax antiserum. After 5 minutes, a ring of precipitate forms around the juncture of antiserum and antigenic extract (Olani et al**.** 2020).

Isolation on Culture Media

Following culture media options are available for the isolation of *B. anthracis:*

Blood Agar

After an overnight incubation on blood of sheep, *B. anthracis* develops, dry wrinkled, non-hemolytic colonies with a frosted glass look. Fringed edges or curled protrusions may appear on occasion in colonies (tailing). The appearance is called as "Medusa head appearance".

Red Line Alert Test

The 'Red Line Alert Test,' an immune chromatographic test for identification of surface present in *B. anthracis* proliferative cells, can be used to presumptively identify non-hemolytic *Bacillus* colonies.

Stab of Gelatin

According to gelatin liquefied, which happens predominantly on top surface and subsequently slows down towards the bottom, growth appears as an upright tree.

Selective Media for *B. anthracis*

Solid Medium with Antibiotic Penicillin

Cells become larger and spherical due to their looser cell walls underneath the influence of penicillin, and cells tend to form in a chain on the surface of agar, giving colonies the appearance of a string of pearls.

PLET Medium

The heart infusion agar contains polymyxin, lysozyme, EDTA, and thallous acetate. It was developed to distinguish *B. anthracis* from spore-bearing bacilli in a mixed culture (Kumar et al. 2019).

Necropsy Examination for Suspected Anthrax Cases

The post-mortem of large animals may divulge the following changes as described in the literature (McInnes 2015):
(1) Fast disintegrating dead body;
(2) Fresh blood from all natural orifices including the nose, mouth, or anus;
(3) No rigor mortis if an infected carcass is unintentionally opened
(4) Dark, tar-like un clotted blood;
(5) Lesions suggestive of widespread septicemia;
(6) Splenomegaly with a "blackberry jam" consistency.

Precaution for Sample Handling

✓ Send a purple top tube of blood for the blood smear.
✓ The red capped blood tube, blood culture vial, or culture swab for culture.
✓ Swabs of nose and pharynx of companion animal are also be acceptable for culture.
✓ All culture swabs should be placed in Amies transport medium, either with or without charcoal (Palacio 2017).

Shipping of Sample

✓ Put the original sample within an additional leak-proof container (95pKa rated), with adequate absorbent material to absorb any liquid contents in the case of a leak or damage (swab in microbial transport media; blood in vacuum blood collection tube).
✓ This secondary container includes cooling sachets. Put the documentation in a zipper-lock bag. All of the things listed above should be placed in an insulated inner box or bag, which should be labelled "Anthrax suspicious specimens."
✓ Put a label on it that says Biological Substance Category B.

Precautionary Measures to be Opted to Handle the Necropsies

As this is a highly fatal zoonotic diseases, so while handling the dead body, latex gloves, aprons, and boots with no perforations are utilized as protective, impenetrable apparels. No flesh should be exposed, especially if it has any sores or scrapes. Disinfection is accomplished by autoclaving if disposable safety equipment is not accessible (Bengis 2021). Oxidizing substances such as peroxides, ethylene oxide, Sandia Foam, chlorine dioxide peracetic acid, ozone gas, hypochlorous acid, sodium persulfate, and liquid bleach solutions containing sodium hypochlorite may be used in chemical procedures for cleaning anthrax-contaminated places or items. Non-oxidizing compounds that have been demonstrated which includes Methyl bromide, formaldehyde, and metam sodium to be useful for anthrax decontamination (Kim et al. 2011). Bacterial spores are degraded by these chemicals. After usage, disposable equipment is burnt or buried. All infected bedding or apparel is segregated and processed as biological garbage in double plastic bags. The spore medium, external factors such as temperature and humidity, and microbiological components such as spore species, *B. anthracis* strain, and test results are all factors to consider. Procedures utilized all influence decontamination strategies for *Bacillus anthracis* spores. Many countries have opted partially or all of the following measures to reduce the risk of anthrax spore-contaminated items being imported or disseminated (Nerandzic et al. 2013; Mushayabasa et al. 2017):
✓ A certificate issued by a veterinary officer in that the products has been imported from anthrax-free countries.
✓ Items that are thought to have a high risk of carrying anthrax spores are monitored wisely and tracking is done.
✓ If the country is not anthrax free then trade limitations are there to import from such regions and prior sporicidal treatment of the raw or finished good products is mandatory to ensure the safety.

Anthrax Suspected Carcass Handling and Disposal

Treatment with Formaldehyde

Anthrax carcasses are treated with 10% formalin and left in place for a few days before being disposed of, allowing natural decomposition processes within the body to kill the vegetative anthrax organisms. The formalin would kill anthrax germs excreted by the deceased animal while also maintaining the skin, allowing the decaying body to maintain an anaerobic environment. This might also stop scavengers from opening up the carcass and spreading the disease, as well as flies from disease transmission (Hassim et al. 2017).
When burial, incineration, or rendering are not viable due to low income then final alternative may be to leave the body motionless in place and ensure that it is unavailable to other animals, notably scavengers, or humans. It is accomplished by draping plastic tarps, tree branches, corrugated iron, or other readily accessible materials over the area. In this instance, warning signs should be placed across the area. It permits the decay activity to resume, while remnant environmental pollution may still exist, and the site should either be burnt or treated with 10% formalin when decomposition is complete. Fences, topping with stone or other impermeable material, covering with brushwood, or establishing inaccessible undergrowth are all options for making it inaccessible to other creatures indefinitely (More et al. 2017).

Disposal Through Burial

The burial of the infected carcass can be done by following the guidelines mentioned by Sidwa et al. (2020) and be careful that local or provincial government officials may demand burial permits.
✓ To excavate an appropriate hole, heavy excavation equipment (such as a backhoe) is required

✓ Make sure the hole is about two meters below, with the water table high above the bottom (minimum 3 feet)
✓ Evaluate the level of the ground water and the type of the soil – topsoil is preferred above fine sand.
✓ To keep predators out, at least 3.2 feet of clay at the deepest point, and a minimal amount of 3.2 feet mud and soil over the body
✓ Decontaminate the body and any dirt going into the burial hole a disinfecting containing 10% formalin and 5% lye solution (sodium hydroxide) or some other appropriate disinfectant.

Limitations of the Method

Regular reports of live anthrax spores at burial sites of animals that died several years ago, as well as occurrences and breakouts in animals linked with such locations, have proven that burial practices are unreliable for long-term disease management. Disruption of these locations, such as farming or installing sewerage, is thought to bring the spores to the top. Scavengers may dig down to access the remains, and in dry, dusty environments, the digging process can disseminate the tainted material far (Sitali et al. 2017).

Disposal Through Incineration

The recommended procedure of disposing (usually when a corpse is mistakenly opened for necropsy investigation and scavenging) is to burn or burn by pyre or pit. Incineration should be handled with care to achieve full burning from the bottom. This usually entails lifting the corpse off the ground before beginning the operation. Mobile commercial incinerators are available to ensure this. Prevent utilizing items that are potentially damaging to the environment (e.g., rubber tires). Assess if there is enough fuel to thoroughly decompose the body into ash. Incorporate any dead animal bits or debris that comes out during burning during the re-entry onto it and flames for total combustion. Infected items should be burned together with the infected dead animal (González and Chaigneau 2017).

a) Wood Burning:

Deep layer: Massive timbers, wooden poles, chain-link fences, and zigzag timber boards spaced 8–10 inches apart to allow air to enter from below. Arrange the materials with the line of the wind blowing**.**
Middle layer: Place smaller bits of wood or coal on top of the lower side.
Upper layer: Place any topsoil that is possibly polluted, then place the carcass on its back by placing the animal/secretions on top of the burn material.
b) Straw: On the bottom layer, place heavy wood, wooden poles, railway ties, and wood pallets 8–10 inches apart in a segment arrangement to allow air to access the fire from underneath. Align these materials to the direction of the current wind.
Inner layer: Each carcass has two enormous round bales weighing roughly 1200 pounds (545 kg). Bales can be stacked on their sides or on their ends. To construct a platform for the carcass, top the bundles with a layer of wood skids. Airflow into the pyre will be improved by using pallets positioned between the bales.
Upper part: Raise up the carcass and place it on its back, covering the pyre with any dirt that may have been polluted by the animal/exudates.

Disposal Through Trough

Usage of trench allows for burying of remnants while also preventing the spread of fire. Airflow to the fire is aided by the pit's slanted sides (Dippenaar et al. 2018)

Considerations

✓ The ditch for an adult animal should be 18–20 inches deep and stretch about 2.5 feet beyond the side of the flame.
✓ On each side allow for airflow around the carcass, the pit should be roughly 10 inches wider than the flame.
✓ Floor of trough is coated with flammable liquid (e.g. gasoline, kerosene, etc.) other materials that allow for airflow.
✓ To sustain the pyre, large lumbers (or other beams) are laid across the trench.

Rendering

Rendering is a heating procedure that leads in the disinfection of animal-derived raw materials, allowing sections of carcasses to be used safely for economic purpose. The rendering process may be classified into two categories: group procedures and continuous processes. Generally, the raw ingredients are finely sliced before being burned in a steam-heated chamber for 10–60 minutes at temperatures ranging from 100 to 150 degrees Celsius (Pantha et al. 2016) The rendering technique requires proper execution at each of three stages:
✓ Carcass pickup,
✓ Shipping,
✓ Treatment.
The body must be packaged, and the container, as well as the collecting equipment, and devices, as well as the carcass site itself, should be thoroughly sterilized and cleaned. To prevent rear contamination, the rendering plant should be adequately split between "unclean" and "clean" portions that are not connected by a single drain. The unclean end has to be well-equipped for sanitizing the trucks and certain other apparatus. Veterinary officials should be in responsibility of all this process (Kisaakye et al. 2020).
To remove the spores, sewage from the dirty end must be collected and treated with heat or chemicals (ideally heat). Carcasses should be cut down into its constituent parts no bigger than 10 cm^3 before being heated. In case of anthrax fatalities, it should be handled with great caution, with the requisite sanitation and cleaning of the rendering premises, tools, clothes, waste run-off, and other areas. The heat, intensity, and timeframe of decontamination are then recorded as part of a regulated thermal processing (Pantha et al. 2016).

Late Disposal

The quick disposal of diseased carcasses may be problematic under certain climatic conditions, such as continuous precipitation; carcass inaccessibility (e.g. stagnant mud, dense forest); or logistical issues (e.g. lack of needed items, staff, etc.). In these conditions, review the problem to determine an acceptable plan of action within a reasonable schedule for disposal in order to reduce anthrax environmental

contamination. Decontaminate the body and the nearby region using cleaning agents such as 10% formalin or a 5% lye (sodium hydroxide) solution, and continue as required (Mushayabasa et al. 2017).

Anthrax Control through Vaccination; Past, Present and Future Perspectives

Louis Pasteur, a French scientist, was tasked with developing a vaccine for anthrax. In May 1881, Pasteur exhibited his concept of vaccination in a public experiment at Pouilly-le-Fort, with the help of his collaborators Jean-Joseph Henri Toussaint, Émile Roux, and others. He'd prepared two flocks of animals including 25 sheep, single goat, and very few cattle. The animals which belong to group one were given two injections of Pasteur's anthrax vaccine, separated by 15 days, whereas the animals in the control group were not immunized. Thirty days after the first injection, both groups received a culture containing live anthrax bacteria. All of the animals in the unvaccinated population died, whereas those in the vaccinated group all lived (Liang et al. 2016).

In 1954, the human anthrax vaccination became accessible. This was cell-free vaccine, as opposed to the live-cell Pasteur-style inoculation used in animal medicine (Zohora et al. 2011). In 1970, a better cell-free vaccination became available. Vaccines containing dead bacilli and/or capsular antigens do not provide considerable protection (Gogoi et al. 2018) In livestock, a non-capsulated toxigenic strain has proven to be successful. People that come into contact with exotic animal skins, furs, bone, flesh, wool, animal hair (especially goat hair), and bristles are at risk at their workplace, as well as those involved in diagnostic or exploratory activities that may expose them to anthrax spores, should be vaccinated (Adone et al. 2016).

Bacillus anthracis strain Sterne generates sub-chronic levels of the toxin that causes the development of protective antibodies (Chitlaru et al. 2016). A new form of passive anthrax vaccination is currently being developed. Mice injected with a human adenovirus producing a single-chain antibody aimed against protective antigen (PA) developed anthrax immunity in about 24 hours (Manish et al. 2020).

Limitations

Passive immunotherapy using vectors which belong to adenovirus producing anti-protective antigen antibodies, either alone or in conjunction with antibiotics, might be a quick, easy, and effective way to defend against or cure anthrax in a bioterrorism strike (Deal et al. 2021).

Active vaccinations do not lose their efficacy over time, but passive vaccines do. The passive vaccine may provide protection for a few weeks, but it would also create a buffer for the active vaccination to induce the formation of more active, long-term immunity (Kumar et al. 2020) In the United States, the anthrax vaccine is made from the protective antigen extracted from the culture filtrate of virulent, non-capsulated type of *Bacillus anthracis* that generates protective antigen during active growth. Because anthrax vaccines are active vaccines that require repeated doses over many months to show protective immunity against anthrax, their effectiveness in a bioterrorism strike is restricted (Phaswana 2015; Kravchenko et al. 2021).

Control Measures in Humans

Anthrax is transferred to humans, but in rare case of cutaneous secretions from skin, anthrax could be transferred. However, anthrax spores can infect a person's clothing and body. Humans can be effectively decontaminated by complete washing with antibacterial soap. Bleach or another antimicrobial agent is used to treat wastewater (Kortepeter 2020). Boiling goods in water for 30 minutes or more is an effective way to decontaminate them. Formaldehyde is more efficient than chlorine bleach by killing spores and proliferative cells on floors. Clothing that has been burned is particularly good in damaging bacteria. It is not essential to vaccinate, treat, or isolate contacts of anthrax patients after decontamination unless they were also subjected to the similar cause of contamination (More et al. 2017). Individuals who have not been vaccinated should be given antibiotics if they are exposed to inhalation anthrax. Anthrax must be treated with antibiotics as soon as possible; waiting too long reduces the odds of survival (Clark and Wolfe 2020). High dosages through venous route and oral antibiotics, such as fluoroquinolones (ciprofloxacin), doxycycline, erythromycin, vancomycin, or penicillin, are used to treat anthrax and other bacterial infections. Ciprofloxacin, doxycycline, and penicillin are all FDA-approved antibiotics. If given previously the commencement of spread through lymph and toxin spread through blood, which is thought to be around one day, penicillin, tetracycline, and fluoroquinolones are beneficial (Williams et al. 2018). Antibiotic therapy has also been shown to reduce the severity of anthrax infection in people who get it through the skin. Previously, it was considered that inhalation anthrax was virtually always lethal despite antibiotic therapy, especially if treatment began after symptoms appeared. Early antibiotic prophylactic therapy is critical in suspected instances of pulmonary anthrax to avoid mortality. Many efforts have been made to produce new anti-anthrax medications, although current treatments are effective if treated promptly (Owen et al. 2015).

Control Strategies in Animals

Annual preventative vaccination is the primary control method for animal anthrax; however, if an epidemic has occurred, further control strategies include ring vaccination, careful body disposal to limit further pollution in the environment, and quarantine (Rahman et al. 2020; Firstova et al. 2021). The weakened strain of *B. anthracis* is utilized in domestic cattle immunization all over the world. Since this vaccination is live-attenuated, using antibiotics at the same time can significantly reduce its effectiveness (Sidwa et al. 2020; Sheykhsaran 2022). Proper safety carcass disposal is critical for managing anthrax outbreaks in enzootic habitats because it enriches the earth with spores and elevates the risk of recurring epizootics. The corpse should be set to fire immediately, a burning or other approach that merely produces trash and permits the dirty soil to be destroyed also (i.e., “burned till it is entirely decomposed"). When a dead animal cannot be incinerated, it is recommended that it be buried deep. Lime should not be added as it has been done in the past. *B. anthracis* spore survival is aided by high soil calcium levels, which may be achieved by adding lime to the soil or occurring naturally in some parts of the world. This increases the possibility of repeated outbreaks (Islam et al. 2021)

Scavenging procedure also distribute the spores and raise future vulnerability risks for sensitive animals, therefore leaving the corpse in place is the least preferred form of disposal. In regions, where the traditional suggestions of burning or burying carcasses are unfeasible, other carcass disposal options are required. This is especially important if there are a lot of sensitive wildlife those aren't immunized or where domestic hoof stock availability of vaccine is low (Carlson et al. 2019).

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CHAPTER 11

PESTE DES PETITS RUMINANTS IN A WIDE RANGE OF DOMESTIC, WILD AND UNUSUAL HOSTS: A POTENTIAL CONSTRAINT IN DISEASE CONTROL EFFORTS

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INTRODUCTION

Peste-des-petits ruminants (PPR) is an infectious viral disease affecting a wide range of susceptible hosts, including domestic small and large ruminants, camel and, some wild ungulates (Aziz-ul-Rahman et al. 2018; Rahman et al. 2020). Owing to the potential of spread, substantial economic losses, and genetic depletion due to high infection and death rate among all susceptible host species, particularly in an endemic region, both OIE and FAO classified PPR disease as A-listed transboundary animal disease (FAO 2015, 2017; OIE 2017a, b). The causative agent of PPR infection is the PPR virus (PPRV) which belongs to the genus *Morbillivirus* under the family *Paramyxoviridae* (Kuhn et al. 2020). This virus has a close antigenic association with some viruses from the same genus including CDV, RPV and MV. These morbilliviruses such as CDV and MV have the capability to the cross-species barrier for adaptation towards novel host species and subsequently potential to infect diverse naïve and unusual hosts (Leonard et al. 2008; Beineke et al. 2015). Among all morbilliviruses, RPV had the highest antigenic relationship to PPRV and RPV has the capability to infect large ruminants and wildlife species as a result of cross-species transmission and spill over events (Barrett and Rossiter 1999). Considering this evidence of morbillivirus, it has been postulated that PPRV may also show the propensity of inter and intra-species transmission to cause infection in wild animals (Mahapatra et al. 2015; Aziz-ul-Rahman et al. 2018).

It was first assumed that PPRV can only affect small ruminant species including sheep and goats (Banyard et al. 2010). However, recent evidence of PPR infection (Clinical and pathological), seroconversion, genomic identification, and viral antigen detection have been observed in small ruminants, wildlife species, large ruminants, camels, and unusual hosts (Kinne et al. 2010; Ratta et al. 2016; Aziz-ul-Rahman et al. 2018; Rahman et al. 2019, 2020). All susceptible host species are at high risk to get PPR infection due to the transboundary nature of the virus targeting small ruminants, wildlife species, and large ruminants, particularly at livestock-wildlife interface zones (Aziz-ul-Rahman et al. 2016). There is a possibility of PPRV dissemination from infected wild host species to other susceptible hosts including wild animals, small and large ruminants (Munir 2014; Schulz et al. 2018). Data related to PPR infection in domestic small ruminants are available. However, information on the susceptibility of wild ungulates, large animals, camels and unusual host species is scarce. It is, therefore, imperative to investigate the potential of all susceptible hosts in disease dissemination towards strengthening PPR control interventions for eventually disease suppression, particularly in disease-endemic settings. After the successful eradication of rinderpest, the primary goal of both FAO and OIE is to suppress PPR till 2030 under a joint progressive control program. However, the capability of PPRV to infect a wide range of susceptible hosts concerning disease epidemiology may create hurdles in strengthening disease control strategies, particularly in disease-endemic regions. It is, therefore, imperative to robust and continuous disease surveillance investigations considering all susceptible hosts with appropriate disease control measures for the eventual global eradication of PPR.

Geographical Distribution of PPR Virus

PPR was first investigated in Cote d' Ivoire in 1942, and was initially termed as Bluetongue, due to ulcerative stomatitis and later on known as small ruminant plague (PPR) based on the clinic-pathological representation (Gargadennec and Lalanne 1942). Initially, PPRV was considered an RPV variant, but later on, it was recognized as a novel virus member of the genus *Morbillivirus*, based on its biological and biochemical characteristics (Appel et al. 1981). Regards to PPR virus geographical distribution, it has shown significant and dramatic terrestrial expansion of the disease occurred over the last 30 years since 1982, beyond its original endemic region in Western Africa to Central Asia, South Asia and East Asia (Banyard et al. 2014). Notably, around 70 countries have reported the existence of the PPRV infection and another 50 are suspected to be infected or are considered at risk. Among these infected countries, Africa is considered highly endemic following Asia and the Middle East (OIE 2017a, b). The occurrence of PPR is now causing worse effects in many Asian countries including, Afghanistan, Bangladesh, India, and Pakistan (Abdollahpour et al. 2006).

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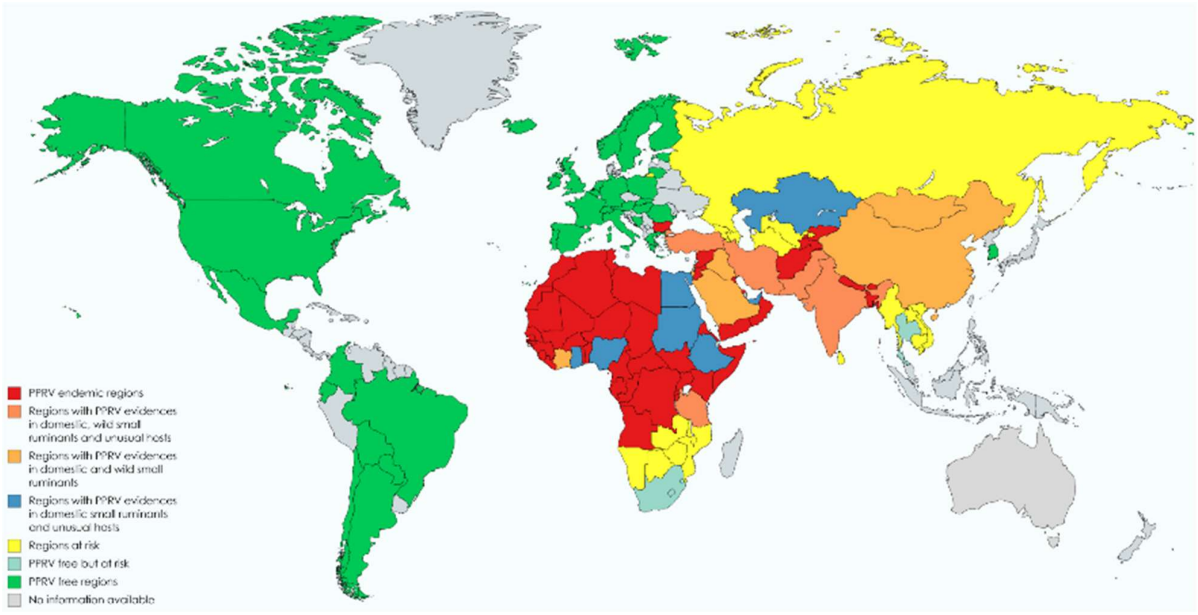

Fig. 1: Geographical distribution of PPR in context of wild ungulates, large ruminants and unusual hosts

PPR caused heavy losses in the region of Africa, apart from Egypt, but endemically in the girdle between the Sahara and the Equator before 2007. For that year, PPR has officially been recognized in the Central, East African countries and onwards, the disease steadily expanded its geographical distribution towards the Western African countries. The disease severely affected the various region of North Africa and was considered endemic including Morocco, Tunisia and Algeria (OIE 2017a, b). Although the available data on PPR in sheep and goats are immense, information about the geographical distribution of this disease in wild ungulates, large ruminants, and unusual hosts is rare. Findings of PPRV or antibodies to PPRV in wildlife have mostly been reported in countries/areas where PPR outbreaks commonly occur (Figure 1). In addition, PPRV as an emergent virus, caused infections in different wild animals of Mongolia, Tanzania, China, Pakistan, KSA, Turkey, and in unusual hosts including large ruminants and camels of West African countries, India, Pakistan, and Sudan, increasing the host spectrum of PPRV in those areas, considered as PPRV endemic regions. Such remarks have highlighted the current need for time to understand the host-related factors and virus-related aspects that regulate the stream of the infection for its eradication on both local and global levels.

Host Susceptibility of PPRV

Along with natural PPRV infection in typical host species, the natural and experimental infection has also been observed in a wide range of wildlife species, cattle, buffaloes, camels, and atypical hosts (Aziz-ul-Rahman et al., 2018; Rahman et al. 2019; 2020). Its infection can pose critical health concerns among various wild ruminant species and shows a series of acute infections with fatal disorders to subclinical infection. Such evidence indicated the transboundary nature of PPRV towards the expansion of the host range particularly in endemic regions around the globe. Due to the expansion of the host range dynamic of PPRV, wild ungulates, large ruminants, camels, and unusual hosts are now considered susceptible to variability in clinical presentation. Altogether, the importance of widening the host dynamics in relation to potential PPR control strategies enforces exploring the disease pathogenesis in all susceptible hosts.

PPR Evidence in the Typical Host or Domestic Small Ruminants

PPRV infection has been reported from a wide range of animals but regularly and severely affects small ruminants around the globe (Banyard et al. 2010). According to the OIE and FAO, over 70 countries are now considered PPRV endemic regions. There are many reports about small ruminant species including sheep and goats across Asia, Africa and the Middle East. Based on pathogenesis, variations in disease have been reported between different species of small ruminants. Thus, detailed research is required to explore disease susceptibility and dynamics of PPRV transmission. In this context, the identification of cellular mechanisms and pathogenesis of PPRV in the naturally-resistant host/s could provide deep insight into selective breeding. A completely-resistant host may serve as an end host in endemic regions to complement and succeed in the disease eradication program.

PPR Evidence in a Wide Range of Wild Ungulates

In endemic regions, it is considered that small ruminants intermingle with wildlife for possible cross-species transmission of PPR during free-range grazing and sharing water sources, particularly at livestock-wildlife zones (Abubakar et al. 2011; Aziz-ul-Rahman et al. 2016). Notably, a previous study from Pakistan investigated a PPR outbreak in wild ibex in the wildlife conservation area and speculated the possible spillover of the virus from infected small ruminants residing nearby to them (Abubakar et al. 2011). Likewise, spill over event of PPRV has been noted from infected sheep and goats to wildlife species in Tibet and Northern Tanzania (Bao et al. 2011; Mahapatra et al. 2015). Considering this aspect, it is emphasized that PPRV has the

Table 1: Serological, molecular, and clinical evidence of PPR in a diverse wildlife species from diverse geography (Aziz-ul-Rahman et al. 2018)

Family	Subfamily	Wildlife species	Binomial name	Country	References
Bovidae	*Aepycerotinae*	Impala	*Acepyceros melampus*	United Arab Emirates	Kinne et al. 2010
		Impala⊥	*Acepyceros melampus*	Tanzania, Kenya	Mahapatra et al. 2015; Jones et al. 2021
	Alcelaphinae	Blue wildebeest⊥	*Connachaetes taurinus*	Tanzania	Mahapatra et al. 2015
		Bubal hartebeest	*Alcelaphus buselaphus*	Cote d Ivoire	Couacy-Hymann et al. 2005
		Kongoni⊥	*Alcelaphus buselaphusTanzania*	Tanzania	Jones et al. 2021
		Tiang⊥	*Damaliscus lunatus tiang*	Sudan	Aguilar et al. 2020
		Topi⊥	*Damaliscus lunatus jimela*	Tanzania	Jones et al. 2021
		Wildebeest⊥	*Connochaetes taurinus*	Kenya	Jones et al. 2021
	Antilopinae	Arabian mountain gazelle	*Gazella gazelle cora*	United Arab Emirates	Kinne et al. 2010
		Blackbuck	*Antilope cervicapra*	Pakistan	FAO 2017
		Blackbuck⊥	*Antilope cervicapra*	Qatar	Haroun et al. 2021
		Defassa waterbuck⊥	*Kobus ellipsiprymnus*	Cote d Ivoire	Couacy-Hymann et al. 2005
		Dorcas gazelle⊥	*Gazella dorcas*	United Arab Emirates, Kingdom of Saudi Arabia	Furley et al. 1987; Abu-Elzein et al. 2004
		Dorcas gazelle⊥	*Gazella dorcas*	Nigeria, Sudan	Bello et al. 2016; Intisar et al. 2017; Asil et al. 2019
		Gerenuk⊥	*Litocranius walleri*	Kenya	Jones et al. 2021
		Goitered gazelle	*Gazella subgutturosa*	Mongolia	OIE 2017b; Pruvot et al. 2020
		Goitered gazelle	*Gazella subgutturosa*	China	Li et al. 2017
		Goitered gazelle	*Gazella subgutturosa*	United Arab Emirates	Kinne et al. 2010
		Goitered gazelle⊥	*Gazella subgutturosa*	Qatar	Haroun et al. 2021
		Goitered gazelle⊥	*Gazella subgutturosa*	Turkey	Gur and Albayrak 2010
		Grant's gazelle⊥	*Nanger granti*	Tanzania, Kenya	Mahapatra et al. 2015; Jones et al. 2021
		Kob	*Kobus kob*	Cote d Ivoire	Couacy-Hymann et al. 2005
		Kob⊥	*Kobus kob thomasi*	Uganda	Aguilar et al. 2020
		Mongolian saiga antelope	*Saiga tatarica mongolica*	Mongolia	OIE 2017b; FAO 2017; Pruvot et al. 2020
		Nile lechwe	*Kobus megaceros*	Sudan	OIE-WAHID 2008
		Spring buck⊥	*Antidorcas marsupialis*	United Arab Emirates, Qatar	Kinne et al. 2010; Haroun et al. 2021
		Thomson gazelle⊥	*Eudorcas thomsonii*	Kingdom of Saudi Arabia	Abu-Elzein et al. 2004
		Thomson gazelle⊥	*Eudorcas thomsonii*	Kenya	Jones et al. 2021
		Waterbuck⊥	*Kobus ellipsiprymnus*	Qatar	Haroun et al. 2021
	Bovinae	Bushbuck	*Tragelaphus scriptus*	United Arab Emirates	Kinne et al. 2010
		Chowsingha	*Tetracerus quadricornis*	India	Jaisree et al. 2018
	Caprinae	Argali	*Ovis ammon*	Mongolia	Pruvot et al. 2020
		Argali	*Ovis ammon*	China	Li et al. 2017
		Bharal⊥	*Pseudois nayaur*	China	Bao et al. 2011
		Nubian ibex	*Capra nubiana*	Israel	OIE 2017a; Berkowitz et al. 2019
		Nubian ibex	*Capra nubiana*	United Arab Emirates	Furley et al. 1987; Kinne et al. 2010
		Siberian ibex	*Capra ibex sibirica*	Mongolia	OIE 2017b; Pruvot et al. 2020
		Sindh ibex	*Capra aegagrus blythi*	Pakistan	Abubakar et al. 2011
		Wild goat	*Capra aegagrus*	Kurdistan, Iran	Hoffmann et al. 2012; Marashi et al. 2017
		Wild ibex	*Capra ibex*	China	Xia et al. 2016; Zhu et al. 2016; Li et al. 2017
	Cephalophinae	African grey duiker⊥	*Sylvicapra grimmia*	Nigeria	Ogunsanmi et al. 2003
	Hippotraginae	Addax⊥	*Addax nasomaculatus*	Qatar	Haroun et al. 2021
		Arabian oryx⊥	*Oryx leucoryx*	Qatar	Haroun et al. 2021
		Gemsbok	*Oryx gazelle*	United Arab Emirates	Furley et al. 1987
Camelidae	*Not categorized*	Alpacas⊥	*Vicugna pacos*	China	Liu et al. 2021
Cervidae	*Capreolinae*	White-tailed deer*	*Odocoileus virginianus*	United States of America	Hamdy and Dardiri 1976
	Cervinae	Water deer	*Hydropotes inermis*	China	Zhou et al. 2018
	Cervinae	Deer	*Cervus elaphus*	Qatar	Haroun et al. 2021
Elephantidae	*Elephantoidea*	Elephant⊥	*Loxodonta africana*	Sudan	Aguilar et al. 2020

*Experimental infection of PPRV: ⊥Detection of PPRV antibodies in wildlife species: Note: Species names are arranged according to the alphabetic manner

potential for inter and intra-species spread among domestic and wild small ruminants, however, the expansion in host dynamic and mechanisms of spill over event is ambiguous. The possible reasons for the virus transmission are the transfer of wildlife from free-range habitat to zoological collection or seasonal migration of animals from one place to another even across country boundaries. Notably, similar PPRV strains have been isolated from affected wild ungulate showing the same clinical presentation as observed in sheep and goats living in the same region (Kinne et al. 2010; Abubakar et al. 2011; Hoffmann et al. 2012). The involvement of respiratory and digestive systems causes lacrimation, nasal and ocular discharge, crust forming over the nostrils and around the lips commissar, erosion of the oral cavity, unilateral corneal opacity, and death due to respiratory arrest in a wide range of wildlife species (Furley et al. 1987; Abu-Elzein et al. 2004; Kinne et al. 2010; Sharawi et al. 2010; Abubakar et al. 2011).

On the basis of detection of viral antigen and antibodies from domestic small ruminants, a bulk of data is available, but in wild ruminants, there is limited data. Species of wild ruminants reported to have seroconverted to PPRV are prominently Saiga antelopes (*Saiga tatarica mongolica)*, Sindh Ibex (*Capra aegagrus blythi)*, African Gray duiker (*Sylvicapra grimmia*), Arabian Oryx (*Oryx leucoryx*), Goitered Gazelle (*Gazella subgutturosa subgutturosa*) and many other species (Table 1). These reports revealed that wildlife hosts are seroconverted to PPRV, most likely due to spill over from PPR outbreaks in sheep and goats. However, since there is little or no molecular epidemiology of PPRV in wildlife, the role of wildlife in disease transmission needs further clarification. Till up-to-date, earlier studies highlighted the possible epidemiological role of wildlife species in the spread of PPR or vice versa that can likely be a threat to endangered species.

Table 2: Serological, molecular, and clinical evidence of PPR in large ruminants, camels, and unusual hosts from diverse geography (Rahman et al. 2020)

Family	Genus	Animal species	Country	References
Evidence of PPR clinical presentation, antibodies detection and nucleic acid identification in Asian countries				
Bovidae	*Bos*	Cattle	Bangladesh	Anowar and Madir 2004
			China	Li et al. 2018
			India	Balamurugan et al. 2012b; Sen et al. 2014
			Iran	Rasooli et al. 2019
			Kazakhstan	Lundervold et al. 2004
			Nepal	Prajapati et al. 2021
			Pakistan	Rashid et al. 2008
			Pakistan	Abubakar et al. 2017; 2019
			Turkey	Ozkul et al. 2002; Albayrak and Gur 2010
	Bison	Water buffalo	India	Govindarajan et al. 1997; Balamurugan et al. 2014
			Pakistan	Khan et al. 2008
Camelids	*Camelus*	Camel	India	Rajneesh et al. 2011
			Iran	Zakian et al. 2016
			Kingdom of Saudi Arabia	Hemida and Al-Ghadeer 2019
Evidence of PPR clinical presentation, antibodies detection and nucleic acid identification in African countries				
Bovidae	*Bos*	Cattle	Cote d Ivoire	Couacy-Hymann et al. 2019
			Ethiopia	Abraham et al. 2005; Agga et al. 2019
			Mauritania	Cosseddu et al. 2021
			Nigeria	El-Yuguda et al. 2013
			Ghana	Anderson and McKay 1994
			Sudan	Haroun et al. 2002; Intisar et al. 2017; Ali et al. 2019; Hekal et al. 2019
			Tanzania	Lembo et al. 2013; Herzog et al. 2019
	Bison	Water buffalo	Cote d Ivoire	Couacy-Hymann et al. 2005
			Tanzania	Mahapatra et al. 2015
			Kenya	Jones et al. 2021
			Uganda	Aguilar et al. 2020
Camelids	*Camelus*	Camel	Egypt	Ismail et al. 1992
			Ethiopia	Roger et al. 2000, 2001; Abraham et al. 2005; Saeed et al. 2015
			Kenya	Omani et al. 2019; Chemweno et al. 2019
			Libya	El-Dakhly 2015
			Morocco	Fakri et al. 2019
			Nigeria	Daneji et al. 1997; El-Yuguda et al. 2013; Bello 2013; Woma et al. 2015
			Sudan	Haroun et al. 2002; Khalafalla et al. 2010; Saeed et al. 2010; Intisar et al. 2017; Kwiatek et al. 2011
			Tanzania	Swai et al. 2011
Evidence of PPRV infection or nucleic acid identification in unusual host species				
Suidae	*Sus*	Pig*	Germany	Schulz et al. 2018
		Pig*	Nigeria	Nawathe and Taylor 1979
	Phacochoerinae	Warthog	Kenya	Jones et al. 2021
Canidae	*Canis*	Dog	India	Ratta et al. 2016
Felidae	*Panthera*	Asiatic lion	India	Balamurugan et al. 2012a
Muridae	*Mus*	Mice*	Ireland	Galbraith et al. 2002
Ceratopogonidae	Not categorized	Biting midge	Turkey	Sevik et al. 2019

Note: Countries' names are arranged according to alphabetic manner: *Experimental infection of PPRV

PPR Evidence in Large Ruminants

Several studies have reported PPR evidence in large ruminants around the globe (Table 2). A previous study from India reported a similar clinical PPRV infection with an outbreak in water buffaloes with the involvement of respiratory and digestive systems showing hypersalivation, respiratory distress, conjunctival congestion, and fever reminiscent of symptoms as observed in naïve hosts (Govindarajan et al. 1997). The outcome of that study highlighted the susceptibility of buffalo to getting infected by PPRV as a result of natural infection. Moreover, the experimental infection of PPRV has been shown susceptibility of cattle to this virus with/ without showing the clinical presentation of the disease (Sen et al. 2014; Couacy-Hymann et al. 2019). A previous study claimed that cattle could be sub-clinically infected after contact with PPRV-infected goats and all four genetic lineages (I-IV) PPRV strains have the potential to induce tissue tropism and subsequently the production of antibodies (Couacy-Hymann et al. 2019). However, there is a lack of evidence about PPR virus shedding from cattle' secretions and excretions and emphasizing the cattle as a dead-end host of PPRV (Agga et al. 2019). Moreover, there is no evidence of the virus shedding in water buffalo' secretions and excretions however, it is assumed that such an event could occur during the upsurge of an outbreak (Govindarajan et al. 1997). Taking in this view, cattle, and buffaloes may have the capability to play a vital role in the virus transmission to all susceptible hosts, particularly in livestock dense regions of Asia and Africa where mixed farming of small and large ruminants are practiced.

PPR Evidence in Camels

Evidence of clinical infection and the presence of antibodies in the result of natural and experimental infection in camel is being reported from endemic regions of Asian, African and Middle East countries (Table 2). The clinical presentation of PPR in camel was observed as similar to those observed in naïve typical hosts (Khalafalla et al. 2010; Zakian et al. 2016). In previous studies, PPRV antigen was detected using traditional and molecular assays from tissue samples collected from camels presenting clinical signs (Kwiatek et al. 2011; Saeed et al. 2015; Intisar et al. 2017). There is more possibility of viral shedding in the feces and nasal discharges, however likely to be verified in future studies. It is can be assumed that the PPRV antigen was detected using PCR in ocular discharge from a camel presenting clinical features of PPR in Kenya (Omani et al. 2019). In this study, outbreaks were investigated among the camel population after indication of the PPR clinical presentation, including fever, conjunctivitis, nasal and ocular discharges, diarrhea, and high morbidity rate in affected camels. Along with the similar clinical presentation of PPR in camels as what was observed in sheep and goats, lineage III PPRV strain was also detected from camel and one goat living nearby the camel population (Omani et al. 2019). On the contrary, a previous study induced PPR infection experimentally in camels and observed no clinical presentation of diseases without showing any virus shedding for epizootiology of disease among susceptible hosts (Fakri et al. 2019). Regarding virus transmission from infected camels, little evidence has been reported and it is, therefore, still controversy about disease pathogenesis and spread mechanism.

PPR Evidence in Atypical/ unusual Hosts

Being morbillivirus, PPRV showed the capability of host switching by affecting various host species other than naive hosts even to unusual hosts including dogs, asiatic lions and pigs (Table 2). Despite several reports, information about PPR infection and possible virus transmission among atypical host specie is scarce. As the result of the experimental investigations, pigs showed subclinical infection of PPRV without virus shedding in body secretions and excretions (Nawathe and Taylor 1979). On the contrary, a recent study detected PPRV in pigs and emphasized that pig may have the potential to shed the virus and is capable to play an epidemiological role by crossing species barriers (Schulz et al. 2018). Besides, previous studies indicated the susceptibility of dogs and asiatic lions towards PPRV after detection of the virus genome in nasal and tissue samples during routine screening (Balamurugan et al. 2012a; Ratta et al. 2016). The possible reason for the expansion of the host dynamic is the nature of PPRV as being a morbillivirus and having a close antigenic relationship to another morbillivirus known as CDV: a causative agent of canine infectious disease. Thus, such evidence indicated that there is more chance of PPRV targeting morbillivirus susceptible hosts. Both studies also postulated that the possible reason for detection of the PPRV genome in nasal swabs and tissue samples from the dog and the Asiatic lion respectively might be due to the feeding of PPRV-infected animals. In most developing disease-endemic settings, dogs are usually lived in close proximity or livestock farms in companionship, and thereby, it is a likely chance of occurrence of spill over event or virus transmission from an infected animal to dogs. Remarkably, the detection and identification of the PPRV genome from biting midge samples also indicated the potential of PPRV to transmit through a vector (Sevik et al. 2019). Considering previous studies on persistent transhumance and pastoralism of PPRV among all susceptible hosts and potential vector involvement (if any), future investigations are prerequisites to explore competent or mechanical vectors of PPRV as such events may generate constraints in disease eradication, particularly in disease-endemic regions.

Cross-species Transmission Potential of PPRV

Natural/ experimental infection and the detection of PPRV genome in typical and atypical susceptible hosts indicated the potential of PPRV to cross-species barriers for expansion of host dynamics (Table 1, 2). It is assumed that the spill over event is most likely to occur following PPRV-infected animals to other hosts and subsequently leads to cross-species spread, particularly in livestock-wildlife interference regions. For instance, the previous study investigated the outbreak in the ibex population and emphasized that PPRV infection in the ibex population is most likely the result of spill over events with the involvement of the nearby living goat population (Abubakar et al. 2011). Thus, the cross-species transmission of PPRV is not uncommon and may provoke constraints in disease control efforts and subsequently disease eradication, particularly in disease-endemic regions. A previous study also observed similar findings of virus spill over from infected small ruminants to wild ungulates (Frolich et al. 2005). The deaths of endangered wild Chowsingha in India are also attributed to virus spillover from closest living PPRV-

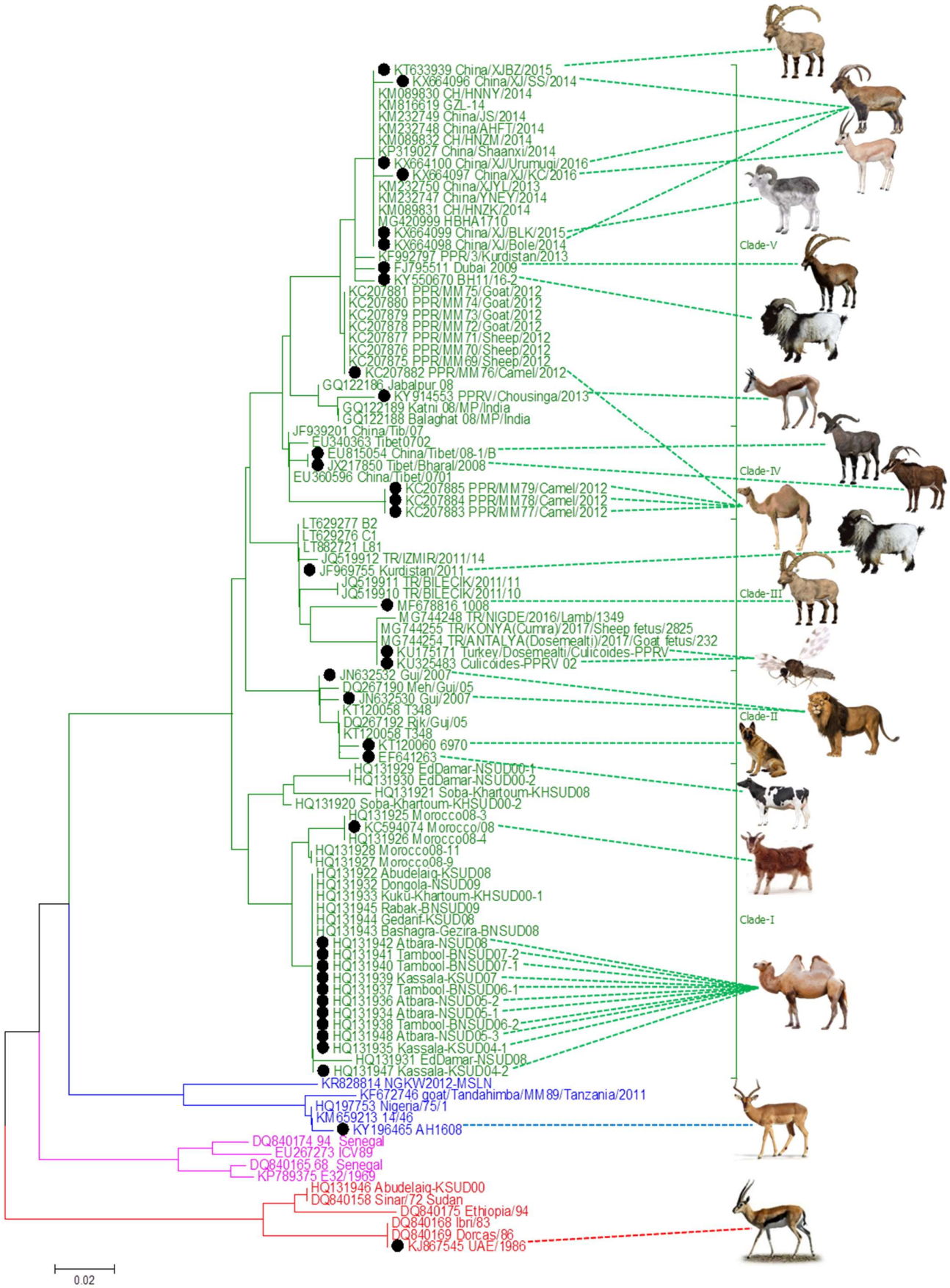

Fig. 2: Evolutionary and phylogenetic relationship of PPRV strains isolated from wildlife species, large ruminants, camels and unusual hosts with those isolated from small ruminants (Rahman et al. 2019).

Fig. 3: Global strategy of regional roadmaps in Asian and African countries developed by OIE [PPR Global Eradication Programme (GEP); https://www.oie.int/en/disease/peste-des-petits-ruminants/#ui-id-4].

infected sheep and goats to them (Jaisree et al. 2018). The PPRV may transmit *via* indirect and direct contact with the infected animal to wild ungulates and large ruminants particularly in developing endemic countries in which the chance of cross-species transmission may likely occur. Despite a large number of reports, mechanisms of virus transmission and factors involved in disease epizootiology with possible spill-over from one susceptible host species to another species are largely indefinite.

Genomic Forthcomings of PPRV Strains Originating from Diverse Animals

PPRV is genetically highly variable, similar to other RNA viruses. The causative agent of PPR infection is a negative sense, mono-partite ssRNA virus having approximately 16 kilobases long genome (Appel et al. 1981). Based on the genetic characterization of F and N, it is divided into four distinct lineages, lineages I-IV (Muniraju et al. 2014). Lineages I and II have been isolated in Western and Central Africa, whereas lineages III and IV in Eastern Africa and Asia (Mahapatra et al. 2015; Xia et al. 2016). Perhaps, the apparent expansion, the viral genome, and partial fragment sequencing of PPRVs in domestic small ruminants have been widely performed to determine the spreading status and to analyze the genetic variability. For wild ungulates, large ruminants and unusual hosts, data related to genome sequencing are scarce. Based on complete and partial sequencing of PPRV from diverse animals except for domestic small ruminants, a total of 37 isolates have been documented till now. Based on phylogeny analysis, most of the isolates from diverse animal origins have been revealed in their association with lineage IV (Bao et al. 2011; Rahman et al. 2019) except a previous study which has shown the relationship with lineage III (Muniraju et al. 2014). So, the genetic relatedness of PPRV can be understood by its phylogenetic relationship as shown in Figure 2. Besides, a few mutations were also observed in conserved and non-conserved regions of the fusion protein (Rahman et al. 2019). Genomic homology analysis revealed that PPRV sequences from different host species have high genetic similarities to PPRV sequences isolated from infected small ruminants (Bao et al. 2011; Rahman et al. 2019). Regarding the genetic makeup of PPRV strains reported from small ruminants, wild ungulates and large ruminants from the same region revealed a marked genomic homology (Rahman et al. 2019). Such outcomes highlighted that similar PPRV strains are prevailing among small ruminants, wild and large ruminants. Owing to the disease-causing potential of PPRV strains among wild and large animals, the IUCN impended that rare wildlife species are at risk of genetic depletion (Osofsky et al. 2005). Recent high mortalities in wild animals in free-ranging regions and zoos efficaciously highlighted this aspect (FAO 2017; OIE 2017a). Considering the role of wild, large and unusual hosts in the epidemiology of PPRV, shifting of only PPRV negative animals with the implementation of quarantine strategies may help to reduce the incidence of disease in livestock-wildlife interface and free-ranging regions.

Future Perspectives and Disease Control Strategies

PPR is an infectious transboundary disease and therefore, its eradication in developing endemic regions needs to be prioritized to secure the genetic depletion of endangered species, for the food security of large animals and poverty alleviation. To strengthen disease eradication measures, there is a dire need to get insight into the potential of all susceptible hosts in disease transmission either under similar or various livestock/ animal production systems. Indeed, mass infection and die-offs in domestic small ruminants, wildlife species, cattle buffaloes, and camels, particularly in disease-endemic regions, are strictly influencing poor communities livelihood, livestock productivity and wild ecosystem. Several analytical studies about the incidence of PPR disease, disease epizootiology, and genetic characterization of fieldprevailing PPRV strains in a wide range of hosts are needed to prompt global scientists interest in the restructuring of disease

control policies for the eradication of PPR by 2030. The susceptibility of diverse host species indicated the spatial and temporal host dynamics and heterogeneities and raises concerns over the strengthening of disease surveillance programs at national and international levels. Such expansion of host heterogeneity makes the situation even more complicated for disease control which aspects take a long process and more time that cannot solely rely on mass vaccination programs. Considering the transboundary nature, disease endemicity, and expanding host dynamic of PPRV in Asian and African countries, OIE has developed a global strategy by dividing affected countries into various 9 regional roadmaps to attain progress toward disease eradication at national and regional levels (Figure 3). A new framework, consisting of investigations on the involvement of unusual hosts in disease epidemiology, is obligatory to include in the global strategy for PPR eradication. To investigate the impact of PPR on natural wild conservation with the involvement of wild animals in the epidemiology of disease, continuous disease surveillance programs should be employed along with the establishment of epidemiological modelling investigations and realistic host heterogeneities models for decision making on strengthening disease control strategies.

Concluding Remarks

Together with typical/ naive hosts, PPRV has the capability to target a wide range of wild ungulates, large animals and camels. In this chapter, we tried to summarize the scattered information and current knowledge on the epizootiology of PPR among wild ungulates, large ruminants, and camels and highlighted the possible epidemiological role of all susceptible hosts for the happening of cross-species transmission events. Concluding, the spill-over events and cross-species transmission can be evaluated and prevented by profound investigations on disease pathogenesis and field-based disease surveillance. Moreover, there is a dire need to explore the possible impact of PPR infection on the genetic depletion of wildlife species.

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CHAPTER 12

BACTERIAL DISEASES AFFECTING SHEEP AND GOATS

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INTRODUCTION

Ruminant farms have gained importance as with the population growth of the country. Dynamics of the animal rearing is changing very fast as the demand of high producer animals increases with micro calculations of economics. Small ruminants play a major role in economy of rural people. Most of the animals are found in the plain areas of the country where average climate is warm and humid due to its geographical location. This type of environment supports the growth of many infectious agents including bacteria. Commercialization of the livestock sector have put pressure on the animal's health. Bacterial diseases have been playing damaging role in terms of morbidity and mortality of the animals. Many bacterial diseases spread in the form of outbreaks that can make people out of business. Some other environmental risk factors like housing, ventilation, water and feed management also play important role in these diseases. Different bacterial infections produce different typical symptoms but mixed and confusing are also not uncommon. Vaccination and antibiotics are the only solutions along with supportive therapy for the bacterial diseases. Bacterial resistance against antibiotics is emerging issue of the time in tackling the infection. So, development of effective vaccines is the viable solution to deal with these maladies. This chapter is focusing on the major diseases of the small ruminants that commonly prevailed in the Pakistan.

Mastitis

Mastitis is a disease of economic importance in ruminants because it decreases the quantity and quality of the milk. It reduces weight gain in lambs and meat kids reared on milk. It can likewise influence the prosperity of animals (Machado 2018). Inflammation of the udder tissue due to any infection or injury, rendered any or multiple following conditions: milk production loss, abnormal milk, changes in the size of gland, or its uniformity, and systemic disease may occur even leading to death. Most of the times transmission route of infection is ascending from teat sphincter to teat cistern and finally glandular tissues (Wan-Azemin et al. 2021). The pathogenicity of the microorganisms leads to serious illness. The infectious agents produce toxins that are absorbed by the circulatory system or it may invade the alveoli and interstitial tissues causing inflammation. Mastitis could be clinical or subclinical in nature. Clusters or changes in milk consistency are indications of clinical mastitis (Chase et al. 2017). Moreover, the udder might become enlarged and temperature of the mammary tissues increases. Subclinical mastitis needs biochemical testing like California Mastitis Test (CMT) or cell count in the milk or culturing. There are few microbes which are known to cause mastitis most of the times in sheep and goats as displayed in Table 1.

Table 1: Common bacteria involved in mastitis in sheep and goats (Chase et al. 2017)

Clinical Mastitis	Subclinical Mastitis
Escherichia coli	*Arcanobacterium pyogenes*
Klebsiella spp.	*Bacillus spp.*
Mannheimia haemolytica	*Coliforms*
Pseudomonas spp.	*Corynebacterium pseudotuberculosis*
Staphylococcus aureus	*Pseudomonas spp.*
	Staphylococcus spp.
	Streptococcus spp.

For the detection of any abnormality in the udder, it starts with the physical inspection of mammary glands, visual investigation of the milk. Visually milk can be evaluated using dark or white background cup plate that make milk changes pus discharge, lumps, blood streaks visible. Diagnosis of clinical mastitis can be possible through clinical signs and symptoms; but subclinical mastitis can only be diagnosed by using tests; it may be California mastitis test or surf field mastitis test (Hussain et al. 2017).

By assessing the somatic cell counts in the milk, risk of the disease can be checked. Somatic cell count of the milk sample can be done easily through automatic counter (Coulter Counter®) or flow cytometry-based counters (Somacount® or Fossomatic®). Molecular techniques like Polymerase Chain Reaction (PCR) and culturing of milk samples are important tools in diagnosis and identification of microorganisms that are responsible for mastitis (Machado 2018).

Hygienic conditions and environment during milking procedure are the key measures for successful control of mastitis in ruminants. Dipping at pre and post milking with disinfection of teats is extremely vital. To screen instances of subclinical mastitis; all instances of clinical mastitis ought to be

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treated as soon as possible. Mostly Intra-mammary route is used for treatment of mastitis. For small ruminants, numbers of licensed antibiotics are there for treatment. Antibiotic treatment along with adequate hygienic condition is necessary (Contreras et al. 2007). Precautionary measures should be adopted because injudicious use of antibiotics increase the antibiotic resistance in the bacteria, which is major public health issue now a days. The use of vaccines is an economic decision for veterinarians and breeders since it reduces costs and has positive effects on milk quality and public health, reducing the need for antibiotics. Vaccines for small ruminants against mastitis are commercially available. Vaccine against S. aureus has been proved in sheep but not for goats (Tollersrud et al. 2002). Mastitis cases in goats has been decrease with vaccinations in relation with antibiotic treatment against staphylococcal infection. For success in controlling and prevention of mastitis in small ruminants, it is essential to have strong mechanism for diagnosis and consistent monitoring of mastitis (Machado 2018).

Enterotoxaemia

Enterotoxaemia in goats and sheep is due to *Clostridium (C.) perfringens*, that is gram-positive, non-motile bacterium. It is spore-forming bacilli that propagate well in anaerobic environment. Enterotoxaemia, usually called "over-eating disease", is a typical issue in sheep and goats, particularly in ruminants in less than 1 year of age animals (Karthik and Prabhu 2021). There are three forms; per-acute, acute and chronic. Common types of *C. perfringens* are C and D. These microbes are usually found in the soil and as common microflora in the gastrointestinal tract of small ruminants (sheep and goats). These microbes under specific environments rapidly reproduce in the GI tract and produce toxins in large amount. *C. perfringens* type C is commonly seen in kids and lambs less than 3-4 weeks of age (milk colic). *C. perfringens* type D is usually present in older animals. Entertoxemia was mostly found in the lambs fed on rations containing high concentrates; however, cases are also there in the animals grazing on green lush pastures. Sometimes it is called overeating diseases but animals affected by this condition do not "overeat" all the times as is the case with grain overload. Animals growing simply at ideal conditions can get the disease. *C. perfringens* grows rapidly in the intestines and produce toxins that are actually involve in the disease (Chase et al. 2017). Usually, abdominal pain, sudden onset of depression, nervous signs and sudden death are common clinical signs of enterotoxemia. Death normally happens promptly after the beginning of signs. Commonly, in lambs onset of nervous signs followed by sudden death, while in kids signs of diarrhea is noted before death. (Karthik et al. 2017).

For evidence of disease, necropsy of animal can be done. On necropsy findings, small intestine portion can be seen dark red to purple. Contents present in the intestines are bloody with necrotic debris and sometime with fibrin clots. Petechial lesions on the epicardium and endocardium may or may not be present (Uzal et al. 2016). Culturing of bacteria can be perform in the anaerobic conditions followed by Polymerase Chain Reaction (PCR) for confirmation of typing. Treatment is seldom successful or accessible. Anti-microbial medicines (antibiotics), pain relief drugs, anti-bloating agents are used for treatment. Along with these treatments supportive therapy is important which include fluid therapy, thiamine (B1) and probiotics for repopulation of microflora in gastrointestinal tract after antibiotic therapy (Murray 2013). Prevention of disease is far better than treatment, because after onset of disease there will be some sort of production losses it may be in form of meat, milk production or effects growth of young ones. Clostridium vaccines are commercially available. Routinely vaccination in lambs and kids is very viable. But vaccinated animals required booster dose after three to four weeks of initial dose. In the end various smart feeding strategies are also needed to be employed in order to get an appropriate control of the disease (Karthik and Prabhu 2021).

Tetanus

Tetanus is also known as "Lockjaw". It is a highly fatal, acute disease of the animals including humans caused by micro-organism *Clostridium tetani* (Karthik and Prabhu 2021). However, all types of domesticated animals are susceptible, small ruminants (sheep and goats) are more susceptible than cattle while, horses being the most vulnerable. Tetanus is described by hyperesthesia, tetanic spasms and convulsions. *C. tetani* is a rigorously anaerobic, gram positive, rod shaped, motile, slender and spore forming ('drumstick appearance') bacterium. On the basis of flagellar antigens, ten serotypes of *C. tetani* have been depicted and all the serotypes produce neurotoxins called tetanospasmin. Autoclaving is the procedure that can destroy the endospores of *C. tetani* but these spores are resistant to different chemicals. Tetanus (Lockjaw) is sporadic disease and distributed worldwide. The bacterium is typical inhabitant of digestive system of animals and spores continues to survive in the soil, dung and manure (Harish et al. 2006). Spores are brought into the tissue through injuries, explicitly profound wounds which are ideal places for anaerobic conditions. Most outbreaks can occur after contamination of animals during improper castration, ear labeling, docking, vaccinations and other surgeries (Pugh and Baird 2012). Neurotoxin in necrotic tissue produced by *C. tetani* caused toxemia in sheep and goat. Grazing on unpleasant and spiky fields might harm the oral mucosa and thus might work with the intrusion of the microbes. Spores of *C. tetani* remain inactive/dormant; on getting favorable conditions they proliferate to produce toxins. The incubation period this disease varies from 4 to 21 days. Prolapse of third eyelid, tremors and stiffness of muscles are initial signs. In the later stage rigidity and extension of the limbs develop that leads to stiff gait and abnormal flexion of the joints. Lock jaw, saliva drooling from mouth is due to tetany of masseter muscles. The animals might suffer from bloat, difficulty in chewing, and hyperthermia. Animal becomes hypersensitive to external stimuli and its posture shows 'saw-horse' appearance. The fits of alimentary and urinary muscles might cause urine retention (Pugh and Baird 2012). Opisthotonos, curve of spine and twisting tail symptoms appear due to contraction of musculature. If untreated, mortality rate may reach up to 100 % as the respiratory system collapse due to muscle paralysis. Death of animal occurs within 3 to 10 days. Necropsy finding are not specific normally except inflammatory reactions at the site of wound. Clinical disease diagnosis can be done easily with the signs and symptoms of tetani. It includes muscular fits, saw horse posture, 3rd eyelid prolapse and hypersensitivity to external stimuli. History of injury or surgical procedure could be helpful in determining the disease. For laboratory testing, the Gram-positive (rods) with terminal spores isolated from

necrotic tissue of the wound (Popoff 2020). In normal conditions, anaerobic culture from wounded tissue might appear ineffective for culturing. Molecular diagnosis through PCR for neurotoxin gene of the bacterium would be confirmative. Treatment most of the times focus on anti-sera, management/ disinfection of wound along with supportive therapy. While, control of the disease is necessary through vaccination. Wound dressing with antiseptics and debridement is necessary. Hydrogen per oxide is flushed upon wound that cause debridement as well as provide aerobic conditions at the site which assist in stopping the further growth of the bacteria. Antibiotics (Penicillin in large doses) is proved handy locally as well as systemically to control the bacterial growth (Lotfollahzadeh et al. 2019). Animals with the disease should be provide cool and calm environment. Noise increases the stress to animals. Fluid therapy with sedative and muscle relaxant can improve clinical condition of diseased animal. Antitoxin serum is available for animals to neutralize the unbound tetani toxins. It can be injected either through intravenous route or in the subarachnoid for three consecutive days treatment regime. Prevention of the disease is easy through adopting hygienic measures during surgery, tail docking, castration, vaccination, naval disinfection of young ones and other invasive procedure. Animals should be vaccinated regularly with toxoid that gives lasting immunity.

Booster doses should also be provided during lifetime. Animals should be vaccinated before lambing and kidding season to provide passive immunity to the young ones. A booster dose can be provided to the animals inflicted with the deep wound (Hamborsky et al. 2015).

Blackleg

Blackleg is also known as black quarter. The causative agent of blackleg is *Clostridium chauvoei (C. chauvoei)*. It is a gram-positive, anaerobic bacterium. *C. chauvoei* makes lemon shaped endospores in unfavorable environment and need enriched media for culture growth (Disasa et al. 2020). Sheep are more susceptible to *C. chauvoei* as compare to goats. It is soil origin infection; spores persist in the environment for years and resistant to climate changes and disinfectants. Organism enters the body of animal through food tract mucosa if feed on contaminated diet or injury to mucosa lining. It is manifested by inflammation of the muscles, toxemia and high mortality. Blackleg occurs when spores which are held up in ordinary tissue and multiply by the process like injury or toxemia. In sheep most of the times disease occur due to wound injury. These wounds may develop at time of shearing, docking or through navel infection after birth. Vaginal or valvular infection in ewes by ram may also cause wound development by ram (Frey and Falquet 2015). Spores enters the body from soil through gastrointestinal tract and travel through blood reach the muscle where they stay inert in the white blood cells for a long time (Kriek and Odendaal 2004). Any injury or ischemia to muscle may start germination of the spores. Bacteria produce cytolytic toxins that necrotize the tissue, myofibers and vascular endothelia lining. These toxins also enter the general blood circulation and systemic disease occur with sudden death. As the bacterium grow quickly, it also produces gas which could be felt as air pockets between muscle bundles. There are clear cut signs and symptoms of the disease but when the disease is per acute or acute in nature some of the signs may not appear and death occur in the field cases. infected animals most of the times shows fatal disease course and found dead before any support. In some of the cases lameness occur and swelling of the muscle become visible. It produces crepitating sound upon pressing of the muscle part. Any of the striated muscle of the body may get infected but heavy muscles are more susceptible (Aiello et al. 2016). Some others bacteria like *C. septicum, C. novyi, C. sordellii,* and *C. perfringens* may also produce same changes to the muscles tissue upon infection. Both *C. septicum* and *C. chauvoei* might be isolated from blackleg affected tissues especially if the sample is taken after 24 hours of the death. Through anaerobic culturing and biochemical identification *C. chauvoei* is confirmed in affected tissue of muscles. Muscle tissue samples ought to be collected quickly after mortality. The fluorescent antibody technique for the diagnosis *C. chauvoei* is quick and reliable. Immunohistochemical diagnosis is performed on formalin-fixed tissue samples. A PCR (Polymerase Chain Reaction) test is also available and reliable for clinical samples but not for the environmental samples. Ewes ought to be immunized twice. Booster can be given one month before the lambing. During outbreak, administration of penicillin and antisera is recommended as prophylaxis. Young sheep ought to be vaccinated prior to going to pasture. At younger age immunity sheep is somewhat short in duration (Aiello et al. 2016). Vaccination against clostridial diseases is reported to produce weaker immune response in small ruminate (sheep and goats) than in cattle. To avoid the spore dispersal in the environment carcass of dead animal should be incinerated or buried too deep (Tagesu et al. 2019).

Brucellosis

Brucellosis is known to be a pernicious reproductive malady which is caused by a facultative, gram-negative, bacterium. Caprine are mostly infected by only *B. melitensis* while Ovine are susceptible to both *B. ovis* and *B. melitensis*. From an epidemiological stand point, *B. melitensis* has been rarely reported in Oceania, Southeast Asia and Europe during last couple of decades while *B. ovis* is far more pervasive and is considered endemic in all the sheep rearing regions of the world (Gompo et al. 2021). This disease has an immense zoonotic potential as humans are also susceptible to *B. melitensis* infections.

Brucella could be transmitted to susceptible individuals through contact with contaminated body fluids of infected animals. Goats suffering from brucellosis are often presented at the clinic after aborting a four-month fetus. Partial lameness and mastitis are concomitantly reported as well upon physical examination (Li et al. 2021). While in the case of Sheep, fetus is aborted at a much later stage. However, unlike goats, placental retention is common in the cases of Sheep. Both bucks and rams develop orchitis as a consequence of infection. Systemic signs are negligible amongst either species during initial phases, yet certain researchers have associated a sudden decrease in milk yield and compromised sperm count with the clinical onset of the disease (Maquivar et al. 2021). Some animals may also develop arthritis in chronic cases of infection. Death is uncommon as a consequence of this disease, but the enduring reproductive and fertility losses render that animal practically useless. Necropsy findings for infected patients are generally unique to respective species. Placentitis and inter-cotyledonary thickening has been repeatedly reported in Sheep while Placenta has been found to be unceremoniously ordinary in case of goats. However, presence of severe

bronchopneumonia has been persistently reported in both ovine and caprine fetuses (Galluzzo et al. 2021). In small ruminant clinical practice, brucellosis must be differentially diagnosed from listeriosis, vibriosis, toxoplasmosis, leptospirosis and salmonellosis.

Serological tests, such as ELISA, plate agglutination and Complement fixation may be employed to diagnose Brucella infection in small ruminants. Brucella is a highly infectious agent and considering its long-term reproductive ramifications, most developed countries have opted to cull the infected animal instead of treating it, thereby avoiding transmission of disease to healthy individuals (Dadar et al. 2021). To date, a feasible treatment plan has not been devised for brucellosis however, a prolonged therapy with an antibiotic regimen comprising Oxytetracycline (25 mg/Kg) and Streptomycin (20 mg/Kg) could be administered intramuscularly for favorable outcome (Radwan et al. 1992). A Rev-1 live, attenuated *B. melitensis* vaccine has been made commercially available to immunize sheep and goat in certain western countries (Mali et al. 2022) while development of a *B. ovis* based vaccine is underway in New Zealand (Sidhu-Muñoz et al. 2018). However, further development and research is required to control worldwide endemicity of this disease.

Contagious Caprine Pleuropneumonia

Two different bacterial agents *Mycoplasma capricolum capripneumoniae* (Mycoplasma biotype F-38) and *Mycoplasma mycoides capri* (type strain PG-3) have been attributed to the clinical manifestation of contagious caprine pleuropneumonia (CCPP) (Parray et al. 2019). CCPP is one of the most contagious and virulent goat diseases. Ailment due to *Mycoplasma capricolum capripneumoniae* is febrile and respiratory in nature, marked by labored breathing and exasperate coughing (Ma et al. 2020). Difficulty in breathing would cause patients to adopt a distinctive posture whereby neck is extended and legs are spread wide. While in case of CCPP caused due to *Mycoplasma mycoides capri*, several systems in addition to respiratory are affected leading to septicemia. Animals remain carriers even after recovery and mortality is extremely high in case of acute infections (Ahaduzzaman 2021).

Carrier animals of CCPP transmit contagion through respiratory secretions. In several situations, infected patients may also be subjected to secondary viral infections and other clinical complications (Parray et al. 2019). Mycoplasma biotype F-38 has been reported far more frequently than type strain PG-3, but either of them are considered endemic around the world. Stressful conditions and exposure to carrier animals facilitate spread of disease in the herd. Post-mortem findings often reveal straw-colored phlegm and pea-sized nodules in the thorax (Ma et al. 2020). In most cases massive hepatization of infected lungs is seen along with mononuclear infiltration on histopathological slides. CCPP has to be differentially diagnosed from Peste des petits ruminants, Viral pneumonias and Pasteurellosis (Parray et al. 2019; Khaliq et al., 2020).

On herd basis, serological tests like ELISA, passive hemagglutination or complement fixation, may be employed to detect antibodies associated with Mycoplasma F-38 (Parray et al. 2019). While diagnosis could be confirmed by culturing fluid samples from lungs. Quinolones, Tetracyclines, and Macrolides could prove efficacious against *M. capricolum capripneumoniae* but animals may remain carriers for this disease (Ma et al. 2020).

A lyophilized saponin-inactivated F-38 vaccine has been proven effective in Kenyan field tests (Ahaduzzaman 2021). However further investigations are warranted in this regard.

Caseous Lymphadenitis (CLA)

Corynebacterium pseudotuberculosis, a gram-positive, hardy bacterium is responsible for causing caseous abscessation of lymph nodes and viscera in goats, sheep and camelids. Researchers have identified that the biotype of bacteria in which proliferation is independent of nitrates are responsible for CLA. It is endemic to all regions of the world where sheep or goat are reared (de Farias et al. 2019). Clinical manifestation of the disease is usually observed in animals aged around 3 months, but disease has been reported in lambs as young as 6 weeks. Mortality rates are quite low, but it often remains undiagnosed and thereby continues to cause chronic wasting (Li et al. 2018). Consequently, this disease could pose devastating implications at herd level. *C. pseudotuberculosis* is known to pose zoonotic concerns as well, but transmission of infection is reportedly uncommon.

Diseased animals are often presented with external swellings and chronic wasting. Other than the aforementioned indications, animals are generally afebrile, alert, and possess a variable appetite (Guerrero et al. 2018). Clinical examination reveals firm abscesses externally, which soften as they mature. Bacterial transmission is believed to be through abraded skin or mucous membranes. After entering the host, bacterium succinctly travels to the lymphatics and starts producing leukotoxic, phospholipase D (PLD) exotoxin, thereby causing deterioration of endothelium (Odhah et al. 2019). Abscesses are formed as a result in lymph nodes, but they are pervasive and therefore may regress or drain to infect other organs. The disease is known to impede weight gain and wool production as well. CLA most commonly affects parotid, cervical, and submandibular lymph nodes in housed sheep and goats, while popliteal, mediastinal and parenchymal lymph nodes are often abscessed in the case of pastoral herds. Occasionally, animals are suddenly discovered dead without demonstrating any clinical signs (de Farias et al. 2019). Congregating animals in tight spaces and shearing them, without disinfecting afterwards are the most frequent abysmal managemental practices associated with the spread of Caseous Lymphadenitis. Abscessation attributed to *Fusobacterium necrophorum*, *Mannheimia haemolytica*, *Staphylococcus aureus,* and *Escherichia coli* should be differentially diagnosed from CLA. Ovine progressive pneumonia (OPP) and Lymphosarcoma may also be confused with CLA. Blood or Biochemistry panels do not generally, offer much insight for disease confirmation in the case of CLA (Li et al. 2018). However, culturing samples collected from abscesses along with ELISA and synergistic hemolysin inhibition (SHI) tests are very insightful in this regard (Guerrero et al. 2018). Ultrasonography and radiography of thoracic region are quite useful as well in identifying the number and size of abscesses. Despite the fact that several antibiotics including penicillin are efficacious against this bacterium, abscess formation prevents effective therapy. In emergent cases, surgical intervention may be performed to drain retropharyngeal abscesses, if value of animal warrants it.

Vaccines against Caseous Lymphadenitis are commercially available, but they perform best when animals are quite young, before they are exposed to the said contagion. In goats, vaccinating while PLA toxin is already present in their blood

stream could pose serious health risks, so extreme caution is warranted during immunization (Odhah et al. 2019).

Johne's Disease

Mycobacterium avium paratuberculosis (Map) has been known to cause a chronic, diarrheal disease called Johne's disease (paratuberculosis), in several wild and domestic ruminant species. Molecular typing has revealed that "C strain" is capable of infecting cattle and goats while "S strain" is responsible for Map in sheep (Selim et al. 2021). However, cross-species transmission of Map between cattle and sheep has been previously reported as well. Fecal-oral route is believed to be the path for disease and younger animals (<30 days) are far more susceptible to contagion (Morales-Pablos et al. 2020). Zoonotic impact of disease has been implied in some studies but concrete significance has yet to be established.

Animals are most frequently presented with chronic weight loss and diarrhea. In sheep, poor coat and hypoproteinemia leading to intermandibular edema are occasionally observed as well (Zhao et al. 2021). Periods of stressful conditions exacerbate or cause clinical manifestation of the disease. Disease should be differential diagnosed from Caprine arthritis and encephalitis (CAE), Copper deficiency and Ovine progressive pneumonia (OPP). Histopathological findings reveal mild to severe corrugation of mucosa of distal small intestine and cecum along with edema of associated lymph nodes (Selim et al. 2021). Mortality rates are not that high but animals suffer loss in production as a long-lasting consequence of this disease. Disease can be controlled at herd level by implementing sound managemental practices.

Blood and biochemistry panel often reveal marked hypoalbuminemia and hypoproteinemia in clinically ill patients (Zhao et al. 2021). Culturing of fecal samples and subsequent antibiotic sensitivity testing can relay significant information regarding Map strain and efficacious antibiotics. Serologic tests such as Agar Gel Immunodiffusion Assay and ELISA could also be performed as a diagnostic tool but their sensitivity improves in the later stages of disease (Selim et al. 2021). While supportive therapy may prolong life, no practical treatment is available.

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CHAPTER 13

BRUCELLOSIS: VIRULENCE FACTORS, PATHOGENICITY AND TREATMENT

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INTRODUCTION

There are nearly 100 different types of organisms that can cause human diseases (Balloux and van Dorp, 2017). Brucellosis is considered as one of important zoonotic diseases, especially in developing countries that is caused by Br. species such as *Br.suis*, *Br.melitensis* and *Br.abortus* are the most important members of the family because they can cause human disease (Franc et al. 2018; González-Espinoza et al. 2021). The British army surgeon David Bruce (1855-1931) isolated a coccobacillus called *Micrococcus melitensis* from some spleen tissue of a man who had died of "Malta Fever" in 1886. The disease was endemic but confused with other diseases, particularly malaria. In Malta during (1901-1906), annually reported 652 civilian cases and 605 military cases, with mortality rates of 10.4% and 2.3% respectively (Rahman et al. 2006; Liu 2015).

The disease in humans is prevalent in those who consume goat milk and have other close contacts with goats. The organism was quickly isolated from the goats. Similar microbes were isolated from cow udder in 1897, as well as from swine udder in 1914 (Ndegwa et al. 2001; Zhao et al. 2015). In approximately 1920, Brucella was renamed and each species was given its own name: *Br. melitensis, B. abortus,* and *Br. suis.* There are not all pathogens that are specific to a particular species e.g., cattle can be infected with *B. suis*. There have been numerous names for the disease, with "undulant fever" becoming dominant in the United States until the 1940s when was named Brucellosis (Alton and Forsyth 1996).

An Overview of Brucella's Characteristics

Brucella species are microorganisms that measure between 0.5-0.7 x 0.6-0.15 micrometers and are gram-negative coccobacilli. Usually, single forms are common; pairs and chains are rare. These bacteria do not produce spores, do not have capsules or flagella, and cannot move. They do not harbor plasmids naturally, even though they readily accept plasmids with broad target ranges (Alton and Forsyth 1996).

Partially acid-fast, do not decolorize when treated with 0.5% acetic acid used in modified Ziehl-Neelsen (MZN), retain carbol fuchsin, and exhibit red coloration under a microscope (al Dahouk et al. 2003; Köse et al. 2005).

Ideal temperature for growth is 37°C, with growth taking place between (20°C - 40°C), and a pH of 6.6-7.4. The majority of them are aerobes, although some species such as *Br. ovis* and *Br. abortus* need an environment with added carbon dioxide (5-10%). Brucella species are included in fastidious bacterial species that require rich culture medium to thrive (Alton and Forsyth 1996).

Growth occurs on Brucella agar, Trypticase soy agar, sheep blood agar, MacConkey agar and standard nutritional agar at (25-42 ° C). Colonies on translucid media are convex, transparent and have an entire edge. After two -three days of incubation of a fresh inoculum they are usually small (0.5–1.0 mm), but variations depend on the strain and medium (Boussetta 1991; de Miguel et al. 2011; Ledwaba et al. 2020) . Cultures can be identified as Brucella by examining colonial morphology, staining, and slides agglutination with anti-Brucella serum, smooth or rough. Many of the Brucella strains are catalase- and superoxide dismutase-positive; they are also mostly oxidase-positive. With cytochrome-based electron transport, aerobic metabolism is the mode of metabolism (Araj 2010; Tekle et al. 2019)

In conventional media, brucellae mostly use oxidative metabolism and show little activity with carbohydrates, though they can hydrolyze urea in many cases (Padilla Poester et al. 2014; Tekle et al. 2019).

There are no classical pathogenic factors produced by Brucella organisms, such as exotoxin, cytolysin, exoenzymes, exoproteins, capsules, plasmids, fimbriae, and drug-resistant forms (Głowacka et al. 2018)

Types and Classification of Antigens

It is still believed that Brucella species, despite a century of research and extensive analysis, are major animal pathogens that cause Brucellosis. These gram-negative bacteria affect various terrestrial and aquatic mammals, such as sheep, goats, cattle, dogs, swine, dolphins, whales, seals and desert woodrats. Within the Brucella genus, there are six species and these species are classified primarily based on their pathogenicity and host preferences (Cardoso et al. 2006). *Br. abortus* affects cattle, *Br.melitensis* affects sheep and goats, *Br.ovis*

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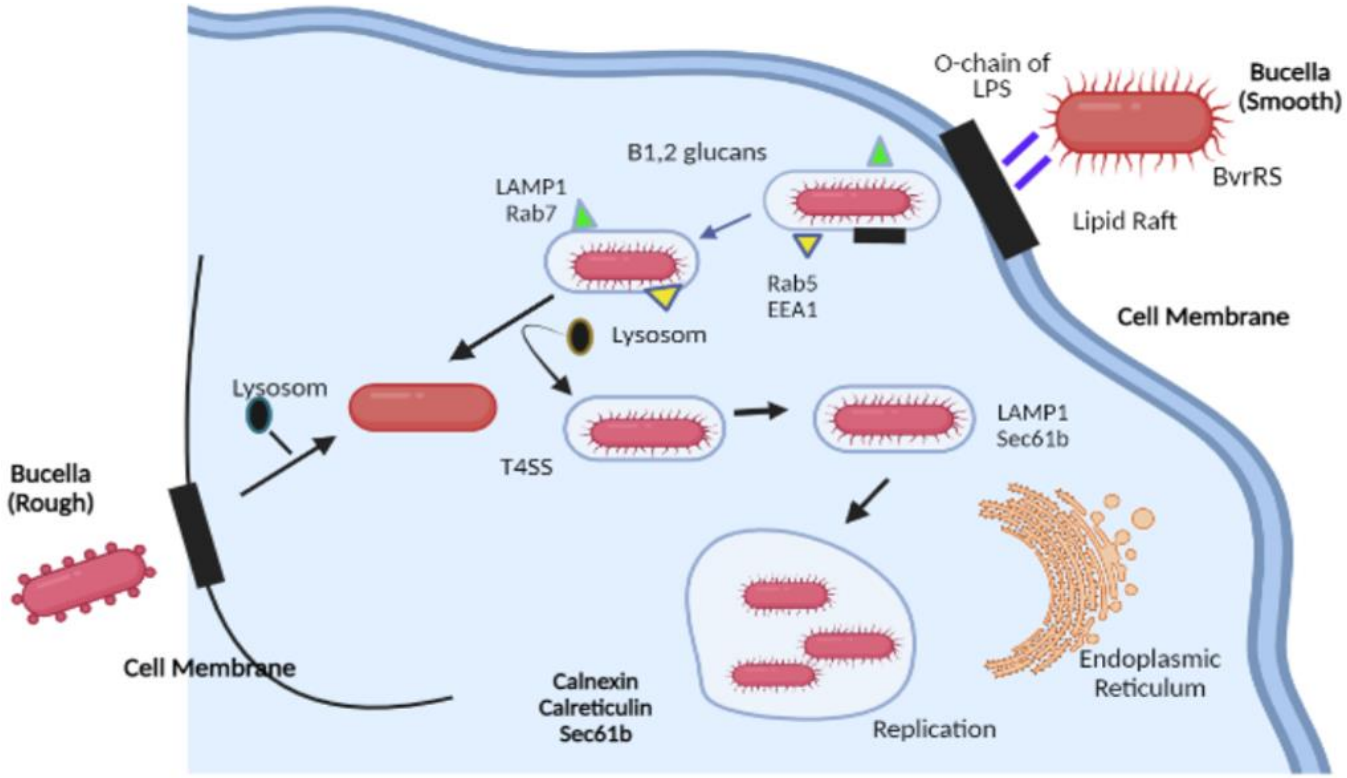

Figure 1: Invasion and Intracellular Trafficking of Mammalian Cell by Brucella (Created in BioRender.com)

Table 1: Brucella species and biovars with host range

Brucella species	Biovar	Host
Br.abortus	9	Cattle, dogs, horses, sheep and man
Br.suis	5	Pigs, cattle, dogs, hares and man
Br.melitensis	3	Sheep, goats, cattle and man
Br.ovis		Sheep
Br.canis		Dogs and man
Br.neotomae		Desert wood rat

affects sheep, *Br.suis* affects pigs, *Br canis* affects dogs, and *Br. neotomae* affects wood desert rats. Recent isolates from human *(Br.inopinata),* (*Br.inopinata*), two aquatic mammals (*Br.pinnipedialis* and *Br.ceti*), and a common vole (*Br.microti*) are now recognized as new species in the genus (de Figueiredo et al. 2015). Biovars occur in some species (Table 1) most of these species infect specific hosts.

Current research suggests that species and biovars can be differentiated based on lipopolysaccharide antigens, CO2 requirement, dye sensitivity, phage typing, hydrogen sulfide production and metabolic properties (Alton et al. 1989; Morgan 1990)

This bacterium is similar to other Gram-negative bacteria in its dominant lipopolysaccharide (LPS) component and three main protein groups in its outer cell membrane (Maldonado et al. 2016)

There are smooth and rough Brucella abortus, melitensis, and suis strains, with smooth LPS (S-LPS) and rough LPS (R-LPS) as major surface antigens. *Br.ovis* and *Br.canis* are naturally rough species that express R-LPS (Cardoso et al. 2006; Maldonado et al.2016)

The LPS of brucellae with smooth colonies has two kinds of O chains. Antigens A and M correspond to *Br. abortus* and *Br.melitensis*, respectively. (Informally,' since some *Br.abortus*, biovars carry M antigens while *Br.melitensis* carry A antigens) They are both homopolymers of 4,6-dideoxy-4-formamido-d-mannopyranose, but the A chain is linked 2-1, whereas the M chain often has three-one linkages. According to routine serology, smooth brucellae cross-react almost entirely with the same species, but not with the rough Brucella, and vice versa. Cross-absorption of A and M monoclonal sera produces monoclonal antibodies specific for each antigen, indicating that each chain contains a distinct epitope (de Figueiredo et al. 2015)

The Clinical Manifestations

Infection with Brucella causes Brucellosis, which is commonly found in domestic, wild, and feral animals, and some strains are pathogenic to humans. The Brucella genus causes the disease (Brucellosis), which is widespread and causes infertility and abortion in domestic and wild animals (Alton and Forsyth, 1996)

The manifestations of brucellosis in humans are typically variable. Sometimes it is difficult to determine how long the incubation period is, but it is usually between two and four weeks. It may occur slowly or suddenly. Subclinical infections are common, and it is characterized by undulant fever (38°C to 40°C), polyarthritis, meningitis, pneumonia, anorexia, endocarditis, splenomegaly, depression, weight loss, and hepatomegaly. There is unusually severe leg and back pain, excessive sweating, and fatigue and other less common clinical manifestations (Sauret and Vilissova 2002). A human with an untreated infection will suffer from a debilitating flu-like illness with chronic complications (González-Espinoza et al. 2021)

In domestic animals, like cattle, sheep, goats, and swine, significant effect includes abortion and metritis in females, and orchiepididymitis and infertility in males, resulting in reduced fertility and a significant decline in milk production (McDermott et al. 2013; Elderbrook et al. 2019).

Epidemiology

Brucellosis is an endemic zoonotic disease typically found in the Middle East, Central Asia, South and Central America, Africa, the Mediterranean region (Portugal, Spain, Greece), and other parts of the world with a high dairy consumption and little of animal health protection (Gwida et al. 2010; Fouskis et al. 2018). There are several species of animals that are infected with Br. abortus and Br. suis, including bears, bisons, caribous, camelids, elks, ferrets, deer, foxes, rats, and wolves, as well as dolphins, dugongs, manatees, otters, and sea porpoises (Głowacka et al. 2018)

People become infected through various routes, including contaminated dairy products, non-pasteurized cheeses, handling of infected animals, and exposure to uterine secretions or aborted fetuses at work (Khurana et al. 2021). As human brucellosis is essentially a zoonotic disease, control and prevention of brucellosis in animals is essential for eradicating the disease in man (Gwida et al. 2010).

Studies have documented *Br.melitensis* infection in ibex and chamois in the Alps (Assenga et al. 2015). There has not yet been evidence of prevalence of *Br. ovis* or *Br. canis* in European animals. Br. pinnipedialis and Br. ceti appear to be the most common causes of infections in marine animals. In contrast, *Br. pinnipedialis* and *Br. ceti* appear to be the most common causes of infections in fish. Birds are not affected by brucella infection. It is spread through close contact and sharing of pastures (Makita et al. 2011; Muma et al. 2007).

Brucella is an accidental human pathogen that is spread mainly through direct contact with infected animals, inhalation of airborne agents, or consumption of contaminated dairy products (Godfroid et al. 2013; López-Santiago et al. 2019). It is possible that human-to-human transmission can happen during organ transplantation, blood transfusions, or vertical transmission through breastfeeding (Ay et al. 2016). Several Brucella species can be fatal to humans, including *Br.melitensis*, *Br.suis, Br. abortus*, and *Br. canis* (López-Santiago et al. 2019).

Virulence Factors

There are several virulence factors of Brucella species, contributed to its pathogenicity like:

Lipopolisaccharide (LPS)

Lipopolysaccharide from Brucella is unique and nonclassical, unlike Gram-negative bacteria such as Escherichia coli (Cardoso et al. 2006; von Bargen et al. 2012). Brucella LPS have distinct structures and properties, several of these properties may contribute to Brucella's ability to survive and replicate inside cells (Lapaque et al. 2005). Brucella is known for their high resistance to macrophage degradation, low endotoxicity, and resistance to immune response (Moreno et al. 1981).

Brucella lipopolysaccharide is less active and less toxic than classical Escherichia coli. In addition, classical LPS induces high pyrogenicity, while nonclassical LPS induces low pyrogenicity, which is a weak indicator of tumor necrosis factor (Christopher et al. 2010). Three features distinguish lipid A in *Br. abortus* from other Gram-negatives: diaminoglucose instead of glucosamine, more extended acyl groups, and lipid A is connected to the core by amide bonds, instead ester and amide bounds (Conde-Álvarez et al. 2012; Corbel 1997). There are three components of smooth LPS (S-LPS) found in smooth colonies: i) lipid A, which contains two types of aminoglycosides in addition to β--hydroxymiristic acid; ii) a core of mannose, glucose, and quinovosamine; and iii) 4-formamido-4,6-dideoxymannose with an O-chain (Alton and Forsyth 1996; Lapaque et al. 2005)

R-LPSs differ from S-LPSs in that their O chains are absent or reduced(Conde-Álvarez et al. 2012) . The O-chains of bacteria attach to lipid rafts on the macrophage surface and enter the cell. Brucella strains with R-LPS, such as *Br. ovis*, and *Br. canis* are not associated with lipid rafts and rapidly adhere to lysosomes. The O-chain of S-LPS strains inhibits host cell apoptosis through interaction with TNF-α (tumor necrosis factor). Therefore, dying cells do not produce specific factors. Thus, Brucellae cannot be detected by the immune system (Celli et al. 2003)

T4SS (Type IV Secretion System)

T4SS is a multiprotein complex involved in the secretion of macromolecules by bacteria. Brucella species have the virB operon, which encodes 12 proteins (11 860 bp), which has many similarities to the T4SS found in rhizobia, such as in phytopathogenic Agrobacterium (Boschiroli et al. 2002)

The expression of the virB operon is controlled by the VjbR quorum sensing regulator (Sieira et al. 2010). Brucella species that lack the VirB gene are unable to replicate within the endoplasmic reticulum, either because they are incapable of reaching the ER or because they are incapable of multiplying within (Boschiroli et al. 2002)

As part of Brucella-containing vacuoles (BCVs), Brucella rods are localized in macrophages; these organelles interact with the ER and are thought to be responsible for the formation of specific compartments. T4SS, which is virB's secretion system, is important for the acquisition of an endoplasmic reticulum membrane (Xiong et al. 2021)

The Superoxide Dismutase and Catalase Enzymes

The macrophage produces reactive oxygen intermediates (ROI) in response to Brucella consumption, which is the primary mechanism by which Brucella is destroyed, and prevents Brucella from replicating in the cell (Gee et al. 2004; Seleem et al. 2008).

Reactive oxygen intermediates are O_2– (superoxide), H_2O_2 - (hydrogen peroxide), and OH– (hydroxyl radical) which are extremely detrimental for the structure of the cell. A major defense against reactive oxygen intermediates is the production of enzymes. These enzymes include catalase and superoxide dismutase (Hasanuzzaman et al. 2020)

This enzyme is encoded by the *sod* (metalloenzyme) sequence. A variety of metals are found at the active sites of enzymes, such as iron, magnesium, zinc, and copper. As a result, SOD converts O^{2-} (superoxide) into H_2O_2 (hydrogen peroxide) and O_2 (oxygen) - transferring from one molecule to another ($2O^{2-} + 2H^{+} \rightarrow H_2O_2 + O_2$).

Water and oxygen are produced by catalase, an enzyme that breaks down hydrogen peroxide. Combined with Cu-Zn SOD, catalase activity is restricted to the periplasmic space, which leaves external sources of ROI unchanged. Other enzymes can compensate for the absence of catalase in catalase mutants, for example alkyl hydroperoxide reductase or enzymes involved in DNA repair. The sequence encoding this enzyme is similar to that of the Escherichia coli *katE* gene of Escherichia coli. (Gee

et al. 2004) found that Br. abortus, *Br. melitensis*, and *Br. suis* make more catalase when exposed to increasing amounts of H_2O_2.

Cyclic β-1-2-glucans (CβG)

Brucella CβG is an OPG (Osmoregulated Periplasmic Glucan) II family. By interacting with lipid rafts on macrophage surfaces, Brucella abortus CβG influences intracellular trafficking. Glucans are essential to the bonding of phagosomes and lysosomes. Mutants are destroyed in phagolysosomes and cannot reproduce. A further advantage of mutants treated with CβG is that they control lysosome fusion and vacuole maturation, which allows them to replicate when reach the endoplasmic reticulum (Roset et al. 2014)

Urease

There are two different urease operons in two different genomes of Brucella. The enzyme breaks down the urea into carbonic acid and ammonium, increasing the pH. This characteristic allows it to survive in acidic environments. Two urea operons (ure-1 and ure-2) are found on the 1 chromosome. The ure-1 and ure-2 genes encode structural proteins: ureA, ureB, ureC, and accessory protein genes: ureD, ureE, ureF, ureG. It has been suggested that the urease enzyme protects Brucella from destruction during its passes through the gastrointestinal tract (stomach), particularly when it enters orally (López-Santiago et al. 2019). Brucella species able to produce urea, except *Br. ovis* (al Dahouk et al. 2010).

The Cytochrome Oxidase Enzyme

Brucella can survive in macrophages in low-oxygen environments through the action of the enzyme cytochrome oxidase. In the genome are two operons that encode high oxygen affinity oxidase types: the cytochrome bd (ubiquinol oxidase) oxidases and the cytochrome cbb3 type. A cytochrome cbb3 oxidase that functions in vitro colonizes anoxic tissues (maximal effect during microaerobiosis). During intracellular multiplication, cytochrome bd oxidase is expressed, allowing cells to adapt to the replicative niche by reducing free radicals' production and eliminated the mechanism of cellular detoxification (Endley et al. 2001; Loisel-Meyer et al. 2005).

The Alkyl Hydroperoxide Reductase Enzyme (AhpC, AhpD)

These enzymes AhpC, AhpD protect cells from oxygen radicals and reactive nitrogen. One promoter control both AhpC and AhpD in an operon. The mutants of AhpC are more sensitive to peroxide killing and spontaneous mutation (Głowacka et al., 2018)

The Nitric Oxide Reductase (NorD) Enzyme

Brucella can use nitric oxide (NO) that infected macrophages produce. There are four types of NorD enzymes in Brucella: the nitrite reductase (Nir), the nitric oxide reductase (Nor), the nitrate reductase (Nar), and the nitrous oxide reductase (Nos), also known as the nitrogen island. When oxygen inside the cell is insufficient the Nitrate is reduced to dinitrogen gas by bacteria, allowing them to respire nitrate. Brucella is able to produce these enzymes to protect itself from low oxygen conditions within the macrophage (Loisel-Meyer et al. 2006)

BvfA (Brucella virulence factor A)

Brucella-specific periplasmic protein; there are no homologous sequences in GenBank. In macrophages, phagosome induces bvfA expression. Possibly, this protein plays a role in establishing the intracellular replication niche. It has not been precisely identified how BvfA functions (Hamdy and Zaki 2018)

The Base Excision Repair (BER)

DNA base excision repair is performed by XthA, a gene that encodes exonuclease III. The Brucella genome contains two different XthA sequences (xthA-1 and xthA-2), this enzyme plays an important role in the prevention of oxidative damage. xthA-1 mutations cause the cells to become more susceptible to reactive oxygen species (ROS) (Poncin et al. 2019).

BvrR/BvrS System

There are two identified open reading frames (ORF) : (*bvrR* and *bvrS*) of the Brucella genomic. The BvrR gene encodes the BvrR protein (237 amino acids) while th BvrS gen encodes the BvrS protein (601 amino acids). (Viadas et al. 2010). BvrR shows similarities to response regulators because its N-terminal domain contains highly conserved amino acids: aspartic (pos: 14, 15, 58) and lysine (pos: 107). A high degree of similarity was found between the C-terminus sequence and OmpR family, so this protein belongs to this family. There are three highly conserved domains in the protein: the N-terminal sensing domain, the periplasmic domain combined with the transmembrane component, the cytoplasmic domain containing histidine residues, and the C-terminal ATP-binding domain (Bialer et al. 2020).
In Brucella, BvrR and BvrS are virulence factors that are best characterized; mutants cannot invade, prevent of phagosomes fuse with lysosomes, or replicate inside cells (Bialer et al. 2019) BvrR / BvrS system are regulate multiple genes. These proteins influence the transcription of membrane proteins: Omp3a (Omp25a) or Omp3b (Omp22) and influence other non-protein membrane molecules and thus, functional and structural membrane homeostasis (Zhang et al. 2017). The BvrR/bvrS mutants show structural changes in LPS, but the O-chains remain intact. Since they are unable to activate GTPase (Cdc42) before entering cells, these mutants persist extracellularly and, consequently, do not infect the cells. The BvrR/BvrS fusion proteins play a role in lysosome fusion and intracellular trafficking (Guzmán-Verri et al. 2001)

Pathogenesis

Both animals and humans are affected by Brucellosis because the same event takes place when a bacterium interacts with its host cell. Brucella can multiply inside macrophages and survive in them, which makes it pathogenic (Liu 2015)
The severity of Brucellosis depends on the number and virulence of the infecting organisms, as well as the host's susceptibility. Proliferation is the goal of Brucella pathogens in the cell (de Figueiredo et al. 2015). Brucella species, as well as other intracellular pathogens, require adhesion, invasion,

establishment, and dissemination to establish themselves and spread throughout the host (Bialer et al., 2020). The smooth and rough strains of Brucella species are both capable of invading epithelial cells, enabling infection through mucosal surfaces, and are both capable of invading phagocytic and non-phagocytic cells (López-Santiago et al. 2019)

It replicates in macrophages, dendritic cells, and placental trophoblasts, showing a strong tissue tropism. Despite this, the pathogen can replicate in many types of mammalian cells, including microglia, fibroblasts, epithelial cells, and endothelial cells. Brucella's main targets are macrophages, dendritic cells (DCs), and trophoblasts (Ahmed et al. 2016)

Additionally, Brucella has the ability to multiply in epithelioid cells (HeLa) and murine fibroblasts (NIH3T3). Brucella invasion, survival, and replication were studied in great detail in phagocytes but not very well in trophoblasts (Kim 2015)

Invasion of the Cell by Brucella

Animal oral mucosa and M cells from mucosa-associated lymphoid tissue of the human digestive tract are the primary entry points of Brucella species (Paixão et al. 2009)

A professional phagocyte (macrophages and DC cells) engulfs a bacterium when it passes through the mucosal epithelium. Following infection, brucellae remain in nonphagocytic cells for up to seventy two hours, then cross the epithelial barrier and enter phagocytic cells. In this initial phase, 10 percent of the bacteria will survive. By breeding and spreading in macrophages, pathogens are able to escape the immune response of the host; therefore, they are able to multiply and invade other tissues. There is a zipper-like mechanism by which Brucella strains invade host cells (Stranahan and Arenas-Gamboa 2021)

Brucella species are spread by the lymphoid tissue of the region, then localized and produced in lymph nodes, before being transported via the bloodstream to parenchymatous organs and tissues. The localization of the bacteria occurs primarily in joint reproductive organs and related glands

During the third trimester of animal pregnancy, there is a high concentration of erythritol, which supports the growth of intra-trophoblastic Brucella, which compromises placental integrity and causes fetal infection, resulting in abortion or weak offspring. Brucella causes acute or chronic infections of the reproductive tract that lead to abortions or severe reproductive diseases (González-Espinoza et al. 2021)

Opsonized organisms are internalized via complement and Fc receptors while Non-opsonized Brucella organisms are internalized via lectin or fibronectin receptors. Pathogens attach to sialic acid residues and sulfated residues on epithelial cells when they come into contact with them (Moreno and Barquero-Calvo 2020).

To penetrate epithelial cells, actin polymerization is necessary. Brucella abortus activates Rho, Rac, and Cdc42 GTPases by adhering to the cell surface. These proteins regulate the cytoskeletal system and regulate the internalization of parasitic bacteria. The only GTPase activated by *Br. abortus* in response to nonphagocytic cells is Cdc42. Other GTPases (Rho or Rac) are believed to be indirectly activated by their inhibition, which prevents invasion into host cells. Additionally, cGMP, PIP3-kinase, MAP-kinase, and tyrosine kinase are involved in adhesion between bacteria and host cells as second messengers (Kim 2015).

Adhesion

Activation of small GTPases plays a role in adhesion to macrophage surfaces and polymerization of F-actin (transient and rapid F-actin accumulation). A protein called Annexin I, implicated in membrane fusion, is also involved in the early stages of adhesion (Kusumawati et al. 2000). The microdomains (lipid rafts), found on the cell membrane of macrophages, are also responsible for bacterial internalization. These structures facilitate the intracellular trafficking of Brucella (Xavier et al., 2014). Through lipid rafts, human monocytes and murine macrophages achieve internalization of nonopsonized Brucella strains. For this process to take place, TLR4 and PI3K must be activated. However, in human dendritic cells, however, lipid rafts are only partially responsible for this process. Strains of Brucella that lack O-polysaccharides in LPS (R-LPS) cannot penetrate eukaryotic cells and are therefore eliminated by macrophages. These lipid rafts contain cholesterol, glycosylphosphatidylinositol (GPI), and ganglioside GM1. Several proteins associated with lipid rafts: GPI and GM1, as well as cholesterol, inoculate with Brucella-contained macropinosomes and facilitate internalization with macrophages.

Intracellular Trafficking

Generally, intracellular trafficking among professional phagocytes and non-professional phagocytes is not remarkably different (Arenas et al. 2000). The bacteria attach to an early endosomal network called a Brucella Containing Vacuole (BCV) after invasion. Early endosomal antigen 1 (EEA1) and GTP-binding protein (Rab5) are markers for this compartment (de Figueiredo et al. 2015)

β-1,2-glucan regulate BCV maturation in macrophages and epithelial cells, also contributes to the formation of cholesterol-rich lipid rafts on the surface of Brucella Containing Vacuole membranes. It takes about 10 minutes to interact with the early endocytic network (Starr et al. 2012). Acidification of BCV at this stage leads to changes in bacterial gene expression and allows intracellular survival of bacteria. By preventing fusion of lysosomes with β-glucans and LPS occurrence, Brucella Containing Vacuole does not react with late endosomes. It indicates interaction with endosomes and lysosomes is required when early BCV transforms into intermediate BCV loaded with LAMP1 and Rab-7 (late endosomal/lysosomal markers) (Jiao et al. 2021).

A Rab-7 effector called Rab-interacting lysosomal protein (RILP) is responsible for acquiring BCV during this process. The interaction between late endosomes/lysosomes and BCV is transitional and controlled (Cantalupo et al. 2001). Subsequently, BCV is acidified and acidic contingent bacterial factors, such as virB, are expressed, while cathepsin D action is prevented. The virB operon encodes the type IV secretion (T4SS), which is required for transporting intracellular materials from the autophagosome to the endoplasmic reticulum in the cell (Ke et al. 2015)

Brucella bacteria are present inside multi-membranous autophagosomes with LAMP1 and Sec61β (calreculin) within an hour of internalization, It occurs only in epithelial cells and is also known as a late BCV. LAMP1 function is unknown, but it appears to contribute to bacterial survival within the cell. Calnexin, Calreticulin, and Sec61β are endoplasmic reticulum markers acquired by BCV during intercellular trafficking. However, BCV loses its ability to make LAMP-1 during this

phase. Bellaire et al. (2005) reported that this protein is detected always in the large vacuoles of human monocytes, where Brucella opsonized reproduces. Endoplasmic reticulum is the only suitable compartment for Brucella multiplication. However, the BCV-ER connection remains unclear. Trafficking of Golgi-bound vesicles to the ER is controlled by Coat Protein Complex I (COPI) and PKCI. Brucella replication in the endoplasmic reticulum is influenced by a variety of factors, including Coat Protein Complex I, GTPase (Rab2), glyceraldehyde-3-phosphate dehydrogenase, and PKCI (Fugier et al. 2009)

Diagnosis

Many Brucella species have been isolated using Thayer's, Martin's, and Farrell's as enrichment and selective media, and after 4 to 6 days the colonies growth when of incubated at 37 °C. However, at 28 °C, they grow slowly and poorly. Additionally, these bacteria can grow with or without 10% carbon dioxide, but they grow better without CO_2 on serum dextrose agar (Yagupsky et al. 2020). Bacteria can be cultured in many media such as Tryptone soya, Triptic soya, Triptcase soya and Bacto tryptose. In addition, Biphase Castaneda medium used for blood and body fluid culture (Yagupsky, 2015). The liquid Castaneda medium contains between 1 and 2% sodium citrate. An antibody level in serum is measured as part of a serological test to detect infection. Brucella infection in the 1st week is characterized by IgM titers, whereas IgG titers dominate in the 2ed week. After two months, both antibodies IgA and IgG are at their peak; excessive IgG levels may indicate mistreatment (Yagupsky et al. 2020)

In serology, enzyme-linked immunosorbent assays (ELISAs) and serum agglutination tests (SATs) are useful tests for diagnosing Brucellosis (Hajia et al. 2013)

Enzyme-linked immunosorbent assays detects antibodies in serum against the S-LPS antigen (Asfaw et al. 2015). However, through molecular techniques such as classical PCR, RT PCR can be used to detect Brucellosis by different pair of primers. Among genes used for identifying Brucella species are the omp2 gene (primer: JPF/JPR), rRNA sequences from 16S (primers: F4/R2), and BCSP 31 (primers: B4/B5), (Yu and Nielsen 2010).

Treatment of Brucellosis

Brucella vaccines for humans are not yet available, but there are many Brucella vaccines for livestock (Lalsiamthara and Lee 2017). Live, attenuated vaccinations that lack virulence components (e.g., the Live *Br.abortus* vaccine strain RB51, the Rev-1 Live *Br.melitensis* vaccine strain Rev-1, and the Live *Br.abortus* vaccine strain 19), yet still have residual pathogenicity (Aragón-Aranda et al. 2020). The use of subunit vaccinations has been shown to be generally safe and cause fewer complications than live immunizations. The immune system is stimulated by purified proteins or DNA, so they do not induce infection. In addition to developing vaccines for animals, researchers are also finding new ways to prevent human disease (Yang et al. 2013).

There are several therapies available to treat Brucellosis, which rarely causes death. In order to treat Brucellosis successfully, an antibiotic must penetrate macrophages and be active in acidic environments. However, does not respond to single antibiotic therapy, leading to relapses.

As with single agents such as oxytetracycline, rifampin, or doxycycline, the rate of relapse with these therapies can reach 9–25% and prolonging the therapy does not have any significant effect. In 30% of cases, trimethoprim-sulfamethoxazole causes relapses, while ciprofloxacin causes relapses in 83% (Gültekin et al. 2021).

The combination of two antibiotics is more effective than monotherapy in treating Brucella-induced infections. In the WHO guidelines (1986), doxycycline and rifampicin should be combined for six weeks and then switched to tetracycline and streptomycin (Alavi and Alavi, 2013). A number of antibiotic combinations and chemotherapy are currently available to treat Brucellosis, including fluoroquinolones, streptomycin with doxycycline (SD), and co-trimoxazole with rifampicin (RCTM) (Colmenero et al. 1994; Hosseini et al. 2019)

Brucellosis treatment by using doxycycline (SD) with streptomycin resulted in a relapse rate of 4.8% and a failure rate of 7.4% (Solís García del Pozo and Solera 2012). Children treated with doxycycline and gentamicin (DG) fail therapy on average by 5.2%, with relapse rates of 5.9% (Alavi and Alavi 2013) . Children treated with co-trimoxazole and rifampicin (RCTM) fail therapy on average by 0% to 16.4% with relapse rates of 3.1% to 10% (Alavi and Alavi 2013).

According to three clinical trials, the relapse rates varied from 3.2 -26 % (average 11.4%) and failure rates ranged from 3.2 - 26% (average 12.2%) with ciprofloxacin or ofloxacin and doxycycline, co-trimoxazole, and rifampicin used (Alavi and Alavi 2013).

Three clinical trials used doxycycline, rifampicin, and aminoglycosides. No evidence exists to support the superiority of triple-drug treatments over two-drug treatments. Triple drug therapy prevents relapses better, but is not effective for treating short-term symptoms, according to (Solís García del Pozo and Solera 2012) . It can be effective to administer triple therapy for eight weeks in arthritis or spondylitis cases.

If the condition is chronic or acute, or if endocarditis, spondylitis or arthritis have not developed, doxycycline and aminoglycosides are recommended. For simple condition, or gentamicin, doxycycline or streptomycin, may be recommended (Alavi and Alavi 2013).

A new strategy for treating Brucellosis was developed by (Smith et al. 2013). For BCV to bind to the ER, the endoplasmatic reticulum must be remodelled to alter the ER structure during the host stress response, which is called the Unfolded Protein Response (UPR). Brucella replication can be inhibited by tauroursodeoxycholic acid, a drug that disrupts UPR. A novel mechanism for treating Brucellosis may involve UPR (Smith et al. 2013)

Research has been conducted on the antibrucellosis effects of RGSF-A (ginseng saponin fraction A). Asia considers ginseng (a valued plant) to be a panacea for a variety of diseases. Researchers found that treated cells by RGSF-A inhibits the polymerization of F-actin and the invasion of bacteria into cells, decreased bacterial adhesion and internalization compared to control cells, inhibiting MAPKs (mitogen-activated protein kinases).

The RGSF-A protein enhances *Br.abortus* intracellular trafficking as well as the interaction between Brucella abortus-containing phagosomes and LAMP-1 (Arayan et al. 2015). A transmembrane protein, LAMP-1 controls the fusion of lysosomes with phagosomes, allowing BCPs to connect with lysosomes and eliminate bacteria. According to (Huy et al. 2017), RGSF-A has been shown to be the most effective

inhibitor of Brucellosis through its component of ginsenoside-panaxadiol saponin. Furthermore, several plants are effective against brucellosis, that contain bioactive elements (flavonoids, flavones, tannins, and anthocyanins), these plants including Teucrium polium, Scophularia deserti, Alhagi, Eucalyptus, garlic and roots of barberry (Alizadeh et al. 2018).

Conclusions

Brucella is a bacterium that is particularly hazardous to domestic animals, causing widespread infections and, as a result, enormous economic loss. Furthermore, people who work with animals that are infected, such as farmers, veterinarians, or laboratory technicians, are susceptible to contracting the disease. In humans, Brucellosis causes vague symptoms, so it is impossible to estimate how many people are infected. Brucella is a curious etiological agent that lacks traditional virulence determinants. Infection is a complicated process with many unexplained problems. As a result, more research is needed on infection pathways is necessary.

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CHAPTER 14

BOVINE RESPIRATORY DISEASE COMPLEX (BRD)

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INTRODUCTION

Despite of advances in veterinary medicine and animal welfare, the economic impact of cattle diseases on the livestock industry still remains important. Bovine respiratory disease complex (BRD) is one of the most important disease prevalent in both dairy and beef production farms (Amat 2019). BRD defines the cases of pneumonia associated with inflammation, consolidation, lung abscesses, and fibrosis caused by one or more infectious agents (Guterbock 2014). This disease complex affects both the upper airways causing rhinitis, pharyngitis, tracheitis and bronchitis and the lower airways/lungs (Woolums et al. 2015; Taylor et al. 2010). Post-weaned calves are most affected by BRD. Respiratory system infections lead to a decrease in feed efficiency, a decrease in development/growth performance, a loss of workforce and productivity for the animal enterprise/producer, and even death in severe cases. It also negatively affects reproductive performance, milk yield, and carcass quality in the long term (McGuirk 2008). The economic effects of BRD increase with the severity of the disease and treatment practices (Amat 2019). It has been reported that the rate of antibiotic use, which has taken an important place in BRD control, is 91.9% (USDA 2010), and this may lead to an increase in drug-resistant bacterial strains (Woolums et al. 2018). In addition, inappropriate and intensive use of antibiotics creates serious public health problems due to antimicrobial drug residues in animals consumed as food (Klima et al. 2014; Woolums et al. 2018).

Etiology

The anatomical and physiological structure of the respiratory system of ruminants makes them more susceptible to BRD. In contrast to other species, the lung capacity of cattle is small compared to their bodies and their functional capacity is low. In addition, low respiratory rate, breathing by mouth, excess lung lobes, limited lung lysozyme and phagocytosis capacity reduced ventilation capacity in cold weather and being sensitive to environmental temperature cause respiratory system diseases to be more common (Cooper and Brodersen 2010). The immune status of the host, environmental risk factors, mismanagement practices, and infectious agents play vital role in the development of BRD (Amat 2019).

Predisposing Factors

The main host-related risk factors predisposing calves to BRD are immune status, age, body weight, and genetics (Taylor et al. 2010). It has been reported that BRD is the most important disease of calves older than 30 days (McGuirk 2008; Woolums et al. 2015) and especially 50.4% of the cases occur in the post-weaning period (USDA 2010). As the weaning age and body weight of calves increase, their susceptibility to BRD decreases (Sanderson et al. 2008). Transfer of calves to group pens, sudden climate changes, poorly ventilated and crowded housing are important environmental risk factors predisposing calves to BRD. Shipping adversely affects the immune system of the animal due to stress and malnutrition during transport and predisposes to the BRD. In addition, exposure of cattle to different pathogens after transport or hierarchical social stress in group pens is an important management risk factor (Taylor et al. 2010; Amat 2019).

Viral Pathogens

The most important role of viral pathogens in BRD is to increase the susceptibility to secondary bacterial infections by inhibiting the defense mechanisms of the lungs (Taylor et al. 2010; Grissett et al. 2015; Amat 2019). The most common viral agents associated with BRD are *bovine herpesvirus type-1* (BoHV-1), *parainfluenza virus-3* (PI-3), *bovine viral diarrhea virus* (BVDV), *bovine respiratory syncytial virus* (BRSV), and *bovine coronavirus* (BCV). Although the viral-bacterial synergism in BRD is well known, the clinical signs of viral infection are absent in most cases. Viral pathogens cause primary infection, which is usually accompanied by mild clinical signs of BRD (Panousis 2009; Panciera and Confer 2010).

Bacterial Pathogens

Bacterial pathogens are responsible for severe clinical findings, chronic disease, and deaths in the BRD. The most important bacterial pathogens associated with this disease complex are *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Mycoplasma bovis*, *Arcanobacterium* (*Trueperella*) *pyogenes*, and *Bibersteinia trehalosi*. Each of these bacteria has different virulence factors such as biofilm, capsule, adhesin, toxin, and enzyme that increase their ability to colonize the lower respiratory tract, escape from the immune system, antimicrobial resistance, tissue damage, and inflammatory response (Confer 2009; Panousis 2009; Panciera and Confer 2010). A small number of *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* are naturally present in the nasal cavities of healthy cattle (Ackermann et al. 2010; Griffin et al. 2010). These microorganisms become opportunistic pathogens when host defense mechanisms are disrupted (Confer 2009; Ackermann et al. 2010; Gorden and Plummer 2010).

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***Mannheimia haemolytica* (*M. haemolytica*)**

M. haemolytica is a gram-negative, fermentative, non-motile, non-spore-forming, oxidase-positive, and facultative anaerobic coccobacillus from the Pasteurellaceae family. It usually produces weak hemolysis on sheep or cattle blood agar plates. The virulence factors of *M. haemolytica* make this bacterium the main bacterial agent of BRD, as it causes high mortality and loss of yield in young calves. Pneumonia caused by *M. haemolytica* is characterized by lesions that begin with acute cranioventral fibrinous pneumonia and progress to fibrinopurulent pleuropneumonia. The prevalence of *M. haemolytica* is estimated to be around 15% in suckling or young calves. Important virulence factors of *M. haemolytica* are capsular polysaccharides, lipopolysaccharide (LPS), protein adhesins, secreted enzymes, iron-binding proteins, and leukotoxin (LKT). Lipopolysaccharide and LKT are the two most important virulence factors responsible for most of the devastating lesions of *M. haemolytica* infection (Rice et al. 2007; Assie et al. 2009; Griffin et al. 2010).

***Pasteurella multocida* (*P. multocida*)**

Pasteurella multocida is a gram-negative, non-motile, aerobic coccobacillus belonging to the Pasteurellaceae family. It is found as a facultative pathogen in epithelial cells of the upper respiratory tract of healthy cattle. *P. multocida* isolates are classified into 5 serogroups (A-F) according to their capsular antigens and 16 serotypes (1-16) according to their somatic antigens. *P. multocida* A:3, the most commonly isolated serotype in BRD, is responsible for severe suppurative bronchopneumonia of calves. *P. multocida* is responsible for 40% of enzootic and shipping fever pneumonia cases. In *P. multocida* infections, typically cranioventral bronchopneumonia lesions are determined in the lungs. These lesions are characterized by acute fibrinosuppurative, subacute-chronic fibrinopurulent, fibrinous-fibrinopurulent, suppurative, and fibrino-necrotic pneumonia. The main virulence factors of *P. multocida* are various adhesins, lipopolysaccharide, and a thick polysaccharide capsule (Welsh et al. 2004; Dabo et al. 2007; Confer 2009).

***Histophilus somni* (*H. somni*)**

Histophilus somni (formerly *Haemophilus somnus*) is a gram-negative coccobacillus belonging to the Pasteurellaceae family. It is one of the main causes of BRD, especially in beef cattle. Like other bacteria, *H. somni* is found in the normal nasopharyngeal flora, but it colonizes mainly in the lower respiratory tract. In cattle, the infection can develop with *H. somni* directly or with the presence of other opportunistic viruses or bacteria. The tendency to disease is increased in overcrowded and poorly ventilated barns and poorly fed cattle. The bacterium alone causes fibropurulent bronchopneumonia. In mixed infections, it can cause thromboembolic meningoencephalitis, polyarthritis, abortion, abscessed laryngitis, fibrinous pericarditis, and sudden death associated with septicemia. *H. somni* virulence factors are lipooligosaccharide, immunoglobulin binding protein, outer membrane proteins, and exopolysaccharides. Histamine production also plays a role in the pathogenesis of *H. somni* (Confer 2009; Dabo et al. 2007; Rice et al. 2007).

***Mycoplasma bovis* (*M. bovis*)**

Caseonecrotic pneumonia of calves is defined as characteristic Mycoplasma infections. However, the causative agent in this disease is *M. bovis*, which is different from other *Mycoplasma* spp. and is more virulent (Haines et al. 2001; Shahriar et al. 2002). In various studies related to BRD, *Mycoplasma* spp. were isolated in combination with other bacteria in 70% of cases while in 20% cases, it has been isolated alone (Gagea et al. 2006). *M. bovis* infections in calves cause chronic pneumonia, lameness, and weight loss. Such cases usually do not respond to antibiotic therapy. *M. bovis* infections typically present multiple miliary caseous (cheese-like) abscesses with a cranioventral distribution, varying in diameter from a few millimeters to several centimeters. Histologically, caseousnecrotic foci are seen in the airways, alveoli, or interlobular spaces (Maunsell et al. 2011; Fulton and Confer 2012). Variable surface proteins form the virulence factors of *M. bovis*. Responsible for phenotypic changes among *M. bovis* strains, these surface proteins enable *M. bovis* to escape from the host immune response. It also functions as an adhesin by allowing *M. bovis* to colonize the bronchioles. Other virulence factors of *M. bovis* are biofilm formation and hydrogen peroxide (McAuliffe et al. 2006).

Pathogenesis

The respiratory tracts of healthy cattle have various mechanisms that prevent the colonization of harmful microorganisms. These mechanisms include retention of microorganisms and particles by mucus and cilia, physical removal, mucosal immune response, and maintenance of the saprophytic bacterial population (Ackermann et al. 2010). Viral agents affect the mucosal barrier, disrupting the clearance of respiratory tract pathogens, damaging the pulmonary parenchyma, and suppressing immune responses. It also facilitates the multiplication of opportunistic bacterial pathogens in the upper respiratory tract and their translocation to the lung, causing pneumonia. In a recent study on humans, it has been reported that viruses can weaken the host's resistance to bacterial pathogens by affecting the structure and composition of the nasal microbiota (Grissett et al. 2015; Korten et al. 2016).

Bacterial pathogens inhaled by the respiratory tract first colonize in the bronchoalveolar junction, overcoming the host defense system and causing inflammation in the region. It spreads through the airways or lung tissue adjacent components, causing suppurative bronchopneumonia (lobular bronchopneumonia), fibrinous pneumonia (lobar pneumonia, fibrinous bronchopneumonia), or caseonecrotic pneumonia (mycoplasmal pneumonia) (Caswell and Williams 2007). The type of bronchopneumonia is classified according to the rate and path of the spread of the infection in the lung, the type of exudation, the place of onset of bacterial colonization, the variety of bacterial virulence factors, and host resistance (Panciera and Confer 2010).

Clinical Findings

Symptoms of the respiratory system diseases in cattle, usually develop within 2 weeks after exposure to stress factors (weaning/transport). The clinical symptoms differ depending on whether the disease is acute or chronic. In the early period of

the disease; mild depression, serous ocular, and nasal discharge and an increase of body temperature up to 39.5-42 °C may be observed. In bacterial cases, the body temperature reaches 40.5-41 °C with signs of toxemia. While signs of toxemia are not observed in viral cases, an increase in body temperature (viremia period) and partial or complete loss of appetite is observed. In the later stages of the disease, unwillingness to move, loss of appetite, varying degrees of dyspnea, and harsh/persistent cough are observed. Nasal and ocular discharges are the mucopurulent-purulent character. Hardened sounds in the anteroventral lobes may be heard on lung auscultation. Clinical symptoms are variable in chronic BRD cases. Impaired hair coat and weight loss are observed in animals. The respiratory rates are above normal, and there is a slight or moderate intermittent fever. Bilateral mucopurulent nasal discharge and chronic productive dry cough are common. Abnormal lung sounds (ronchus and wheezing) can be heard in the entire lung area during auscultation examination of the lungs, but these sounds are most often heard in the ventral region of the lungs (Joshi et al. 2016; Kumar et al. 2018).

Diagnosis

Early diagnosis and accurate determination of etiological agents are significant for effective control of respiratory system diseases (Poulsen and McGuirk 2009; McGuirk and Peek 2014). Early diagnosis increases the effectiveness of treatment and eliminates most of the herd's problems. Delayed diagnosis in respiratory tract diseases may cause long-term antibiotic use, increased disease recurrence rate, lung abscess (chronic case), and otitis. As a result, affected and poorly treated calves may cause endemic herd problems when they enter collective calf shelters after weaning (Panousis 2009; Burgess et al. 2013; Buczinski et al. 2014).

Diagnosis of respiratory system diseases can be made by identifying the causative agent and laboratory analysis by evaluating clinical findings, osculopercussion, hematology, radiography, ultrasonography, nasal-pharyngeal swab, tracheal wash, and the tracheobronchial secretions collected by bronchoalveolar lavage procedures. In addition, more invasive procedures such as thoracocentesis and lung biopsies can be utilized (Burgess et al. 2013; Buczinski et al. 2014; Abutarbush et al. 2019).

Clinical Diagnosis

Diagnosis of respiratory tract diseases is usually made with clinical findings in field conditions. Instead of comprehensive and equipment-requiring scanning devices, different scoring systems have been developed recently by standardizing clinical examination findings and scoring each of the clinical parameters according to the degree of importance (Poulsen and McGuirk 2009; Ider and Maden 2021). For this purpose, the Wisconsin (WI) scoring system based on five clinical parameters, including rectal temperature, cough, nasal discharge, ocular discharge, and ear position, was started to be used by Poulsen and McGuirk (2009). According to this scoring system, calves with a total respiratory score of five or higher (if there are at least two abnormal parameters) are considered sick. It is stated that screening calves with the WI score system at least twice a week before weaning provides a significant advantage in the early diagnosis and control of respiratory system diseases (Poulsen and McGuirk 2009; McGuirk and Peek 2014; Ider and Maden 2021).

Hematological Analyzes

Blood gas analyzes are shown as an important analyses method that provides useful information in the evaluation of BRD severity and in making therapeutic decisions. Compensable respiratory acidosis develops in most calves with respiratory system disease. Ventilation, pulmonary diffusion, pulmonary hemodynamic disorders, and/or deterioration in ventilation-perfusion balance in the pathogenesis of BRD cause a decrease in blood pO_2 levels. It has been stated that the decrease in blood pO_2 level and the increase in pCO_2 level concurrently indicate obstructive changes and ventilation disorders in animals with signs of catarrhal and catarrhal-purulent bronchopneumonia. The decrease in blood pCO_2 levels in calves with pneumonia is associated with tachypnea and hyperventilation, frequently seen in respiratory system diseases (Nagy et al. 2006; Šoltésová et al. 2015; Ok et al. 2019; Ider and Maden 2021).

Leukocytosis and neutrophilia are observed in severe bacterial bronchopneumonia, while viral cases of pneumonia, leukopenia, and lymphopenia can be determined. In the biochemical analyzes of calves with BRD, it was determined that ALT, AST, CK, LDH, BUN, and creatinine levels were high, while albumin, glucose, magnesium, phosphorus, iron, and zinc concentrations were low (El-Bahr and El-Deeb 2013; Šoltésová et al. 2015; Ok et al. 2019; Ramadan et al. 2019).

Recently, some potential biomarkers, cytokines, acute-phase proteins (APP) and biochemical parameters involved in the pathogenesis of pneumonia have been investigated as diagnostic or prognostic markers in bronchoalveolar lavage (BAL) fluid and serum/plasma in BRD (Ider and Maden 2019). Haptoglobin (Hp), serum amyloid A (SAA), albumin, fibrinogen (Fb), and Lipopolysaccharide Binding Protein (LBP) are the most commonly used APPs in BRD. Most of cases (94%) in cattle with BRD have Hp levels above 0.15 mg/mL (Wolfger et al. 2015). When evaluated together with SAA, it has been reported to be more useful than hematological tests in the differential diagnosis of acute and chronic inflammation. In addition, it has also been reported that Hp increases significantly in viral and bacterial diseases and can be used to differentiate viral/bacterial diseases (Horadagoda et al. 1999). Lipopolysaccharide Binding Protein has been indicated as an earlier and more sensitive biomarker in respiratory tract diseases compared to other APPs (Nikunen et al. 2007; Ider and Maden 2019).

Analysis of Body Fluid Sample

Specific determination of the etiologic agent associated with pneumonia is made through nasal or pharyngeal swab, transtracheal fluid aspirate, BAL fluid, autopsy, or serology of lung tissue samples. Samples are taken from the lower respiratory tract, bypassing the nasopharynx in the transtracheal washing technique. However, this technique is difficult to apply in the field because it requires operative preparation of the ventral part of the neck. In addition, it is not suitable for routine examinations in the field because it is an invasive technique. The nasal swab technique is easy to apply and is a useful technique especially used to detect acute viral infections such as BHV-1. However, it is not a reliable technique because most of the microorganisms associated with the BRD complex are found in the normal upper respiratory tract flora (Caldow 2001). BAL fluid analysis is the best

technique used to accurately determine the etiologic agent (in the field or the clinic) in live calves (Panousis 2009).

Bronchoalveolar lavage (BAL) is the process of collecting fluid for examination from the lower respiratory tract, especially the alveolar cavity of the lung, as a result of giving fluid to the lung alveolo-bronchial system (Ok et al. 2019; Ider and Maden 2019). BAL is a relatively safe procedure that helps to diagnose respiratory diseases. Although it requires some training, it is a safe and efficient technique. It can be performed by using fiberoptic endoscopes or simple commercially available catheters (Caldow 2001). In addition to the isolation of the agent, this procedure is also used to evaluate antimicrobial drug therapy for lung diseases. Lung inflammation and damage cause changes in enzyme activity and cellular components in BAL fluid. Changes in BAL fluid are useful tools in determining pulmonary damage (Abutarbush et al. 2019; Ider and Maden 2019).

Diagnostic Imaging

Radiography and ultrasonography are diagnostic imaging devices used in the diagnosis of BRD. Although these are non-invasive methods for the antemortem diagnosis of pneumonia, the use of radiography for the diagnosis of BRD is impractical. Today, ultrasonography has taken its place because it is more practical in field conditions. Thoracic ultrasonography has a sensitivity of 79.4% (66.4-90.9) and a specificity of 93.9% (88.0-97.6) in the diagnosis of BRD (Buczinski et al. 2015). Thoracic ultrasound detects non-ventilated or consolidated lung lesions and diagnoses pneumonia in all its stages. Lung consolidation can be observed ultrasonographically only a few hours after infection. Thoracic ultrasonography allows consolidation of the lung as well as visualization of the pattern of pneumonia, abscesses, and extrapulmonary air/fluid. Ultrasonographic examination of both sides of the thorax may reveal an anechogenic or hyperechogenic area due to fluid in the pleural space in the ventral region of the thorax in cattle with pleuropneumonia (Siegrist and Geisbühler 2011; Buczinski et al. 2014; Ollivett and Buczinski 2016).

Treatment

Antimicrobial and adjunctive therapy (anti-inflammatory, bronchodilator, antitussive, expectorant/mucolytic, diuretics, immunomodulators, and vitamins) applications are included in the treatment of BRD. Antibiotic therapy is aimed at reducing and controlling bacterial proliferation by preventing the further release of bacterial virulence factors. For an effective treatment protocol, it is important to determine the causative agent of the disease, the time to start treatment, choose antibiotics that reach and maintain an effective therapeutic concentration and follow accurate dosage and duration protocol. Macrolides, tetracyclines, phenicols, fluoroquinolones, cephalosporins, and penicillins are widely used antimicrobial agents in the therapy of BRD. Among these antibiotics, macrolides (tilmicosin, spiramycin, erythromycin, tulathromycin, tylomycin, tildipirocin) can be used alone, as well as macrolides and florfenicol; macrolide and tetracycline (doxycycline or oxytetracycline); quinolones (enrofloxacin, marbofloxacin, danofloxacin), and cephalosporins (ceftiofur, cefquinome) or penicillin or ampicillin (sulbactam), amoxicillin-clavulanic acid combinations are also used (Güreli 2009; Tütüncü et al. 2017; Ok et al. 2019). Antimicrobial therapy in bacterial pneumonia can be successful if it is used for a sufficient time and most importantly at an early stage of the BRD complex. In the late stage of disease, antibiotics and other treatments may not be successful in the regeneration of normal lung parenchyma (Woolums et al. 2009). The efficacy of metaphylaxis in pneumonia is variable. In a study conducted in North America, it was shown that metaphylaxis reduces the rate of mortality and morbidity (Lekeux 2007; Wileman et al. 2009).

The second component of the BRD therapeutic strategy is anti-inflammatory agents (steroidal and non-steroidal). Anti-inflammatory therapy targets the control of local and systemic inflammatory processes. Steroidal anti-inflammatory drugs (SAIDs) are recommended to be used in a single dose because of their immunosuppressive effects. For this purpose, a single dose of dexamethasone (5-25 mg/animal) can be used. The use of non-steroidal anti-inflammatory (NSAIDs) drugs reduces fever, clinical signs, and lung pathology, and increases daily weight gain. The most commonly used NSAIDs in the treatment of BRD are flunixin meglumine, meloxicam, ketoprofen, carprofen, tolfenamic acid, and metamizole sodium (Lekeux 2007; Joshi et al. 2016; Tütüncü et al. 2017).

In calves with respiratory distress syndrome, nebulized bronchodilators such as salbutamol (0.025 mg/kg/6h), formoterol (15 µg totally/12 h), ipratropium bromide (2 µg/kg/12h) can be used to improve pulmonary functions in a short time (Ok et al. 2020). Parenteral form theophylline (1-10 mg/kg), clenbuterol (0.8 µg/kg) and atropine sulfate (2.2 mg/45 kg) can be used to relieve bronchospasm in BRD cases. Mucolytics are used because they facilitate mucociliary clearance. For this purpose, N-acetyl cysteine, bromhexine, and ambroxol are administered orally or intramuscularly for 5 days at a dosage of 0.25-0.4 mg/kg/day. If pulmonary edema is suspected in severe BRD cases, diuretics (furosemide 1 mg/kg) may be used (Joshi et al. 2016; Tütüncü et al. 2017).

Regardless of the cause, there is localized or generalized immunosuppression in respiratory system diseases. Immune modulator therapy provides rapid recovery and prevents relapses, especially in cases where viral pathogens are at the forefront. For this purpose, levamisole and inactivated *Parapoxvirus ovis* strain D1071 are commonly used in cattle. Levamisole (2.5 mg/kg) can be administered at 3 times intramuscularly or subcutaneously, and *Parapoxvirus ovis* (zylexis) can be administered in 3 doses, 2 days, and 1 week later as a single dose. For supportive purposes, vitamin A and C supplementation and limited fluid therapy can also be performed (Lekeux 2007; Tütüncü et al. 2017).

Prophylaxis

The protection practices related to the control of respiratory diseases in calves include the development of a strong immune system by providing sufficient amount of good colostrum, proper vaccination, healthy nutrition, biosecurity, and ensuring adequate ventilation. The importance of vaccinating pregnant cattle and colostrum management in the control of respiratory diseases is emphasized. Ensuring adequate passive transfer to calves and proper care of the umbilical cord are practices that reduce the rate of respiratory system diseases. Vaccination against the pathogens that cause BRD is a frequently used method of protection to control the disease. For this purpose, many vaccines have been produced commercially against BRD agents. In animals with good colostral immunity, firstly modified live vaccines are administered at the age of 3-4 months. The

combination of the vaccine with intranasally modified live PI-3 and infectious bovine rhinotracheitis (IBR) viruses in newborns, provides specific and nonspecific protection against respiratory diseases that can affect calves in the first week of life. Intranasal vaccination of one-week-old or older calves is beneficial in rapidly stimulating immunity by avoiding the undesirable effects of circulating maternal antibodies. An early vaccination program may be recommended for animals with insufficient colostral immunity (McGuirk 2008; Gorden and Plummer 2010).

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CHAPTER 15

TREATMENT OF BACTERIAL INFECTIONS OF ANIMALS: A SHIFT FROM ANTIBIOTICS TO NANOFORMULATIONS

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INTRODUCTION

The history of animal survival is mostly linked to the several existing lethal ailments. However, those caused by bacterial infections, impose the potent threat to animal health and surveillance (Gandhi et al. 2010). The prevention and control of infectious diseases is of basic economic importance in the animal husbandry. The causative agent of bacterial infection is the bacterial species that reside either over the surface or inside the organism, causing damage or death of cells /tissues via metabolic substances and other toxins (Fukuda et al. 2011). Antibiotics have been considered as the first-line therapeutic agent to fight against the invading pathogen hence, reducing the rate of morbidity and mortality of mammals (Martens and Demain 2017) and ultimately leading towards a dramatic progress in the animal production. Moreover, antibiotics have also been introduced as growth promoters in the form of additives to the animal feed leading to further economic advantage of animal production especially in the poultry industry (Wierup 2000).

Up till now, antibiotics have been playing an irreplaceable role in treating bacterial infections and promoting growth in animal production industry. However, the abusive and unattended use of antibiotics lead to increased resistance to them. Another major factor inducing drug resistance is the use of high dose of antibiotics with the common thinking that high dose may provide effective therapy against bacterial infections. However, increasing dose of antibiotics may cause more serious antibiotic resistance (Chen et al. 1999).

The emergence of resistant strains indicated that the antibiotic use should not be the ultimate and only way to control the bacterial infections (Bilal et al. 2021). Multi drug resistant invading organisms are imposing great threat to both animal and human health and need special attentions to control (Lee et al. 2019). The use of antibiotics in animal production need special measures as the emergence of resistant bacterial strains impose a potential threat to human health (Ma et al. 2021). It has also been predicted that the death rate via antibiotic resistance (10 million/year) will exceed to that caused by cancers (8.2 million/year) by 2050 (Liu et al. 2019). The use of antimicrobials especially fluroquinolones and other growth promoting antibiotics has been highlighted because of emergence of resistant microorganisms in animals (Fair and Tor 2014). It has been estimated that the bacterial infection may become the biggest cause of death if new effective antibiotic or other effective therapeutic agents have not been discovered in the next prevailing years. However, unluckily the speed of bacterial resistance against antibiotics is so high that it lagged behind the speed of development of new antibacterial agents and if the resistance issue remains unattended it will lead to return to the pre-antibiotic era, where minor cuts and a common cold could be lethal. Statistically, it was found that the number of newly synthesized therapeutic agents against bacterial infections, decreased sharply since last 30 years and there is even a gap of discovery of new drugs (Liu et al. 2019). World Health Organization (WHO) recommended and endorsed that antibiotics should not be administered as growth promoters and alternative treatment strategies should be used for therapeutic purposes (Manyi-Loh et al. 2018).

There is ultimate requirement to develop new therapeutic agents via advanced technologies (Ling et al. 2015). New treatment strategies using advanced nanotechnology, are immediately needed to tackle the problem of antibiotic resistance. This chapter describes the potent and effective nanomaterials that can considered for treatment of bacterial infections to improve animal production. Excellent properties of nanomaterials such as easy modification, specific physicochemical activity, size effect and so on, make them potent candidates to be considered for treatment of severe drug-resistant bacterial infections. Major bacterial diseases in animals are elaborated in Table 1 along with their potential nanotherapeutics.

Synthesis of Nanoparticles

Several strategies have been considered for the synthesis of nanoparticles. High temperatures are required for physical methods. Other disadvantages of this method are required space, time and harmful environmental effects. Physical methods include the advantage of absence of contamination of solvent and the uniform distribution of NPs in comparison

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Table 1: Major bacterial diseases in animals along with their potential nano-therapeutic agents

Bacterial disease in animals	Synonym	Animal species	Causing organism	Treatment	Reference
Actinomycosis	Lumpy jaw	cattle, swine	*Actinomyces bovis* or *Streptothrix bovis*	Silver and gold nanoparticles	Hu et al. 2018; Gajdács and Urbán 2020
Anthrax	Splenic fever, Wool-sorter's disease and Malignant pustule	All domestic animals, poultry, cattle and birds	*Bacillus anthracis*	PLGA-dendron nanoparticles, Viral nanoparticles	Manayani et al. 2007; Ribeiro et al. 2007
Bacillary White Diarrhea	Pullorum disease	newly hatched chicks, turkeys, pheasants, ducks and other wild birds.	*Salmonella pullorum*	Silver nanoparticles	Farouk et al. 2020; Schat et al. 2021
Black Quarter	Black leg, Quarter-ill, Emphysematous gangrene, Quarter evil, Symptomatic anthrax	Cattle and Sheep	*Clostridium chauvoei, Cl. septique* and *Cl. oedematiens,*	Not formulated yet	Ziech et al. 2018
Braxy	Braxy	Sheep especially weaned lambs	*Clostridium septique*	Not formulated yet	Alves et al. 2021
Botulism	Food poisoning	Animals and birds	*Clostridium botulinurri*	Silver nanoparticles	Aminianfar et al. 2019
Contagious abortion of Cattle	Brucellosis; Bang's disease; Infectious abortion.	Cattle	*Brucella abortus bovis* or *Bang's bacillus.*	Silver nanoparticles	Alizadeh et al. 2014; Khurana et al. 2021
Actinobacillosis	Wooden tongue	Cattle and sheep	*Actinobacillus lignieresis* *Actinomyces viscosus*	nano-ZnO loaded eggshells	Chen et al. 2021
Botryomycosis	Discomycosis	Horses	*Staphylococcus aureus*	silver nanoparticle-containing polymer composite, Silica nanoparticles	Bhardwaj 2015; Quintero-Quiroz et al. 2020
Pasteurellosis	A group of diseases like hemorrhagic septicemia, fowl cholera, plague, arthritis, influenza	Animals and Birds	Pasteurella group of bacteria: *Pasteurella multocida* Cause of hemorrhagic septicemia in cattle *Pasteurella aviseptica* Cause of fowl cholera *Pasteurella suiseptica* Cause of Swine plague *Pasteurella canis* Cause of otitis, bacterial rhinitis, vertebral osteomyelitis, meningomyelitis, bronchopneumonia, tracheitis, paranasal sinus inflammation and toxicosis in canines. *Pasteurella bovine* Cause of hemorrhagic septicemia in cattle		Csébi et al. 2010; Griffin 2010; Malayeri et al. 2010; Sellyei et al. 2011; Ashraf et al. 2019; Chatelier et al. 2020; Shyam et al. 2020

to chemical method (Abou El-Nour et al. 2010). Chemical reduction is another most common process for preparation of silver NPs by inorganic and organic reducing substances. Other chemical methods of NP synthesis include the micro emulsion assay, photo induced reduction, ultraviolet initiated photo reduction, irradiation methods and electrochemical synthetic methods. Chemical methods provide higher yield as compared to physical methods (Raghavan et al. 2016).

Biological methods of NP synthesis harness the reducing ability of microbial cells, enzymes and biological molecules. The green plants, bacteria and fungi are recently being used to biologically synthesize metal NPs via environment friendly method. For instance, silver nanoparticles have been successfully synthesized from *Pseudomonas aeruginosa* (Peiris et al. 2017), *Staphylococcus aureus* and *Escherichia coli* (Peiris et al. 2019).

Moreover, the fungus Fusarium based silver nanoparticles were analyzed to have long term stability as the fungus secretes an enzyme i.e., nitrate reductase which help in reducing the Ag ions and also involved in secreting capping proteins, which contribute to the long term stability (Ingle et al. 2008). Recently several plants have been considered for biological synthesis of NPs (Ahmed et al. 2016). The extracts of plants are rich in enzymes and phytochemicals, which assist in reducing metal ions into nano sized particulate material. This procedure definitely provides a cost effective and environment friendly alternative method for large scale production of NP over the environment unfavorable chemical and physical methods (Loo et al. 2012).

Nanoparticles Act as Antimicrobials

In the medical field, nano-scale particles (Medina et al. 2007) are being considered as suitable agents for the treatment and diagnostic purposes (Surendiran et al. 2009) . The recent advances in the development of medicinal nanomaterials may have beneficial role to cover the purpose of antibiotics. The nanoparticles may be considered as a modern class of bacterial antimicrobials. Nanoformulations as antibiotics may play role to efficiently reduce resistance and kill the pathogenic bacteria (Rai et al. 2009). Latest research studies elaborated that several antimicrobial - activated metal

Table 2: Mechanism of action of different types of nanomaterials against specific bacteria

Nanomaterial	Size (nm)	Mechanism of Action	Target organism	Reference
Carbon mediated nanoformulations				
Carbon Nanotubes	1-100	Inhibition of energy metabolism, bacterial membrane and respiratory chain damage	*Escherichia coli*, *Klebsiella pneumonia*, *Yersinia pestis*, *Enterococcus faecium*, MDR *Streptococcus* spp. *Salmonella enterica, Acinetobacter baumannii, Burkholderia cepacia*	Shvero et al. 2015
Fullerene	200	Inhibit energy metabolism and destroy the respiratory chain	*Salmonella, Streptococcus* spp. and *E. coli*	Heredia et al. 2022
Graphene Oxide nanosheet	12	RNA effluxes by damaged cell membranes, methiciline resistantg MDR	*E. coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus faecalis, Staphylococcus aureus* *Methicillin-resistant Staphylococcus aureus* (MRSA)	Gao et al. 2021
In-organic nanoformulations				
Silver Nanoparticles	1-100	Inhibit cell division and damage the respiratory chain	almost all bacteria, eukaryotic organisms and viruses as well	Bruna 2021
Gold nanoparticles	1-100	Cell damage via induction of local hyperthermia in the presence of a magnetic field	*Staphylococcus aureus, Enterococcus faecium, Pseudomonas. aeruginosa, E. coli* and *Candida albicans*	Su et al. 2020
Ferric oxide nanoparticles	1-100	Production of reactive oxygen species leading to cell wall disruption	*Methicillin-resistant Staphylococcus aureus,* multi drug resistant *E. coli* and *K. pneumoniae*	Shvero et al. 2015
Aluminum oxide nanoparticles	10-100	Production of reactive oxygen species leading to cell wall disruption	*E. coli*	Manyasree et al. 2018
Magnesium oxide NP	15-100	Generation of reactive oxygen species, Alkaline effect, Peroxidation of lipids	*S. aureus, E. coli*	He et al. 2016
Silicon NP	20-400	ROS production leading to membrane damage	*MRSA*	Selvarajan et al. 2020
Titanium dioxide NP	30-45		*S. aureus, Enterococcus faecium, E. coli, P. aeruginosa,*	Dicastillo et al. 2020
Super-paramagnetic iron oxide nanoparticle (SPIONS)	15-25	NO release Production of reactive oxygen species.	*S. aureus, E. coli*	Gholami et al. 2020
Zinc-oxide nanoparticles	10-100	Lipid and protein damage Adsorption to cell surface ROS production, disruption of membrane	*K. pneumoniae, E. coli, Enterobacteraerogenes, MRSA, Klebsiellaoxytoca*	Emamifar et al. 2010
Copper NP	2-350	DNA damage, Generation of ROS, Cell membrane potential dissipation Lipid and protein peroxidation	*A. baumannii, MDR E. coli*	Chen et al. 2019
Organic nanoformulations				
Poly-ε-Lysine	1-100	Disrupt the cell wall and membrane integrity. Destroy cell membranes or cell walls	Gram-positive and Gram-negative bacteria. Additionally, it acts against spores of *B. subtilis, B. stearothermophilus*, *B. coagulans, S. cerevisiae* *E. coli*	Rodrigues et al. 2020
Quaternary Ammonium Compounds	1-100	Interfere with the function of the cell membrane Lysis, or destruction of the cell Affects DNA ROS release	*Pseudomonas* *Pseudoalteromonas* *Erwinia* *Enterobacter*	Xue et al. 2015
N–Halamine Compounds	1-10	Disrupts the functionality of bacterial cell membrane leading to inactivation	*S. aureus, P. aeruginosa*	Padmanabhuni et al. 2012
Chitosan nanoparticles	200	Loss of permeability of membrane	*S. aureus, E. coli*	Alqahtani et al. 2020
Quaternary bis-phosphonium and ammonium	1-100	Inhibits the growth of bacteria disruption of thecell division mechanisms	*S. aureus, E. coli, B. subtilis, S. epidermidis*	Nikitina et al. 2016
Composite mediated nanoformulations				
Ceramic Matrix Nano-Composites	1-100	Inhibit the growth of bacteria and hinder physical interaction	*S. aureus* *E. coli*	Baaloudj et al. 2021
Polymer Matrix Nano-composites	1-100	Inhibit the growth of bacteria and hinder physical interaction	*A. baumannii* *K. pneumonia* *E. coli* *S. aureus*	Giraud et al. 2021
Metal Matrix Nanocomposites	1-100	Hinder physical interaction and inhibit the growth	*S. aureus* *A. baumannii* *K. pneumonia* *E. coli*	Yang et al. 2018

nanomaterials play significant role to treat infectious diseases (Goodman et al. 2004). Such synthetic nanomaterials may indeed provide benefits of lower cost, lower toxicity and good pharmacokinetic factors while supporting to eradicate drug-resistant bacteria. Their major advantage is that they provide longer efficacy than traditional antibiotics, which is

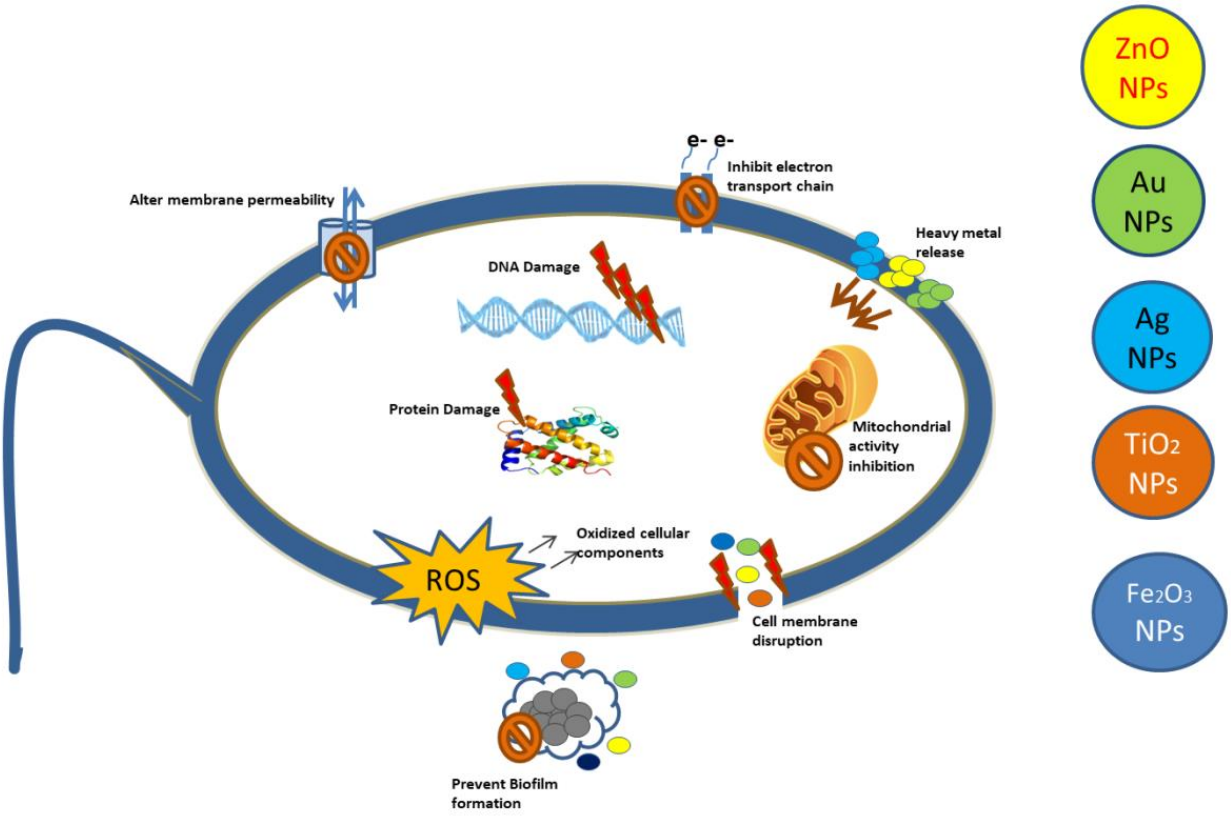

Figure 1: Different pathways for nanoparticles antimicrobial behavior.

highly required in the long-term sustained therapeutic effect (Pal et al. 2007). Moreover, the synthetic therapeutic nanoparticles are biomimetic to biological molecules and are highly specific in their action (Medina et al. 2007). Thus, these features of nanomaterials enhance the effectiveness and reduce adverse effects associated to them. These nanomaterials are categorized into several categories including carbon-based, organic, inorganic, polymeric and composite-based nanoformulations (Table 2).

Possible Mechanism of Action of Antibacterial Nanomaterials

Nanomaterials are used as a fairly modern strategy to resolve the global problem of antibiotic resistance. These nanosystems show antimicrobial activity without the involvement of antibiotics and show their actions via several destroying pathways involving multiple targets. The antibacterial mechanisms of these nanoparticles involve ATP depletion, reactive oxygen species (ROS) production, membrane disruption and inhibition of DNA synthesis (Slavin et al. 2017; Tamara et al. 2018). The adsorption of nanoparticles to surface of the cell causes bacterial wall degradation, leaking out the cytoplasmic material (Shmarakov et al. 2014) (Figure 1).

The metallic nanoparticles are well-known for their effective antimicrobial activity against a wide variety of resistant bacterial species. They work through several mechanism of actions to fight against bacteria. The silver nanoparticles show their action by producing large number of silver ions, which alter the cell membrane permeability and inhibit energy transport chain of electrons. Moreover, silver ions have also been identified to damage the microbial cell DNA (Donaldson et al. 2006; Kumar et al. 2019; Aunkor et al. 2020). Zinc oxide nanoparticles emit Zn^{+2} ions in the cells and are involved in production of hydrogen peroxide and destruction of membrane of the cells (Pinto et al. 2013; Karwowska 2017). Titanium dioxide nanoparticles work by producing reactive oxygen species and ultimately affect the stability of cell membrane (Klasen et al. 2000; De Aberasturi et al. 2015; Liao et al. 2019). In addition to metal nanoparticles, there are several other nanomaterials including liposomes, polymers, or carbons, each of which has its own antibacterial action against bacterial pathogens. Chitosan nanoparticles show their action by boosting permeability and inactivating the enzymes of the microbial cells (Li et al. 2008; Zhang et al. 2008). Carbon nanotubes show their action primarily by membrane damage via production of reactive oxygen species (Sondi and Salopek-Sondi, 2004). Another group of nanoparticles involving fullerenes functions by enhancing neutrophil infiltration and disruption of cell membrane (Dastjerdi and Montazer 2010; Tania and Ali 2021). Thus, there are various action pathways in which nanoparticles can effectively attack the machinery of microbes (Figure 1).

Nanoparticles Derived Antibacterial Vaccines

Vaccines may provide protection and treatment against bacterial infections by linking the host's immune system. Successful control over former epidemics world widely has been counted as the most effective public health intervention ever achieved (Plotkin 2005; Germain 2010). The prevention and treatment strategy via vaccine development, provides a promising effect to control antibiotic resistance by decreasing the use of antibacterial agents (Wenzel and Edmond 2000; Mishra et al. 2012). On the other hand, the majority of

existing vaccines predominantly neutralize or opsonize antibodies against invading pathogenic organism, a mechanism that is not effective to prevent or treat a variety of bacterial infections (Levy and Marshall 2004). The development of vaccine is further become challenging due to lack of complete understanding of complex human immune system and the principal protective mechanisms (Fauci and Morens 2012). To overcome these hard challenges, nanoparticles have been considered as strong antibacterial candidates bearing unique feature of immune modulation against microbial infections (Swartz et al. 2012; Irvine et al. 2013).

Nanoparticles may also have characteristics to overcome the undesirable systemic biodistribution, instability, and toxicity associated with the soluble molecule administration (Tan et al. 2010; Gu et al. 2011). It has been analyzed that nanoparticle surfaces conjugated with antigens and facilitated the activation of B-cell (Villa et al. 2011) as higher number of antigens were provided to antigen presenting cells (APCs) (Nembrini et al. 2011). The improvement of antigen loading has been achieved by the advancement in nanotechnology leading to establishment of fabrication technique for formulation of nanoparticle-based drug delivery systems such as facile spray-drying process (Dierendonck et al. 2010), layer-by-layer assembly procedure (De Rose et al. 2008; De Geest et al. 2012) and soft lithography-based PRINT technique (Perry et al. 2011).

Moreover, cell membrane-enveloped nanoparticles have also been coated to impede membrane-damaging toxins and distract them away from their specific cell targets (Hu et al. 2013). Such a toxin-detainment technique was applied to safely deliver staphylococcal α-hemolysin to antigen presenting cells and induced immunity against toxins in the mice in comparison to vaccination of heat-denatured toxins (Hu et al. 2013). This methodology aid in maintaining a faithful antigenic presentation while removing virulence of toxins.

In addition to delivery of antigens, nanoparticles may also involve in carrying adjuvants to mimic natural microbes to improve the efficacy of vaccine (Little 2012). Predominantly, several ligands of toll-like receptor (TLR) such as DNA, RNA, carbohydrates and other small molecules in combination of antigens may be delivered via nanoparticles, leading to equivalent response of immune system in comparison to soluble antigen formulations (Demento et al. 2009).

More notably, nanoparticles especially designed to assist in programmable presentation of adjuvants and antigens to immune cells to get desirable immune responses. One such example is of combinations of TLR agonists which have been concomitantly loaded into specific nanoparticles to get the combinatorial TLR activation, similar to that occurring in natural infections, leading to more vigorous immune responses (Mount et al. 2013; Orr et al. 2014).

Another example involves encapsulation of antigens and TLR agonists in prepared nanoparticles to induce effector T-cell responses (Zhu et al. 2010; Mount et al. 2013; Orr et al. 2014) in the same manner as the antigen processing takes place in the dendritic cells (Blander and Medzhitov 2006). On the other hand, delivery of both TLR agonists and antigens in separate nanoparticles showed beneficial effect in producing antibody responses (Kasturi et al. 2011).

Developing vaccines for specific required target sites to induce effective and safe immune responses is a major advantage of use of nanoparticles for delivery of vaccine. For example, *Clostridium botulinum* type-A neurotoxin loaded cationic nanogel has been analyzed to assist antigen to persistently adhere to the nasal epithelium and then effectively up taken by mucosal dendritic cells (Nochi et al. 2010). The newly synthesized nanogel not only showed immune responses, but also protected central nervous system and upper respiratory tract from exposure to toxic antigens. Moreover, nanoparticles sensitive to the pH of the gastrointestinal tract play protective role and prevent antigen deterioration in the stomach and release antigens as soon as nanoparticles reach the lower gastrointestinal tract of high pH medium, for successive translocation across the epithelium of intestine (Zhu et al. 2012). A similar approach has also been considered for nanoparticle-mediated vaccines which may efficiently target lymph nodes. Nanoparticles which are smaller in size can be transported faster to the lymph node (Reddy et al. 2007), whereas, larger nanoparticles may stay for longer duration in the lymph node (Jewell et al. 2011). Such distinct features show the size optimization importance in lymphatic system for inducing required immune responses.

Conclusion

A number of promising nanotherapeutic agents have been discovered and developed for treatment of resistant bacterial infections in animals. Optimal strategies and measures must be adopted related to prevent and treat antibiotic resistance. Studies elaborated that the nanotherapeutics and nanovaccines contributed in upgrading the status of health and economy of animal production, without introducing antibiotics to the animals. There is dire need to rethink that antibiotics should not be introduced as first line agents. The use of antibiotics should only be considered when other management strategies have failed or are not responsive.

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CHAPTER 16

JAPANESE ENCEPHALITIS VIRUS

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INTRODUCTION

Japanese encephalitis (JE) is an acute vector born viral disease that cause encephalitis in human, equids and reproductive disease in swine. Rare clinical cases are also reported in other domesticated animals such as cattle (Katayama et al. 2013). The disease is caused by Japanese encephalitis virus that belongs to Flaviviridae. JE has high peaks in horses during late summer and autumn in temperate regions, while in tropical areas virus circulates around the year. Most of cases remain asymptomatic but clinical cases tend to manifest sever encephalitis leading to death and many survivors left with neurological disorders. The morbidity and mortality rate is high in unvaccinated population during an outbreak. The case fatality rate in horses can be as high as 5-15% but in some outbreaks it has been reported up to 30-40 % (Prow et al. 2013; Kumar et al. 2018). The transmission is mainly caused by mosquito bite and life cycle is enzootic. Pigs are amplifying host and/or reservoirs, while water birds act as carrier and humans, equids and other domesticated animals serve as dead end host. The first outbreak was reported in 19th century in Japan and then extended its geographic area to Asia and western pacific region.

Etiology

Japanese Encephalitis Virus (JEV) belongs to arbovirus within the genus Flavivirus and family Flaviviridae. Genome is RNA in nature that is single stranded, positive sense, non-segmented and length of genome is about 11kbs. As genome is positive sense it acts directly as mRNA and encode a single open reading frame. The genome is translated into a single polyprotein, precursor of 3432 amino acids that undergoes proteolytic cleavage to form three structural proteins [C (capsid), pr M (pre-membrane), E (envelope)] and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). To date there is only one serotype and five genotypes [GI (GI-a, GI-b), GII, GIII, GIV, GV] of JEV are reported (Figure 1) (Gao et al. 2017; Yun et al. 2018). Genotyping is based on envelope (E) protein of virion and is considered as potential antigenic part, involved in the binding of virus to the cell receptors, virion assembly and fusion activity. Amino acid substitution in E protein plays key role in determining the neurovirulence and/or neuro invasiveness. The JEV is closely related to other arthropod borne viruses' i.e West nile virus and Murray valley encephalitis virus (Rice et al. 1986).

Host and Transmission

Disease mainly occurs in equids i.e., horses, donkeys and pigs. Domesticated and feral pigs and ardeid water birds (e.g., herons, bitterns and egrets) serve as main amplifying hosts in JEV endemic areas, as they develop high viremia and involve in disease transmission through mosquito vectors. Asymptomatic infection have also been reported in other domestic and wild mammals (cows, goats, and dogs), reptiles (snakes) and amphibians (frogs) and considered as dead-end host because they do not contribute in spread of infection as they develop low viremia that is not capable of transmitting to mosquitoes. Humans are also susceptible to the disease and considered as dead-end host (Kobayashi et al. 1948; Soman et al. 1977; OIE 2019).

The virus is transmitted through mosquitoes, the genus Culex, several species are reported but Culex (C.) tritaeniorhynchus and C.annulirostris are primary vector species involved in transmission of virus. *C. tritaeniorhynchus* is important in spreading of virus to humans and domesticated animals around the globe, while *C. annulirostris* are important vectors in Australia. *C. tritaeniorhynchus* breeds in rice fields, wells, fish ponds, connecting canals and also has been reported in urban areas close to human proximity such as water storage tanks in houses (Scherer et al. 1959; Hanna et al. 1996; Su et al. 2014). The JEV is mainly transmitted through mosquito bites but can also through mucous membrane, inhalation of aerosols or acquired through needle injuries. Human and most of domesticated animals are incidental host and are not considered important in virus transmission. Although birds, suids and pigs are amplifying host and involve in disease transmission (Figure 2). Boars are reported to transmit the virus through semen. The JE virus does not survive well outside a living host and is sensitive to ultraviolet and gamma rays. Virus can be destroyed by heating at 56°C for 30 minutes, inactivated at low pH (3) and can be killed by organic and lipid solvents like iodine, 70% alcohol, 3% formaldehyde and 1% sodium hypochlorite (OIE 2019).

Incubation Period and Clinical Signs

In experimentally infected horses' incubation period is approximately 4-14 days, while in pigs clinical signs appear after 3 days but high t'rature and viremia is detected within 24 hours. Infection ranges from subclinical to symptomatic in horses and disease severity varies. Three syndromic manifestations have been described in horses:

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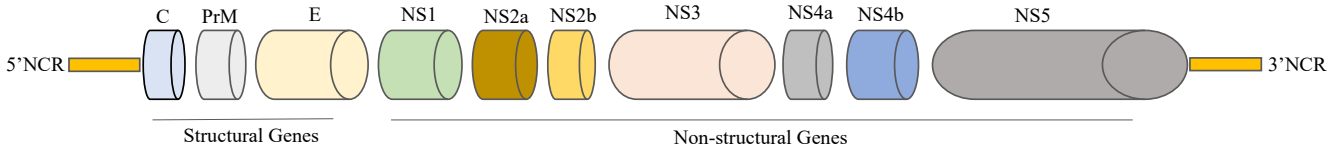

Fig. 1: Genome organization of Japanese encephalitis virus. 5'NCR (Non coding region) followed Single open reading frame and 3'NCR (Non coding region).

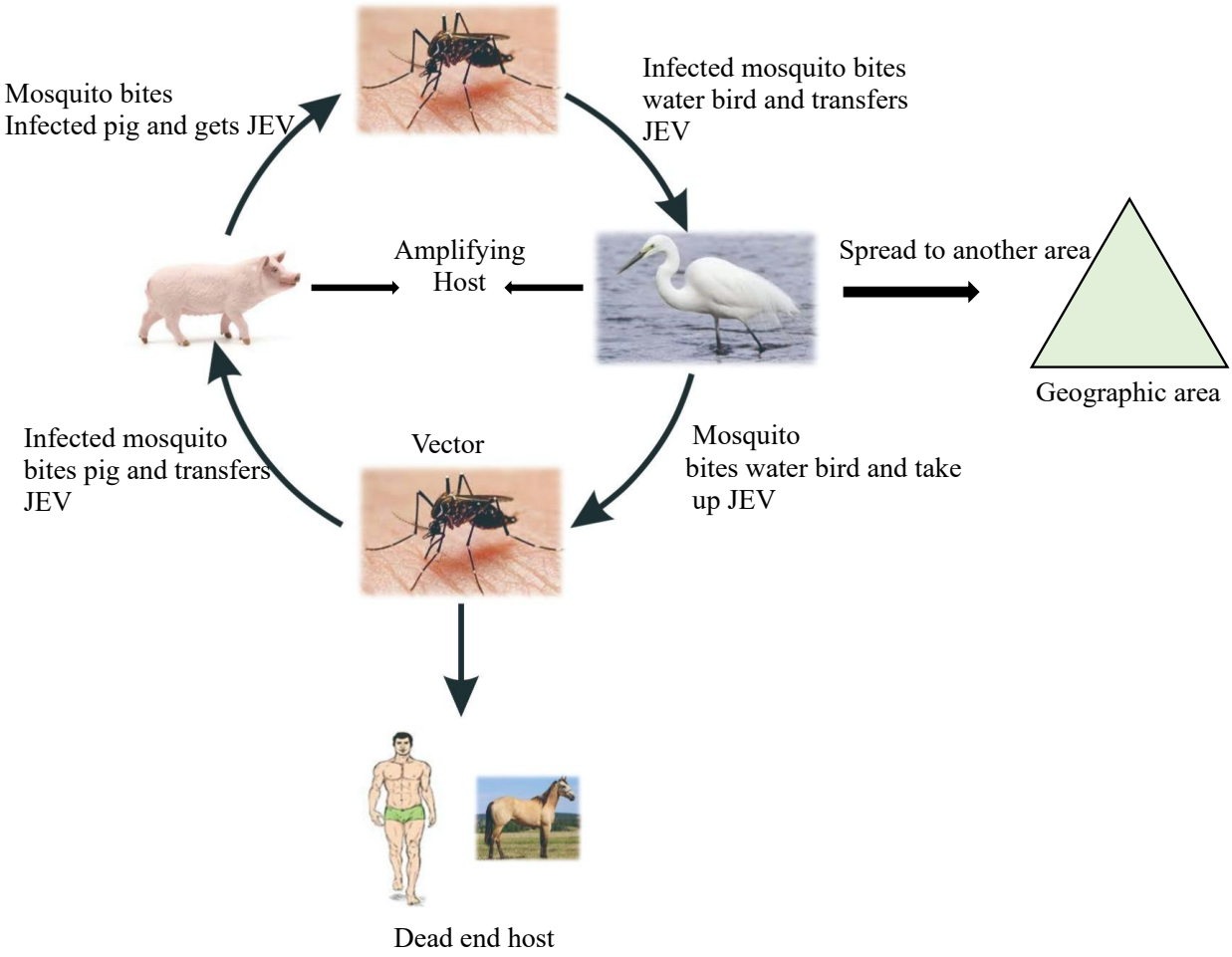

Fig. 2: Transmission cycle of Japanese encephalitis virus.

1) Transitory type syndrome: Infected horses develop mild fever lasting for 2-4 days along with loss of appetite, immobility and congested mucosa.
2) Lethargic type syndrome: Infected horses develop moderate fever, difficulty in swallowing, chewing motion, incoordination, compromised vision and paralysis. Recovery usually occurs within one week.
3) Hyperexcitable taype syndrome: Horses develop high grade fever accompanied with profuse sweating, muscle tremors, loss of vision, coma and death (Van den Hurk 2009; Go et al. 2014; OIE 2019). Mortality rate is up to 30%. Risk of abortion, still birth and congenital deformities is higher in pigs (Li et al. 2010). JEV infection in cattle is also reported but cases are very rare. Clinical signs observed in cattle include depression, loss of appetite, circling movement and inability to walk. Most JEV infections are asymptomatic or cause mild flue like disease in human that lasts for 5-15 days but some infections result in encephalitis accompanied with headache, fever, hemorrhagic lesions in brain, inflammation of meninges followed by neck stiffness, coma, paralysis of upper limbs and ultimately death. Mortality rate in human can be as high as 30% and recovered patients may have lifelong physical and mental impairments such as epileptic seizures. Miscarriages have been reported in pregnant women infected first time during pregnancy (Solomon and Vaughn 2002; Hollidge et al. 2010).

Pathogenesis and Gross Lesions

After transmission through infected mosquito bite, replication of virus occur in skin dendritic cells (Langerhans cells) and gets transported into the lymphatic and peripheral tissues resulting in viremia. Then lymphocytes harboring virus cross the blood brain barrier and enter into endothelial cells of central nervous system. The JE virus replicates in neurons which results in neural cell's death (Li et al. 2015). Although viral entry into brain is not well understood but once the virus enters brain cells, JEV is detected in cerebrospinal fluid and nervous tissues. The JEV can cross the placental barrier and cause abnormal neural development in fetus (Mathur and Chaturved 1982). Postmortem lesions in infected horses and cattle show diffuse

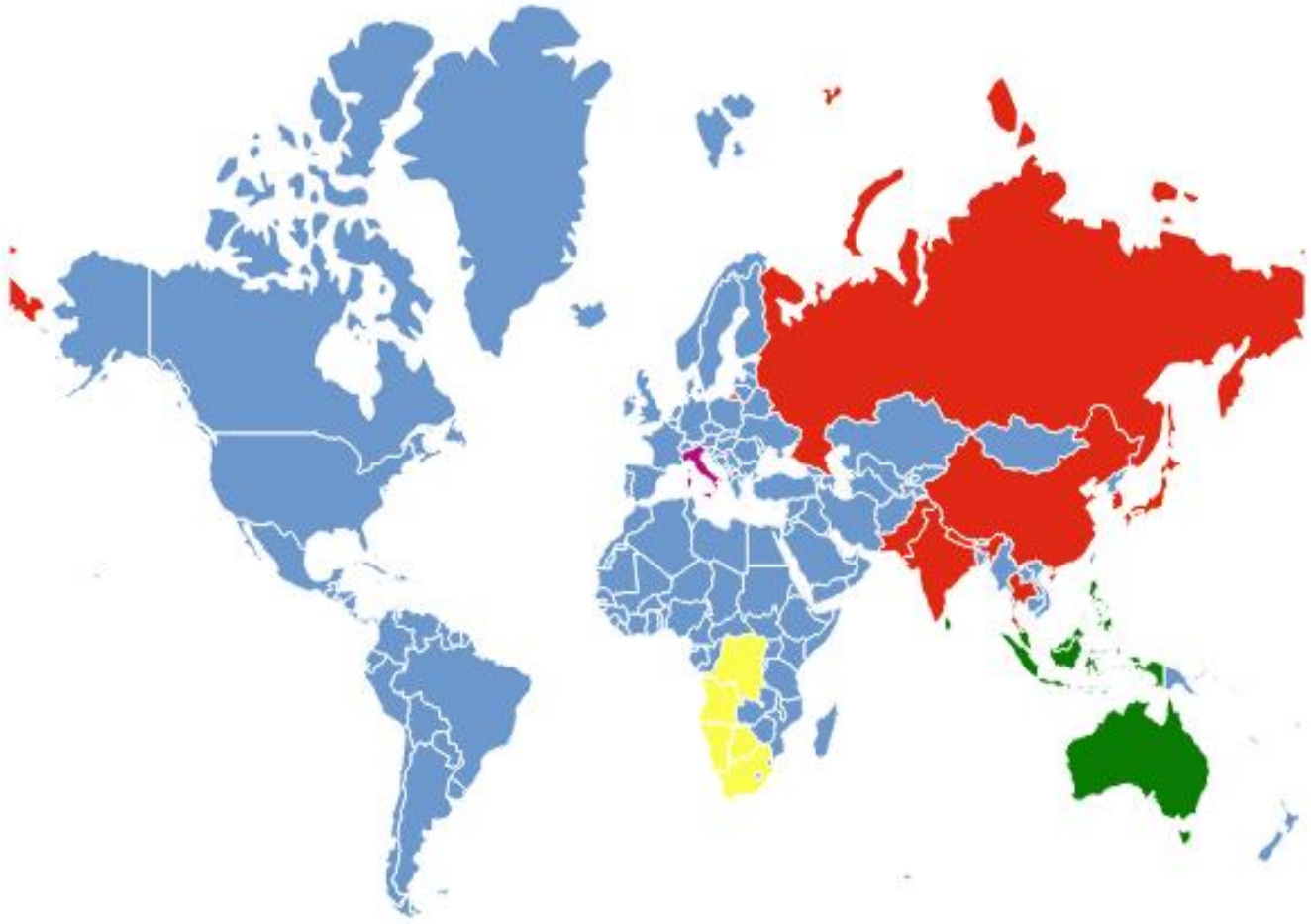

Fig. 3: Geographic distribution of Japanese Encephalitis Virus. Red color highlights countries where disease is epidemic (China, northern India, Japan, Pakistan, Korea, Nepal, Thailand and Russia). Green color highlights countries with endemic status (Australia, Indonesia, Malaysia, Singapore, Philippines, Sri Lanka and southern India.). Yellow color highlights African countries. Purple color highlights European countries**.**

non-suppurative encephalomyelitis that is characteristic microscopic lesion along with hemorrhages and congestion (Katayama et al. 2013; Kako et al. 2014). Congenital neurological defects including hydrocephalus, cerebellar hypoplasia and spinal hypomyelinogenesis has also been observed in mummified or still born fetuses from infected sows (Desingu et al. 2016).

Epidemiology and Geographic Distribution

Japanese encephalitis virus, a serious vector based viral disease is found worldwide especially in Asia, Western Pacific region and Northern Australia (OIE 2019). JEV has also been isolated from mosquitoes and birds in Italy, where human cases are not reported (Preziuso et al. 2018). A mixed infection with yellow fever has also been reported in African countries. Presence of virus in other regions is still unclear due to lack of surveillance and/or cross reactivity with other Flaviviruses. In Asia, JEV is wide spread in temperate and tropical regions especially in areas of rice farming and pig farming. Mainly two epidemiological patterns of this virus have been recognized. Epidemic pattern has been reported in China, northern India, Japan, Pakistan, Korea, Nepal, Thailand and Russia. While endemic pattern has been reported from Australia, Indonesia, Malaysia, Singapore, Philippines, Sirilanka and southern India (Figure 3). In northern region, GI and GIII are most prevalent genotypes, while in southern region GII and GIV are reported and GV is putative (Ladreyt et al. 2019).

Diagnosis

Asymptomatic infection, acute nature and cross reactivity with other viruses made difficulties in diagnosing Japanese encephalitis virus (JEV) in the past. But now with the combination of classical and advanced diagnostic techniques, it became easier to diagnose the virus (Mansfield et al. 2017).

Virus Isolation

Virus can be isolated from the CNS of diseased or dead animal. In vivo and in vitro techniques are employed for this purpose. Virus is collected from different parts of the brain including corpus striatum, cortex, or thalamus. It can also be isolated from spinal cord and blood. In vivo isolation of virus is done in mice by inoculating affected tissue intracerebrally. Mice will develop symptoms during the incubation period. In vitro primary cell cultures prepared from minced chick brain, chick embryoblasts, porcine or hamster kidney cells, or with established cell lines such as Vero (African green monkey kidney), BHK (baby hamster kidney) or C6/36 (mosquito – Aedes albopictus) are used. Discrete plaques are visualized after dyeing with crystal violet (Stear 2005, Mansfield et al. 2017).

Serology

Several serological techniques are used to detect JEV in the cerebrospinal fluid (CSF) or blood of the animal. These include

ELISA (enzyme linked immunosorbent assay), virus neutralization test (VNT), hemagglutination inhibition tests (HI) and complement fixation tests (CFT). Determination of IgM against surface proteins through ELISA is the readily available marker for current infection, within a week of the start of infection, and IgG to track previous infection. This test is also handy and suitable to perform field survey for epidemiological investigations. Drawback of this test is cross reactivity with the other flaviviruses. To overcome this issue, plaque reduction neutralization test (a variant of VNT) is an alternative which gives a reliable result and considered as gold standard for the detection of JEV. Commercially available indirect immunofluorescence test (IIFT) is also available to detect IgM and IgG using the immunofluorescence and is more sensitive. CFT is also a cheap source of identification which comes with good sensitivity. HI is also used for the detection of JEV, but it has low specificity and sensitivity that makes it a bad choice (Cardosa et al. 2002, Feng et al. 2018).

Molecular Methods

Now a days molecular methods are the advantageous over conventional methods due to number of reasons, including high specificity, sensitivity, rapid and convenience. There are five genotypes of JEV based on the viral envelop E gene, each of which can be individually detected in a single setting of assay. Genotype GI (GI-a, GI-b) is most prevalent among all. Molecular methods are based on the specific primers which amplify the nucleic acid and yield a qualitative and quantitative measure of the virus. RT PCR (Reverse transcriptase polymerase chain reaction) and multiplexed PCR techniques are used to amplify RNA of JEV. This assay can amplify genome present in samples, like serum, CSF, tissue, or any other body fluid. Primers for these assays are reported by Mansfield et al. (2017).

Prevention and Control

JEV infection can be prevented if strict measures should be taken in time. Though vaccine development against in JEV in human is under process but it is available in market for the livestock and pets. Live attenuated and killed vaccines are recommended by The World Organization for Animal Health. Vaccine is highly effective in preventing infection in livestock and rare animals like horses. This has a huge impact in the revenue generation from the production of livestock. Other than vaccination, strict mitigation of vectors is highly desirable to prevent the spread of infection in animals and human. Isolation of infected animals is also a strategy to control the spread of the virus to healthy animals. Availability of rapid, cheap, easy to handle and convenient diagnostic techniques in endemic areas can mainly help in prevention and control of the infection which can save huge losses (Fischer et al. 2008).

Biosafety

JEV has the potential biosafety threat to handlers of the infected animals, as virus can be transmitted while in contact with blood, cerebrospinal fluid, tissues, infected arthropods, needle and inhalation. So, standard biosafety measures should be taken while handling the infected material either in or outside the laboratory. These include the use of PPE and BLS level3 laboratories while culturing the virus, proper disposal of the waste and incarnation of infected material (Artika et al. 2017).

Treatment

No specific antiviral therapy is available for treating human and animals. Disease can be prevented though management and vaccination (Zhang et al. 2014).

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CHAPTER 17

RECENT APPROACHES IN DEVELOPMENT OF RNA AND DNA BASED VACCINES

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INTRODUCTION

Vaccines are success story in modern medicine from decreasing the incidence of some diseases like polio virus to completely eradicating others like smallpox. The milestone in history of vaccine was discovery of small pox vaccine by Edward Jenner in 1798, this innovation leads to immunization of animals and humans against many diseases in more effective way. Vaccines prepare the body to fight against foreign pathogens that invade body to cause infection. Conventional vaccines use harmless piece of particular pathogen (virus, bacteria) that triggers host immune reaction (Paoletti et al. 1984).

Vaccine development is the most important invention in the last century. It saved the people from many infectious diseases and plays a significant role as life saver. Vaccination is considered as one of the most effective way to combat infectious diseases in human and animals (Smith 2011). Advancement in field of vaccinology leads to the development of many types of vaccines on the basis of principles like inactivated vaccines, live attenuated vaccines, recombinant vaccines, subunit vaccines, RNA/DNA and peptide vaccines (Hasson et al. 2015).

Conventional Vaccines

Live attenuated, killed or inactivated, subunit vaccines (toxins and proteins) are traditional vaccines commonly used for the prevention of disease from many years, however, with the emergence of new pathogens, pathogenic strains or variants there is a dire need to develop novel and better vaccines. Conventional vaccines have shown effectiveness in preventing many of the infectious diseases but these also have many disadvantages; time consuming, difficulty in cultivation of many microorganisms, adverse immune response, booster required, storage and efficacy (Rappuoli 2000). Inactivated vaccines are most widely used traditional vaccines. These are safer but necessitate the administration of multiple doses in order to achieve strong and long-lasting immunity. Live attenuated vaccines develop a strong immune response, but always have a risk of infection by becoming a less virulent pathogen to more virulent one (Bouazzaoui et al. 2021).

Nucleic Acid Vaccines

Nucleic acid (NA) vaccines, as the name indicates are constituted from nucleic acids of a pathogen. NA vaccines can be either DNA vaccine or RNA vaccine. The aim to form and administer a NA vaccine is to stimulate an immune response against the particular from which the NA was taken. Both DNA and RNA vaccines work on the basis of central dogma i.e DNA after entering the cell transcribes into RNA and then is translated to protein whereas RNA vaccine translates to specific protein of pathogen (Gurunathan et al. 2000). This protein acts as immunogen and activates immune system within as well as outside host cell. Eventually host cell's machinery keeps on making the pathogen specific protein which later on triggers and activates immune response for a longer period of time. NA vaccines have several advantages over conventional vaccines including; these vaccines are quick and easy to develop, provide both antibody mediated and cell mediated immunity, no risk of the vaccine to trigger disease and relatively easy to develop (Table 1).

NA vaccines are also known as "third generation vaccines". This is a relatively new technology. Although DNA and RNA vaccines are being developed against various diseases, including HIV, Zika virus and COVID-19, so far none of them have yet been approved for human use. However, two RNA vaccines (Pfizer BioNTech and the Moderna COVID-19) have been authorized for emergency use, in the UK at present. Several DNA vaccines are licensed for animal use, including a horse vaccine against West Nile virus (Chen et al. 2014; Regalado 2016).

Later in the chapter DNA and RNA vaccines will be discussed separately in detail.

DNA Vaccines

Simply the concept of DNA vaccine is that the inserted plasmid is expressed in the host cells using cell's machinery and immune response is initiated due to the production and later detection of foreign protein in the body and works as vaccine (Wolff et al. 1990). DNA vaccine like live attenuated vaccine has the ability to activate both types of immune responses; cell mediated and humoral immune response (Gurunathan et al. 2000). DNA vaccine works as antigen in the host body analogous to viral infection. It leads to activation of major histocompatibility complex (MHC) class I type (cytotoxic T cell) response. The activation of humoral immune responses is indicative that mechanisms exist in vivo for antigen presentation by the (MHC) class II pathway upon

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Table 1: Difference between Conventional vaccines and Nucleic acid based vaccines

	Conventional Vaccines	Nucleic Acid Vaccine
1.	Also called traditional or protein vaccines	Also called DNA or RNA vaccines
2.	They contain whole pathogen either virus or bacteria.	They contain genetic material of bacteria or viruses.
3.	When injected, immune system recognizes pathogen to make antibodies for next encounter in future	When injected, genetic material provides instructions to host cell to produce antigen, which is presented by cell of immune system, to prepare for next encounter in future
4.	Weakened or inactivated pathogen is used in vaccine preparation.	Pathogens own genes (DNA or RNA) are used in vaccine preparation
5.	These take longer time to develop as they rely on actual viruses or their proteins.	These develop rapidly in the lab as these rely on genetic code rather than on live virus or bacteria
6.	These require use of mammalian cells or tissue culture or embryonated eggs and expensive to produce	They are cheaper to produce as do not require lots of pathogens, mammalian cells or embryonated eggs
7.	Proteins are more demanding as molecules so slower to produce immune response	Nucleic acid is simpler structure
8.	They do not incorporate themselves into host genomic program	DNA vaccines incorporate themselves into host genomic program
9.	Less stable in environment & needs refrigeration	DNA vaccines are more stable in environment & can be stored in room temperature without losing activity.

Table 2: Differences between RNA vaccine and DNA vaccine.

	RNA Vaccine	DNA Vaccine
1.	These contain RNA as mRNA	These consist of DNA as plasmids
2.	mRNA will enter in cell & cell translates it into pathogens proteins. These are recognized by host immune system & a response is generated.	DNA is transcribed to mRNA, which translates it into pathogen's proteins; detected by host immune system.
3.	mRNA vaccine has one less step in development than DNA vaccine.	DNA vaccine has one more step in its development than that of RNA vaccine
4.	RNA vaccine does not integrates into host genetic material	DNA vaccine integrates into host genetic material so may cause permanent change in host DNA sequence
5.	RNA molecules expose to RNase in tissue that can degrade RNA and deteriorate vaccine.	Greater amount of DNA vaccine is required to produce effective immune response

DNA-mediated immunization. In this way, both of the immune responses get activated and responded to DNA vaccine and make it more attractive and useful for antigen delivery for vaccination purpose (Krishnan 2000).

History of DNA Vaccines

It all started with the strategy devised by Paoletti et al. (1984) to produce recombinant DNA vaccines by using genetic engineering to transform ordinary smallpox vaccine into vaccines that may be able to prevent other diseases. They altered the DNA of cowpox virus by inserting a gene from other viruses (namely Herpes simplex virus, hepatitis B and influenza) (Paoletti et al. 1984). However, direct introduction of genes into tissues in vivo, without the use of viral vectors, would be useful. Later the idea of DNA inserted in expression plasmid, as a vaccine was first described in 1990 when Wolff et al. (1990), found out that a gene inserted in plasmid could be expressed in vivo. It has also demonstrated that the expression of the genes in the muscles tissues at injection site was lasted for almost 60 days (Wolff et al. 1990). Ulmer et al. (1993) described that the direct injection of plasmid DNA that encoded influenza virus antigen to mice protected the animals upon challenge experiments. This discovery opened new doors of research in the field of vaccinology. Many other scientists used this technique in several studies (Prigozy 1993; Wilson and Andrews 2012). DNA vaccine against Zika virus was developed and tested in 2016 (Regalado 2016), DNA vaccine against HIV is also under clinical trials (Chen et al. 2014).

DNA Vaccine for Animals

For veterinary use, five DNA vaccines are available in the market including hematopoietic necrosis virus in salmon, H5N1 DNA vaccine for birds, West Nile virus, melanoma in dogs, growth hormone-releasing hormone (GHRH) gene therapy for swine (Zhang 2015; Myhr 2017).

DNA Vaccine for Humans

At present DNA vaccines played useful role in veterinary medicine but have no widespread achievement in human vaccination. The reason is less immunogenicity however recent studies increased the value of DNA vaccines in priming high-level antigen specific antibody responses. For improvement of DNA vaccine in humans, several approaches have been used e.g. novel non-mechanical delivery methods, improvement in vector design for DNA vaccine, and insertion of genetically engineered cytokine as adjuvants. These techniques increase the immune response in both models of study that are mice and animal models (Suschak et al. 2017). ZyCoV-D first human DNA plasmid-based COVID-19 vaccine, developed by Indian pharmaceutical company was approved for restricted use in emergency only in 2021. The vaccine contains a DNA plasmid vector that carries the gene encoding the spike protein of SARS-CoV-2. As with other DNA vaccines, the recipient's cells then produce the spike protein, eliciting a protective immune response (Momin et al. 2021; Rawat 2021).

The DNA vaccine technology was found highly promising as both cellular and humoral immune responses could be activated against parasites, bacteria and disease-producing viruses in animals as well as humans (Kurath 2005; Coban et al. 2011; Ahmad et al. 2012).

Advantages and Disadvantages of DNA Vaccines

DNA vaccines have many advantages as compared to conventional vaccines due to many reasons including; genetic

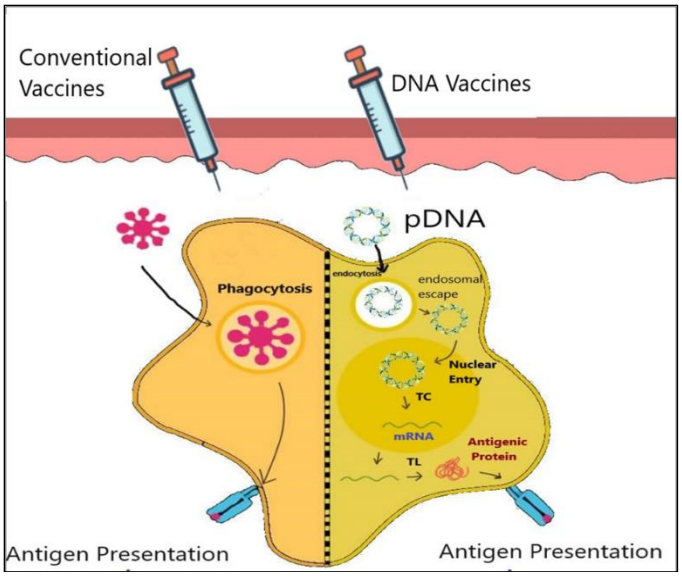

Fig. 1: Schematic diagram showing mechanism of action of conventional and DNA vaccines.

nature, more stable and safer, easy production and mostly no need of cold chain. As discussed above, the major advantage of DNA vaccines is the ability of the vaccine to induce humoral as well as cellular immune response (Fig. 1).

Route of administration or delivery system is very important for the better efficacy of the DNA vaccines but a major disadvantage is also faced due to it. Usually, these vaccines are preferred to be administered through intramuscular (IM) injection. It is difficult for the vaccines to move through cell membranes, and a very small amount of vaccine usually reaches antigen-presenting cells (APCs) to induce immune responses after intramuscular injection. Some clinical trials showed that magnitude of immune response produced by DNA vaccine is much weak and greater amount of DNA vaccine was required for effective immune response (Zhao et al. 2014). Antibody titer was also found lower than that with traditional vaccines. Also, direct injection of vaccine made the process labor intensive.

Mechanism of Action and Components of DNA Vaccines

DNA vaccine mimics the natural infection after entering into host cell, where it uses the host cell protein synthesis mechanism and directs the synthesis of its encoded proteins. Later, this protein is delivered to immune system and produces a non-inflammatory and noninfectious response where antigen is non-proliferating. The APCs with these antigens then travel to the lymphnodes and present the antigen peptide and co-stimulatory signalling molecules to helper T-cells to initiate the immune response. Cytotoxic T and B cells with the help of MHC class I and MHC class II molecules activate cell mediated and humoral immune response respectively (Huebener et al. 2008).

The region of DNA gene being used to insert in plasmid should be immunogenic which can be selected using automatic algorithms to optimize codons. For an effective and long-lasting DNA vaccine, a strong promoter like SV40 in high expression plasmid for better expression of the antigenic gene should be used. Addition of poly A tail to stabilize mRNA can be useful (Soltani et al. 2018).

Recent Strategies in Development of DNA Vaccines

In recent years, several strategies have been proposed to improve the efficacy of DNA vaccines through optimizing the plasmid, improving delivery methods and routes of immunization, target cells and antigens for effective antigen presentation and utilizing powerful adjuvant to enhance immunogenicity.

Routes of Administration and DNA Vaccine Delivery Methods

Similar to other conventional vaccines, DNA vaccines can be administered by a variety of routes. Physical delivery methods for DNA vaccine include intramuscular (IM) needle injection, intradermal (ID) electroporation, mucosal, intranasal and transdermal whereas chemical delivery methods include nanocarriers (lipid based nanocarriers, inorganic nanopaticles, polymeric nanoparticles etc.) and liposomes.

Among physical delivery system, needle injection is the most widely used delivery system. Needle injections help in inducing Th1 immune response, however, for longer and stronger immune response, booster doses are required. Gene gun delivery is another effective method of DNA vaccine delivery into host cells. It requires less amount of DNA (1-3ug). Plasmid DNA is absorbed on gold microparticles and compressed helium gives momentum to the particles to allow penetration into the tissues first and then into the cell. It is often used to induce Th2 responses (Donate et al. 2011).

Just like needle injection, the electroporation (EP) method induces Th1 immune response but this response was found to be much stronger as compared to other vaccination methods (Geiben Lynn et al. 2011). A non-invasive modification of EP known as multi-electrode array (MEA) has been found more effective after intradermal DNA vaccination in guinea pigs against hepatitis B infection (Donate et al. 2011; Saadh et al. 2019). Intradermal and intra muscular EP techniques are usually used for the DNA vaccine delivery. Many cell types have been used for this purpose but muscle cells were found highly effective and expressed the plasmid coded protein for many months. Intradermal DNA EP has also shown to produce high immunogenicity due to presence of APCs (dendritic and langerhans cells) (March 2006).

Intranasal is another method of vaccine delivery. Plasmid DNA is breathed through the nose and absorbed to respiratory tract surfaces. After intranasal inoculation of Plasmid with HIV envelop gene, envelop gene transcripts and proteins have been found in lungs, liver and spleen (Vanniasinkam et al. 2006). Non-needle Co2 biojector is also used as delivery method. It induces Th2 and CD8+ T cell immune response. Topical application of Plasmid DNA vaccine has also been found simple, useful, painless and cost-effective method in case of HIV, HSV and matrix gene of influenza in mouse model but level of immunization achieved was very low (Firouzamandi et al. 2016; Liu 2003).

Safety of DNA Vaccines

DNA vaccines are safe and stable as compared to conventional vaccines. Plasmids are non-living, unable to replicate so has no or little risk of reversion. There are less chances of re-infection or secondary infection. Studies have shown that anti-DNA immune response, genomic integration

and auto-immunity have not been reported in case of DNA vaccination. Overall DNA vaccines are well tolerated and have desirable safety records (Plotkin 2011).

DNA Vaccines and Adjuvants

The term adjuvant is derived from the Latin word "adjuvare" which means "help" or "develop." Adjuvants are usually low in toxicity and can be absorbed slowly from the site of injection into the host body. This helps in the slow release of antigen which gives a long-term immune response against the antigens. Adjuvants are immunostimulants and increase the immunogenicity of vaccines not only due to slow release, inflammatory response but also due to the direct induction of some important cytokines. Two types of adjuvants are being used and tested for improvement of DNA vaccines; chemical and genetic adjuvants (protein encoded by DNA plasmid).

Genetic Adjuvants

Genetic adjuvant found more effective than chemical adjuvant. Cytokines can be used as genetic adjuvants in the DNA vaccines as a part of the same plasmid DNA or a separate plasmid. The effects of plasmid encoding cytokines such as interleukin (IL)-10, IL-12 or IFN-γ together with DNA vaccines have enhanced the immunogenic effect of DNA vaccine in recent studies in animals (Peeridogaheh et al. 2019). A higher level of IgG antibody was also detected in a group of mice which received DNA vaccine and IL-2 expressive plasmid (Flingai et al. 2013). Immune signaling molecules also act as genetic adjuvants and activate the immune response by binding with Toll Like Receptors (TLRs). Two genetic adjuvants MyD88 and TRIF were incorporated into plasmid of DNA vaccine have been found to enhance immune response against influenza and rabies. Cellular immune response of Influenza DNA vaccine was improved by mitochondrial antiviral-signaling protein activated by RIG-1. Some other adjuvants like chemokines, complementary system components and protein aggregating domains are also used for enhancement of immune response (Grunwald and Ulbert 2015).

Chemical Adjuvants

Many adjuvants like saponins, alums, aluminium hydroxide gels etc. have been used to check their effect to increase immunogenicity of DNA vaccine. Adjuvants mixed with antigen form a depot effect (slow release) at administration site. Alum used in many cases but in large animals trials have not been found very effective. A DNA vaccine against envelope gene of HIV-1 was developed in combination with saponin adjuvant. Significantly higher levels of IgG antibodies were found (Sasaki et al. 1998). An adjuvant known as cationic lipid formulation vaxfectin mixed in DNA vaccine, enhanced the immune response against measles and influenza (Gurunathan et al. 2000).

Some carrier molecules like mineral salts, emulsions, liposomes, biodegradable polymer nanoparticles and immune stimulating complexes (ISCOMs) have been found useful for delivery of DNA vaccine. These carrier molecules can entrap the plasmid DNA and convert their soluble nature to particulate nature. Professional APCs can easily capture and process particulate antigens and present the antigen through MHC class I or MHC class II and eventually activate antibody mediated or cell mediated immune system. Most of these carrier molecules can provide a depot effect to facilitate the long-term retention and sustained release of antigens at the site of administration (Marasini 2017; Suschak et al. 2017).

RNA Vaccines

On contrary, scientists have developed a novel type of vaccine in which molecule called messanger RNA (mRNA) is used rather than actual virus or bacteria. Messenger RNA is an RNA type that is required for protein production. It uses information in genes to generate a blueprint for designing proteins. Like DNA vaccines, RNA vaccines show good potential in becoming a novel therapeutic option for many pathological conditions like cancers, allergies and infectious diseases (Blackburn 2018). Cancer vaccines are type of immunotherapy that triggers immune reaction targeting the cancer cells, cancer causing pathogens or gene coding for cancer specific antigens. Many clinical trials for RNA-based vaccines have been done including melanoma, prostate cancer and blood cancers.

History of RNA Vaccine

RNA vaccines share a common history with DNA vaccines (As discussed earlier in the section of "History of DNA vaccines"). In this section, important events related to RNA vaccine will be discussed. In 1990, first report was published about IVT (in-vitro transcribed) mRNA in mice, where mRNAs were injected in body and protein translation was seen. In 1992, it was demonstrated in rats that vasopressin encoding mRNA was administered in hypothalamus and it has produced a physiological response (Pardi 2018). In 2013, the mRNA vaccine for rabies is tested in humans. Up till now, RNA vaccines have been used against many viruses like zika virus, ebola virus, influenza virus and rabies virus and currently against coronavirus.

Mechanism of Immune Response of RNA Vaccine

Conventional vaccines work by introducing inactivated disease-causing organism or its proteins, which inside body mimic the infectious agent. The body recognizes it and stimulates immune response, thus when body is exposed to infectious agent, it is primed to respond in more effective way. Unlike conventional vaccines, RNA vaccines have different approach. They mimic the process that a cell uses to make its own proteins. Generally, body cells use DNA as a template to make messenger RNA that is translated to produce proteins. RNA vaccine responds by introducing mRNA sequence that is specifically coded for a viral protein usually found on outer membrane of virus, into the patients' body. This mRNA molecule tells body's cells to build the antigen protein, which is then presented on APCs, where it confronted by other cells of immune system (Fig. 2). Immune system then prepares body to fight if the real antigen enters (Blackburn 2018).

COVID-19 mRNA Vaccine

During COVID-19 outbreak, in 2020 and 2021, Food and Drug Administration USA has authorized two mRNA vaccines; 1. the Pfizer-BioNTech (BNT162b2) vaccine, and the

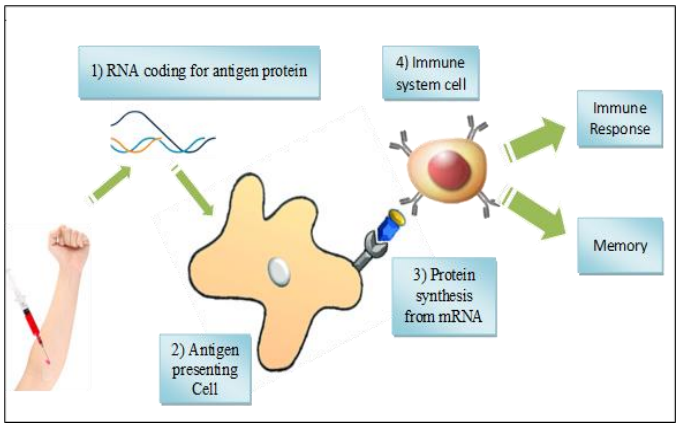

Fig. 2: Schematic diagram showing mechanism of producing immune response of RNA vaccines

Moderna (mRNA-1273 vaccine). These two were given first historic authorization for use in emergency, while another mRNA based vaccine; CVnCoV is still under clinical phase 3 testing (Kadali et al. 2022). Both vaccines, BNT162b2 and mRNA-1273, consist of genetically engineered mRNA that gives instructions to the cells to make viral spike glycoprotein of SARS-CoV-2. After vaccination, patients muscle cells begin making these spike proteins and displaying on cell surfaces. So, if one gets infected by covid-19, body recognizes the spike protein present of SARS-CoV-2 and immediately starts making antibodies against it (Verbeke et al. 2021).

Types of RNA Vaccines

Non-replicating/amplifying mRNA Vaccine

It is a simplest form of an RNA-based vaccine where messenger RNA is injected in the host body, then taken up by body cells to build the antigen proteins. It usually has one gene or one open reading frame which encodes antigen protein (Wang et al. 2021).

In-vivo Self-replicating/amplifying Messenger RNA Vaccine

Additional RNA strands are packaged along with pathogen mRNA that will make sure that pathogen mRNA is copied when vaccine gets inside the cell. Thus, greater amount of antigen proteins are built from a little quantity of vaccine, ensuring a robust immune reaction. Self-amplifying RNA vaccine will be discussed in detail in the section 4.6.1.

In-vitro Dendritic Cell non-replicating/amplifying

Dendritic cells are APCs that present antigen, recognized by other immune cells, stimulating an immune reaction. Dendritic cells are removed from patient's blood and then transfected with the vaccine and later introduced back into body to generate an immune response.

Challenges and Approaches in Development of RNA Based Vaccine

Clinical trials of RNA-based vaccines against different viral infections are conducted in various studies (Scorza and Pardi 2019; Zhou et al. 2019; Maurya et al. 2020). Researchers are facing some challenges. Major challenges encountered were related with the vaccine delivery and its stability due to RNA degradation, cold chain maintenance and its mode of delivery into the cells. RNA vaccines direct their translation in cytoplasm so they can eliminate the chromosomal integration. After injection of vaccine, RNA is exposed to RNase in the tissue that deteriorates the vaccine which can restrict uptake of functional mRNA by cells. It compromises the treatment efficacy (Renuka et al. 2022).

The challenges related to delivery and stability have been tried to address by various techniques including encapsulation of RNA molecules by nanoparticles, lipids and polymers, targeting APCs, modification of RNA structure by altering RNA base sequence to avoid degradation and using self-amplifying RNA (just like ssRNA viruses have self-replicating RNA) (Lundstrom 2018). These RNA improvements are discussed in detail as follows:

Cap Analog

Cap 5'7 methylguanosine triphosphate is important for RNA stability. When cap analogs are used in-vitro for RNA transcription, a reverse orientation cap analog incorporation can disable efficiently transcribe mRNAs. But with designing of anti-reverse cap analogs (ARCAs) having only 3'-OH group rather than two of 3'OH groups in cap analog have prevented reverse orientation incorporation (Wadhwa et al. 2020). Compared with conventional analogs, ARCAs application provides more than double efficiency in transcription. It has been also reported that in-vitro use of ARCAs improves the duration and levels of protein expression, which can eventually help in stabilizing mRNA for optimum vaccine delivery and expression (McNamara et al. 2015).

Poly (A) Tail

For stability of RNA molecules, poly(A) tail is engineered at 3'end of mRNA. It is seen that poly(A) tail acts in harmony with the 5'm7G cap sequences by joining with PABP (poly(A)-poly(A) binding complex). PABP interacts with eukaryotic translation initiation factor (TIF) and makes a TIF complex with 5'm7G cap & eIF4E. These poly(A) tails can be engineered on mRNA either by encoding poly(A) tail on DNA template or by using recombinant poly(A) polymerase to lengthen RNA after transcription in-vitro (Hoang et al. 2018). Drawback of this technique is that by application of recombinant poly(A) polymerase, various lengths poly(A) tails are generated ranging from 33-35 to 120-150 however short or long lengths both gave RNA stability and high translational efficiency (Lima et al. 2017; Kon et al. 2022). With tail engineering, increased length of poly(A) tail enhances efficiency of polysome formation which has impact on level of expression of protein (Trepotec et al. 2019).

5′ & 3′ Untranslated Regions (UTRs)

UTRs have a very significant role in regulation of post-transcriptional gene expression. It includes mRNA transport modulation from the nucleus and RNA translational efficiency, sub-cellular localization and mRNA stability. Selection of UTRs is also needed in mRNA vaccine preparation because important regulatory elements are present in 5′ UTRs and 3′ UTRs. However, incorporation of β-globin at 3' end provides

enhanced translational efficiency and enhanced mRNA stability (Gergen and Petsch 2021).

Chemically Modified Nucleosides

Therapeutic properties of RNA can be improved by addition of natural nucleosides during post translational processing of RNA. It has proven beneficial as it made the RNA less immunogenic in-vitro to stop the stimulation of RNA vaccine. It has been reported that mRNA containing modified pseudouridine has shown improved RNA stability and translation. Normally, RNA has ability to stimulate immune system by stimulation of TLRs (toll like receptors) but incorporation of these nucleosides has reduced this activation, resulting in lesser cytokine levels (Kariko et al. 2005). So, this approach halts identification of RNA by receptors i.e. TLR8, TLR7, TLR3 and decreases immune reactions against in-vitro transcribed RNA (Kariko et al. 2008).

Route of Administration and RNA Delivery System

RNA vaccine can be administered through various routes just like DNA vaccines including needle injections into muscles, blood, lymph node (LN) or directly into organs; by nasal spray. The exact optimal route for vaccine administration is not fully known yet (Blackburn 2018). The delivery of RNA vaccine has more difficulties as compared to that of DNA vaccines section (discussed earlier). An efficient delivery system of RNA vaccine is much needed for RNA stabilization and vaccine development. Many approaches have been tested to improve mRNA delivery like injection strategies, gene gun-based injection, RNA adjuvants, encapsulation of RNA in nanoparticles etc. Some of these have been already discussed in section "DNA Vaccine" and very similar for RNA vaccines. The different ones will be discussed below:
The administration of naked RNA was achieved by needle injection (intra-muscularly), initially done in-vivo in mice by inoculating a reporter gene and getting its expression later (Wolff et al. 1990). But this approach has limitations due to rapid RNA degradation seen in other animal models where antibodies and T cell responses have been elicited (Steitz et al. 2006). To overcome this limitation, gene gun-based delivery was evaluated (Section 3.1.4.1). The gene gun is utilized to bombard the cell wall with many DNA coated metal (like gold) particles by using compressed helium as the propellant. Another approach is protamine condensation of RNA that saves RNA from degradation and reduces stimulation of immune reaction via TLR8 and TLR7 dependent pathways. Protamine condensation stimulated the production of antigen specific Ig-G antibodies and stimulation of cytotoxic T-lymphocyte reaction (Lundstrom 2018). To improve delivery and RNA stability encapsulation techniques have been used. Cationic liposomes have been used in vaccines for RNA entrapment or encapsulation. Nano-particles have also been shown to improve RNA stability, reduces RNA degradation and increases cellular uptake. Targeting dendritic cells using nanoparticles has also been found helpful in stimulation of immune response against vaccine.

Self-Amplifying RNA

Self-amplifying RNA genome is unique characteristic of some families of viruses. These viruses make copies of RNA genome and encode proteins from a single RNA template. Owing to this capacity of RNA for self-amplification, ssRNA viruses have been used for vaccine development. These self-amplifying RNA viruses have a single strand of (+) sense RNA template encapsulated by protein/lipid envelope. Viral RNA encodes RNA replicase. This RNA replicase is autoproteolytic polyprotein that cleaves viral RNA into 4 non-structural components. Upon infection, initially viral RNA translates the replicase complex and which later on causes its own RNA replication. Later (-) strand/ anti sense RNA is synthesized by replicase complex, which acts as template for synthesis of (+) strand RNA and sub-genomic RNA encoding structural viral proteins. As a result, RNA copies are formed, (up-to 200,000 within single cell in 4 hours) and expression of encoded antigen can be detected that is usually 25% of the total cell protein. This replication occurs in cytoplasm of host cell. It is independent of replication system of host. To create powerful replicase based vaccine, gene for antigen of interest can be placed in place of genes for structural proteins of virus (Leitner et al. 1999).

Advantages

Compared with traditional vaccines, RNA vaccines are cheaper and faster to produce. RNA vaccines production is lab based. They are produced from DNA template with readily available materials, thus faster and cheaper than traditional vaccines that require use of mammalian cells or chicken eggs.

Safety

It is safer to use an RNA based vaccine since it is non-infectious. RNA strand neither enters nucleus nor incorporates itself into patients' genome and is deteriorated once protein is produced.

Efficacy

Results of clinical trials have shown that RNA vaccines produce a reliable immune reaction and have fewer side effects, so well tolerated in individuals. The main aim of the RNA vaccines is to provide instant translation in patient's cells, providing a long-term expression of genes and production of antibody for therapeutic purpose.

Production

RNA vaccines are low cost, can be produced more rapidly in laboratory processes that can be easily standardized and scaled, thus improving responsiveness to emerging epidemics and large outbreaks. RNA vaccines are effective against pandemics as they provide more flexibility to treat or prevent rapidly evolving pathogens. According to WHO, it takes approximately 5-6 months to develop an influenza vaccine.

Conclusion

DNA and RNA based vaccines opened new avenues in drug discovery. These have wide application range in prophylactic interventions for number of infectious diseases where use of conventional vaccines has limitations. However, a good and clear understanding of mechanism of immune response

produced by nucleic acid vaccines is necessary for improvement of efficacy of these vaccines. Recent studies suggested that new techniques will enhance the efficacy of vaccines and these are safe to use. Cycokines, chemokines, adhesion molecules, delivery methods, electroporation, adjuvants and many other strategies are used for improvement of these vaccines. These approaches will redefine the field of vaccinology. Still there is lot of research to be done on DNA and RNA stability and delivery methods before these vaccines can truly become standard treatments. Self-amplifying mRNA viruses give this possibility a significant alternative in future medicine development.

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CHAPTER 18

VETERINARY VACCINES: WHERE ARE THEY NOW?

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INTRODUCTION

Livestock vaccination has been used for over a century and is widely regarded as an effective strategy for preventing a wide range of bacterial, parasitic and viral infections. The smallpox vaccine is an excellent example of the prevention and control of diseases through vaccination in both animal and human health. Controlling zoonotic diseases in farm animals, domestic pets, and occasionally wild animals has a significant impact on the spread of zoonotic infections in humans. The relationship between human and animal health in terms of bacterial, viral, parasitic, and fungal diseases continues to have a significant impact. Even a cursory look at the history of virology, bacteriology, parasitology, and mycology demonstrates that the analysis of illness etiological agents, as well as prevention methods and therapies, is highly dependent on the encounter of humans, animals, and microorganisms (Sander et al. 2020). Human vaccinology, with its own principal focus on the individual, appears to be diametrically opposed to veterinary medicine, with its problem for livestock health. Historical, with a few exceptions, immunization appears to have been primarily a public health method, aimed at population densities rather than individual people. A vaccine used to be administered by a veterinarian to household pets, feral animals, as well as farm animals' herds. However, the utility of veterinary vaccines transcends outside these boundaries because most of them furthermore keep people safe from zoonotic diseases that affect both people and animals. There are many differences between veterinary immunization and human immunizations in aspects of moral issues related to research and testing, as well as the importance of financial concerns and even priorities in animal care. There is still a significant shift in the use of viable methods for vaccinating or treating sick pets, such as bulk slaughter, a tactic commonly used in veterinary health care systems that costs and this is due to the unexpected impression created (Kaiser and Alan 2019).

The historical background of veterinary immunization is very similar to that of the human vaccine, and because of the animals, it can serve as a model for the veterinary vaccine as it hosts many bacterial and viral human diseases including sheep pox, smallpox and bovine contagious pleuropneumonia. The smallpox vaccine is the oldest known example of a key link between human and animal vaccines (Esparza et al. 2020). There's no doubt that this strategy drew attention to the possibility of establishing immunity to a severe illness by creating a milder variant of the disease. The substitution of treatment using smallpox fluids, termed variolation, to injection with cowpox, a process established from a British physician, Edward Jenner, could be summed as the historical development of the vaccine against smallpox called vaccinia vaccine (Kaynarcalidan et al. 2021).

Cowpox papules, pustules, and scab have also been applied with variolation and may contribute to human protection against smallpox. This approach was usually employed at the beginning of the nineteenth century to reduce the catastrophic impact of smallpox outbreaks on people. Later, cowpox lymphatic fluid was grown in human arms, notably in children. This approach had a number of drawbacks, including moral dilemmas and lymphatic fluid that had lost its immunogenicity and effectiveness. As a result, calves, the original reservoir, have been used to produce cowpox vaccine while also ensuring an ample and immediately available source of lymphatic fluid (Esparza et al. 2018). The era of modern vaccines began with the scientist Louis Pasteur (1822-1895), who pioneered rabies, anthrax, and chicken cholera vaccines. The identification of bacteria using a microscope, confirmation of their virulence, and, most crucially, laboratory culture opened the door to the long-term establishment of novel preventative strategies while also giving animal studies of human healthcare trials (Iwasaki and Saad 2020). The aphtisation technique is the earliest known method of protecting a herd against FMD. We used FMD-infected saliva and rubbed it against the lips or muzzles of healthy animals in the herd, resulting in long-lasting robust immunity (Berdah 2021).

Friedrich Löffler developed filterable FMD serum as a therapeutic strategy to rescue the herds in 1897. Rinder pest,

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foot and mouth disease, and bovine infectious pleuropneumonia are the key diseases that have influenced the development of veterinary vaccinology. They all devoured human livestock for decades. The technology for making industrial vaccines is directly linked to the history of FMD vaccines (Guzman and Maria 2018). In 1886, Salmon and Smith vaccinated pigeons against the Hog Salmonella heat treatment culture, which led to the idea of a killed vaccine. This method of vaccination was discovered to be quite efficient against dangerous bacteria, and by the end of the nineteenth century, killed vaccines for cholera, plague and typhoid in humans, as well as many bacterial infections in animals, had been developed (Plotkin 2014). In 1888, Roux and Yersin's experiments with diphtheria toxins on animals prompted the invention of toxoid vaccinations. Initially, a toxin and antitoxin balancing formulation was used. However, the use of formalin for toxoid inactivation by Glenny and Hopkins in 1923 may have signaled a revolution in toxoid vaccines. The toxoid vaccine was first used by Roman in 1924 against Marek's disease in poultry (Cavaillon 2018). Subcutaneous rout was initially employed for toxoid Marek's vaccine injection. *In ovo* rout is now employed in embryonated eggs on day 18. The operational costs involved in managing individual chickens after hatching save a large number of eggs from being vaccinated at the same time. *In ovo* injection has no adverse effect on egg production or long-term production of chickens. During the early years of the modern age of immunization formulations, infectious materials such as scab, pustules and others were extensively used as a microbiological source, with adopting various procedures such as grinding, centrifugation, filtering, and inactivation with formaldehyde solutions. Most of these vaccinations were developed in national research centers (Peebles 2018).

Modernization of the vaccine production techniques started in the late 1940s, when Waldmann and colleagues pioneered massive scale, controlled approach to produce foot and mouth disease antigens in Germany. High-volume roller bottle technologies, improvement of inactivation methods, identification of adjuvants, cell line systems and bioreactors for large scale microbial production were established between 1950 and 1960. Since these technological advancements were implemented in the industrial world, various state agencies worked independently and collaboratively to develop legislative framework which formed rules and guidelines for the induction of new vaccines as well as the consistent production of pure, sterile, secure, and effective immunization. The establishment of good manufacturing practice (GPM), as well as parent inoculum, master seed and cell line approaches, has also guaranteed ensure the production of safe, sterile and secure vaccines. As a result, a veterinary doctor can use any authorized commercially prepared vaccines to attain the intended treatment outcomes of the prevention of specific disease (McVey and Jishu 2010).

In this chapter, we discussed significance with historic point of view about vaccines, types of vaccines, public health link of veterinary vaccines, the role of adjuvants used in vaccine design, mode of action of vaccines and challenges to the efficacious use of veterinary vaccines.

Public Health Link of Veterinary Vaccines

Veterinary vaccines have three main goals: to increase the safety and well-being of domestic animals, to promote low-cost livestock farming, and to reduce the transmission of zoonotic disease from both domestic and wild animals (Monath 2013). The expanding global demand for meat, eggs, and dairy commodities is being driven by an increasing human population and high living standards in developing countries. Larger and denser populations of farm animals have resulted from advances in livestock husbandry over the last few decades. Infectious diseases that wreak havoc on cow herd performance and health can be financially devastating and destabilize food supplies. Viral and bacterial microorganisms are transmitted zoonotically from animals through meat, milk skin hides etc., to peoples, posing a significant risk to human health. To prevent infectious and contagious diseases in livestock, a variety of measures are implemented, such as station hygiene, separation or slaughtering of infected animals, screening of illnesses gene pools, chemoprophylaxis, and immunization (Rahman et al. 2020).

Vaccines comprise biological agents which are required to generate adaptive immunity particular to microbial pathogens in the hopes of preventing or reducing deadly infections. Veterinary vaccination has numerous advantages, including being the most cost-effective way to combat infectious diseases, ensuring the availability of nutritious food like meat, milk, eggs and dairy & meat products from domestic animals, protecting companion and pet animals, and preventing the spread of foodborne and zoonotic diseases to humans (Francis 2020). The intimate bond between humans and their companions would have been less secure without immunization. Animal vaccines that are both safe and dependable have now become a requirement in today's life (Pollard and Bijker 2021).

The aim of providing enough animal protein to feed the world's billion people would have been substantially higher and which is not possible without vaccination in food producing mammals. Vaccines improve the efficiency of animal farming by allowing a dynamic business to avoid output losses due to infectious illnesses in food-producing livestock. There are over a hundred different veterinary vaccinations available in the market. Without access to immunizations, farmers, territories and governments would be at greater risk of contracting the deadly diseases of livestock. Rinderpest, a severe highly infectious disease caused by the rinderpest virus, a member of the Morbillivirus genus and the Paramyxoviridae family, is a perfect illustration of the human-animal health relationship. This disease primarily affects cattle and buffalo (Hoelzer et al. 2018). The Rinderpest virus spread from Asia to Africa via cattle transportation. It caused massive mortality in African cattle and sheep, massacring more than 90% of them. The depletion of working animals, household cattle, and wildlife resulted in mass hunger, eradicating one-third of Ethiopia's population and two-thirds of Tanzania's citizens. Thickets formed in pastures as the number of grazers decreased (Roeder et al. 2013). These trees and bushes serve as perfect breeding sites for the growth of tsetse flies resulting in an epidemic of sleeping sickness infections or trypanosomiasis in humans. Several scientists thought the rinderpest outbreak was one of the most devastating natural disasters to ever hit African countries. The Worldwide Rinderpest Elimination Project was a significant multinational teamwork that included immunizations, economic sanctions at the international and regional levels, and monitoring. The rinderpest disease was confirmed to be completely eradicated from the world's domestic and wild animals in 2011, highlighting one of veterinary medicine's most significant successes.

Table: List of Commercially available zoonotic disease vaccines worldwide

Sr. No	Name of Disease	Trade Name	Types of Vaccine	Host	References
1.	Rabies	Lyssavac® / Rabipur®	Inactivated	Humans	Giesen et al. 2015
		Rabisin®	Inactivated	Dogs and Cats	Mantik and Putra 2012
		Imrab®	Inactivated	Cattle, Horses and Sheep	Brown et al. 2016
2.	Avian influenza	Fluzone®	Inactivated	Humans	Robertson and Lauren 2016
		Cevac Flu-Kem®	Inactivated	Broilers, Layers	Kapczynski et al. 2015
3.	Hantavirus	Hantavax®	Inactivated	Humans	Song et al. 2016
4.	Yellow Fever	Yf-Vax®	Inactivated	Humans	Gershman and Mark 2017
5.	Rift Valley Fever	Rift Valley Fever Vaccine®	Inactivated	Cattle, Sheep and Goats	Faburay et al. 2017
6.	Monkey pox	Jynneos®	Live	Humans	Overton et al. 2020
7.	Dengue Fever	Dengvaxia®	Chimeric vaccine	Humans	Salje et al. 2021
8.	COVID-19	Sinovac®	Inactivated	Humans	Chuaychoosakoon et al. 2021.
9.	MERS-CoV	ChAdOx1-MERS	DNA based	Humans, Camels	Alharbi et al. 2019
10.	Hepatitis E	HEV 239®	Recombinant	Humans	Li et al. 2015
11.	ORF Disease	Scabivax® Forte	Live	Sheep, Goats	Silk and Lovatt 2016
12.	Brucellosis	RB-51 CZV	Inactivated	Cattle, Buffalo	Caetano et al. 2016
		Ocurev®	Inactivated	Sheep, Goats	Ponsart et al. 2019
13.	Anthrax	Anthrax Vaccine Adsorbed (AVA)/ BioThrax®	Live avirulent strain	Humans	Sandra and Olga 2011
		Anthravax®/Sterne live spore vaccine	Live	Cattle, Sheep and Goats	Jauro et al. 2020
14.	Leprosy/Tuberculosis	Bacillus Calmette–Guérin®	Live	Humans, Bovine	Cernuschi et al. 2018
15.	Leptospirosis	Spirolept®	Inactivated	Humans	Verma et al. 2013
		Lepto 3-Way®	Inactivated	Cattle	Cave et al. 2014
		VANGUARD L4®	Inactivated	Dogs	Midence et al. 2012
16.	Lyme disease	LYMErix®	Subunit	Humans	Stricker and Johnson 2014
		Nobivac®	Subunit	Dogs	Scott-Garrard et al. 2018
17.	Q Fever	Q-VAX®	Inactivated	Humans	Bond et al. 2017
18.	Leishmaniosis	LetiFend®	recombinant	Dogs	Cotrina et al. 2018

People are continuing to improve and increase the use of animal immunizations, which may have a number of benefits such as improve animal health, effective food output, farmer financial damage, and zoonotic risks. Animal vaccinations must be extensively used in order to have a significant influence on livestock and global health, which implies that vaccines must be inexpensive (Donadeu et al. 2019).

Immunizations to govern zoonotic maladies in farm animals, domestic pets and sometimes wild animals have a significant effect in decreasing the spread of zoonotic infections in humans. Rift Valley Fever, Brucellosis, Salmonellosis, Niphah, Influenza, Rabies, COVID-19, MERS-CoV, Leptospirosis, Q-fever, Hendra, Trypanosomiasis, and Japanese encephalitis are some examples of zoonosis that have been managed using veterinary immunization only (Hasanov et al. 2018; Abd El-Ghany, 2020; Mohapatra et al. 2020; Petrovan et al. 2021).

People are hesitant to keep domestic animals such as dogs and cats if rabies vaccines are not available. Vector-based recombinant rabies vaccines were shown to be helpful in reducing rabies occurrence in feral animals when administered orally. The brucellosis vaccination campaign had been responsible for the eradication of *Brucella abortus* in the United States. Many governments are struggling to control brucellosis in cows, sheep, goats, and humans because of a shortage of the brucella vaccine for cattle. Brucellosis live immunization has occasionally shown signs and symptoms. The latest, safest vaccine is needed to control brucellosis (Khan and Muhammad, 2018). Novel and rare animal diseases are becoming a significant hazard to humans and animals, putting food production in danger. Human and livestock population increases, as well as ecological degradation, global climate change, the spread of insect vectors, and global trade agreements, have all increased the potential for pathogenic transmission of microbes between and across the species. The subsequent maladies are a major issue, either now or in the near future. Rising consumption of animal nutrients resulted in increased economic food producing animals and expanded village farming in many parts of the world (Sekaran et al. 2021).

Disease emergence and management present unique challenges in both commercial and village production systems. Large-scale production systems with a high animal density produce more food for human consumption at a lower cost. These production systems integrate biosecurity, vaccination therapies, and vet services. High densities of domestic and wild animals, on the other hand, can hasten the onset of the disease due to the widespread multiplication of large numbers of animals, resulting in adaptation to the genetic type of the microorganisms (Perry et al. 2013). In village animal farming, sheep, goat, as well as chicken rearing can effectively utilize domestic waste for diet and could be a significant source of nutrients to protein intake and revenue (Reddy 2016). Moreover, close contact to peoples, especially adolescents and children, carries the potential of spread of zoonotic pathogens, such as bird flu, Salmonella species, *Brucella abortus* and *Brucella melitensis.* Biosecurity, biosafety and satisfactory immunization are relatively uncommon in rural animal farming. The use of farmed animals' meat, eggs, milk, and other byproducts have led in the establishment of novel and emerging zoonotic diseases that pose a substantial threat to people worldwide. Superbug outbreaks will undoubtedly continue to haunt the earth in the coming decades. Animal vaccines' continuous improvement has the potential to play a major role in the control of harmful emerging diseases (Renault et al. 2021).

Types of Veterinary Vaccines

Vaccination has been proved to be the most effective technique of controlling bacterial, viral, and protozoan infections in both animals and humans, both in terms of preventing deaths and

reducing severity. Veterinary immunization improves the well-being and prosperity of domestic and pet animal owners while also improving animal health and performance. Vaccination against viral and bacterial zoonotic infections e.g., rabies and brucellosis has boosted animals and human wellbeing. Vaccines for animals can also assist in reducing the usage of veterinary medicines in the food animals and the need for medications by preventing diseases (Hoelzer et al. 2018).

Now days, three categories of veterinary vaccines have been existed such as first, second and third generation of veterinary vaccines. First generation vaccines comprise conventional killed/inactivated and live attenuated vaccines and toxoid vaccines. Pathogenic microorganisms such as bacteria, viruses, and protozoa have been employed in vaccination approaches that are either killed or inactive (Jiskoot et al. 2013). It has been grown in nutrient broth, embryonated eggs and cell culture systems, where it is inactivated using chemicals (glutaraldehyde, binary ethylenimine, formalin, β-propiolactone, Hydrogen peroxide) and physical means (heating, UV radiation), which reduce pathogenicity and hence prevent disease from vaccination. There are numerous advantages of using inactivated or killed veterinary vaccines such as more stable, less storage and transportation issues, no reversion to virulent form, do not replicate and are safe to use in people with weakened immune systems. There are several disadvantages to using inactivated immunization, including the inability to provide adequate long-term safety due to the destruction of microorganism propagation, the inability to deal with prevalent serotypes throughout the field, needing boosting doses, the need for new vaccines to be derived from clinical strains with novel epidemics, and enhancers such as adjuvants are frequently designed to accomplish and establish protective immune responses (Mebatsion 2021). Toxoid vaccines are another type of immunization in which toxin is isolated and inactivated before being used as a vaccine candidate. Toxoid can be produced by suppressing the toxicity of toxins with formaldehyde and heating. Toxoid vaccinations stimulate the immune system and provide protection against certain toxins. Tetanus and diphtheria toxoid vaccines are examples of toxoid vaccines (Fortner et al. 2018).

Live immunizations are sometimes known as modified live attenuated vaccines. A live vaccine is formed by reducing the pathogenicity of a pathogen while keeping it alive. Attenuation is the process of altering a pathogen so that it becomes benign or less virulent. Viruses can be attenuated following evolutionary principles by serially passing the virus throughout a distinct host organism, such as cell cultures, fertilized eggs chicken eggs and live animals. The reverse genetic process is also used for virus and bacterial attenuation. Live vaccines have numerous advantages, including generation of an efficient and long-lasting immune system response, accurately mimicking natural infections, and need of only one or two doses. However, there are significant drawbacks, including live pathogens not always being responsible for protection, reversion to infectivity towards a more aggressive phenotypic expression recurrence, the necessity for refrigerated storage, severe difficulties in immunocompromised patients, and being costly. First generation or conventional immunizations have dominated commercialized human and livestock vaccinations over the past century. Nonetheless, the disadvantages highlighted above have pushed second and third generation immunizations into the focus of study (Baron et al. 2018; Chen 2021).

Second generation vaccines strategy contains recombinant subunit vaccination. A subunit immunization consists of pure pathogenic components that have been infectious or necessary to elicit a protective immune response. Unlike a live or weakened and killed vaccine, this vaccinations method contains only the pathogen's antigenic components, such as peptides and polysaccharides. Cloning and heterologous expression vector methods have been used to produce subunit vaccines. The accuracy of the immune system response elicited is enhanced with this method; however, the intensity of the immune reaction is lesser than with weakened or live vaccines. Immuno-adjuvants, targeted methods, or acute boost protocols could thus be investigated to improve immune function. There are four categories of subunit vaccines such as polysaccharide subunit vaccine, conjugated subunit vaccine, peptide subunit vaccine and virus like particles subunit vaccines (Jorge and Dellagostin 2017).

Vi capsular polysaccharide vaccine (ViCPS), which contained capsular Vi antigen and was used to treat typhoid fever caused by *Salmonella enterica*, is an example of a polysaccharide's subunit vaccination (Hitri et al. 2019). Conjugate is used to boost immunological responses. The advantages of subunit vaccines include inability of reversal to pathogenicity, stability, safety and secure for use in immunosuppressed ones but still it needs adjuvants to boost immunogenicity, requires numerous doses, can be challenging to isolate and manufacturing (Bashiri et al. 2020).

The 3rd generation immunization strategy contains DNA or RNA vaccines, inactivated or live chimeric vaccines and viral vector-based vaccines (Crommelin et al. 2021). A DNA vaccination is a type of immunotherapy in which containing an antigen-coding nucleotide sequence of interest is transfected into living cells to trigger an immunological response. Many DNA vaccines in livestock application have been investigated. In some circumstances, pathogen control in animals has been established, while in others it really hasn't. The method is being researched for bacterial, viral and protozoan infections in people, and also malignancies (Imtiaz et al. 2018). ZyCoV-D was granted urgency status by Indian official authorities in mid of 2021. This is the first DNA vaccine licensed for human use against COVID-19, manufactured by Cadila Healthcare. Some benefits of DNA vaccines were identified, such as no risk of complications, stimulate MHC class I and class II molecules and type 1 or type 2 T-cell response, ease of production and development, stable for storage and transporting and provide long-term immunity (Sarkar et al. 2021). Some disadvantages of DNA vaccines include ineffectiveness against polysaccharides and parasite antigens, as well as cross-contamination when producing several kinds of live attenuated vaccines within the same laboratory (Lee et al. 2018).

Recombinant viral vector immunizations are novel veterinary science approaches that utilize viruses as immunization or vaccinology tools. These immunizations are genetically modified, with necessary antigen nucleotide sequences incorporated into a viral vector. Long-term safety is comparable to that of killed subunit vaccinations, and hence elicits both cell-mediated and humoral immune responses. Many viral vectors, such as canarypox, adenovirus, fowl pox, alphaviruses, and others, have been used to develop a variety of veterinary immunizations, namely those against feline leukemia virus, equine influenza virus, West Nile fever virus, rabies virus, and canine distemper virus (Giles and Vanniasinkam 2021).

Although an effective animal conventional vaccination strategy is the protective way to combat viral infection, it would interfere with disease surveillance systems depending upon the serological assays, thus jeopardizing the nation's disease-free status. The foot and mouth disease is the excellent example in cattle. Despite of the fact that inactivated Foot and Mouth Disease conventional vaccinations methods have been used for a decade and are extremely effective in suppressing clinical signs and symptoms of disease, they are not used in FMD-free countries due to the risk of jeopardizing this status and thus foreign trade. Furthermore, traditional vaccines have lowered illness frequency in highly endemic regions (Singh et al. 2019). Vaccination was employed to prevent the spread of the illness in a recent incident in The Netherlands, but the vaccinates had afterwards slaughtered to allow for the country's speedy reinstatement of FMD-free status. To address this crucial concern, the concept of marker vaccines has gained popularity around the world. Marker vaccines are immunizations strategy that enable for the antibody distinction of diseased and immunized people. This distinction is based on the vaccine's lack or deleted of one or even more viral found naturally in the wild-type microbes. Marker vaccines, when combined with appropriate diagnostic assays, allow differentiating infected from vaccinated animals (DIVA) by distinguishing immunogenicity elicited by the immunization i.e., neither any immunoglobulin produced to erased genomic sequences, by those generated following disease with field virus. These diagnostic tests are now commercially available for a variety of disease such as FMD, pseudorabies, Influenza virus, bovine herpesvirus, leishmania and infectious bovine rhinotracheitis (Wong et al. 2020).

The Role of Adjuvants Used in Vaccine Design

An adjuvant is a substance that is added to a vaccine to stimulate and enhance the magnitude and durability of the immune response. The traditional development of new vaccine adjuvants has been described as one of the slowest processes in the history of medicine. For more than seven decades since initial licensure in the 1920s, insoluble aluminum salts (alum) remained the only adjuvant included in licensed products, such as vaccines against hepatitis B, diphtheria, tetanus and pertussis or human papilloma virus (Pulendran et al. 2021). The ideal adjuvant is stable, effective, non-toxic, well-defined, increase antibody affinity, multifunctional, in expensive, stimulate both innate and humoral immune response (Nooraei et al. 2021). We emphasized on commonly available veterinary vaccination adjuvants like emulsions, saponins, mineral salts, TLR agonists, and other immunoactive substances (Burakova et al. 2018). Emulsions have historically been used as adjuvant systems in veterinary immunizations. When two immiscible solutions are combined, one of them that can assemble into minute particles those are disseminated inside the other and maintained by an intermediate surfactant surface. Emulsions are also a great option for livestock vaccinations since they are easy to make, inexpensive, and successful at producing immune reaction. Various kinds of emulsion formulations such as water in oil (W/O) and oil in water (O/W) have been used in veterinary vaccine manufacturing (Zhang et al. 2018). Freund's adjuvants are the well-studied example of water-in-oil (W/O) emulsion. Its further divides into two categories freund's complete adjuvant (FCA) and freund's incomplete adjuvant (FIA) on the bases of heat-killed and dried mycobacteria (Abdelsadig et al. 2021). Adjuvants elicited a humoral immune response. However, some adverse effects have been reported, including pain, discomfort, and local inflammation. The FIA is marketed under the brand name Montanide™ adjuvants. These are commercially used in ND vaccines, Foot and Mouth disease vaccines, and subunit mycoplasma vaccines all around the world (Young 2019). MF59 is a perfect representation of the oil-in-water emulsion adjuvant. MF59 significantly outperforms than calcium phosphate and aluminum hydroxide in enhancing cellular immunogenicity against flu virus (Tahara et al. 2022). Several commercially available O/W emulsion adjuvants, including MetaStim® and Emulsigen®, have been employed in the development of animal vaccines. Water-in-oil emulsions are better adjuvants when used for the production of viral vaccines than mineral salts (Bhat and Sheikh 2021). Aluminum hydroxide and calcium phosphate, two mineral salt adjuvants, have been utilized in livestock vaccination. Adjuvants comprising aluminum salts were first to be introduced in vaccinations manufacturing. Alum is universally acknowledged for stimulating humoral immunity, providing long term stable immunity, easy in preparation, and an extended history of success of overall safety (Shi et al. 2019). Calcium phosphate adjuvant is commercially available in market as an adjuvant. It has been investigated and used in vaccinations against a variety of toxoids and viral pathogens. Numerous investigations have shown that calcium phosphate causes lower tissue inflammation than aluminum adjuvants, which can be linked to lower immunoglobulin IgE synthesis. Mineral salts, on the other hand, are reasonably safe and effective adjuvants with such a long record in human and livestock vaccine development. These exhibit great adjuvanticity in immunization towards bacteria, and their use in immunizations towards obligate intracellular pathogenic bacteria are weak (Abkar and Sattarahmady 2019).

TLR agonists have received much interest in animal vaccination research as adjuvants; however, their implementations are extremely challengeable. Toll-like receptors (TLR) are an important component of the innate immune system. TLRs have two key receptors, RIG-1 and NOD-like receptors, and are capable of detecting a wide range of infections at places wherever microbes' interactions within cells can occur, such as cell membrane, lysosomes, and the cell cytosol. The discovery of such receptor and associated agonists opens the door to the development of vaccine techniques where both boost and target the immune system response throughout the ways that will benefit animals. The best well investigated examples of TLR agonists are Lipopolysaccharides (LPS), flagellin, lipoprotein and monophosphoryl lipid A (MPLA). MPLA has been used as an adjuvant in hepatitis B vaccine. Some disadvantages of TLR agonist such as septic shock, toxicity and hypersensitivity reaction have been reported. Some immunoactive substances, such as saponins, cytokines, chitosan, carbomers, polyoxidonium, and others, have been employed as powerful adjuvants in the production of veterinary vaccines (Kirtland et al. 2020).

How do the Vaccines Act?

The primary objective of immunization is to stimulate the innate, cell mediated and humoral immune responses which provide long term immunity against infectious pathogens. It appears that vaccine-mediated protection can be a difficult task. Vaccines available today are primarily developed with little or

no understanding of how they activate the immune response. Their initial protective efficacy is mainly due to antigen-specific immunoglobulin activation. Furthermore, antibody-mediated protection is primarily concerned with enhancing vaccine-stimulated antibody response. Immunogens' avidity, sensitivity, or combating capacity has been used to determine their quality and efficacy (Speiser and Martin 2020).

Long-term immunity required the presence of immunoglobulins above the protective level on a consistent basis, as well as the monitoring of immunological memory cells capable of prompt and efficient upregulation in response to subsequent microbial attack. The importance of cell-mediated responses should never be underestimated in light of the central role of humoral immune B cells in the efficacy of current vaccines. T lymphocytes have been required for the formation of high affinity immunoglobulin and immunologic memory cells, and they contribute significantly to the defense achieved by current vaccines such as bacilli Calmette-Guérin (BCG). In scientific jargon, immunogen is used instead of antigen for vaccine. The vaccine was given to the animal via intramuscular injection. First, it was recognized by antigen-presenting cells (macrophages, dendritic cells, B cells, and T cells) via receptors such as MHC-Class I and MHC-Class 11. These are found on the surface of cells that present antigens (Pollard and Bijker 2021).

These antigen-presenting cells then migrate to nearby lymph nodes, from which they 'display' the immunogens to certain other antigen presenting cells of the immune system, eliciting a bigger, more clear response. Specific antibodies are produced as a result of this response. Specific antibodies help to minimize infectious illness by removing extracellular pathogenic microbes in various ways, such as high affinity to toxic substance enzymatic binding sites or blocking their absorption, inactivating viral growth e.g., blocking viral attachment and entrance into host cells, boosting process of opsonization and complement cascade stimulation (Zhang et al. 2021).

Viral immunogen binds with MHC-Class I receptors and presented with dendritic cells to cytotoxic T lymphocytes (CD8+) while bacterial and parasitic antigen attach with MHC-Class 11 and presented by the antigen-presenting cells to T helper cells (CD4+). CD8+ cells induce cell mediate immune response while CD4+ stimulates antibody mediated immunity. Cytotoxic T lymphocyte cells (CD8+) cannot completely eliminate infection but can decrease, govern, and clear intracellular pathogenic organisms. These cells eliminate infected cells by releasing perforins, cytokines, and granzymes. T helper cells (CD4+) further produce cell subsets such as Follicular T-helper (Tfh) cells, T-helper I (Th1), T-helper 2 (Th2), Th9 and Th9 effector cells. These cells secrete different kind of immune mediators such as TNF-α, TNF-β, IL-4, IL-5, IL-12, IL-13, IL-17, IL-21, IL-22, IL-26 and interferons (IFN)-γ. These immune mediators help to remove intracellular as well as extracellular pathogens from body (Abualrous et al. 2021).

Challenges to the Efficacious Use of Veterinary Vaccines

Two types of vaccine challenges have been reported during veterinary vaccination: antigenic component and immunological host reactivity (Chambers et al. 2016). The antigenic and immunogenic structural components of any vaccine is the key to success any immunization strategies. The proper titer of vaccine against specific virus or bacteria during manufacturing process should be maintained. The decrease in titer may be the cause of low protection level in animals. Therefore, standard manufacturing procedures should be strictly adopted during vaccine formulation (Gomez and Robinson 2018). Various circulating serotypes of various viruses provide a significant hurdle to the effectiveness of any immunization effort. Serotyping of numerous dangerous viral and bacterial infections, such as infectious bursal disease, Newcastle disease, foot and mouth disease, and salmonella, has also proven a barrier to vaccination success (Belsham 2020). Several serovars have been widespread with one geographical area but many are ubiquitous in some other. Nonetheless, local microbial pathogens in any place continue to be important for vaccine production. Regional serogroups and immunogens extracted locally are known to be the most effective immunogens for vaccine development. If indigenous immunogens are not exploited, epidemics may occur (Rauch et al. 2018).

Viruses for vaccine production have been grown in embryonated eggs and viable cells such as Vero cells and MRC-5. Bacterial strains used in vaccine production were grown in agar medium such as nutrient broth. A correct vaccine formulation process would result in an optimal immunological response to either the antigen or the vaccination, and hence a systemic immune system. Cold chain maintenance during vaccine delivery from the production to remote regions, especially during hot and developing nations, is a significant impediment to the effectiveness of vaccine initiatives (Chambers et al. 2016).

Temperature has a direct impact on the efficacy and fragility of vaccination strains. The effectiveness of vaccines deteriorates over time. As a result, proper chilly temperatures are essential for them to remain stable and alive for prolonged periods of time. The appropriate storage and cold storage conditions for vaccinations appears to be crucial. Vaccine strains must be stored at temperatures less than 4°C. Foods, pharmaceutical drugs, histopathological samples, laboratory samples, and vaccines should never be stored in the same place (Yakum et al. 2015).

When the vaccinal strains in the vial were exposed to direct sunshine, they perished. As a result, the immunogen titer in vaccinations was reduced, but it became inactive. For oral vaccinations, direct sunlight must be avoided during formulation production, and the top of the vaccinated vials must be removed within water. These vaccines must be diluted in drinkable water inside rooms or in a shaded area; additionally, dark or colored boxes and packages are used throughout vaccine shipment to prevent light from damaging the vaccine strains (Kurup and Thomas 2020). The expired vaccines should not be used at any cost. Stabilizer and adjuvants must be used to enhance the effectiveness of vaccines.

Vaccine should be administered in healthy stress-free animals. The temperature should be normal during immunizations, so early morning is a good time for vaccination, especially in hot areas. In addition, animals must be in good physical condition before being vaccinated. Because stress reduces the immune response of animals, they should never be vaccinated under stress. Animal's discomfort can be reduced by adding minerals and vitamins to animal water before, during and after vaccination. Deworming should be done at least two weeks before immunization particularly in pets (Sánchez et al. 2018).

Nutrition is critical in the development and maintenance of the immune system. Toxin concentrations in commercial animal

feed should be checked on a regular basis. The fungus grows on food, particularly in humid settings, and the fungus's toxins penetrate the animal's body, causing a reduction in immunity, stunted growth, allergies, and low feed consumption. Maternal antibodies, in particular, in poultry chicks, have a negative impact on immunization programmed success. It is recommended that chicks have administered the infectious bursal disease vaccination at 11 days and the Newcastle disease vaccine at 7 days of age. To eliminate financial loss, a record should be kept, and a rigorous immunization protocol regarding disease incidence in the region should be implemented in livestock (Dey et al. 2019; Ma et al. 2019).

Conclusion

Immunization could be referred to as disease protection. The cow pox vaccine is an outstanding historical example of the evolution of human and animal vaccination concepts. The development of novel vaccine production technologies is a prerequisite for meeting the growing human population's demand to prevent zoonotic disease transmission from both wild and domestic animals. Vaccination has been shown to be the most efficient method of controlling bacterial, viral, and protozoan diseases in both animals and people, in terms of the both minimizing mortality and lowering intensity. The ideal adjuvant is stable, effective, non-toxic, well-defined, increase antibody affinity, multifunctional, in expensive, stimulate both innate and humoral immune response. Vaccines must be stimulating the innate, cell mediated and humoral immune responses against particular pathogens. An effective immunization is dependent on a variety of factors, including vaccine processing, vaccine kind and quantity, use of indigenous antigens, immunomodulatory reaction inside the animal's body, and compliance to manufacturer directions. In developing nations such as Pakistan, India, Bangladesh, and Sri Lanka, infectious animal diseases are frequent, and vaccination is the only option to protect animals from all of these diseases. Reduced vaccination failure can enhance the country's supply of foods such as meat and chicken eggs, and animal protein deficits can be eradicated, boosting per capita access to meat and eggs.

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CHAPTER 19

TICK BORNE-BACTERIAL AND VIRAL DISEASES

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INTRODUCTION

Ticks are obligatory blood-feeding parasites that belong to the order Parasitiformes and are widely distributed worldwide, especially in tropical and subtropical regions (Shaw et al. 2001). According to an estimate of total ticks known, about 10 % act as a vector (Jongejan and Uilenberg 2004). Ticks have three main families; Ixodidae, Argasidae, and Nuttalliellidae (monotypic species belonging only to South Africa), and approximately 899 tick species are included in these families (Shaw et al. 2001). The *Ixodidae* are hard ticks having scutum (hard plate) on the dorsal side and has estimated about 700 species and classified into 7 genera. The main genera are *Amblyomma*, *Dermacentor*, *Rhipicephalus, Haemaphysalis*, and *Hyalomma*, *Ixodes.* The *Argasidae* are the soft ticks without scutum and comprises of two main genera *Argas* and *Ornithodoros* and has approximately 193 species (Lagos-Quintana et al. 2001; Gondard et al. 2017). The *Nuttalliellidae* (have only one genus and species) and shares characteristics with the first two families (hard and soft).

Ticks transmit various pathogens of both medical and veterinary importance such as bacteria, rickettsiae, protozoans, helminths, spirochaetes, and viruses when they are compared to other arthropods vector groups (Yu et al. 2015). Furthermore, along with transmitting tick-borne disease, ticks are also responsible for serious allergy, irritation, abscesses, anemia, immunodeficiency, paralysis, hypersensitivity and toxic condition (Jongejan and Uilenberg 2004).

Ticks communicate with their hosts by detecting their breath, scents, body heat, and vibrations. The sites of predilection to bite animals' are ear, udder, and tail areas (Estrada-Peña, et al.,2004). In hard ticks the blood meal is long (3-10 h) depending on the growth phase and tick bites relatively occur in the day time. Conversely, in soft ticks (nymphs and adults) bites rather occur at night and only feed for a few minutes (Sun et al. 2010). They can survive for a long time without sucking blood, determined by the availability of hosts and tick species. Tick go through four development stages; eggs, larvae, nymphs, and adults (male/female). After hatching from eggs, they evolve into six-legged nymphs and then eight-legged adults (Doggett 2004).

In recent years, tick-borne zoonosis has become more common due to climatic change and the transportation or migration of tick-infested animals across the border. Other variables that contribute to the spreading of tick-borne infections include urbanization, deforestation, habitat destruction, biodiversity loss, wildlife immigration, bird migration, and other livestock-related trades that give ideal circumstances for ticks to multiply. The spread of vector-borne diseases is also influenced by variables such as biotic (host density and optimal places for tick species protection) and abiotic (habitat structure and global warming).

In the previous thirty years, the worldwide emergence of tick-borne pathogens, become an immense hazard to human health. The identification of new pathogens continuously is an increasingly globally threat of tick-borne diseases. Furthermore, the development of new molecular techniques has made reliable identification and attribution of correct phylogenetic positions to many pathogens that make tick-borne zoonoses possible (Khamesipour et al. 2018)

Tick surveillance is also becoming more important as the epidemiology changes as a result of climate and habitat, as well as increased host availability and travel of people with their companion animals. Ticks on companion animals have been the subject of investigations in several parts of Western Europe as a result of this, ticks from household pets are being monitored (Jongejan et al. 2019). In this short review of the literature, we will highlight the bacterial and viral diseases of animals that are spread by ticks.

Tick Borne Bacterial Diseases

Lyme Disease

The Lyme disease and its causative agent were reported in 1976 by Burgdorfer and his associates in the city of Old Lyme, Connecticut, USA by performing an epidemiology analysis on many children having complaints of arthritis (Stanek et al. 2012). In animals, this disease is frequently reported in dogs and horses, but its accurate diagnosis is still challenging (Bartol 2013).

Etiology

Borrelia species belongs to phylum Spirochaetes are thin, elongated, motile, gram-negative, flagellated bacteria that consist of 21 plasmids (12 linear and 9 circular), which are the

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highest figure of plasmids in a bacterium (Stricker et al. 2005). *The B. afzelii and lusitaniae* are the predominant species in Austria and Italy respectively (Veronesi et al. 2012) (Muller et al. 2002).

Epidemiology

The black-legged tick (*Ixodes scapularis)* and *Ixodes ricinus* is the major vector of *B. burgdorferi* in the USA and Europe respectively (van der 2016). Along with rodents, birds as avian hosts transport these ticks from one area to another (Humair 2002). The ranges of geographical distributions depend on the vector adaptations (Becker et al. 2016). According to the findings of (Boseret et al. 2013) many strains of *Borrelia burgdorferi sensu lato* have been identified from songbirds' ticks from various regions globally. Canaries present relatively less spirochetemia which was reported in post-experimental infection of *B. burgdorferi* with no or mild clinical signs. So, passerines are not of great importance for *borreliosis* as long-term amplifying reservoirs (Boseret et al. 2013). Clinically healthy horses can carry *B. burgdorferi s.s.* (Chang et al. 2003), while older horses are more susceptible (Ebani et al. 2012; Funk et al. 2016). Along with vector (ticks), the specific climate conditions of an area also play role in diseases spread.

Pathophysiology

Horses may get an infection by the attached nymphs or larvae harboring the *B. burgdorferi* as a reservoir. The ticks attach with the host and the spirochetes from the mid-gut come in the salivary glands, and become part of the tick's saliva which is transferred into the host in two or three days after attachment. This bacterium protects itself from the humoral antibodies by predominant migration within connective tissues. Frequently, inoculation can happen before 2-3 days' period depending if the infected tick already has spirochetes in his salivary glands (van der 2016). In some cases, transmission of *B. burgdorferi* into the host body has been found within 18 to 24 hours of post-attachment (van der 2016). Concurrent infection through other tick-borne pathogens such as *Theileria equi* (Basile et al. 2015) and *Anaplasma phagocytophilum* (van der 2016) may also take place.

Clinical Presentation

The infected horses manifest clinical signs including low-grade fever, fatigue, reduced body weight, behavioral changes, dysphagia, lameness, arthritis, stiffness in the neck, episodic respiratory distress, anterior uveitis, cranial nerve deficits, ataxia, cardiac arrhythmias, meningoencephalitis, abortion and foal mortality. The variation of clinical signs in many infected horses has proved it as a multi-systemic disease and uveitis is the most frequent extra neural manifestation of *Borrelia* infection (van der 2016). The clinically infected horses show variations in the signs and symptoms because of co-infection with other pathogens such as *A. phagocytophylum* (Butler et al. 2005).

The incidence of tick-borne infections is more in dogs as they get more exposure to dirty places where ticks are in large numbers. The infected dogs show anorexia, lethargy, vomiting, reduced weight, generalized pain, joints swelling, fever and lameness. Occasionally, the kidneys are targeted straightly due to the formation of antigen-antibody complexes within the glomerulus which shows the chronic nature of the disease. The disease duration ranged from 2 to 730 days before death (Clark and Bidaisee 2021).

Differential Diagnoses

It is challenging because of the different clinical signs that can be associated with possible co-infection and diverse *B. burgdorferi* genospecies (van der 2016).

Diagnosis

The diagnosis is difficult in various species because *B. burgdorferi* infections are persistent devoid of any clinical signs and symptoms. The antibodies can be confirmed after 5-6 weeks of post-exposure, while the highest titers can be found after 3 months. It is preferred to culture *B. burgdorferi* from suspected horse skin biopsies (Chang et al. 2003) along with a 2-step serology protocol (ELISA or IFAT) (Butler et al. 2005). The new fluorescent bead-based multiplex assay serum analysis is an important quantitative tool for identification of the antibodies to outer surface protein antigens of *B. burgdorferi* symbolic for natural infection with and/or vaccination against the Lyme pathogen (Wagner et al. 2011). It is preferable to combine cytological assessment, antibody, and PCR testing of ocular fluids in highly suspected cases (Divers et al. 2012). Moreover, histopathology is the definitive way for the equine *borreliosis* diagnosis (Sircar et al. 2016).

The histopathology lesions of infected horses with leptomeninges include cranial neuritis, lymphohistiocytic leptomeningeal vasculitis, and peripheral radiculoneuritis with Wallerian degeneration, whereas the spirochetes are identified with the help of immunohistochemistry and Steiner silver impregnation (James et al. 2010; Imai et al. 2011).

Management & Treatment

The infected ponies are cured using the intravenous tetracycline @ 6.6mg/Kg body weight bid for weeks has proven better than per-oral use of doxycycline or sodium ceftiofur (Divers et al. 2003).

Above and beyond, the preventive measures like avoiding interactions with tick-infested areas and vigilant cleaning of the horse for early removal of all ectoparasites like ticks should be adopted. Tick infestation can be protected by several types of insecticidal sprays but many of them have not been approved for horses and also their effectiveness is undocumented up till now (Butler et al. 2005). The canines insecticidal can be used to kill ticks on horses because so far, no adverse effect has been reported (Divers et al. 2001).

Q Fever

This fever is a globally important socio-economic, public health and occupational zoonotic disease (animal searchers, slaughterers, farmers, and veterinarians) (Marmion et al. 2005; van der et al. 2011). This disease was reported by E.H. Derrick in 1937 and the term "Q fever" (query fever) was proposed to describe febrile illnesses in animal slaughter house workers in Brisbane, Queensland, Australia in the same year (Gwida 2012).

Etiology

The *Coxiella (C.) burnetii* (gram-negative) bacterium causes Q fever and is considered as a possible biological warfare source with the capability of windborne spread as well as durability in the environment (for more than 40 months) by adopting form like a spore (Dalton et al. 2014; Asamoah et al. 2020). In Ethiopia, the confirmed case of *C. burnetii* was reported in ticks collected from cattle (Philip et al. 1996).

Epidemiology

As *C. burnetii* is secreted in amniotic fluids, placental discharges, milk, feces, urine, and vaginal fluids of infected animals (Schimmer 2018). It is frequently spread in animals as well as in humans by contaminated dust inhalation, parturition or aborted placenta secretions, infected milk (unpasteurized) or meat, and wool (Salifu et al. 2019). If the environment is contaminated with parturition excretions of infected animal, then there are chances of an outbreak in that locality due to a vast spread of bacteria via contaminated dust particles (Asamoah et al. 2020). Dogs and cats are prone to *C. burnetii* infection and can spread to humans (McQuiston et al. 2002). In Ethiopia, *Coxiella* was suspected as the potential cause of abortion episodes, as it can affect all three ruminant species (Deressa et al. 2020).

Clinical Signs

This infection usually occurs in multiple species including sheep, goats, cattle, dogs, cats, rodents, birds, and other wildlife (Asamoah et al. 2020). The sheep and goat are the important causes of outbreaks in human, whereas cows are also a significant reservoir of the *Coxiella* (Rodolakis 2009). As *C. burnetii* multiplies in the trophoblasts of the allantochorion and placentomes of ruminants (Roest et al. 2012), therefore, Q fever causes reproductive issues like infertility, abortion, stillbirth, premature delivery, and weak offspring (Anderson et al. 2013; Hogerwerf et al. 2013). Generally, infected animals show anorexia, fever, rhinitis, and mild coughing (Asamoah et al. 2020).

Diagnosis

For diseases diagnosis in ruminants, enzyme-linked immunosorbent assay (ELISA) (OIE 2008) and phase-specific serology is a recommended tool to analyze the disease dynamics within herds (Bottcher et al. 2011; Sting et al. 2013). Although in dogs and cats, IFAT is recommended to detect antibodies of *C. burnetii* (Shapiro et al. 2016; Bauer et al. 2021).

Zoonosis

In recent years with an inclining rate, Q fever is reported as a re-emerging zoonotic disease in many European countries, particularly a huge number of human cases attributed to livestock have been diagnosed in the Netherlands (Roest et al. 2011; Georgiev et al. 2013). During epidemic of Q fever (2007 to 2010) 4000 human cases were diagnosed, as a result 50,000 small ruminants were culled with short-term restrictions on animal breeding. This fever causes $1 million losses annually to the Australian meat industry.

Treatment

In small ruminants, antibiotic treatments with two successive injections of oxytetracyclin (20 mg/kg body weight) have been successful (Van den et al. 2015). In many reports doxycycline and fluoroquinolones are also suggested as the most effective drug, hence in 1989 these were used together to treat acute meningitis in Q fever infection. The sulfamethoxazole-trimethoprim salts are also effective and no resistance reported so far (Alemneh and Melaku 2018).

Prevention

In the United States, human and animal vaccines are developed but not available commercially (McQuiston et al. 2002), while in Australia, whole-cell formalin-inactivated vaccine has been produced and licensed (Eldin et al. 2017). The preventive efforts should be considered by avoiding contact with infected animal excretions on the farm and try to apply maximum sanitation practices to reduce the risk of infection while dealing with parturition procedures. Serostatus of researchers, veterinarians, and farm workers should be analyzed periodically (McQuiston et al. 2002). General disinfection of farm and specially parturition pens by 10% sodium hypochlorite or 2% formaldehyde can be done (Gwida 2012).

Bovine Anaplasmosis

Etiological Agent

The highly pathogenic intra-erythrocytic rickettsia *Anaplasma (A.) marginale* causes Bovine Anaplasmosis (Pothmann et al. 2016) that mainly affects the health, reproduction, and production of infected animals (Kocan et al. 2010).

Clinical Signs

The characteristics signs of B. Anaplasmosis include fever, anorexia, decrease milk yield, depression, hemolytic anemia, mild to severe hemolysis, jaundice and abortion (Kocan et al. 2003; Kocan et al. 2010). The lactating and peri-parturient cows are more prone to infection due to associated stress and immunosuppression (Aubry and Geale 2011; Da Silva and Fonseca 2014). The recovered animals become reservoirs and spread the infection in remaining healthy herd (Aktas and Özübek 2017).

Transmission

The biological vector of *A. marginale* is ixodid tick mainly *Rhipicephalus microplus* (Al-Hosary et al. 2020). There are many other sources of transmission like the trans-placental route and direct transmission during tattooing, castration, dehorning or by infected blood transfusion (Inokuma 2007; Aubry and Geale 2011). The persistence of *A. marginale* infection is enabled by antigenic variation. The Major surface proteins (MSP) of *A. marginale* are extremely variable proteins that are responsible for the invasion of host cells (Al-Hosary et al. 2020).

Diagnosis

In acute stages of disease microscopic examination of Giemsa-stained blood smears is reliable but not for carrier or pre-

symptomatic animals (Inokuma 2007; Carelli et al. 2007) however, cross-reactions have been reported (Aktas and Özübek 2017)

Treatment

Earlier to the availability of antimicrobial (tetracycline), many preparations for example dyes, arsenic compounds, antimalarial, and antimony derivatives were implemented to treat acute anaplasmosis. However, due to less therapeutic effects, these compounds could not control mortality (Shaukat et al. 2019)

At initial infection, imidocarb dipropionate @ 5mg/Kg twice, seven days apart and tetracyclines such as oxytetracycline @22 mg/kg o.i.d for 5 days are effective. As these can only control the infection but cannot consistently eliminate *A. marginale*, the animals can become asymptomatic carriers. The extensive diversity and variability of *A. marginale* strains make it difficult to eliminate the disease (Almazan et al. 2018)

Prevention

Antigenic and genetic variations in the outer membrane proteins responsible for transmission make the prophylactic approaches less successful.

Canine Monocytic Ehrlichiosis (CME)

Canine ehrlichiosis is a fatal tick-borne disease caused by an obligate intracellular parasite, *Ehrlichia canis,* which resides and replicates within mononuclear cells (Zhu et al., 2009). At least five bacterial species are found in domestic dogs: *Ehrlichia canis, Ehrlichia chaffeensis, Ewingii, Anaplasma platys and Anaplasma phagocytophilum* (Hmoon et al. 2021).

Etiology & Epidemiology

The *Rickettsiales* group, which has been established by combining the *Rickettsiaceae* and *Anaplasmataceae* families under the genus name *Ehrlichia*, currently contains the etiological agent of ehrlichiosis. This genus presently contains 11 *Ehrlichia* species, which have been divided into three categories based on their serological, morphological, immunological, and genetic characteristics. *Ehrlichia* are small rickettsias with a round shape that can contain a wide range of pleomorphism, particularly in cell cultures, while their cell walls are identical to gram-negative bacteria. Moreover, monocytes, granulocytes, and thrombocytes are among their favorite target cells (Procajlo et al. 2011). The Giemsa staining method is preferable for identification and enables cells to become dark blue or navy blue.

All feeding stages of the brown dog tick, *Rhipicephalus sanguineus*, transmit *E. canis*, although nymphal and adult ticks can disseminate infection for at least 155 days after separating from an infected host. The *E. canis* was the first *Rickettsiales* species to be discovered in dogs and recognized in Brazil (1970s), has global distribution, particularly in tropical and subtropical regions.

Pathogenesis

The animal gets the infection after being bitten by a carrier tick *R. sanguineus*, as a result host's blood circulation is invaded and morulas are formed in blood cells. When an infected blood cell disintegrates, new elementary corpuscles attack the next blood cells, such patterns follow across the body, gaining access to the liver, spleen, bone marrow, and lymphatic nodes, where they can multiply repeatedly. The *E. canis* resides in monocytes while *E. phagocytophila* (act. *Anaplasma phagocitophilum*), and also *E. ewingii* prefer neutrophilic and acidophilic granulocytes of infected dogs.

Clinical Signs & Symptoms

Canine ehrlichiosis occur in three different clinical forms as acute, subclinical and chronic (Ristic and Holland 1993). The acute form of disease exhibits clinical sign and symptom e.g., Anorexia, high temperature, depression, emaciation, reduced weight (Iqbal et al. 1994). In sub-clinical phase the clinical signs and symptoms are not evident but still animal is suffering from diseases and exhibiting anemia, uneven leucopenia and thrombocytopenia (Greene and Harvey 1990). The chronic phase of disease is typically recognized by anorexia, fever, anorexia, emaciation, edema, epistaxis and shock finally reaching to death (Buhles et al. 1974).

Diagnosis

In laboratory examination canine ehrlichiosis is diagnosed by traditional method like hematology, serology and biochemistry parameters. In lab testing, thrombocytopenia, anemia, and leucopenia can be found in infected animal tests reports. The most reliable serological method includes plate latex agglutination and immune-fluorescent antibody test (Greene and Harvey 1990). The CME diagnosis is difficult, and it typically demands the employment of many diagnostic methods at once (Procajlo et al. 2011). Now the most recent and advanced molecular technique PCR (polymerase chain reaction) has been developed and consider as most sensitive as compared to traditional methods (Hmoon et al. 2021).

Treatment

For the management of acute CME, an antibiotic from tetracycline group for at least 28 days are indeed a preferred therapy and has a rapid recovery. The signs of acute ehrlichiosis normally diminish within 48 to 72 hours after the use of the antibiotics. Conversely, chronic ehrlichiosis, might be more difficult to cure since dogs do not react well enough to tetracycline therapy and antibiotic resistance has been reported in such cases (Procajlo et al. 2011). Tetracyclines are the most effective medications for treating ehrlichiosis, independent of the Ehrlichia species or the disease's type (Procajlo et al. 2011). The doxycycline, given orally at a dosage of 11 mg/Kg b.m. daily for at least 28 days, supplemented with imidocarb shots administered after 14 days at a rate of 3-6 mg/kg b.m is the most preferred antimicrobial drug against *E. canis* (McClure et al., 2010; Procajlo et al. 2011).

According to Price (1980), imidocarb's activity is 3 times higher than that of tetracyclines, but it has the added benefit of being effective against *Babesia canis*, which can accompany ehrlihiosis (Procajlo et al. 2011). Other than doxycyclines, oxytetracycline at 25 mg/kg b.m. every 12 hours, can be

administered. Liquids, corticosteroids, and vitamins are used as adjuvant treatments. Blood transfusions may be required in extreme conditions (Procajlo et al. 2011; Sharma et al. 2015)

Tick-Borne Viral Diseases

Approixmately10% of tick species are involved in the spread of tick-borne viruses (Kazimirova et al. 2017) and are generally known as Tiboviruses (Abubakar et al. 2018). These viruses belongs to nine tiboviruses families; among them eight are RNA families (*Flaviviridae, Reoviridae, Rhabdoviridae, Orthomyxoviridae, Nyamiviridae, Phenuiviridae, Nairoviridae*, and *Peribunyaviridae)* and one DNA family *(Asfarviridae*) (Abubakar et al. 2018). They require ticks and vertebrates as their host to complete their life cycles. Until now, about 19 tick-borne viral diseases in animals and 16 in human have been observed. The most common Tiboviruses belong to Flaviviridae family which include tick-borne encephalitis, West Nile, Louping ill, Powassan and Kyasanur Forest virus, that is transmitted by *Dermacentor reticulatus*, *Ornithodoros moubata, Ixodes ricinus, Ixodes scapularis, and Haemaphysalis punctata*, respectively. While *Asfarviridae family* is involved in the spread of the African swine fever virus (Abubakar et al. 2018).

Tick Born Encephalitis Virus

Epidemiology

Tick born encephalitis is a viral disease caused by arbovirus and transmitted via ticks i.e., *Ixodes ricinus* and *spersulcatus* in Asia and Northern Eastern Europe respectively (Leschnik et al. 2002). The mice, deer, foxes, sheep, cattle, and dogs act as a natural host (Stanek and Hofmann 1994). The disease mainly affects the nervous system (Leschnik et al. 2002) and spread depends upon the number of ticks in a specific season (Kirtz 1999).

Clinical Signs

After the virus has completed its incubation period (5-9 days), clinical signs like fever, anorexia, apathy, and neurological symptoms including (acute thalamic and cerebrocortical lesions cause alteration in behavior, consciousness, decreased tone in muscles of limbs, and seizures) start to appear. Further diffused brainstem lesions cause vestibular strabismus, nystagmus, facial paresis, and dysphagia. While meningeal inflammation leads to increased sensitivity to pain in the neck and spinal reflexes may alter due to lesions in the spinal cord (Tipold 1997; Reiner and Fischer 1998; Kirtz 1999). The hematological analysis revealed leukopenia and lymphopenia in the early stage of infection (Leschnik et al. 2002). Prognosis is good if the infected animal survives the first week of infection (Kirtz 1999).

Diagnosis and Treatment

The clinical signs and symptoms along with the history of the tick infestation are determined for the disease diagnosis. The virus identification is confirmed through various tests including PCR, ELISA, cytological, as well as serological tests of blood and cerebrospinal fluid. As no specific treatment is carried out, only supportive therapy is done (Leschnik et al. 2002).

Louping Ill Virus (Ovine Encephalomyelitis)

Etiology

The louping Ill virus caused ovine encephalomyelitis disease that is transmitted through I. *ricinus* tick in sheep. The disease is characterized by encephalitis and have high mortality rate 60% (Reid et al. 1981). However, the disease is less prevalent in cattle, but infected animal can also develop CNS signs and fatal illness (Dobler 2010).

Clinical Signs

The virus gets entry into the body of an animal through the saliva of the infected tick. After replication into lymphatic tissue, the virus comes into the bloodstream and remains there for 1-5 days. During this period, the animal may develop fever. After that, the virus enters the central nervous system of animals and starts replication. The clinical signs like muscle tremors, ataxia, nervous nibbling, and weakness start to appear and animal may collapse after 1-3 days of onset of signs (https://www.msdvetmanual.com/nervous-system/louping-ill/louping-ill-in-animals). The dogs that are guarding a sheep herd, infested with the ticks might develop CNS signs. (Mackenzie 1982).

Diagnosis and Treatment

Clinical signs along with the tick infestation help in the diagnosis of the disease. No specific treatment or vaccines are available (https://www.msdvetmanual.com/nervous-system/louping-ill/louping-ill-in-animals)

Powassan Virus

The Powassan virus caused fulminant meningo-encephalitis in horses. After the incubation period of 8 days, the animal exhibits clinical signs including frothy mouth, excessive chewing, ataxic, tremors in neck and head. Further neurological lesions like encephalomyelitis, brain necrosis, and necrotic foci in parenchyma were seen (Little et al. 1985) Goats don't show any signs however they shed virus in the milk from day 7-15 post-infection (Kokernot et al. 1969).

Diagnosis and Treatment

The diagnosis is made on the basis of clinical signs and symptoms along with the history of the tick infestation. Moreover, laboratory testing of blood and spinal fluid is recommended. There is no specific medication for the treatment of Powassan virus disease.

Kyasanur Forest Disease Virus

The disease is caused by the virus of Flaviviridae family and transmitted through hard tick (*Haemaphysalis spinigera*) is endemic to South-western India. The Blanford rat, striped forest squirrel, and house shrew are the natural acting hosts.

Clinical Signs

After the incubation period (3-8 days), the symptoms including chills, fever, muscle pain, vomiting, gastrointestinal

and bleeding problems start to appear. Animals infected with ticks can show high virus titers in their blood (Trapido et al. 1959). While other livestock and wild animals show no clinical signs (Dobler 2010).

Diagnosis and Treatment

The diagnosis can be confirmed during early stage through molecular tests (PCR) and in later stage by Elisa. As no specific treatment is carried out, only supportive therapy is done.

West Nile Virus

Etiology

The virus is present in birds without exhibiting the clinical signs, however horses may become infected and can develop a severe illness that can lead to death. (https://www.msdmanuals.com/professional/infectious-diseases/arboviruses,-arenaviridae,-and-filoviridae/west-nile-vir)

Clinical Signs

The infected animals manifest clinical signs including anorexia, stumbling, muscle twitching, paresis, hazy vision, noggin, teeth grinding, uncontrolled walking, convulsions, circling, and dysphagia. This may lead to coma and eventually death (https://www.oie.int/en/disease/west-nile-fever)

Diagnosis and Treatment

The disease is diagnosed by observing clinical signs and determining the presence of antibodies in the blood. No specific treatment is carried out, although vaccines in horses are available.

Control and Prevention of TBD

Chemical Control

Acaricides are used to control tick population at different growth phase of their life cycles. Dipping vats, sprays, pour-on and parenteral acaricides are used in chemical control. During the treatment with acaricides, taxonomy of the ticks as well as their susceptibility to that kind of treatment should be considered for effective results. Ticks can gain resistance against a particular type of acaricide which can be transferred genetically to the next generations (Almazan et al. 2018).

Immunological Control

Vaccines are an effective source for the control of TBDs. Depending upon the ticks whether they are one host ticks or multiple host ticks, vaccines are administered accordingly. This is because the immune response against the vaccination varies from host to host. This factor needs to be considered when preparing a vaccine or running its trial. Vaccines against tick-borne pathogens are more effective for the wild reservoir hosts that are transmitted by ticks from an infected host to a healthy host than those pathogens that are transmitted transovarially. Some tick-borne pathogens like bacteria and protozoans require much more time for transmission from the tick to the host during the feeding time of ticks than the viruses that are transmitted as soon as a tick bites the host. This factor should also be considered while designing a vaccine. In the case of viruses, vaccines are prepared by keeping in view tick attachment, its feeding time, and reduction in host tick-borne pathogens. Moreover, vaccines used for wild, domestic, or humans have different considerations (de la Fuente et al. 2017).

Vaccines for Domestic Animals

Vaccines like BM86/BM95 for tick infestation control in cattle are proved to be cost-effective and a reasonable approach. One host ticks like Rhipicephalus spp. which are the main vectors for bovine anaplasmosis and babesiosis, have been effectively controlled by the vaccinations. For designing a vaccine of bovine anaplasmosis, which is also transmitted by other means like biting insects and blood contaminated objects, other factors like tick pathogen infection, tick bite pathogen transmission, host tick bite pathogen should also be considered in addition to the reduction of tick infestation (de la Fuente et al. 2017). BM 86 is derived from a gut membrane protein found in the intestine of *R. microplus*. This vaccine controls the tick population by interfering with blood clotting and cell growth of the ticks. *R. microplus* feeding on cattle vaccinated with BM86 show a marked reduction in females, their weight and reproductive ability. Combined treatment with acricides and BM86 can give 100% tick control. BM95 is homologous protein to BM86, has 39 and 21 difference in nucleotide at amino acid level has 64% efficacy in cattle (Almazan et al. 2018).

Conclusion

Using acaricides for complete tick eradication seems to be impossible. Ticks can also be controlled by the safe formulations with repellency and parasiticidal activity (de la Fuente et al. 2017). Emerging pheromones base control and biological control agents like tick repellents and botanical acaricides respectively are also promising (Benelli et al. 2016). Vaccines are the safest and effective way to control tick-borne diseases, because they can induce a long-lasting immunity in the animal against tick infestation as well as pathogen infection. The vaccines derived from the combination of the tick as well as pathogen antigens seem to be more effective.

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CHAPTER 20

IMPORTANT VIRAL DISEASES OF WILD CARNIVORES

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INTRODUCTION

Wild carnivores are under the threat of habitat fragmentation, climate change and uncontrolled hunting. Infectious diseases are also considered a threat to wild animals. The increase in the human population and the consequent increase in domestic animals around natural habitats increases the risk of disease transmission between domestic and wild animals. Due to close phylogenetic relationship between wild and domesticated carnivores, some viral pathogens infecting domestic carnivores may infect wild carnivores, resulting in population decline. For example, transmission between wild and domestic carnivores, particularly rabies virus, canine parvovirus (CPV-2), canine distemper virus (CDV), feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) has been reported. Viral infections are of great importance to wild carnivores, as this contamination can become endemic in the wild carnivore population.

Canine Distemper Virus

Canine distemper virus (CDV) has appeared as an important wildlife sickness that is very contagious and easily transmitted among susceptible hosts. Originally described as a contagious disease of domestic dogs, it is now recognized as a global multi host pathogen with rapid transmission and causing mass mortality in a wide variety of carnivorous species (Loots et al. 2017).

CDV/ Canine Morbillivirus is a worldwide infectious disease of dogs. Also known as CDV or 'Canine Distemper Virus', it is a lethal viral infection that affects the digestive, respiratory, and central nervous system (CNS) and is especially seen in dogs and can be seen in other carnivores such as foxes and ferrets, and sea mammals (Beineke et al. 2009).

CDV is located in the genus *Morbillivirus* of the family *Paramyxoviridae*, together with measles virus, dolphin morbillivirus, peste-des-petits-ruminants virus, rinderpest virus, porpoise morbillivirus, and phocine distemper virus. CDV is an enveloped, negative-strand virus. Single-stranded RNA virus contains six structural proteins: large (L), phospho (P), matrix (M), nucleocapsid (N), fusion (F) and hemagglutinin (H) protein. The H glycoprotein has a determining role in tropism and cytopathogenicity. Viral M protein binds to surface glycoproteins and nucleocapsid together during viral maturation (Loots et al. 2017; Rendon-Marin et al. 2019).

CDV has a wide host range that includes many different species in the order Carnivora. In the natural host spectrum of CDV; there are members of the family Canidae (dog, fox, coyote, wolf, wild dogs), Mustelidae (mink, badger, weasel, skunk, marten, sable), and Procyonidae (raccoon, panda, bear). All breeds of foxes are susceptible to infection and the clinical manifestation develops (Rendon-Marin et al. 2019). Studies have reported CDV with high mortality rates in non-human primates (rhesus monkey / Macaca mulatta and cynomolgus macaques / Macaca fascicularis). Infections in these primates have raised several concerns regarding the potential zoonotic risk of CDV in humans (Qiu et al. 2011; Loots et al. 2017).

As CDV quickly loses its infectious ability outside the host, the most important transmission route of infection is a close contact of sick animals having fever with other susceptible animals. CDV is easily transmitted by contact among susceptible hosts or through aerosolized with various body (oral, nasal, respiratory) fluids (Appel 1987; Loots et al. 2017). CDV, like other Morbilliviruses, is lymphotropic and epitheliotropic. Therefore, the most severe lesions caused by CDV infection, which is a multisystemic infection, are seen in organ systems rich in lymphoid and epithelial tissue. It takes 1-2 days after the entry of agent into the body of host that the virus is transported to the bronchial, pharyngeal, and tonsillar lymph nodes via the lymphatic route. Primary replication of the virus takes place in respiratory lymph tissue cells (macrophages, T and B lymphocytes) (Kubo et al. 2007; Loots et al. 2017).

Clinical manifestations reported due to CDV infection in wild carnivores are largely similar to those in domestic dogs. However, the severity and outcome of infection may vary between species. This is dependent on several factors such as virulence, host's age, and host immune status. If an animal develops a strong immune response, no clinical signs will occur, so an estimated 50-70% of CDV infections in domestic dogs are thought to be subclinical. Since CDV infection has a multisystemic nature, the clinical symptoms seen during the disease are varied. Clinical findings are especially related to respiratory, digestive, and central nervous systems. It also causes hyperkeratosis (hard pad) on the skin (on the tip of the nose and the surface pad), especially in domestic dogs (Baumgärtner 1993; Loots et al. 2017; Rendon-Marin et al. 2019).

In animals with minimal or no immune response, two clinical forms of CDV can be distinguished: the acute systemic form and the chronic nervous form. The acute systemic disease occurs 2-3 weeks after infection. The virus continues to multiply and spread throughout the body, causing severe clinical signs such as biphasic fever, mucopurulent oculonasal discharge, cough, dyspnea, depression, loss of appetite,

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vomiting, and diarrhea (which may be bloody). At this stage of infection, the virus is present in every secretion and excretion of the body (Appel 1987; Williams 2001).

Neurological symptoms may occur simultaneously or may occur following systemic disease. Symptoms vary according to the affected area of the brain but often include abnormal behavior, convulsions or seizures, gum-chewing movements, blindness, cerebellar and vestibular signs, paresis or paralysis, and incoordination. Infection in the central nervous system results in acute demyelination and most animals die 2-4 weeks after infection. (Williams 2001).

In case of deaths due to CDV infection, cachexia, dehydration and atrophic appearance in the muscles are the first signs that draw attention. In cases where CDV infection is accompanied by upper respiratory tract infections, mucopurulent eye and nasal discharge and exudate ranging from serous to purulent in the nasopharynx, trachea, and bronchi are present. In cases where interstitial pneumonia is formed, it is observed that the lung lobes change color from red to brown, while the lung tissue hardens and takes on a liver-like appearance. As a result of viral and bacterial secondary infections, bronchopneumonia and lesions characterized by the fusion of the cranial and caudal lobes of the lung can be seen (Beineke et al. 2009; Williams 2001).

Since the diagnosis of CDV is confused with diseases with a similar clinical manifestation and As the symptoms of CDV appear late, it is difficult to obtain an accurate report based on the clinical history and clinical symptoms. Therefore, various laboratory diagnostic methods are needed.

In acute CDV infection, the dog's hematological profile such as anemia, thrombocytopenia, leukopenia, lymphopenia, neutropenia and monocytopenia draws attention. By taking samples such as conjunctival, nasal, and vaginal swabs or whole blood and urine from animals with a multisystemic clinical manifestation, methods such as immunofluorescence, RT-PCR, and ELISA can be used for direct virus detection from these samples (Loots et al. 2017).

Maternal antibodies are the most important source to prevent the disease in the early period. Puppies born to vaccinated mothers and do not receive colostrum, they have enough antibodies to protect against infection for 1-2 weeks. Despite the transplacental transfer of CDV antibodies in small amounts, the main antibody transmission occurs by absorption of maternal antibodies ingested with colostrum from the small intestine. Vaccination is the most reliable way to protect against Canine Distemper Virus infection. In puppies considered to have received colostrum, vaccination is started at 6-8 weeks of age and repeated at 3-4 week intervals. Year later, the booster vaccine is administered and repeated every 3 years. Hyperimmune serums is recommended for treating infected animals to provide passive immunization. In addition, antibiotics can be administered to prevent secondary infections. (Pardo et al. 2007; Beineke et al. 2009; Loots et al. 2017).

As canine distemper infection has a wide species distribution in wild carnivores and vaccination is limited in these creatures, eradication of the infection is not considered possible today.

Parvovirus Infection

The family *Parvoviridae* includes two subfamilies, *Parvovirinae* and *Densovirinae*. *Parvovirinae* infects vertebrates, while Densovirinae infects insects. Parvoviruses are small and have a genetically simple structure. Parvoviruses with non-enveloped, cubic symmetric virions are single-stranded linear DNA (ssDNA) and approximately 5,200 nucleotides in length (Decaro and Buonavoglia 2012).

Parvoviruses can cause diseases in various mammals. The most common are CPV-2, feline panleukopenia virus (FPV), mink enteritis virus (MEV), and raccoon parvovirus (RPV). Canine and Feline parvoviruses (CPV and FPV) belong to the genus *Carnivore protoparvovirus-1* in the family *Parvoviridae*, commonly known as carnivore parvoviruses. Parvovirus diseases seen in cats, minks, and dogs are very similar, especially in terms of causing leukopenia and enteritis. Although CPV and FPV are antigenic variants of the same virus species and have 98% genome homology, there are evolutionary and epidemiological differences between them. Although FPV has existed for over 100 years and remains without visible changes today, CPV has undergone significant antigenic changes that arose in dog populations in the 1970s, resulting in sub-antigenic variants. These are CPV-2a, CPV-2b, and finally CPV-2c, respectively (Balboni et al. 2021; Chang and Chen 2021).

The host range of CPV includes nearly all species of wild and domestic carnivores but is more commonly identified in domestic dogs (Canis lupus familyis) and cats (Felis silvestris catus). In Europe, the presence of CPV has been reported to be present in various free-range carnivorous populations from canines such as wolves and foxes to mustelids, by molecular methods or serology. Additionally, FPV has been documented in many European wild carnivores such as badgers and Egyptian mongooses. Transmission between domestic and wild carnivores has been demonstrated by some molecular studies based on sequence analysis. Results of these studies showed that wild and domestic carnivores share the closely related or same parvoviruses. (Calatayud et al. 2020; Balboni et al. 2021; Chang and Chen 2021).

The pathological and epidemiological features of Carnivore parvoviruses are not reported. For this reason, the consequences of the infection at the population and individual levels can't be predicted exactly. While these viruses are present in nearly all carnivorous populations tested, they were reported to be able to trigger reductions in pure wild populations, for example in the outbreak among wolves (Canis lupus) in North America (Calatayud et al. 2020). The severity of disease symptoms ranges from subclinical, acute to fatal, depending on several factors such as host immunity, host's age and virus strain. Clinical manifestations are mainly observed in offspring and assessing offspring health in wild carnivores has major limitations. As a result, the effect of carnivorous parvoviruses in wild ecosystems is not fully understood. (Calatayud et al. 2020).

CPV outbreaks are characterized by high morbidity and mortality rates. Especially the incidence in the area where carnivores are found is high. Acute CPV-2 enteritis can be seen in carnivores of all breeds, ages, and genders, but it was reported that offspring between six weeks and four months are more susceptible to this infection and low body weight increases the mortality rate. The factors that make the offspring susceptible to parvovirus infection are the lack of intestinal parasites, protective immunity, stressful, crowded and unhealthy environmental conditions (Goddard and Leisewitz 2010; Miranda and Thompson 2016).

The most effective mode of transmission for the rapid spread of CPV-2 among carnivores is fecal-oral transmission (direct). Another mode of transmission is oronasal (indirect)

transmission by exposure to substances contaminated with feces. CPV-2 is highly contagious. Parvoviruses are resistant to pH and temperature changes, detergents, anhydrous environments, and solvents. So they can cause infection by carrying agents with dust particles and contaminated materials. If conditions are suitable, CPV can survive on the fecal-contaminated ground for several weeks or more (Goddard and Leisewitz 2010; Decaro and Buonavoglia 2012).

The virus can show high affinity for intestinal crypt epithelial cells, bone marrow precursor cells, lymphoid tissue cells, and cardiac muscle cells due to its rapid proliferation ability. Viral replication can cause cell death and thus cellular loss due to the failure that occurs during mitosis. Histopathological examination reveals pathognomonic lesions for parvoviral enteritis, such as destruction of the lamina propria, emptying of Peyer's patches, necrosis of epithelial cells in intestinal crypts, swelling of crypt lumens containing necrotic enterocytes, and suppressed by infiltration of neutrophil granulocytes and mononuclear cells in the lamina propria. CPV can cause fatal myocarditis by replicating in heart cells in 2-3 weeks old seronegative puppies. Because cardiac muscle cells in neonatal animals are actively dividing cells, the virus infects the cardiac muscle cells directly (Goddard and Leisewitz 2010; Decaro and Buonavoglia 2012; Schoeman et al. 2013).

The disease has two clinical forms. These are acute hemorrhagic enteritis and myocarditis. The most characteristic clinical manifestation of canine parvovirus type 2 is hemorrhagic enteritis, which is mostly dependent on maternal antibody titers of CPV-infected offspring. Clinical symptoms such as anorexia, depression, vomiting, mucoid or bloody diarrhea, often dehydration, and fever occur after 3-7 days of the incubation period of the virus. Concomitant pulmonary infections may lead to the onset of respiratory distress. Mortality rate in puppies can be high (up to 70%) but is generally less in adult dogs. At initial stage of the disease, there is a slight increase in body temperature. Although there is no obvious characteristic feature of the stool, it may be watery, light yellow, or bloody in severe cases. Intense fluid and protein losses through the gastrointestinal tract can cause severe dehydration, resulting in hypovolemic shock. Usually dogs presented with severe vomiting and diarrhea may lead to death within 3 days of the onset of symptoms. The course of the disease and its clinical findings are variable depending on the infectious dose of the virus, but clinical symptoms usually appear 3-5 days after ingestion of virus and last for an average of 5-7 days (Goddard and Leisewitz 2010; Decaro and Buonavoglia 2012; Schoeman et al. 2013).

Other clinical form of canine parvovirus type 2 is heart syndrome or myocarditis, which is more prevalent in puppies younger than 3 months. CPV-2-origin myocarditis is infrequently identified because infected offspring die soon after clinical symptoms appear or before clinical symptoms appear (Goddard and Leisewitz 2010).

The diagnosis of the disease is made by obtaining a good history, clinical, laboratory, and histopathological findings, and using virological tests for antigen detection and serological tests for antibody detection. Various diagnostic methods have been developed for CPV antigen detection using feces or intestinal contents from infected animals. Hemagglutination test, electron microscopy (EM), virus isolation, ELISA, latex agglutination test (LAT), fluorescent antibody test (FAT), virus neutralization test as well as PCR and real-time PCR, loop-mediated isothermal amplification (LAMP), nucleic acid hybridization, or dot blot, nucleic acid sequence analysis tests are used for diagnostic purposes, but these tests have different rates of sensitivity and specificity (Dik and Simsek 2021).

Puppies receive immunity just after birth through colostrum. Successful immunization with vaccine can only be achieved in seronegative puppies or puppies with very low antibody titers. Vaccination is the most important practice to prevent CPV infection and control the spread of the disease. Because of short-term immunity with inactivated vaccines, modified live vaccines are preferred (Goddard and Leisewitz 2010; Chang and Chen 2021).

The effect of CPV diseases on wild populations is not yet fully understood. However, it is known that disease symptoms mainly develop in offspring and there are great limitations in protection against parvovirus infections in wild carnivores due to the incomplete maternal antibody uptake or vaccination of the offspring.

Adenovirus Infection

The family *Adenoviridae* has a double-stranded DNA genome and causes significant infections in humans and animals (birds, reptiles, fish, mammals) all over the world. The viral genome encodes about 40 proteins. About 1/3 of these proteins are structural proteins. Many adenoviruses can agglutinate erythrocytes. They do this by forming bridging bonds between the ends of penton fibers and cellular receptors. Each adenovirus and the type of erythrocyte that it agglutes are defined (Saraç 2016).

There are 5 genera in the family *Adenoviridae*, namely *Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus*, and *Ichtadenovirus*. Canine adenovirus 1 was isolated from dog, fox, bear, and skunk while Canine adenovirus 2 was isolated from dog, wolf, fox, and marine mammals. Adenovirus 1 causes hepatitis, interstitial nephritis, encephalopathy, ocular and respiratory disease, while Adenovirus 2 causes tonsilitis, pharyngitis, tracheitis, bronchitis, and bronchopneumonia (Bulut et al. 2013).

Clinical signs ranging from fever, reduction of white blood cells, congestion of mucous membranes, and even death can be observed in infected animals. In recent years, infection is rare in areas where regular vaccinations have been carried out. Although respiratory and systemic infections are reported most of time, involvement of central nervous system has been reported sometimes in foxes. Adenoviruses are significantly species specific. They usually cause subclinical, persistent, productive infections. Lymphoid tissues of the pharyngeal region, in particular, are sites of persistent infection associated with antibodies for most adenoviruses. It is thought to persist in enteric adenoviruses, probably by the same mechanism. Transmission is by droplet or fecal-oral route (Dowgier et al. 2016).

Initially, adenoviruses reproduce in the bronchial epithelial cells, nasal mucosal epithelial cells, pharynx, conjunctiva, or small intestine and usually do not spread beyond the associated mesenteric, cervical, auricular, or lymph nodes. The incubation period is between 5-8 days. Canine adenovirus 1 (CAV-1) causes Hepatitis Contagiosa Canis (HCC), which can be observed in wild canidae, wolves, coyotes, skunks, foxes, and bears. Immune complexes formed after recovery from acute or subclinical infection cause chronic liver lesions and corneal opacification (blue eye manifestation). Deaths can be observed as a result of harm caused by the virus in liver hepatocytes.

Kennel cough disease, which can be observed in dogs, coyotes, bears, pandas, skunks, mongooses, is caused by canine adenovirus-2 (CAV-2). During the disease bronchitis, bronchiolitis, and interstitial pneumonia are observed (Thompson et al. 2010; Walker et al. 2016; Oleaga et al. 2021). In HCC, tonsil crypts and Peyer's patches are affected first. Subsequently, viremia and disseminated infection develop. Primarily vascular endothelial cells, kidney, liver, lung, and spleen are affected. In the systemic disease, 5 forms are observed (Walker et al. 2016).

In the peracute form, offspring die within 1-2 hours as soon as symptoms of the disease begin. Two-phase fever rising to 40°C is observed. One day after the rise in temperature of animal, the leukocyte count decreases. Fever, depression, loss of appetite, and yellowish mucous membranes are detected in the acute form. Mild fever, loss of appetite, and laryngitis are observed in mild form of the disease. In ocular form, opacity occurs in the eye as a result of corneal edema and uveitis due to immune complexes formed during the disease. The disease is also called "blue eye" because of this corneal opacity, which usually heals by itself. In rare cases, CNS form is observed with symptoms of convulsions, ataxia, and blindness due to forebrain damage. This form is generally observed in foxes. Animals affected by CAV-2 develop pneumonia. The disease may be exacerbated by secondary bacterial infections. Sudden onset of dry cough is the most characteristic finding of the disease (Saraç 2016).

Urine, saliva, blood, and stool samples are collected for diagnosis. CAV-1 and CAV-2 differentiation can be made by PCR or RFLP methods. Also, virus isolation can be done in canine cell cultures. Antigen detection can be made by ELISA from infected tissues and by immunohistochemical staining from lymph nodes, spleen, liver, kidney samples of deceased animals. ELISA, hemagglutination inhibition, and neutralization tests are available for serological diagnosis (Dowgier et al. 2016).

There may be temporary opacity after getting attenuated CAV-1 vaccines or viral shedding in the urine. For this reason, attenuated CAV-2 live vaccines are recommended to provide cross-immunity. Maternal antibodies can protect offspring up to 12-16 weeks. Afterward, protection should be provided by vaccination. Although annual repetition of the vaccines is recommended, studies reported that the immunity generated by the vaccine can last longer than 3 years. Considering that domestic dogs are a potential source of the virus as a reservoir, it is recommended by researchers to expand routine vaccination of dogs and reduce their circulation in rural areas to protect many wild carnivores, such as the endangered brown bear (Schultz 2006; Lamberski 2012).

Influenza Virus Infection

The family *Orthomyxoviridae* consists of seven genera and nine species. It was reported that influenza A viruses (IAV) infect leopard, tiger, raccoon, ferret, chicken, duck, horse, pig, bird, human, seal, mink, whale, dog, and cat. Influenza B strains (IBV) are generally found in humans but have also been reported in pigs and seals. Influenza C strains (ICV) have been reported in humans and pigs, while Influenza D strains (IDV) have been reported in Equids and Artiodactyles. The single-stranded negative-sense RNA genome has a segmented structure. Influenza viruses are known to evolve continuously and overcome species barriers. The genetic diversity of these viruses is constantly increasing with new subtypes being discovered. Influenza viruses evolve by antigenic shift and antigenic drift. Antigenic drift is caused by point mutations in the amino acids of the HA and NA genes. Regional and periodic flu epidemics occur with these changes. Antigenic shift is the exchange of RNA genome segments between two or more subtypes. Canine influenza virus (CIV) is a highly contagious viral infection characterized by cough that affects canines of all ages and breeds. Two subtypes, H3N8 and H3N2 infect hounds and dogs. The H3N8 virus was adapted through transmission from horses and the H3N2 virus from birds to dogs. Cats are infected with H3N2, H7N2, H5N1, and H1N1. Influenza virus A (H1N1) has been detected in skunk, raccoon, hyena, and avian influenza (H5N1) in fox, tiger, and leopard (Keawcharoen et al. 2004; Bouvier and Peter 2008; Reperant et al. 2008,).

Transmission of CIV is usually by droplet infection, aerosols, or nasal discharge. Close contact and closed environments facilitate transmission. About 20% of infected animals are asymptomatic, but they can still spread the virus. Influenza virus has affinity for epithelial cells in the respiratory tract. Significant amounts of virus are found in nasal discharge, pharyngeal secretions, and trachea. While low pathogenic strains such as H1N1 and H7N2 infect only the upper respiratory tract, the highly pathogenic H5N1 virus affects both the upper respiratory tract and gastrointestinal tract epithelium (Weber and Stilianakis 2008).

H3N8 and H3N2 cause disease characterized by symptoms of upper respiratory tract infection. Clinical findings such as fever, cough, runny nose, sneezing, weakness, and conjunctivitis are observed. If a secondary infection develops, the runny nose becomes mucopurulent. Pneumonia and bronchopneumonia may develop in severe cases. While the morbidity rate is about 80%, the mortality rate is between 1-5%. Higher mortality rates are observed in puppies and older animals with weakened immune systems (Watson et al. 2017).

Different methods for virus isolation, viral genome, and antibody detection can be used for the diagnosis of influenza virus infection. Nasal and pharyngeal swabs and lung tissue samples of dead animals can be taken for viral genome detection and virus isolation. For this purpose, PCR (including Real-time RT-PCR) technique are mostly used. Neutralization test and hemagglutination inhibition test can be used for serological diagnosis (Trombetta et al. 2018).

Both cellular and humoral immunity are involved in immunity against influenza virus infections. The first defense mechanism of the host is mucosal secretory antibodies (IgA). Animals usually recover within 2-3 weeks unless a secondary infection is involved. Neuraminidase inhibitor antiviral drugs can be used in the early stage of infection. It is recommended to use oseltamivir against highly pathogenic viruses (H5N1). Further studies are needed to determine the influence of commercial vaccines against H3N8 and H3N2 on wild carnivores (Keawcharoen et al. 2004; Bouvier and Peter 2008).

Coronavirus Infection

Coronaviruses have become popular since there are different mammalian and poultry reservoirs, and that they cross the species barrier from these reservoirs and lead to epidemics in humans. The existence of many unidentified coronaviruses raises concerns that many human and/or animal diseases may cause epidemics in the future. The family *Coronaviridae* is in the order *Nidovirales*, in the kingdom Riboviria. *Alphacoronavirus* in

the family *Coronaviridae* has been reported to infect seals (HSCoV), dogs (Canine Coronavirus; CCoV), cats (Feline enteric coronavirus, FCoV; Feline infectious peritonitis virus, FIPV), leopards, cheetahs, hyenas, lions, wolves, humans, and pigs (Transmissible gastroenteritis virus, TGEV), and Betacoronavirus has been reported to infect mice, rats, cattle, horses and pigs, and Gammacoronaviruses infect dolphins, sea lions, foxes, chickens, and turkeys (Goller et al. 2013).

Coronaviruses are positive-sense, single-stranded RNA (ssRNA) and enveloped viruses. The viral genome encodes 4 structural proteins: Envelope (E), spike (S) Nucleocapsid (N) and membrane (M). The S protein, which is responsible for species and tissue specificity, is the main factor determining the pathogenicity of the virus. Viruses in this family generally show an affinity for epithelial cells for replication. The frequency of recombination shaped within the family allows for an increase in genetic diversity (Núñez-Nogueira et al. 2021).

FIPV affects all domestic and wild felines and is fatal especially in young and very old animals. The disease is transmitted to susceptible individuals by the oral-fecal route (Goller et al. 2013).

The main event that initiates infectious peritonitis is the productive infection of enteric coronavirus variants in monocytes and macrophages. The characteristic lesions of the infection are formed in the small blood vessels. Vascular disorders and leakage of blood fluid from the vessels result in pathogenesis specific to wet form. Cytokine and arachidonic acid-derived inflammatory regulators cause changes in vascular permeability and trigger a viral infection in macrophages. In the wet form of the disease, symptoms such as accumulation of fibrous, viscous, clear yellow-colored fluid in the abdominal cavity and gray-white nodules 1-10 mm in diameter on the surface of the liver, spleen, omentum, kidney are observed. Fibrinous polyserositis is not observed in its dry form although lesions and pathogenesis are similar. Although the mechanism that determines which form of FIP will develop in a cat is not known exactly, it is thought that individual differences between strains and animals may cause this. Central nervous system (CNS) findings are more common in dry form FIP in cats under 2 years of age (Benetka et al. 2004; Pedersen et al. 2008).

Affected cats exhibit anorexia, chronic fever, weight loss, and malaise. Ulcerative colitis due to the involvement of the colon and caecum is a typical finding of dry FIP. In the wet form of the disease, death mostly occurs within a month with accumulation of fluid in the abdominal cavity, while the clinical course is much slower in the dry form (Benetka et al. 2004).

As no single method is sufficient for diagnosis, all tests and clinical symptoms should be evaluated together. Necropsy is known as 'gold standard' especially for the diagnosis of FIP infection. Antigen detection by RT-PCR, immunofluorescence, immunoperoxidase tests, serum neutralization test, fluorescent antibody technique are diagnostic methods. ELISA can be used to detect antibodies from blood and fluids accumulating in the body cavity. Cat-derived cell cultures are used for virus isolation (Hartmann et al. 2003).

It is not easy to manage the disease, as the virus must be eliminated in all felines. Although there are commercial vaccines used primarily to protect pet animals, their effectiveness is disputed. Antiviral drugs (Remdesevir), which are long-term nucleoside analogs, and preparations containing interferon have a positive contribution to the treatment (Takano et al. 2020).

Immunodeficiency Virus

Viruses in the family *Retroviridae* are positive-sense, single-stranded RNA (ssRNA) and are enveloped. Retroviruses with a diploid genome of approximately 8-11 kb have two genomes in their virions (Pseudodiploid genome). The genome of viruses in the *Retroviridae* family has 4 different gene regions characteristically, gag (synthesis of group-specific antigens such as matrix protein, nucleoprotein, capsid), pro (protease synthesis), pol (reverse transcriptase and RNase-H synthesis), and env (envelope and receptor binding sites). There are 5 genera in this family: *Alpharetrovirus* infects chickens, *Betaretrovirus*, which infects sheep and goats; *Gammaretrovirus* causes "feline leukemia" of domestic and wild cats; *Deltaretrovirus* infects cattle; *Lentivirus* infects sheep, goats, Equidae, domestic and wild cats, cattle and buffaloes. Virus in *Retroviridae* family use reverse transcriptase enzyme during replication (Friend et al. 1990; Yamamoto et al 2007; Frankenfeld et al. 2019).

Feline Acquired immunodeficiency syndrome (Feline AIDS, Feline immunodeficiency virus-FIV), which is similar to AIDS in humans, has been detected in both domestic and wild cats all over the world. FIV, which is in the genus Lentivirus, persists in cats and causes immune system damage. Infection-related deaths are common (Friend et al. 1990).

FIV can be isolated from blood, plasma, serum, saliva and cerebrospinal fluid. In areas where cat populations are dense and cats stray freely, transmission through bite wounds is common. Since male cats bite more than females, it is more common in males. Also, it can be transmitted through intrauterine, venereal, perinatal, and breast milk (Beebe et al. 1994).

The agent can reproduce in B lymphocytes, helper T lymphocytes (CD4), cytotoxic T lymphocytes (CD8), macrophages, astrocytes and microglia cells (Roukaerts 2017). While the primary receptor of FIV is CD134 (OX40), both human and feline CXCR4 can serve as co-receptors as chemokine receptors. During virus replication, the virus integrates its information into the host chromosome. This DNA is called "proviral DNA" and contains all the genetic information of the virus. Proviral DNA can be detected in lymphocytes as early as 5 days post infection and 10 days later in various organs (Zhang 2002).

Clinical findings of the disease include fever, diarrhea, conjunctivitis, gingivitis, icterus, neutropenia, secondary bacterial sepsis, and generalized lymphadenopathy. Mesenteric and peripheral lymph nodes enlarge. Clinical signs continue from a few days to a few weeks. Later, the infection in cats passes into the latent stage (subclinical). In this way, the virus can persist for 5 years or longer. During this period, virus isolation can be done. Some cats can carry the virus for life with very few clinical signs. Afterward, non-specific changes such as fever, lymphadenopathy, leukopenia, anorexia, anemia, and weight loss can be detected in the period that can extend from 6 months to several years. Opportunistic infections come into play in the last stage of the disease (Norris et al. 2007; Yamamoto et al. 2007).

While the virus can be detected by tests such as virus isolation from blood, PCR, ELISA, tests such as immunofluorescence (IF), ELISA, Western Blot (WB) can be used for serological diagnosis (Nichols et al. 2017; Frankenfeld et al. 2019).

It is recommended to neuter domestic cats and keep them away from stray cats. Deveoplemnt of vaccine is under process,

but it seems very difficult to produce effective vaccines against retroviruses due to their biological properties. Chemotherapy methods learned from HIV studies can be applied to cats infected with FIV. In the present day, azidothymidine (AZT) preparations are used in veterinary medicine. The drug increases both life expectancy and quality of life (Hartmann et al. 2015).

Rabies

Within the family *Rhabdoviridae*, there are *Lyssavirus, Vesiculovirus, Novirhabdovirus*, and *Ephemerovirus,* which infect humans and animals, and *Nucleorhabdovirus* and *Cytorhabdovirus*, which infect plants. Classical rabies virus, Lagos bat, Mokola, European bat rabies virus-1, European bat rabies virus-2, Duvenhage, Australian bat rabies virus, which is in the genus Lyssavirus, are closely related viruses. All of these viruses cause rabies-like disease in animals and humans. The Rhabdovirus genome is in the form of negative polarity single-stranded RNA. Although rhabdoviruses are resistant to environmental factors in alkaline PH conditions, they are not resistant to UV rays and heat (Consales and Bolzan 2007; Pantha et al. 2020).

According to The World Health Organization (WHO), 35,000-60,000 people die annually due to rabies. Although the majority of human cases are caused by dog bites but skunks, foxes, and raccoons play an important role as reservoirs in the wild animals. In many regions, rabies and rabies-like viruses are transmitted by bats (Bilal 2021).

Rabies virus has many reservoir hosts such as dog, bat, fox, wolf, raccoon, skunk, coyote mongoose. Dogs are most common reservoir and about 95% of human deaths due to rabies were associated with dogs. After the reservoir species, final hosts such as humans and animals are infected. Although the most important source of infection is the transfer of the virus in the saliva to sensitive individuals by biting, there are various ways of transfer of virus such as tissue and organ transplantation, aerosol scattering, and contamination of open wounds with infected saliva (Hampson et al 2009). The disease is endemic in all continents, except for Australia and Antarctica. The disease raises an important public health concern in Asia and Africa. In the Asian subcontinent, it is most common in India and Bangladesh, and moderately common in Bhutan, Nepal, Thailand, Indonesia and Myanmar. Cyprus, Bahrain, Hong Kong, Lakshadweep, Japan, Maldives, Qatar, Malaysia, Singapore, Andaman, and the Nicobar Islands of India and East Timor, located in the Asian subcontinent, are rabies-free countries. Antigua and Barbuda, Bahamas, Belize, Falklands, Barbados, Jamaica, Saint Kitts, and Tobago, Nevis, Trinidad, Albania and Uruguay, Macedonia, Greece, Iceland, Finland, Gibraltar, Malta, Isle of Man, Portugal, Norway (except Svalbard), Spain (except Melill + Ceuta) and United Kingdom have rabies-free status. The group of islands such as Cook Islands, Fiji, Vanuatu, French Polynesia, Guam, New Zealand, Solomon Islands, New Caledonia, and Papua New Guinea are also rabies-free. According to the definition of the World Health Organization (WHO), a country can claim rabies-free status if it has not had a case record for animal or human rabies within two years. The susceptibility of animals varies according to the genetic structure, age of the animal, species, biotype of the virus, dose, and exposure route. In Rabies, which is endemic worldwide, they have managed to reduce the number of cases with the control programs implemented by some countries such as the United States. Low human mortality in many developing countries is due to cultural beliefs, under-reporting, inadequate or poor rabies diagnostic units, and insufficient knowledge of the mode of prevention and transmission of the disease. (Leung et al. 2007; Morters et al. 2013; Pantha et al. 2020; Bilal 2021).

At start, virus reproduces in the muscle cells (myocytes) in the bitten area and then it reaches the peripheral nerve endings and enters the nervous system with acetylcholine receptors located here. Various cell receptors such as G protein nicotinic acetylcholine play an important role in interaction with p75 neurotrophic receptors on neural cell membranes and neural cell adhesion molecules. With the infection of the neurons, the virus reaches the central nervous system (CNS) via a centripetal route along the axons at a retrograde rate of 12 to 100 mm/min. Clinically observed aggression is observed at this stage. Whether the disease is in an aggressive or calm form is related to how widely the virus spreads and reproduces in the CNS. Factors such as genotype of the virus, the dose, the severity of the bite, and the distance from the bite site to the brain are important in the emergence of disease symptoms. The virus multiplying in the CNS spreads to peripheral tissues and organs by the centrifugal route. Virus reaching the salivary glands in this way is shed in high concentrations from the mucous cells on the apical surfaces. Clinical symptoms occur within 10 days after the virus reaches the salivary glands from the CNS and then animal dies. Eosinophilic intracytoplasmic inclusion bodies (Negri bodies) in neurons observed in histological examinations of brain tissue of animals that died due to rabies are pathognomonic. Negri bodies are particularly observed in Purkinje cells of the hippocampus and cerebellum (Consales and Bolzan 2007; Abraham et al. 2017; Singh et al. 2017).

The incubation period is between 30-90 days on average but can extend from few days to several years. In general, multiple deep lacerations to the head and bites from the neck area are associated with short incubation times. The prodromal period lasts 2 to 10 days and has been associated with viral invasion of the central nervous system. Non-specific prodromal symptoms include influenza-like symptoms and hyperesthesia, paresthesia, pain, and itching at the viral entry site. It can occur in encephalitic (aggressive) or paralytic (paralysis) forms. The brain stem is involved in both clinical forms, but there are no clinical signs of dysfunction in this part of the brain. Differences in tropism in the CNS or inoculation site, differences in spreading pathway, or triggering of the immune cascade in the brain stem are thought to be responsible for clinical variations. The progression of the disease is of two types: the encephalitic (aggressive) form, which is seen in about 80% of the patients, and the paralyzed form, which is seen in about 20%. T-cell immunity to rabies and high serum concentrations of interleukin-2 and interleukin-6 are observed in the aggressive form while in paralytic form such responses are lacking. Autonomic dysfunction (lacrimation, hypersalivation, mydriasis, sweating, and hyperpyrexia), hydrophobia, hyperexcitability and aerophobia are characteristic features of encephalic rabies patients. Hydrophobia and aerophobia develop due to painful contraction of the pharynx and larynx while drinking water. (Consales and Bolzan 2007; Hampson et al. 2009).

For the diagnosis of rabies in dead or killed animals, the viral antigen is usually sought in brain tissue by immunofluorescence or immunohistochemical tests. For genome detection, viral nucleic acid detection can be made from brain tissue, cerebrospinal fluid (CSF), biopsy material from the hair follicle

in the nape, saliva, and cornea by RT-PCR and real-time RT-PCR methods. Besides this, virus isolation can be performed in BCC cell culture and experimental animals. Indirect diagnosis can be made by determining the antibody titer in CSF and double blood serum samples taken at 3-4 days intervals. Neutralization test (Rapid Fluorescent Focus Inhibition Test (RFFIT) and fluorescent antibody virus neutralization (FAVN)) are accepted as reference tests for antibody detection by WHO and World Organization for Animal Health (WOAH). However, ELISA, avidin-biotin dot ELISA, blocking ELISA, MAb based capture ELISA, sandwich ELISA tests can also be used. Serological methods are used especially during international transport of pet animals, in-field controls of wildlife after oral vaccination, and to evaluate post-vaccination immune response in humans (Morters et al. 2013; Singh et al. 2017).

WHO and WOAH recommend the use of inactivated vaccines produced in cell culture for parenteral immunization. In countries where it is endemic, these vaccines are recommended to be started at the age of 3-6 months and repeated once a year in animals. The first rabies oral vaccination campaign was carried out in Switzerland in 1978 for wildlife and then spread to other European countries. In Europe, strains of rabies virus such as SAD Bern, SAD B19 and SAG 2 are used for oral immunization in the wildlife. Exotic Asian raccoon dog, arctic fox, the red fox have been regularly vaccinated with recombinant vaccinia virus expressing rabies virus glycoprotein G in oral wildlife vaccination campaigns for the last 15 years for rabies control in North America. And Europe. Countries such as Yemen, Israel, Iran, Saudi Arabia, Oman, and Turkey in the Middle East are dealing with increasing problems due to wildlife rabies. In these countries where rabies is endemic, it is recommended to monitor antibody titers every 2 years together with vaccination (Pfaff et al. 2019).

Although there are still high-risk countries for rabies, there are many countries which are declared as rabies-free. This shows us that rabies is a preventable disease, with effective protection and control measures, public awareness on this issue, and of course high-level political commitment.

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CHAPTER 21

PATHOGENESIS OF GLUTEN-SENSITIVE ENTEROPATHY IN DOG

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INTRODUCTION

Celiac disease (CD) is the name given to gluten-sensitive enteropathy (GSE), which was first discovered in humans (Batt et al. 1984). The disease has been frequently recorded in Irish Setter dogs in England since 1984 (Batt et al. 1984, 1987). Partially villous atrophy, the intestinal mucosa infiltrated with lymphocyte plasma cells, and abnormalities of brush border are all symptoms of this naturally occurring SGE (Batt et al. 1987; Hall & Batt 1990a, b). Gluten triggers an endogenous toxicity mechanism that initiates a cascade of immunological reactions that result in intestinal lining destruction and celiac disease (Stamnaes & Sollid 2015; Juhász et al. 2018).

Increased intraepithelial goblet cells and lymphocytic-plasmacytic infiltration are the first morphological changes detected in the intestinal mucosa of Irish Setters fed gluten at the age of four months. This occurs before villous atrophy and selective changes in brush border enzymes (Hall & Batt 1990b; Hall et al. 1992). These findings have been compared to celiac disease in humans. It has also been validated that susceptible dogs on a gluten-free diet are resistant to gluten challenge, implying that intestinal damage is a result of gluten sensitization, which may be an age-related occurrence (Hall & Batt 1991c; Hall & Batt 1992).

Genetic studies concluded that gluten sensitivity in Irish Setters does not resemble celiac disease in humans (Polvi et al. 1998) and that inheritance is autosomal recessive (Garden et al. 2000).

Due to genetically transferred, Irish Setters acquire wheat-dependent incomplete-villous atrophy with lymphocyte infiltration in intestinal mucosa, and so this model most closely resembles early stages of Celiac disease pathogenesis in newborns that are related with MHC II-dependent (Elli et al. 2015). When on a wheat-containing diet, the Irish Setter model had no elevated levels of antigliadin antibodies, this is showing a purely innate (non-adaptive) response to gliadin (gluten) in these animals, this argument supports the hypothesis that a strong aberrant innate response to gluten may activate or enhance the $CD4^+$ T-cell response to gluten and gliadin (Hall et al. 1992).

GSE is incredibly difficult to diagnose. A non-invasive technique for screening both at-risk patients and the broader public serological assays established in the previous two centuries. The diagnosis is made based on the presence of particular autoantibodies (anti-transglutaminase type 2 IgA) and corresponding features on affected intestinal structures (Ensari 2010; Osman et al. 2012; Elli et al. 2015). The class of drugs had been evaluated or will shortly undergone through clinical trials for GSE. Making modified grains free of immunostimulatory sequences is one option for treating the gluten antigen. This might be done through traditional breeding or, more likely, through genetically modified organisms (Gottlieb et al. 2015).

Keywords: GES, Gliadin, Gluten, Irish Setter, Zonulin.

Epidemiology

Prevalence

The Irish Setter was the first animal model of GSE. Studies conducted in the 1980s discovered that when the Irish setter was fed a wheat-containing diet as a puppy, it developed incomplete villous atrophy and intraepithelial lymphocyte infiltration (Batt et al. 1985). Garden et al. (2000) claimed that GSE is inherited as an autosomal recessive trait.

They add more grains especially those rich in gluten, during processing of feed for economic reasons. Eating gluten in large quantities has resulted in a rise in the prevalence and incidence of gluten allergy (Volta et al. 2013). Sometimes even gluten was detected in samples of food labeled as 'free from grains' and 'free from gluten' diets as a result of productive side contamination (Meineri et al. 2020).

Gluten sensitivity is the cause of sensitive enteropathy. Because the dog's immune system reacts when the dog eats gluten-containing items, gluten-sensitive enteropathy is classified as an autoimmune illness. The disease is idiopathic, but genetic factors are thought to have a role in the development of CD (Verlinden et al. 2006). GSI has been seen in a small number of dogs, but not in cats (Gaschen & Merchant 2011). The diseased pups came from a single-family line of Irish Setters and a group of Border Terriers, and the ailment may be shown in puppies as young as three months old. In Irish Setters, digestive issues including inappetence, chronic diarrhea, and weight loss, as well as growth retardation in young animals have been reported in association with gluten consumption. Clinical indications usually appear at six months of age (Daminet 1996). Gluten intake, on the other hand, has been linked to paroxysmal gluten-sensitive dyskinesia (PGSD) in Border terriers (Lowrie 2017). Park et al. (2014) reported a case of PGSD in a nine-

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month-old Yorkshire Terrier. A gluten-free diet is beneficial to these pets. Gluten-free diets are not found to be better for healthy pets than other nutritionally full and balanced diets (Gujral et al. 2012). Canine epileptoid cramping syndrome has also been connected to the consumption of gluten-containing foods in Border Terriers (Black et al. 2014).

Gluten in Grains

Grains are nutrient-dense food for pets. Grains are the seeds of cereal grasses like oats, barley, and corn, which assist the body to meet its critical requirement for glucose, an energy source (Sharma et al. 2013). All grains have 65-75 percent complex carbs and less than 2% sugar. Protein, fiber, vital fatty acids, B vitamins, and minerals are all present in them (Lafiandra et al. 2014).

Wheat's particular baking characteristic is due to gluten proteins. They are made up of two primary categories of linked glutamine and proline-rich proteins (Wieser 2007) which are insoluble in aqueous alcohol glutenins (glutelins) and alcohol-soluble gliadins (prolamines) respectively. Gliadins are monomeric proteins with a variety of secondary configurations. Glutenins are characterized by big, clumpy features (Schalk et al. 2017). Hordeins in barley, avenins in oats, and secalins in rye are all gluten-like proteins that are comparable to wheat gliadins. The two gluten-protein fractions, gliadins, and glutenins were quantified in wheat, barley, rye, and oat flour, each containing a proportional mixture of four cultivars, using analytical reversed-phase high-performance liquid chromatography (RP-HPLC) (Schalk et al. 2017).

Gluten in Dog Food

Glutens are a type of protein found in grains that can cause allergic reactions, but not all glutens are created equal (Biesiekierski 2017). Gliadins are proteins found in wheat, barley, rye, and spelt that can cause celiac disease symptoms (Lacorn et al. 2018).

Gluten-free is generally grain-free, but grain-free is not necessarily gluten-free. Only non-gluten grains, such as corn and/or rice, are allowed in dog food. Gluten hypersensitivity in many dog owners is likely to hunt for gluten-free food or be drawn by this characteristic (Allred & Park 2012). Corn gluten is listed in certain dog food ingredient lists. Gluten immunoassays do not recognize any of the proteins found in corn or rice (Morón et al. 2008). There is no evidence of canine hypersensitivity to corn, rice, barley, rye, or oats, however, wheat allergy has been recorded in dogs (Verlinden et al. 2006).

Several commercially available immune-chromatographic gluten assays are accessible (Allred & Park 2012; Lacorn et al. 2019). Gluten-free test kits are used to check for gluten in meals and beverages. Antibodies that respond with both gliadins and glutenins are used in specific testing (Allred & Park 2012). Uncontaminated rice and corn were found to be gluten-free in the tests, which identify gluten levels as low as 10-20 mg/kg product (Allred & Park 2012; Lacorn et al. 2019).

Pathogenesis

The pathophysiology of canine enteropathies has been discovered to be multifactorial in the last 15 years, involving an abnormally powerful cell-mediated immune response in genetically vulnerable dogs induced by a lack of tolerance to antigens in the diet and a complex commensal microbiota (Day et al. 2008).

Immunogenicity of Gluten Associated with the Pathogenesis

Dogs lived for thousands of years by eating raw, fresh, and entire food. Since the last era, commercially processed food was brought to provide accessible food. In developed countries, dogs' lifestyles and environments have changed. As a result of these changes, improved hygiene, and a lack of exposure to diverse microbes, "dogs diseases" have grown by 80 percent in the last few decades. Inadequate dietary nutrients and processed commercial dog food have been linked to an increase in sickness, allergies, enteropathy, inflammation, autoimmune disease, obesity, kidney/liver disease, and digestive problems (Verdu et al. 2015).

Indeed, the recent increase in the number of GSE diagnoses may have been impacted by the introduction of novel grain types for technological rather than nutritional reasons (de Lorgeril & Salen 2014; Lowrie et al. 2015).

GSE in dogs has similarities in morphology and biochemical feature with Celiac disease in humans and pathogenesis appears to be mediated by cell-mediated immunity rather than humoral immunity (Biagi et al. 2019).

Gluten consumption activates autoimmune enteropathy to gluten-sensitive enteropathy in dogs that are hereditary susceptible. Moreover, the increased prevalence is associated with other factors that include the amount and quality of dietary gluten and the introduction of novel grain types for technological modification in feed (de Lorgeril & Salen 2014; Leonard et al. 2020).

Gluten is made up of many non-digestible peptides which are immunogenic and resist being digested regularly (Silano et al. 2009). Gliadin is made up of peptide sequences called epitopes that are resistant to proteolytic processing in the gastrointestinal tract, enabling it to avoid destruction in the intestine. This occurs due to high quantity of amino acids, glutamine, and proline in gliadin which cannot be broken down by many proteases (Wieser 1996). In the period of enteric illness, the immune system become active and this condition may aid in breaking the tolerance to gluten dietary antigen (Silano et al. 2009).

In result of incomplete digestion, combination of peptides are obtained that cause host reactions, increase intestinal absorptivity, also stimulate innate and adaptive immune responses similar to those triggered by the presence of potentially hazardous microbes (Shan et al. 2002).

It's been proposed that a fundamental permeability problem could be the root of gluten-sensitive enteropathy pathophysiology (Menzies et al. 1979). According to this view, an increase in mucosal permeability permits ingested gluten or peptides formed by gluten digestion to pass through the intestinal epithelium, causing mucosal damage by direct toxicity or immune-mediated manners (Behrens et al. 1987).

The presence of two alpha-gliadin receptors has been discovered, and it is possible that they alter gut barrier activity by attaching to chemokine receptor 3 and inducing the synthesis of zonulin, which causes the interepithelial junctional complex to disintegrate. If gluten tolerance is compromised, gluten can potentially get through the intestinal

barrier via the transcellular pathway (Tripathi et al. 2009; Caio et al. 2019).

Importantly, given the availability of dietary cereal, the absence of alkaline phosphatase and aminopeptidase N action in brush border was a unique result of this enteropathy, as disaccharidases were intact. In an investigation of pups reared solely on a cereal-free diet, no structural or biochemical evidence of intestinal damage was detected. An experiment involving two groups of littermates, revealed significant permeability to 51Cr-ethylenediaminetetraacetic acid (EDTA), implying that impairment in permeability may be implicated in the disease development (Hall & Batt 1991a).

The role of the Gut Microbiome in the Pathogenesis of GSE

Considering that the immunity of the intestine may frequently be exposed to a diverse spectrum of antigens derived from food and xenobiotic substances, endogenous microflora, and pathogenic organisms, immunopathology in wounded mucosa develops (Dandrieux et al. 2008).

The enteropathy affects the permeability to nurturing absorption resulting in the changes in the intestinal environment which ultimately causes changes in the structure of a dog's gut microbiome. These factors may influence the maintenance of a healthy microbiome. The gut microbiome is linked directly and indirectly to their general health (O'Mahony et al. 2015).

The gut flora interacts with the host on a spectrum ranging from health to disease; in the initial section of the gastrointestinal tract, dietary components have an impact on the microflora's composition and metabolism (Eissa et al. 2019). This proposed that intestinal microbes play role in the pathophysiology of gluten-sensitive enteropathy. The specific microorganisms implicated and the underlying processes in gluten-sensitive enteropathy remain unknown. New evidence from gnotobiotic studies suggests that intestinal microbiota has a complicated monitor in responses of host immunity to gluten (Belkaid & Hand 2014; Verdu et al. 2015).

Mechanisms of Gluten Trafficking from Lumen to Lamina propria and Enhancing Signaling Pathway

The digestive and absorptive processes of the gastrointestinal system have been studied extensively. In phases of gluten's biological effects, scientists found that repeated gluten uptake changes the villi to finger-like structures to become chronically inflamed and damaged. These changes prevent them from acting their normal function of breaking down food and diverting nutrients through the gut wall to the blood circulation (Fasano 2011). The stimulation of the zonulin pathway by food-derived environmental triggers or alteration in gut microbiota, along with genetic susceptibility, miscommunication in both innate and adaptive immunity, and exposure to environmental stimuli, all appear to play a role in the pathogenesis of inflammation and autoimmune disease as seen in Figure 1. It's the only situation in which the presence or absence of a single environmental trigger, gluten, can switch the disease progression on or off. GSE is an extremely useful model for understanding autoimmune conditions (Fasano 2011).

Zonulin is a protein found in both animals and humans that helps to maintain intestinal barrier's integrity. In genetically predisposed cases, deregulation of the zonulin signaling is postulated to increase intestinal leakage and possibly autoimmune syndromes. The basic autoimmune disorder paradigm comprising of unique gene composition, dysbiosis of the microbiota, defective innate-adaptive immunity interactions and exposure to environmental variables, induces tight junction displacement that causes loss of the intestinal barrier junction. The mechanism by which aberrant antigen is transited from the luminal intestine, generates an inflammatory reaction and is assumed to be intestinal hyperpermeability, which is associated with autoimmune disorders. This innovative and creative treatment technique suggests that autoimmunity is not self-perpetuating and that autoimmune impairments can be prevented or treated by altering the interplay between genes and environmental triggers by restoring intestinal barrier function (Fasano 2012).

Prior studies in similar animals, however, had abortive to prove the existence of antigliadin and anti-tTG antibodies, as well as a link between MHC class II and illness (Polvi et al. 1997; Polvi et al. 1998). Antigliadin and anti-tTG immunoglobulin G (IgG) were found in the sera of dogs with enteropathy in one investigation; nonetheless, the link between canine enteropathy and human CD remains largely unknown (Vincenzetti et al. 2006).

Further research found that wheat-sensitive Irish Setters exhibit higher permeability to 51CrEDTA and probe sugars, as well as lower anti-gliadin antibody concentrations, than healthy Irish Setters, unrelatedly of whether they were fed a wheat-free or wheat-containing diet (Hall & Batt 1991b; Garden et al. 1998). GSE in Irish Setters isn't the same as CD in humans, according to genetic studies (Polvi et al. 1998), and that inheritance is autosomal recessive (Garden et al. 2000).

In terms of the pathophysiology of gluten-sensitive enteropathy in humans, competent tight junctions that limit macromolecule transit prevent gliadin from reaching submucosal tissue under normal physiological conditions. Tight junctions are disintegrated in people with the HLA-DQ2 or DQ8 haplotypes, permitting gliadin to pass through and prompting gliadin-induced immunological reactions, ultimately developing CD (Lammers et al. 2008). A set of processes that occur after gluten exposure are hypothesized to promote autoimmune disease. Gliadin and its immunomodulatory and inflammatory fragments bind to chemokine C-X-C receptor 3(CXCR3) in the intestinal lumen, inducing MyD88-dependent zonulin release and subsequent TJ disintegration. When the functional amide group is eliminated by tTG, gliadin peptides pass through the epithelium across opening TJs and connect to HLA molecules on the antigen-presenting cell surface (APC). As a result of this, Zonulin and cytokines that rely on My88 are released. HLA-gliadin peptide complexes presented by APCs activate T-lymphocytes, resulting in humoral (B cell activation leading to plasma cell release of anti-gliadin antibodies (AGA), N-arachidonoylethanolamide (AEA) and anti-tissue transglutaminase (tTG) antibodies) and cell-mediated (natural killer cells that destroy epithelial cells via cytokine release) feedback (Smyth 2017).

In the ideal situation, pattern recognition receptors such as toll-like receptors (TLRs) will continuously select antigens from food and the microbiome in the intestinal lumen. This procedure triggers antigen-presenting cells in the lamina propria, causing T regulatory cells to generate anti-inflammatory cytokines interleukin-10 (IL-10) and tissue growth factor (TGF) when only food antigens and typical

commensal microorganisms are noticed. Even though no infections have been discovered in the instance of dog enteropathy, sampling of typical commensals and dietary antigens causes T-lymphocytes to proliferate and produce pro-inflammatory cytokines (Day et al. 2008).

An underlying permeability issue appears to exist in groups of Irish Setter dogs who were given a gluten diet challenge, which may have allowed gluten antigens to enter and result in intraepithelial lymphocyte infiltration. In all sensitive setters in response to gluten, high permeability can increase intraepithelial lymphocyte density which does not always predict instantaneous intestinal harm (Hall & Batt 1992). There has been speculation that there is no concrete evidence that intraepithelial lymphocytes are directly responsible for disease development, and that their role may even be helpful. In untreated gluten-sensitive enteropathy, their increased quantity concerning the number of enterocytes is mostly due to an increase in the density of suppressor T cells (Freedman et al. 1987). Gluten's potential to cause significant intestine damage in Irish setters appears to be linked to early gluten exposure. An early gluten exposure allows the manifestation of intestinal damage, while the absence of early exposure appears to diminish gluten's harmful effect (Hall & Batt 1992).

Diagnosis

Accurate diagnosis of GSE may lead to rapid gluten-free diet treatment, restoring health, and preventing the progression of GSE-related problems (Shakeri et al. 2009). Therefore, several tests are performed to obtain a preliminary diagnosis of GSE (Gheller-Rigoni et al. 2004).

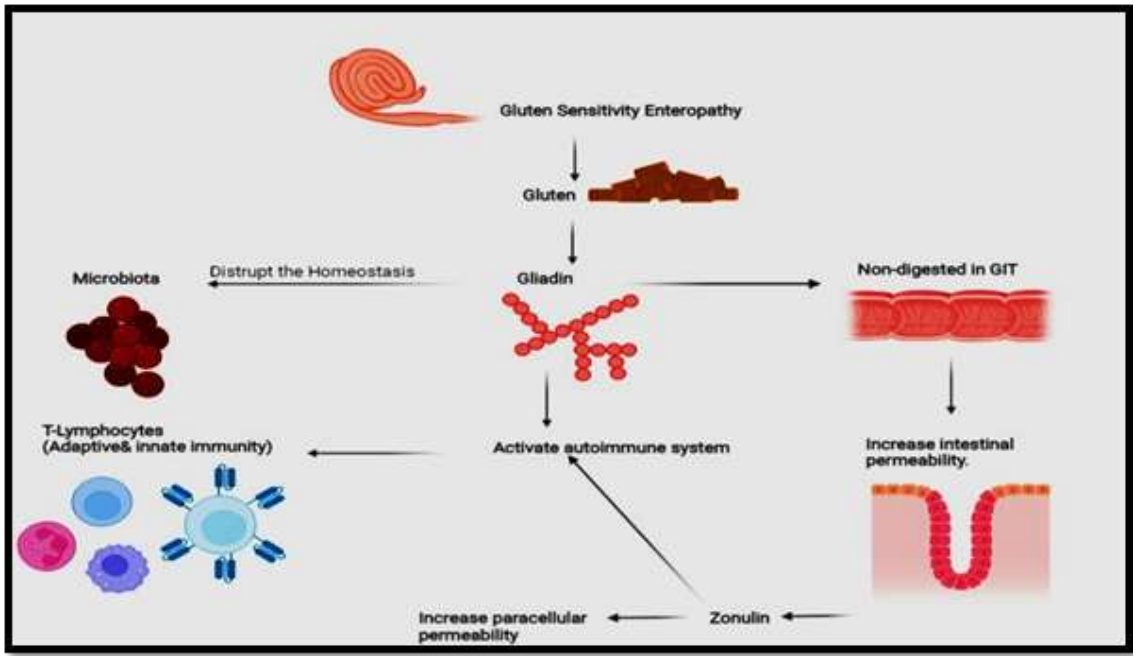

Fig. 1: The pathogenesis of GSE, Gladin important component of Gluten that activates the immune system via disturbing GIT homeostasis and increasing intestinal permeability. Author designed graph by using biorender.com.

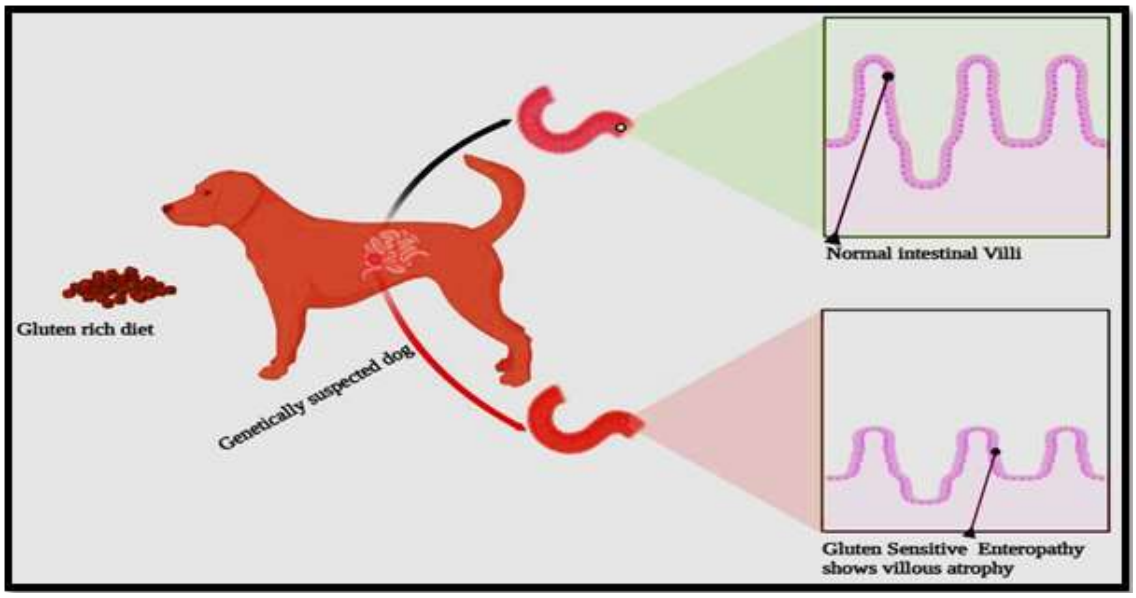

Fig. 2: Graphic presentation reveals the differences between healthy dogs with genetically suspected animals that demonstrate the reduction in the villi length as the mucosal involvement. Author designed graph by using biorender.com.

Clinical Signs

Affected dogs may show the signs of classic chronic enteritis such as intermittent diarrhea, inappetence, weight loss or inability to gain weight, and occasional vomiting (Garden et al. 2000).

Clinicopathologic Examinations

GSE can manifest itself in a variety of ways, thus veterinarian should be aware of the different clinical findings it might cause. As a result of improved awareness of the disease's processes, more canines are being diagnosed through utilizing different tests (Lohi et al. 2007). Moreover, the majority of clinical alterations can occur in result of GSE-induced damage to the small intestine, as plasma protein leaks into the intestine's lumen, resulting in hypoproteinemia. Biochemical tests can indicate low levels hypoglobulinemia, hypopal-buminea, hypocholesterolemia, hypocalcemia, hypokalemia, hypomagnesemia and hypochloremia (Donnini et al. 2021). Furthermore, hematological examination determines an iron shortage, neutrophilia, and thrombocytosis, as well as a significant drop in serum folate and cobalamin concentrations due to malabsorption (Allenspach et al. 2007; Craven et al. 2009). Also, many serologic indications can be utilized to assess the dogs with gluten-sensitive enteropathy or subsequent reactions to gluten-free diet therapy. IgA tissue transglutaminase antibody (IgA-tTG) test and IgA endomysial antibody (IgA-EMA) test are two serological tests that are now used in clinical practice. IgA and IgG (AGA) antigliadin antibodies show considerable sensitivity but are less used than IgA-EMA and IgA-tTG antibodies. Antigliadin antibodies are no longer suggested as a screening test as the existence of higher sensitive and specific EMA and tTG antibody tests replaced it. Anti-tTG immunoglobulin G (IgG) and antigliadin antibodies were detected in the serum of dogs with GSE in a prior study (Gheller-Rigoni et al. 2004; Vincenzetti et al. 2006).

Histopathology

Gluten-sensitive enteropathy can be diagnosed by using tissue samples from the distal section of the duodenum or the proximal jejunum, which are susceptible to the highest amounts of dietary gluten. Extensive lymphocyte infiltration from the lamina propria ascended to the surface epithelium has been observed microscopically. Additionally, the villus lengths are reduced with hyperplastic changes in the crypts (Figure 2). Also, the morphology of enterocytes changes from columnar to cuboidal, with numerous vacuoles visible in their cytoplasms (Lowrie et al. 2016; Matsumoto et al. 2018). The malabsorption is most likely caused by the reduction of mucosal and brush-border surface area caused by villous atrophy. Furthermore, greater epithelial turnover, as seen by increased crypt mitotic activity, may impair absorptive enterocytes' ability to properly differentiate and express proteins required for terminal digestion and transepithelial transport. Increased number of plasma cells, eosinophils, and mast cells, exclusively in the upper section of the lamina propria, are the other pathologic characteristics of gluten-sensitive enteropathy. It is important to keep in mind that intraepithelial lymphocytosis and villous atrophy can occur in multiple situations, including viral enteritis. As a result, the diagnosis of gluten-sensitive enteropathy is most specific when histologic and serologic evidence is combined (Freeman et al. 2011; Simpson & Jergens 2011).

Differential Diagnosis

Finally, dogs should be examined for any concurrent clinical problems that could indicate chronic enteritides such as inflammatory bowel disease, lymphangiectasia, metabolic disorders, parasitic infestation (roundworms, hookworms, and Giardia), and dietary allergic reaction (Simpson & Jergens 2011).

Treatment

Changing the Food Component

All GSE therapies begin with a gluten-free diet, suggested to all patients after a correct diagnosis has been established (Sollid & Lundin 2009).

Cereal grains such as wheat, barley, rye, and closely related cereals such as spelt are not included in a gluten-free diet (a wheat breed). Despite uncommon occurrences of oat sensitivity, oat is a related grain that is widely accepted. Oats are intolerant in almost all celiac, and there have been rare occurrences of oat intolerance. Many commercially available oat products are tainted with additional contaminants (Saturni et al. 2010).

Wheat starch as a bread base increases the gluten in the overall load, while wheat starch as a flour adds gluten to the entire bag (Huang et al. 2021).

Certain states, but not all, allow wheat starch to be used as a bread ingredient, which adds gluten to the total load. Another gluten-free food is beer, which is produced from malted barley protein (Lynch et al. 2016).

Alternative and Novel Treatments

Two of the treatments intend to block gluten from interacting with the mucosal immune system that include oral enzyme supplementation to accelerate up the breakdown of gluten into non-immunostimulatory pieces and the addition of a polymer to sequester the gluten proteins (Caputo et al. 2010; Van Buiten & Elias 2021).

Two medicines were tested to deal with the issue of oral glutenase supplementation. ALV003 is made up of microorganisms that produce a glutamine-specific cysteine protease (EP-B2) and a proline-specific prolylendoprotease. Both enzymes function together in the gastrointestinal tract and have a gastric effect (Geßendorfer et al. 2011; Wei et al. 2020).

Another enzyme that breaks down gluten and has gastric activity is Aspergillus niger's prolyl endoprotease (Salden et al. 2015).

For GSE, this class of medicines was examined or was going to be evaluated in clinical studies. Making modified grains devoid of immunostimulatory sequences is one strategy that also addresses the gluten antigen itself. This might be accomplished through traditional breeding or, more likely, through genetically modified organisms (Gottlieb et al. 2015). This technique is difficult to implement due to the vast number of unique peptide epitopes found in many different classes of gluten proteins and gliadins, as well as glutenin,

which were encoded at separate loci in the wheat genome (Sharma et al. 2020).

Another strategy tries to overcome a GSE-related epithelial barrier defect. More immunostimulatory gluten peptides and other compounds that may play a role in intestinal homeostasis have been reported due to increased barrier permeability (Cardoso-Silva et al. 2019).

AT-1001, a zonulin inhibitor, was in phase II research to reduce gluten-induced barrier dysfunction (Bakshi et al. 2012). Drug development is being conducted to develop medicines that disrupt these systems. Peptide-like substances bind to HLA-DQ2 and DQ8, preventing T-cells from identifying gluten peptides, and TG2 inhibitors available in a variety of types. The identification of gluten epitopes implicated in the $CD4^+$ T-cell response to gluten has opened the path for gluten-reactive T-cell peptide vaccines. The discovery of dominant epitopes that react to all GSE instances was a critical step in putting this idea into practice (Yoosuf & Makharia 2019).

Furthermore, a phase II clinical trial in GSE has been conducted to explore whether an intestinal hookworm infection modifies the mode of local immune response and suppresses gluten sensitivity in patients with CD (Daveson et al. 2017; Pearson et al. 2019).

Interfering with cytokines has been used effectively to treat various autoimmune diseases, and a slew of new treatment approaches are being developed in this sector (Moudgil & Choubey 2011). However, due to the unacceptability of complications in GSE therapy, most treatments are unlikely to be used as the main indication for GSE. Anti-IL15 therapy, proposed as a treatment for refractory GSE, may represent an exception to this rule. Uncomplicated GSE may become an indicator if refractory GSE is successful (Rashtak & Murray 2012).

Conclusion

- Gladin is the most cause of host reactions, increases intestinal absorptivity and also stimulates innate and adaptive immune responses.
- Gluten sensitivity in Irish Setters dogs isn't the same as celiac disease in humans, according to genetic studies.
- Antigliadin and anti-tTG immunoglobulin G (IgG) were discovered in the sera of dogs with enteropathy; although, the relationship between canine enteropathy and human CD seems to be mostly unexplained.
- Drug development is now conducted to develop medicines that disrupt immune system systems, which block T-cells from detecting gluten peptides.

Recommendation

Reducing the need for gluten in dog food may be a good choice in the amelioration of GSE development. More studies are needed to fully explain the zonulin pathway that plays important role in the trafficking of cells and activation of the immune system.

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CHAPTER 22

PATHOGENESIS OF FELINE INFECTIOUS PERITONITIS

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INTRODUCTION

Infection with the feline coronavirus (FCoV) caused a rare, deadly and invulnerable expanding illness in cats called Feline infectious peritonitis (FIP) (Sherding 2006). Although the occurrence of FCoV in domestic and non-domestic feline populations around the world (seroprevalence 20-100%), FIP will only affect about 10% of FCoV-positive felines (Kennedy et al. 2002). Observational reports of felines with FIP have recognized a numeral of possible causes for the illness' progression. The infection is most common in young felines (3-36 weeks old), with the type of instances (75-90%) occurring in multi-feline families or, less frequently, in aging felines (Pedersen 2009). Extensive pyogranulomatous sores in the brain, liver, lymphatic tissue, and lung, as well as substantial fundamental systemic inflammatory degradation of serosal membranes, distinguish FIP pathogenesis. End-stage FIP patients have considerable T-cell reduction from the periphery and variations in cytokine expression, suggesting that the immune system plays a role in the pathophysiology kof this illness (Kipar et al. 2006b). There haven't been any viral genetic variables connected to FIPV pathogenesis discovered yet. *De novo* virus mutation gives rise to virulence according to the *in vivo* alteration shift paradigm. It Has been proposed in a different study that variations in the sequences of the spike protein, nonstructural protein (NSP) 7b, and NSP3c may triggers illness (Rottier et al. 2005). In light of *in vitro* examinations determining the FIPV strains fondness for macrophages, the theory was protracted to suggest that the intestinal Covid (FECV) endures a mutational revolution in the structures of the gastrointestinal tract, enabling contamination of macrophages, foundational scattering, and lethal illness appearance (Brown et al. 2009; Brown 2011).

TNF, IL-1 and CD11b and CD18 as adhesion molecules are abundantly expressed by circulating activated monocytes, making it simpler for monocytes to interface within small and medium veins particularly inside activated endothelial cells (Kipar et al. 2006b; Takano et al. 2007a; Takano et al. 2007b). In addition, is thought that the main cause of increased vascular permeability in normal bodily coelom is the production of vascular endothelial growth factors in FIPV-infected monocytes and macrophages (Takano et al. 2011). The illness provides numerous challenges for veterinarians because it is problematic to identify, there is no successful cure, and sufficient prophylactic mediation measures are not available (Chang et al. 2017).

Epidemiology

Feline infectious peritonitis (FIP) is a significant issue in multi-cat families. The virus spreads in settings where large numbers of cats are kept in a small area (e.g., catteries, shelters, and pet shops (Horzinek and Osterhaus 1979). Although feline coronavirus is relatively common in free-moving local cats, since they do not always dispose of their excrement in the appropriate locations, shared rubbish bins are a significant transmission route in multi-cat households (Benetka et al. 2004).

FIP disease is most common in domestic cats. In addition, it was found in African lions, mountain lions, leopards, cheetahs, Jaguars, Lynx, Servals, Caracals, European Wild Cats, Sand Cats, and Pallas Cats (Stuetzer et al. 2014). Despite the prevalence of FCoV infection in multi-cat households, only about 5% of these cats develop FIP, compared to a much lower percentage in single cat households. FIP is more common in young and immunosuppressed cats because FCoV replication is less tightly regulated in these animals, making the crucial mutation more likely. FIP affects more than half of all cats under the age of a year. Males, purebred cats, and sexually intact cats are more likely to get FIP. According to epidemiological research, the cat's genetic history may have a role in developing FIP. Researchers have established breed resistance and sensitivity to FIP. In Persians and Birmans, sensitivity to FIP is a polygenic genetic characteristic. FIP is more common in the Abyssinian, Bengal, Birman, Himalayan, Ragdoll, and Rex breeds (Pedersen 2014).

The bulk of FCoV is excreted from the body through feces. The most prevalent infection route is through the oronasal cavity. Within one week of a common disease, cats release the virus into their feces. Saliva, respiratory secretions, and urine may contain it in the early stages of the disease (Andrew 2000). When naive cats in multi-cat households are exposed to FCoV for the first time, they are likely to get infected and produce antibodies; many will discharge the virus intermittently for weeks or months (Pedersen et al. 2009). Most felines develop into chronic FCoV shedders, allowing new cats to get infected regularly. Antibody-negative cats are unlikely to shed the virus, even though only roughly a third of FCoV antibody-positive cats do (Felten et al. 2020). Cats with high antibody titers are more susceptible to shedding FCoV. They're also most likely to spread the virus more widely and frequently. The majority of FIP-affected cats shed non-mutated FCoV (Pedersen et al. 2008). For naive cats, the main

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sources are trash bins shared with shedding cats. Furthermore, chronic reinfection via an infected cat's dirty litter box appears to be essential for the virus's endemic survival (Pedersen et al. 2008). Saliva, reciprocal grooming, eating from the same plate, and, in rare situations, personal contact is all ways for viruses to spread. Droplet transmission by a sneeze is also uncommon, although it does happen. It is unclear whether cat shows contribute significantly to the spread of FCoV (Pedersen et al. 2008). The infection is unlikely to be spread by lice or fleas. Although the transplacental transfer is conceivable, it is uncommon in natural circumstances. Most kittens are not affected until they are 5-6 weeks old, after being removed from adult cats shedding the virus (Pedersen et al. 2008). Kittens are most likely to become infected between the ages of 6-8 weeks, when their mothers' antibodies are waning, and are infected mainly through contact with their mothers' feces or other sources. FCoV is a reasonably harmless virus that may be treated with the most common disinfectants and detergents. It can survive outside the cat for up to 7 weeks in cold or dry conditions (for example, on a carpet). Indirect fomite transmission can transfer FCoV from one person to another. It was spread from person to person for a short time through clothing, toys and grooming products (Addie 2019).

Infectious Peritonitis in Cats: Pathogenesis

The pathophysiology of feline infectious peritonitis (FIP) has been a hot topic of research for quite some time. Even though the image is still unsatisfactory, the aftereffects of both *in vivo* and *in vitro* research, while occasionally doubtful, have provided an ever-increasing number of pieces to the puzzle. For the improvement of FIP injuries, three main factors have been identified as fundamental essentials: FIPV-infected monocyte activation, effective and long-term FIPV replication in monocytes, and systemic infection with a virulent FCoV (i.e., FIPV) (Kipar and Meli 2014).

Virulent FIPV that Induce Systemic Infection

The "*in vivo* mutation transition" or "internal mutation" idea, as well as the "distinct circulating a virulent and virulent strains" hypothesis, have both been presented as explanations for the infection of the host (Brown 2011). The main concept predicts that in infected animals mostly FIPVs emerge *in vivo* from FECV transformations, and there is undeniable proof that the Feline Enteric Coronavirus (FCoV) of maximum felines isn't an FIPV in the first place. Early genomic analyses of FECV and FIPV research office and field strains revealed that they appear to be inexorably connected sets of viruses. (Chang et al. 2011). In addition, several studies have shown that FIPV and FECV phylogenetic grouping is based on geographic distribution rather than illness phenotype (Pedersen et al. 2009; Barker et al. 2013). FIP-affected animals produced more viral quasi-species in spontaneous illnesses than healthy cats, showing that for the development of pathogenic mutants a sophisticated viral mutation rate is required (Battilani et al. 2003). This is further supported by the fact that felines with FIP are less likely to initiate viral replication (Dewerchin et al. 2005).

Changes in two genes have been linked to improved FIP such as; accessory genes and the S gene of FCoVs. As a result, additional gene 3c was most likely the most notable concentration. Early research revealed that FECVs had usually good 3c quality, while over 66% of FIPV-determined 3c configurations include alterations (e.g., deletion or point mutation) that prevent full-length complete protein translation. As a result, mutations in 3c were formerly assumed to constitute an overall FI-related harmfulness signature (Addie et al. 2009). The past investigations affirmed these prior perceptions, with 3c being vigorously mutated in most of FIPV confines and conceivably associated with FIP advancement because of expanding viral wellness in monocytes/macrophages (Bálint et al. 2014). Nucleotide sequence studies of the 7a gene, the study concluded that deletions in the 7a gene of FECV/FIPVs are concomitant to the pathogenesis of FIP (Kennedy et al. 2001; Lin et al. 2009). The inquiry of FIP pathogenesis has lately progressed to include the evaluation of the S gene. The S protein of the crown virus is required for receptor restriction and infected propagation. Alterations in the S gene alone or conjunction with alterations in other genes could lead to the biotype flip because the FECV-FIPV progression includes a change in target cell tropism (Lin et al. 2009).

FIPV Replication in Monocytes that is Effective and Sustained

The second fundamental requirement for FIP appears to be the capacity of the virus to replicate well and long-term in the infected host's monocytes. In isolated feline peritoneal macrophages, FIPV can proliferate in *in vitro* conditions (Dewerchin et al. 2005; Rottier et al. 2005). Furthermore, significant changes in monocyte persistence and infection vulnerability have been identified in cats, showing that host factors play a role (Dewerchin et al. 2005). These findings back with *in vivo* studies show that FIP is associated with considerably greater virus levels in tissues (Simons et al. 2005; Kipar et al. 2006a; Hornyák et al. 2012).

Viruses after being entered into the body, can reproduce unrestrictedly within monocytes, get entrance to the bloodstream, and infiltrate in the body. The principal targets are the lymph nodes in the mesenteric regions, serosal surfaces of the GIT tract and to a lower degree in the omentum and pleura. FIPVs can even reach the brain parenchyma, the eyes' leptomeningeal arteries, and venules (Falck et al. 2010). Virus-infected circulating monocytes are not only critical for viral propagation, but they also start the formation of the typical extensive granulomatous vascular lesions when they become activated (Coyne et al. 2006). Furthermore, immunopathological damage was seen in FIP by FIPV-infected macrophages. In FIP lesions, these cells seem to be the most infiltrated (Berg et al. 2005).

Infected macrophages cluster around small venules after being infiltrated into the body, where they proceed to multiply resulting in multiplication of the virus. In reaction to virus replication, more monocytes are drawn from the circulation and sent to the objective tissues, culminating in the typical FIP symptoms of vasculitis and the production of pyogranulomas. Besides the virus, infected and dying macrophages generate inflammatory mediators such cytokines, leukotrienes, and prostaglandins (Adlercreutz et al. 1991). These substances increase vascular permeability and offer an extra chemotactic boost to neutrophils and monocytes. These entrained cells produce more mediators and cytotoxic substances in response to inflammation, leading to increased local virus

production and tissue destruction. The vascular lesions appear as circular and localized infiltrates dominated by venous and perivenous macrophages, huge numbers of macrophages are infected, and infection is restricted to these cells neutrophils and T lymphocytes address the minority of inflammatory cells in FIP granulomas, whereas B lymphocytes surround and gradually replace macrophages (Solheim et al. 2005). Despite viral determinants, an individual cat's immunological competence has a role in the consequence of the infection (Coyne et al. 2006).

Stimulation Monocytes Via Infection of FIPV

Granulomatous phlebitis and periphlebitis induced by highly activated monocytes is the morphological confirmation and start of disease of FIP, most likely throughout high-level monocyte-related viremia with a strong viral replication rate (Simons et al. 2005; Hornyák et al. 2012). According to investigations based on real-life examples, a direct interaction between monocytes and activated endothelial cells leads to phlebitis. TNF- and interleukin-1 (IL-1) are cytokines produced by monocytes, as well as adhesion molecules like CD18, which allow them to engage with activated endothelium cells. They also produce enzymes that break down the vascular basement membrane at monocyte emigration locations, such as matrix metalloproteinase-9. The endothelial cells appear to be activated all over the body, and the limited dispersion of vascular lesions is most likely due to the endothelium's selective sensitivity (Kipar et al. 2005). According to a recent flow cytometry study, adhesion molecules are upregulated in monocytes, T-cells, and B-cells in response to FIP (Olyslaegers et al. 2013). Triggered monocytes alone can stimulate both macrophages in hemolymphatic organs and vascular endothelial cells if they produce enough cytokines (Kipar et al. 2006a). Inflammatory exudate in peritoneum from FIP-infected cats have high TNF- mRNA levels, also published reports been revealed exudate collected by bronchoalveolar lavage contain a high amount of released IL-1 and IL-6 and alveolar macrophages from FIP infected cat, feline represents a considerable overexpressed of IL-6, TNF-α, granulocyte (G)CSF, GM-CSF, and other B-cell differentiation factors (Takano et al. 2007a; Takano et al. 2009). In contrast, as established that FIPV infection of monocytes seems to be a crucial preconditioning step in vitro in isolated feline monocytes and macrophages. FIPV triggered the p38 mitogen-activated protein kinase (MAPK), which also directly regulates the expression of proinflammatory cytokines by phosphorylating a range of signaling molecules in PBMCs, most likely during the entrance and, albeit less strongly, between 6 and 12 hours PI (HPI), when the virus is being generated in peripheral blood mononuclear cells (PBMCs0) (Dewerchin et al. 2005; Regan et al. 2009). This was linked to the stimulation of TNF- and IL-1b production, as revealed in the peripheral blood mononuclear cells supernatant at 24 HPI (Regan et al. 2009). VEGF transcription was remarkably increased in isolated feline monocytes and alveolar macrophages at 48 HPI, while feline alveolar macrophages generated higher TNF at 48 and 72 HPI, both in association with viral replication (Takano et al. 2011).

Pathological Findings

Referring to the 20th century, the term peritonitis was given to FIP in the 1960s as it denotes the most common changes in the peritoneal cavity during necropsy (Rissi 2018). Two types of FIP are distinguished; effusive (wet or non-parenchymatous) and non-effusive (dry or parenchymatous). The most frequent type is wet which induces a diffuse inflammatory reaction on the serosal surfaces of internal organs with infiltration of inflammatory fluids to the peritoneal cavity (Figure 2) (Norris et al. 2005; Tasker 2018).

A second form (Dry FIP) produces granulomatous lesions in the parenchyma of different organs like the liver, kidneys, lymph nodes, wall of the intestine, eye, and central nervous system (Figure 2). FIP granuloma is known as dry (parenchymatous or non-effusive) because of the lack of inflammatory fluid in the body cavities. In general, cats seldomly display both forms simultaneously. Despite indications that the dry form has become more prevalent in recent decades, the majority of cats still exhibit the wet form (Diaz and Poma 2009; Pedersen et al. 2009; Pedersen 2014; Malbon et al. 2019).

The lesions are variable based on the form and organs involvement. The predominant gross lesion in the wet FIP is abdominal enlargement, which accounts for a higher percentage of ascites caused by neoplasia, liver disease, and cardiovascular disease (Cannon et al. 2005; Barker and Tasker 2020). By using percussion, the collected fluid might be easily moved inside the abdomen. The abdomen may contain more than 1 liter of fluid of various colors (straw-colored or blood-tinged) after being opened (Figure 2). Also in wet FIP, scrotal distention may occur as a result of descending peritonitis to the testes, or there may be indications of liver lipidosis and skin fragility (Giori et al. 2011; Redford and Al-Dissi 2019). Additionally, as the result of the migration of infected macrophages into the synovium, synovitis can be detected (Pedersen 2014).

The most prevalent lesion of wet FIP is a pyogranuloma, which is defined as a manifestation of vasculitis (i.e., phlebitis). The abdomen is the most frequent site for wet FIP pyogranulomas, near the cranial mesenteric artery as they appear in the omentum and the serosal surfaces of different organs. As a result, the omentum is engorged with inflammatory fluid and showed evidence of necrosis and inflammatory cell infiltration (Cannon et al. 2005; Addie et al. 2009). Microscopically, the pyrogranulomas are composed of a significant number of macrophages accompanied by a rich-protein inflammatory fluid and including neutrophils, lymphocytes, as well as plasma cells (Figure 3). Virus antigen is identified in a considerable number of the macrophages in the wet FIP pyogranulomas. However, the pyogranulomatous lesion is predominantly surface-located, beneath muscle or organ parenchyma may show isolated lesions of phlebitis and mixed inflammatory cell infiltration (Kipar et al. 2005; Ziółkowska et al. 2017).

In dry form, the abdominal and thoracic effusions are either lacking or present in a minimal amount. Regarding the abdominal abnormalities of dry FIP are greater, fewer in numbers, and less extensive in comparison to the abnormalities of wet FIP (Kipar and Meli 2014). The utilizing term parenchymatous for dry form comes from the invading lesions from the pleural and serosal surfaces to the underlying tissues (Drechsler et al. 2011; Anis et al. 2014).

Kidney and mesenteric lymph nodes are the main sites to demonstrate the abdominal changes in dry FIP. On the contrary, the minimum changes are denoted liver and hepatic

lymph nodes. In addition, dry form is characterized by inflammatory changes in the walls cecum and colon, as well as associated caeco-colic lymphadenopathy with symptoms similar to ulcerative colitis. As well, thoracic involvement occurs in around 10% of cats with dry FIP but the lesions are generally confined and only one aspect of a larger systemic infection. Both pleura and parenchyma of the lung may be affected by small granulomas and additionally, pericardium can be involved (Autieri et al. 2015; Fujii et al. 2015; Felten et al. 2019).

Moreover, the lesions of the dry form of FIP, also known as pyogranuloma, resemble granuloma in their outgrowth, particularly in the abdomen. Dry FIP lesions are similar to granulomas in the effusive form in that they have accumulated macrophages around vessels. As in typical granulomas, these accumulations are accompanied by extensive infiltrates of lymphocytes (mostly B-cells) and plasma cells that spread into adjacent tissues. On the contrary, hyperemia, edema, necrosis, protein exudation, and fibrin deposition are not as prominent as in the pyogranulomatous lesions of wet FIP (Garner et al. 2008; Gnirs et al. 2016).

It has been reported that 60% of cats with dry FIP have ocular and cerebral lesions, while 40% have abdominal lesions with or without cerebral and ocular involvement. FIP affects more than half of cats with the central nervous system (CNS) inflammatory disease, as well as, one-sixth of all cats with CNS symptoms from any cause. It has been well documented that FIP is the most frequent spinal disease among cats under the age of two years old, and it is one of the three primary causes of spinal disease in cats of all ages, along with lymphosarcoma and vertebral neoplasia. Similar to CNS involvement, ocular lesions are most commonly detected in dry FIP than in cats with wet forms. Moreover, chorioretinitis and Uveitis are the fundamental characteristics of dry form. In dry FIP, the ocular disease appears alone or in conjunction with CNS or peritoneal cavity changes. An early indication of ocular FIP is the alteration in the color of the iris. The pathognomonic lesion of FIP is keratic precipitates, which are caused by the deposition of fibrin and inflammatory cells on the caudal part of the cornea (Addie et al. 2009; Ziółkowska et al. 2017; Pedersen 2021).

Diagnosis of Feline Infectious Peritonitis Infection

FIP can be hard to diagnose even if having a high level of clinical suspicion history, clinical manifestations, and basic laboratory tests. FIP is different in a variety of clinical cases due to the vast range of clinical symptoms. History and clinical indications, on the other hand, might be utilized to raise the index of suspicion. FIP is more prevalent in cat under ten years old (Yin et al. 2021).

Given its tendency for younger cats, its high affinity for involving catteries and shelters, the typical physical with history findings and multiple distinctive laboratory abnormalities, the detection of FIP should be quite easy. Despite this, many veterinarians find it one of the most difficult diagnosis to make. While veterinarians have minimal difficulty placing FIP just above their diagnostic list, they have a lot of problems, if not downright reluctance, proving their diagnosis. However, not only does the veterinarian faces challenges; the owners are also hesitant to give up without a definitive diagnosis (Pedersen 2009).

A histopathological analysis is conducted to make a conclusive diagnosis of FIP since the virus is frequently discovered inside the lesions utilizing immunohistochemistry for virus antigen. It is possible to detect the cytological and biochemical properties of the wet form using virus antigen immunohistochemistry (Tasker 2018).

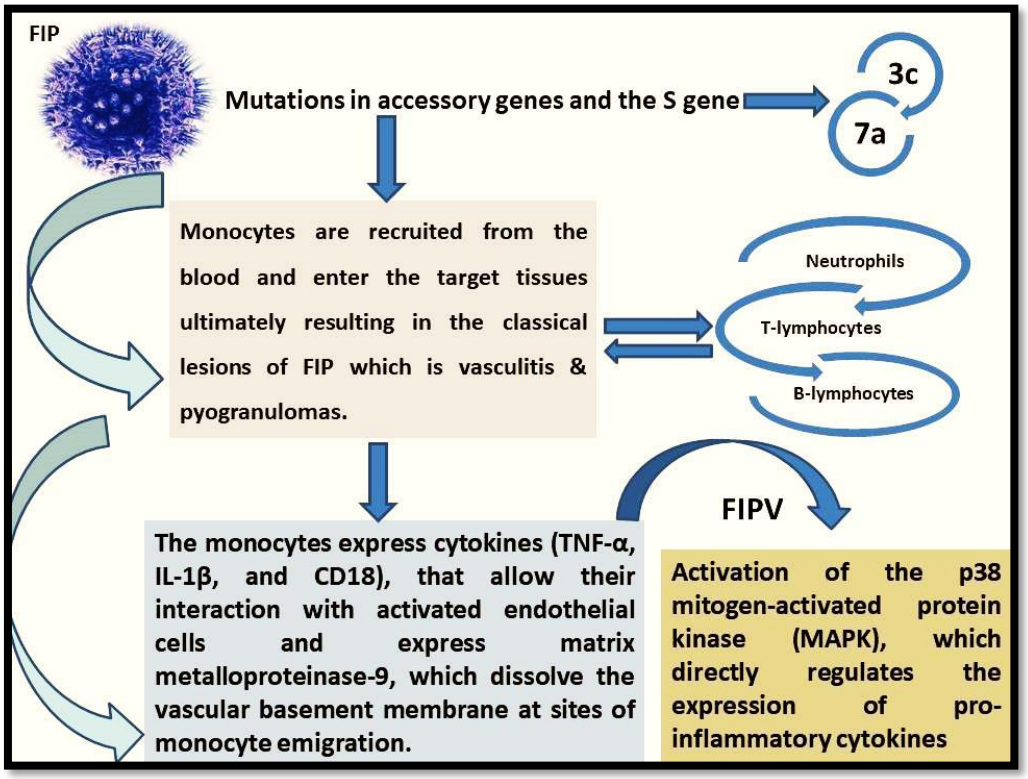

Fig. 1: Pathogenesis of FIPV. Mutation in the S gene, migration of monocyte from the bloodstream to the tissue, and releasing of pro-inflammatory mediators.

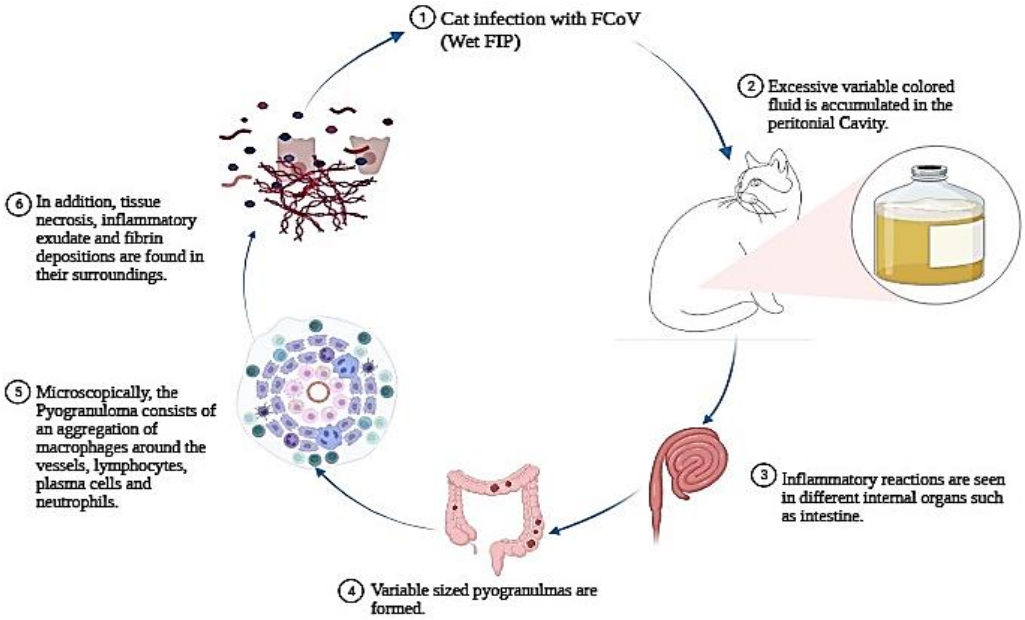

Fig. 2: Graphic represents the most noticeable lesions in wet FIP. Authors designed graph by using biorender.com.

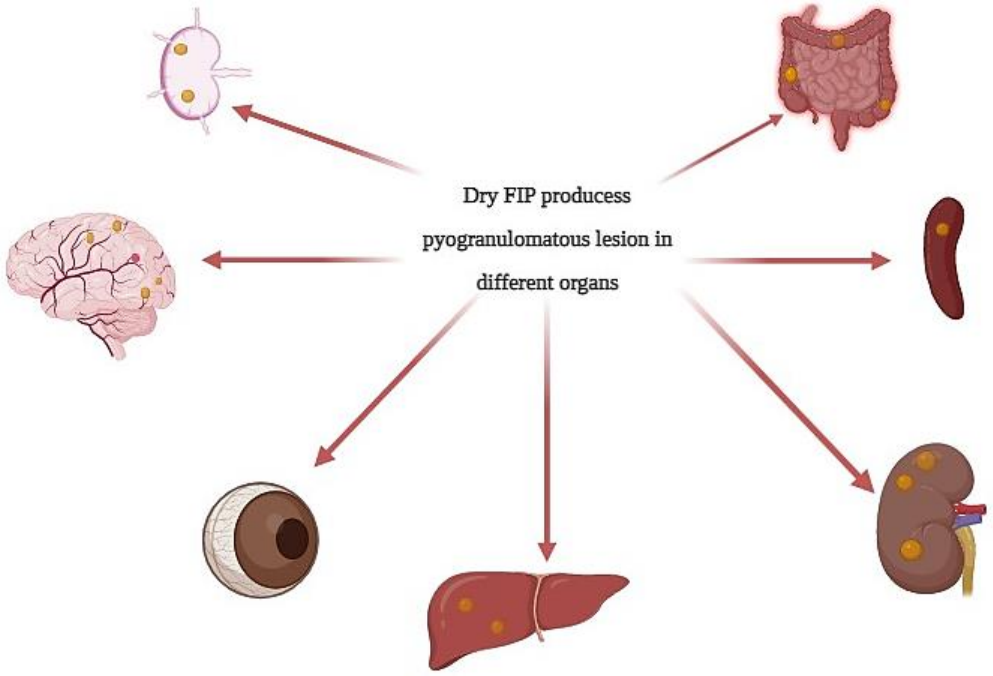

Fig. 3: Graphic demonstrates the most predilected organs for pyogranulomatous lesions during infection with FCoV dry form. Authors designed graph by using biorender.com.

Magnetic resonance imaging of the brain can be used to show anomalies in situations of miscellaneous diagnostic analysis in cases of neurological clinical manifestations. Foramen magnum herniation, obstructive hydrocephalus, meninges, syringomyelia, third ventricle, mesencephalic aqueduct, and brainstem contrast enhancement have already been associated with FIP (Penderis 2009; Crawford et al. 2017).

Finally, for approaching a diagnosis of FIP generally these protocols should be followed:

It's necessary to take into consideration that FIP is more frequent among kittens (those under the age of three, and especially those under the age of two), with a lower peak of cases in cats over the age of ten. Male cats are generally at a minor threat (Riemer et al. 2016).

The following protocols are available to reach the goal of diagnosis (Addie et al. 2009; Chang et al. 2017; Felten and Hartmann 2019):

1. History and Clinical Signs
2. Blood Tests include the following;

a. Hematology

b. Serum Biochemistry

c. FCoV Serology: Enzyme-linked immunosorbent assays (ELISAs) or indirect immunofluorescence antibody (IFA) tests have been commonly utilized in commercial testing of serum FCoV antibodies.

3. Analysis and evaluation of body cavity effusions:

This method can be quite helpful in determining whether or not one has FIP. Although ascites are the most frequent body

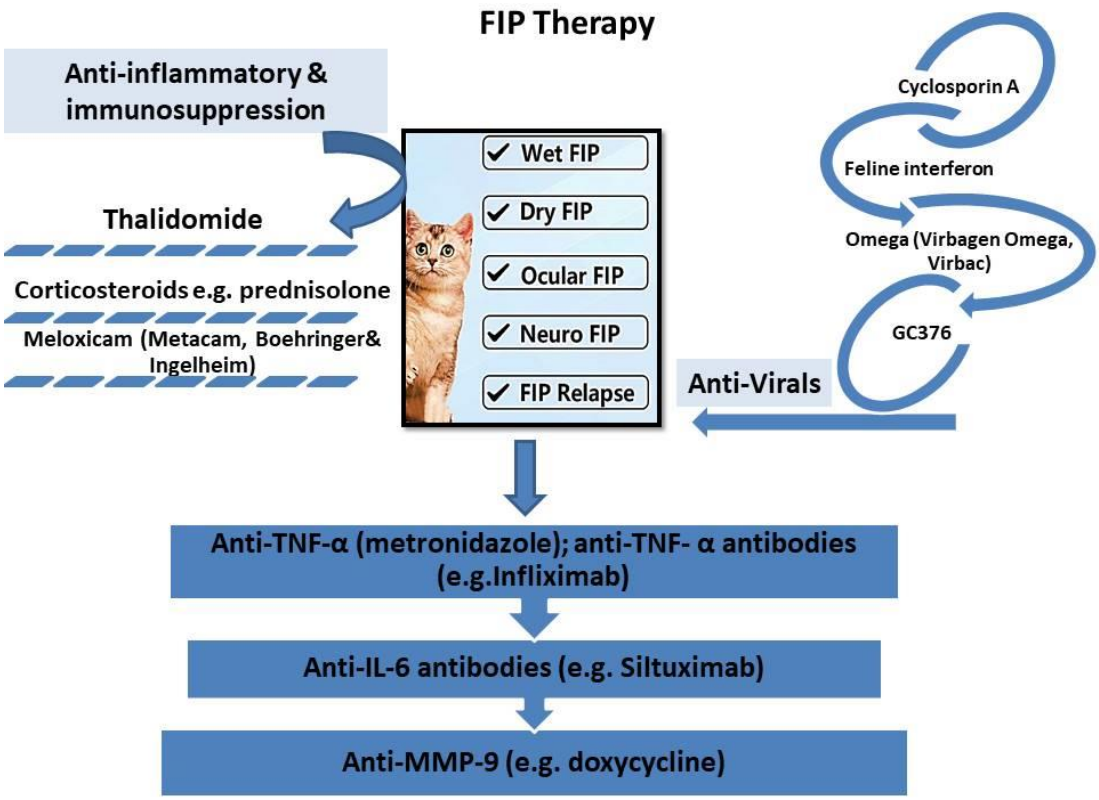

Fig. 4: FIP treatments fall into one or more categories.

cavity effusion, repetitive imaging (especially ultrasonography) can reveal pleural effusion and/or pericardial effusion in the presence or absence of ascites.

4. Histopathological examination of tissues
a. Routine histopathology (hematoxylin and Eosin)
b. Immunological staining of FCoV antigen (IHC)
5. FCoV detection utilizing reverse transcriptase (RT-) polymerase chain reaction (PCR).

Treatment

Antiviral medicines, anti-inflammatory, immunosuppressive therapies, and immunostimulant drugs have all been utilized in the treatment of cats with FIP. Antiviral medications work by preventing viruses from replicating. Anti-inflammatory and immunosuppressive medicines are used to treat inflammatory conditions such as pyogranulomatous inflammation (Lindemann et al. 2016) or antibody-enhancement (Takano et al. 2008).

Immunostimulant medications, boost the immune system's ability to fight the virus. As well as possible virus-based and host-based therapy methods are all aimed at interrupting the FIPV replication cycle. Most categories of drugs used for FIP therapy are found in figure 4.

Conclusion

- At least 3 distinct forms of mutations have been linked to FIP virulence acquisition, with more likely to be uncovered.
- Although the synthesis of numerous cytokines and other inflammatory proteins has been widely investigated in the FIP pathogenesis, little is known about how these elements are triggered and their impact on pathology.
- FIP cannot be diagnosed as an antemortem only on the results of a single diagnostic test. Every cat's organ should be examined for history, clinical symptoms, and histopathology and immunohistochemistry malformations if there is a suspicion of FIP.
- Antiviral, anti-inflammatory, immunosuppressive, and immunostimulant medications have all been tried in the lab to treat cats with FIP, but no therapy has yet been demonstrated to be effective in curing cats with spontaneously acquired FIP.

Recommendations

Further research into the pathogenesis that controls the virus-host interaction during FIP infection is required, as is the advancement of molecular techniques, especially IHC and fluorescence techniques, in the hopes that diagnostic tools for FIP will be simplified in the foreseeable future to explicitly diagnose FIPV and provide effective FIPV treatment.

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CHAPTER 23

BOVINE VIRAL DIARRHEA: A CHALLENGE TO DAIRY INDUSTRY AND FOOD SECURITY

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INTRODUCTION

Bovine Viral Diarrhea (BVD) remains a disease of great concern to dairy and beef producers around the world. The broad nature of the disease, transmissibility, and sparse treatment regimens render BVD a global pandemic, and one of the most significant cattle diseases in the world (Gunn et al. 2005). Coupled with diverse clinical signs, symptoms, and death loss caused by this disease make it a sickness that can no longer be overlooked. But major impediment is economic rationality and anonymous status of BVD because of lack of diagnostic centers (Gohar et al. 2013).

BVD is commonly seen in the young cattle of 6-24 months old. On the basis of clinical signs, generally it appears in subclinical to acute and severe form. It is extremely fatal and described by severe enteritis along with classic lesions on mucosal surface. It has the symptoms like fever and diarrhea. Clinical signs of the disease has a wide spectrum from being asymptomatic to the death of the animal in severe cases. The degree of variability depends on type of virus harbouring the animal body and extent of the infection. Generally, it is believed that a non-cytopathic strain of virus will have less deleterious effects than it's counterpart cytopathic strain. It has treacherous nature and commonly exist in all over the world. Bovine viral diarrhea virus (BVDV) is related to genus *pestivirus*, family flaviviridae (Wegelt et al. 2011). The viruses that cause BVD are currently divided into 3 species within the Pestivirus genus; Pestivirus A (Bovine viral diarrhea virus 1, BVDV-1), Pestivirus B (Bovine viral diarrhea 2, BVDV-2), and Pestivirus H (HoBi-like pestivirus, atypical ruminant pestivirus) (Smith et al. 2017). Its virus belongs to heterogonous group and divided into 2 main genotypes BVDV-1, BVDV-2 (Becher et al. 2003). Every genotype exists in a cytopathogenic (CP) and non-cytopathogenic (NCP) biotype. It comprises 4 recognized species BVDV-1, BVDV-2, Border disease virus BDV, classical swine fever virus CSFV. Giraffe pestivirus is uncertainly considered as 5th specie. Its prevalence is high in cattle as compared to other species. The virus is sensitive to chloroform ether and trypsin depending on the stage of gestation and strain, virus induces acute transient infection (TI) or persistent infection (PI). Cytopathogenic virus involves in outbreaks of mucosal disease while non-cytopathogenic virus leads to persistent infection (Ahmad et al. 2012). BVDV produces mainly GIT, reproduction and respiratory system related problems (Mishra et al. 2011). It is one of the most important virus distributed over worldwide (Gunn et al. 2005). Besides cattle other species i.e sheep, goat and camels are also infected.

Usually, infection is subclinical in cattle depending upon the strain involvement, on close examination generally mild fever and leukopenia is seen. Neutralizing antibodies are generated after 2 to 3 week onset of infection (Howard 1990). While infection in pregnant cow may lead to delivery of infected calf, abortion, stillbirth, congenital problems, birth of calf with persistent infection.

History and Epidemiology

This disease was first described in 1946 with isolation of the BVDV. Initially it was observed in Ithaca New York considered as winter dysentery. Morbidity ranges 33-88% and mortality 4-8%. Since then extensive studies have been conducted to understand this disease including its biotype and genotype identification, monoclonal antibodies (Mabs), immune tolerance and determining the pathogenesis of this disease. In 1957, *in vitro* experiments revealed that virus from acute BVD did not cause cytopathology. A cytopathic virus is also isolated from mucosal disease in the same year (Goyal and Ridpath 2008). Huge economic losses are reported in dairy sector in terms of reduced milk yield, retarded growth, reproduction losses, high mortality in young stock and culling (Houe 1999).

Antibodies were detected by the serological diagnostic procedure in which it came to know that BVDV is regularly found all around the world in approximately all countries having livestock. Certain countries have only BVDV as a principle viral infection. Housing, stocking density, vaccination program and control measures of various countries and geographical zones differ from each other, as a consequence, the prevalence of disease is vastly different in all such regions. Onset of PI is widely different in various part of world. The expected average prevalence for the PI with BVD is about 1-2%, however it may turn to 4% in endemic state. Generally, BVD spotted in same age group of the herd. In Europe its prevalence is 0.75-1.4% (O'Neill et al. 2009).

In Pakistan, a research study was conducted on its detection, 184 samples were collected from different farms of Punjab, and results showed that 21 samples were seropositive

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(11.4%) for BVDV. This prevalence was quite higher than European countries as they adopted better management, regularly vaccine protocol and culling of PI animals. If we do comparison of farms, we get to know that more positive cases reported in Public sectoral farm rather than private farms. Possible reason behind this scenario is poor screening test, failed vaccination and inadequate budget to diagnose BVDV at herd level. In addition to these, comparison between buffalo and cattle is also made that reveals that disease is endemic in cattle i.e. 16.85%. This is equivalent to prevalence reported in Greek herd i.e. 14% (Billinis et al. 2005). Research reports revealed that incidence of BVDV is more in our native breed Sahiwal as compared to cross bred. The philosophy behind the high positivity in Sahiwal is the lack of BVDV vaccine in Sahiwal. The investigation depicts that there is no even single positive case of pure breed of Holistein/Fresian. Hence, we may conclude that BVDV is present in the dairy herds of Pakistan and it should be eradicated to prevent its spread to susceptible animals (Gohar et al. 2013).

Symptoms & Clinical signs

Usually, the disease affects cattle between 6-24 month of age. Cattle infected from non-cytopathic strains of the virus will exhibit mild clinical signs if any. This form of infection has good tendency to produce PI calves if infection occur in pregnant cows. On the other hand, transient infection is more likely to occur if contracted in older animals. This form of the virus is relatively milder, enabling the animal to elicit an effective immune response capable of neutralizing the virus to considerable extent. Though up to 50% of these animals will appear normal (Larson et al. 2004), to know the source of transmission, it is very essential to determine the presence or absence of the PI animals in the herd. These PI animals shed virus throughout their lifetime, play an essential role in BVD pathogenesis and represents one of the main sources of viral infection (Goyal and Ridpath 2008).

In certain cases of BVD, the animal might be having a rough hair coat, unthrifty, mild respiratory disease, At close physical examination, erosion may be seen on oral cavity, tongue, nasal and buccal cavity. Up to 75% cases have these lesions. In few cases, there may be a peeling off muzzle with severe crusting and purulent discharge (Brownlie 1985). High rise of temperature, depression, hyper salivation, discharge from nose, hemorrhage, ulcer, erosion in GIT and intense bloody diarrhea are observed. In addition, other signs are included such as decreased milk production and fetal death within 10 days to 3 months after entry of pathogen (Goyal and Ridpath 2008), while progressive emaciation, chronic bloat, anorexia, and intermittent diarrhea have also been reported. Lesions on skin are unable to heal which are important finding and these lesions lead to hoof deformities and lameness (Jubb 1985). Both of the genotypes produce persistent as well as acute infections, however type 2 leads to more severe acute signs as compared to type 1 (Pellerin et al. 1994).

Sources and Transmission of Infection

PI cattle are observed as a principal source of infection for BVDV. They have an excessive amount of this virus. An ample amount of virus is seen even after diluting the serum sample up to 10^6 by using virus isolation technique (Brock et al. 1991). Carrier animals (PI) take off large amount of virus in their natural secretions and excretions such as discharge from nose, semen, saliva, feces, urine, milk and tear (Coria 1978; Straver et al. 1983; Brock et al. 1991).

Cattle with acute infection may also become a source of infection. Usually virus is excreted from day 4 to day 10, but may be excreted from acute infected cattle for longer periods of time (Brownlie 1987). Virus may be isolated from blood of other animals (other than cattle), for example, sheep and goat are also the promising source of infection. Thus, virus transmission between cattle and sheep is also described in publications (French et al. 1974; Gibbons et al. 1974; Carlsson 1994). It can be categorized into two ways, horizontal and vertical transmission. In vertical transmission, the transmission of the disease occurs from dam to offspring while in horizontal transmission, the infection takes place between members of same specie due to direct contact, but there is no transmission via sexual contact involvement. Hence, it may be concluded that the PI animals are produced as a result of vertical transmission. In most of the cases, the horizontal transmission of the disease occurs at first in herds of dairy animals and then the vertical transmission occurs among these animals. Therefore, it is difficult to differentiate the outbreaks based on horizontal and vertical transmissions (Houe 1995).

Vertical Transmission

BVDV is excreted out from semen in TI and PI cases of BVDV. If healthy cattle is inseminated with a semen having BVDV, the chances for the production of a PI fetus are very minute. If the PI semen is introduced into 12 healthy cattle then all animals will be seroconverted within 14 days, but the calves from these cattle will be healthy except one of them will be the PI (Meyling and Jensen 1988). PI dams produce the PI-calves for the rest of dam-life, and these PI calves will remain infected for the next several generations (Radostits and Littlejohns 1988; Houe 1992). Infection may also be transferred to the next animal, if either donor or recipient is PI. By following the proper method of embryo washing, one may prevent from later infection (Wentink et al. 1991a; Ballasch 1993).

Diagnostic tests were performed on semen of 1538 bulls in four different artificial insemination centers, and results manifest only 12 PI bulls (Howard et al. 1990). Hence, care should be taken before using bulls for semen collection. Although high mortality rate was reported in BVDV infected animals, but most of the bulls spent their adult life to propagate the breeding practices through artificial insemination (Houe 1993).

Horizontal Transmission

Two ways are important in this regard, one is direct and another is indirect. In case of the direct contact, effective way of BVDV transmission is through nose-to-nose contact (Tråvén et al. 1991). Aerosol transmission is yet not studied properly and even not proved by experiment. It is noted that if the infected animals are kept closely with negative animals, disease spreads rapidly (Houe et al. 1993). If the distance between PI animals and healthy animals is large then chances of the spread of infection is negligible (Wentink et al. 1991b; Ballasch 1993). Transmission of infection from animals having

acute infections is slow as compared to PI infected animals. Some studies reveal that there is no transmission of infection from acute infected animal to vulnerable specie even after kept in close environment (Meyling and Jensen 1988). Semen could be good source of horizontal transmission. In contrast to direct transmission, horizontal transmission needs a medium to transfer the pathogen from PI animals to susceptible host animals. This method of transmission relies on the integrity of pathogen outside the body of animal.

Thermo stability of BVDV is below 10°C in the lab, pH ranges between 3-9 (Duffell and Harkness 1985). In slurry, BVDV becomes inactivate within 3 hours at 35°C , 3 days for 20°C , 3 weeks at 5°C (Bendixen 1993). If pregnant cattle is vaccinated with live BVDV vaccine, it leads to fetal infection by crossing placental barrier. The outcomes of this infection is just like the natural infection as observed in PI dam (Liess et al. 1984). Some live vaccines consist of CP BVDV (Peter et al. 1967; Donis 1993). It is reported that BVDV spreads via contaminated vaccines as well (Lohr et al. 1983;Løken et al. 1991). Transmission may take place during embryo transfer technique because of contaminated wash fluid by BVDV. Commercial available serum for calf may be the source of BVDV (Rossi et al. 1980).

BVDV could be separated from the blood sucking flies within 96 hours after they had fed on PI animals. Moreover, it was revealed that infection may spread after foraging of 50 flies on PI animals for 5 minutes and biting of these flies to susceptible animals (Tarry et al. 1991). It is experimentally proved that use of equipment and appliance by human leads to indirect transmission of BVDV. Nose tong may be the source of transmission after using it on PI animals (Gunn 1993). Cloths are regarded as the origin for transmission of BVDV.

Economic Importance

BVDV has the treacherous nature, that's why, it leads to the considerable losses in dairy and beef sectors (Duffell et al. 1986; Houe et al. 1993). BVDV causes direct losses in the form of decreased reproductive performance and indirect losses i.e. high efforts regarding control of disease (Otte and Chilonda 2000). Harmful effects of the BVD disease involve decreased reproductive capacity, milk production, growth rate, respiratory problems, increased disease incidence, unthrifty, higher culling rate, high mortality in young animals. The expected losses in individual herd ranges from few thousands to $100000. Estimated loss at national level lies between 10 to 40 million $ per million calving (Houe 1999).

BVDV infection has a significant economic impact. High morbidity and high mortality is just because of suppressed immune system and other losses include decreased 1st service conception, prolonged calving interval and early embryonic death (McGowan et al. 1993a; McGowan et al. 1993b; Baker 1995; Houe 1999; Kozasa et al. 2005). BVD occurs in majority of the countries which are producing cattle breeds (Truyers et al. 2010) and are also enlisted by the world health organization (Richter et al. 2017).

Animal health economics is comparatively new discipline for research field that makes architecture, procedures and tools to support the decision making just to design disease control and prevention tools. There are also other thoughts including ethics and politics which are necessary for decision making for the control strategies. Research related to health economics comprises three segments.

1. Quantitative analysis of disease economics
2. Development of techniques to make the optimal decisions
3. Defining the profit (James 2000)

A number of standards are required for assessing the economic effect of BVDV in a herd. The presence of both persistently infected and acutely infected cases are good signs for the determination of infection in the populations. During evaluation of the BVDV economic impact, clinical signs should be kept in mind. In acute cases reproductive problems include abortion, repeat breeding, mortality in newborn and non-flourishing young ones (Baker 1995). Thus, the virulency of disease for two different population and different strain will be dissimilar that will ultimately affect economic loss depending upon these factors. Significance of a disease depends upon the economic impact.

Reproductive Losses

Reproductive losses are the most imperative economic consequences associated with BVDV infection and the evidences are suggesting the occurrence of BVDV related reproductive losses are increasing in the dairy industry day by day (Goyal and Ridpath 2008). When estimating losses of milk production, most data use milk production parameters which also consider fat and protein composition in the total volume of milk. This model is quite identical to the fat corrected milk (FCM) model often used to calculate overall production along milk components. The equation used in a study performed by Heuer et al. (2007) is given below:

FPCM (kg/d) = expintercept + b X lnDIM + c X DIM + d X age

Where FPCM denotes volume of milk X fat (%age)/ 4.5 X protein (%age)/3.2, the variables a, b, c, and d were regression coefficients estimated for each of the herd, and ln is the natural logarithm of days in milk (DIM).

Pathogenesis

The BVD infection has many clinical manifestations, and in order to select an optimal diagnostic technique understanding the pathogenesis is very important. On the basis of cytopathogenicity in cultured cells, BVDV is characterized further into two biotypes, cytopathogenic and non-cytopathogenic. The cytopathogenic subtype causes apoptosis in cultured cells while non-cytopathogenic subtype does not lead such type of lesions (Gamlen et al. 2010). Studies suggest that the main source of persistent infection is the non-cytopathogenic subtype, however it causes acute infection and modes of transmission are majorly the body fluids like semen, nasal discharge, urine, saliva, fetal fluids (Meyling et al. 1990). Virus blocks the neural impulses of intestine leading to diarrheal profiles in infected animals.

Acute Infection

As mentioned above, the acute infection is mainly due to non-cytopathogenic subtype of BVDV that causes transient viremia in non-pregnant and non-vaccinated cattle herd (Howard 1990), transient viremia exists usually 3 day post infection till 2 weeks until the immunity develops in cattle (Meyling et al. 1990). Virus transmission occurs mostly through muzzle-to-muzzle contact of infected dam and calf, sexual contact with

the persistent infected cattle. Few studies suggest that virus spreads through flies, acutely infected animals, aerosolized virus and through the fomites. Workers that are in contact with the infected animals are also responsible for the spread of BVDV (Gunn 1993).

The pathway of entry of virus is through the CD46 receptor on cell membrane of macrophages and lymphocytes of host, it is reported that virus has E1 and E2 protein that are responsible for the attachment on CD46 receptor (Maurer et al. 2004). Some researchers found that endocytosis occurs through clathrin, lysosome associated membrane protein-2, and mannose receptors, through this BVDV is entered in the cells and start its replication, and further progression to cell lysis causes viremia. This leads to temporary leukopenia and thrombocytopenia which leads to immunosuppression in 10-14 days (Howard 1990). BVDV also causes apoptosis in thymus, pyrexia and ultimately ulcerative lesions in the intestine and apoptosis of lymphocytes in the GALT (Gut associated lymphoid tissue) caused diarrhea (Pedrera et al. 2012), it is observed that the infection of mesenteric and submucosal ganglia, blockage of normal neural function of intestine lead to diarrhea (Wilhelmsen et al. 1990) and this immunosuppression precipitates other concurrent infections like respiratory diseases and *Neospora caninum* infection and abortions (Björkman et al. 2000).

Persistent Infection

The birth of persistently infected calf varies from calf to calf, it is suggested that if the dam gets infected as early as 25 days to 98 days up to 128 days that is 1st trimester of gestation, this results in birth of persistently infected calf as the virus can cross transplacental barrier. Some studies also gave information that Persistently infected calf born along a seropositive twin (Möstl et al. 2004).

The ability of non-cytopathogenic subtype of BVDV is to stop the production of Interferon type 1, that helps the virus to replicate easily (Charleston et al. 2001; Chase 2013) and hence the virus survives inside the host and produces persistent infection, animal sheds large loads of virus as blocking the interferon type 1 stops immune response and virus is not cleared from the host body. Shedding of virus is done mostly through body fluids and secretions; milk, semen, saliva, urine, blood, nasal secretions, aerosols (Brownlie et al. 1998). Virus inside the persistently infected animals is mainly present in the brain, skin , lymphoid organs like GALT, lymph nodes, mucosal layer of GIT (Gastrointestinal tract) and lungs (Liebler-Tenorio et al. 2004). Further research suggests that virus is distributed in neurons, oligodendroglia, blood vessel associated cells other than endothelium and astrocytes in the CNS of infected animals (Montgomery 2007).

Screening of the persistent infected cattle herds gives us information that some PI animals appear clinically healthy, but some might seem feeble and thrifty, low weight gain, chronic illness due to immunosuppression and stunned growth (Voges et al. 2006). Researchers found that immunosuppressed animals are frequently possessed with secondary infections, and combination with the mucosal disease leads to a reduced chance of survival of most PI animals (Voges et al. 2006).

Effects of BVDV on Fertility of Cattle

Acute infection of BVD also causes deleterious effects on reproductive performance of animals by reducing conception rates, causing abortions, inborn defects, and early embryonic death (EED) (McGowan et al. 1993). Studies also confirmed that sexually active bulls that have acute infection of BVDV have lower sperm motility, less sperm density and abnormalities in sperm (Paton et al. 1989).

In vitro studies found that the animals infected by non-cytopathogenic subtype of virus incubated with sperm and oocyte expressively declined the chances of fertilization (Garoussi and Mehrzad 2011), the persistently infected bulls had testicular hypoplasia and decreased sperm counts (Borel et al. 2007). Experimental BVDV infection was carried out on heifers by Intra muscular inoculation and found that lymphoplasmacytic oophoritis that persisted 61 days after inoculation and further studies shown that the surge of pre-ovulatory LSH was absent in experimental animals and super ovulated cattle (Ssentongo et al. 1980, McGowan et al. 2003). Researchers found that the virus is localized in the oocytes of persistently infected cows which is perhaps the main reason of persistently infected calves born to persistently infected cows (Meyling et al. 1990).

Effects of Fetal Infection of BVDV

Description of fetal infection of BVDV is intricated as the effects majorly depend on the age of fetus when it gets infection. Studies on this topic found that during 1st trimester (18days), the embryo is not attached, and infection does not arise because BVDV cannot breach the zona pellucida of embryo (Montgomery et al. 2008). Between 29-41 days, the cotyledons develop, and embryo is placed in placenta, if viremia of dam occurs the embryo gets infection and leads to death and decreased fertilization rates, the other outcome of embryo infection is the birth of persistently infected calves (Brownlie et al. 1998). Theriogenology departments of different countries studied the effects of BVDV and found that infection of BVDV in 80 to 150 days of gestation leads to teratogenic defects in fetus, cerebral atrophy, white matter edema, swelling, necrosis of outer germinal layer, and cerebral hypoplasia leads to ataxic calves (Rinaldo et al. 1976). Additionally, degeneration of eyes, colloquially parrot mouth, pseudocyst in brain and thymus (Montgomery et al. 2008), and growth retardation mainly of lungs and bones are also included. It is also reported that BVDV may also lead to fetal death and abortions without showing any apparent clinical signs. Other consequences reported are hydrocephaly and decrease in myelin sheath (Otter et al. 2009).

Endemically infected herd lacking BVDV vaccination concluded that 7% of the death of the fetus is attributed to BVDV (Rüfenacht et al. 2001). Death of the fetus may occur at any time during gestation, post infection to BVDV, however, death is seen more frequently in 1st trimester (Kahrs 1968; Casaro et al. 1971; Kendrick 1971; Done et al. 1980; Duffell and Harkness 1985; Roeder et al. 1986). Generally, death of fetus occurs within the 10-27 days post infection to BVDV and fetus expels out 50 days later to death.

Immunotolerance

If the fetus survives from infection during 1st trimester then it develops the immunotolerance later and becomes persistently infected with bovine viral diarrhea virus (Coria 1978). It is stated that mechanism of development of immunotolerance is not clear as much.

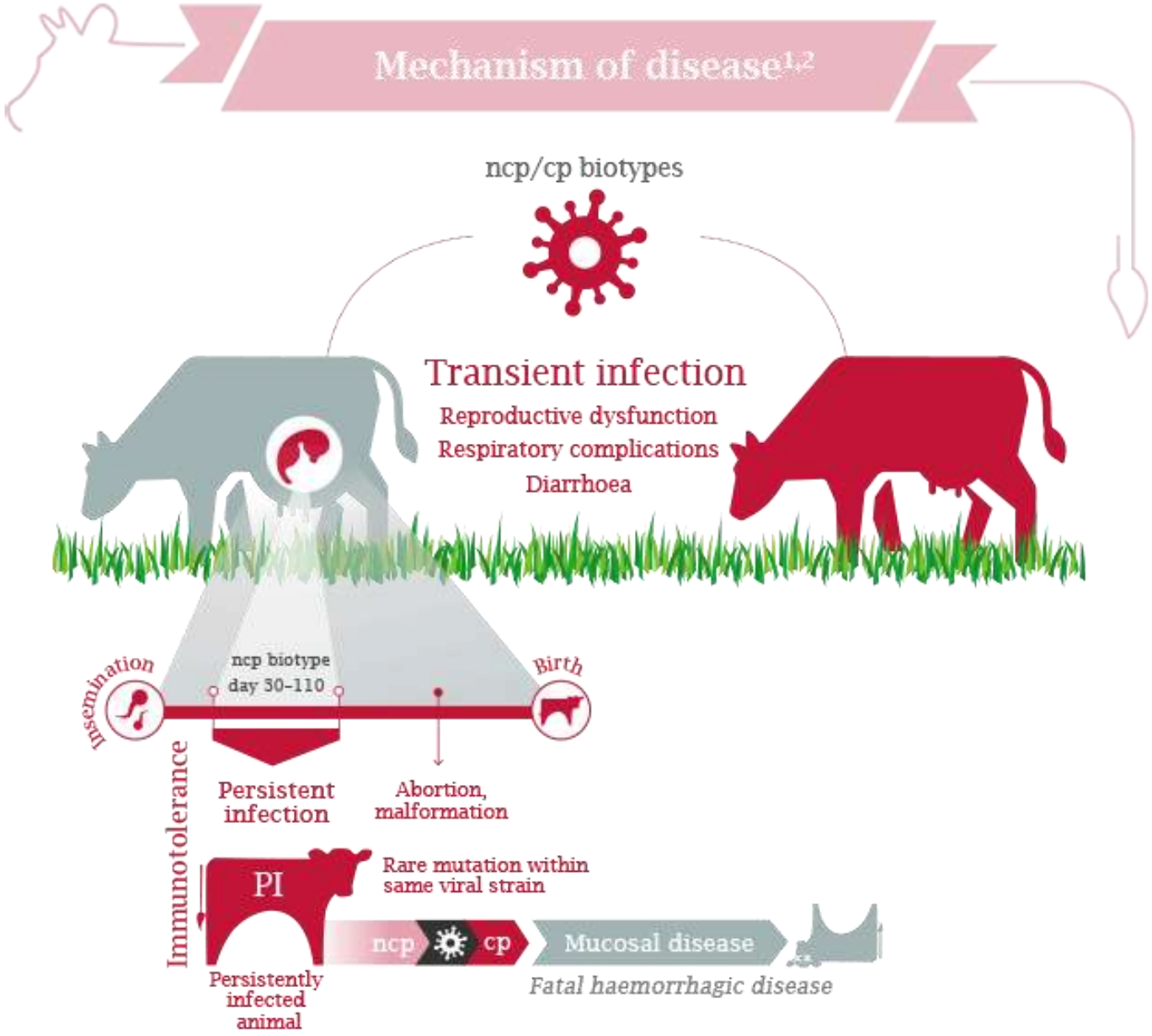
Congenital Defects

Infection at the gestation length of 100-150 days is stated as a congenital infection and leads toward the several defects. At this stage, synthesis of organs and complete development of CNS take place. Direct cellular damage and inflammatory reaction collectively contribute in developing the mechanism of congenital infection and related biological defects in animal body (Castrucci et al. 1994).

Some of the congenital defects seen may be cerebellar hypoplasia and underdeveloped anatomy of organs and musculo-skeletal system, all of which are devastating to the calf. On the other hand, approximately 1 to 2 percent of calves infected during this time may become tolerant to the infection and become the classically described PI calves that will shed the disease throughout their life (Goyal and Ridpath 2008).

Major defects in ocular system comprises of following (Grooms 2004):

- Cerebellar hypoplasia
- Microencephalopathy
- Hydrocephalus
- Hydroanencephaly
- Porencephaly
- Hypomyelination
- Cataracts
- Microphthalmia
- Retinal degeneration
- Optic neuritis
- Thymic hypoplasia
- Hypotrichosis/alopecia
- Deranged osteogenesis
- Mandibular brachygnathism
- Growth retardation



Disease mechanism of BVD. Source: http://www.bvdzero.us/infograph/infograph1.html

Control Measures

When former policies regarding BVDV control were studied, it came to know that at that time there were two main strategies to control BVD i.e., vaccination vs. non vaccination approach. On the other hand, late policy involves the use of systemic vs. non systemic approaches. Non systemic methodology encompasses usage of vaccine to augment the immune status along with slaughter and diagnostic test of PI cattle without any systemic go through, while in systemic mechanism dynamic decrease in the incidence and prevalence of disease at national regional and sectoral level should be carried out by observing herd status to evaluate the timely progress (Moennig et al. 2005).

There are two major principles to control the infectious disease

1. Removal of the reservoir
2. Restricting transmission from clinical hosts

Persistent infected individuals are the main reservoir for BVDV and temporary affected animals are also act as a reservoir to minor level. Consequently, eradication of the PI animals is a vital task. Therefore, for this purpose, a program should be devised that should work effectively for the maintenance of herd health i.e. minimize the exposure of pregnant cattle to BVDV.

BVDV control strategy contains following three facets:

1. Recognition and removal of persistent infected animal
2. Routine vaccination to boost up the immunity
3. Strictly follow biosecurity plan

Above three points are noteworthy because by following these points instantaneously, better result can be expected in the control program of BVDV. Healthy outcomes can be obtained only after implementation on all aspects as discussed above.

Biosecurity

It is the set of methods designed to halt the spread of infectious disease from one individual to other. The prime objective of the biosecurity is significantly decreasing the threat of pathogens being familiarized into herd. Recognition of these threats in timely manner is the backbone feature of the biosecurity at farm level. However, if there is breach in the early detection of the BVDV then control and prevention skill-sets have to manage the further spread of BVDV to other animal populations across the farms. In biosecurity, it is not mandatory to eliminate the pathogen but just to reduce the risk of infections.

- The important and common source of BVDV entry into a herd is the addition of new cattle into a flock. The addition of these cattle may have an acute or PI of BVDV. So these newly introduced animals should be recommended for screening of this pathogen. It is ideal to keep them in isolation until the availability of the screening test report. It is mainly emphasized when you are buying the new young stock in the market, because the young stock at the age of 6-24 month is commonly affected.
- Even if the pregnant cow is negative for BVDV then there may be chances of positive newborn calf. Hence the screening test for newborn calf should also be performed.
- Mating from the bulls of another herd and suspected animals contact with farm fence are included in risk factors. Screening of artificially inseminated bulls is also necessary.
- Ruminants other than the cattle (white tail deer and sheep) are also involved in disease transmission so prevention from their mixing with the herd is also required (Passler et al. 2009).
- BVDV is vulnerable to heat and commonly used disinfectant. Prevent the entry of gumboots, automobile and utensils into a farm because these items also have great role in BVDV transmission (Grooms et al. 2009).

Vaccination

BVD is a well-known and essential disease of cattle that will impact badly on reproduction of cattle and health of calf. BVD vaccine prevents from infection after exposure with BVDV and also prevent from viremic phase which is prerequisite for the crossing of placenta. It means vaccine prevents the spread of infections among dairy herds of buffaloes and cattle in various dairy farming systems (Newcomer et al. 2017).

Vaccine Types

Several vaccines are available for BVD in a combined form i.e. containing the other bacterial and viral agent. There are two main types of vaccine named as killed vaccine and modified live vaccine. Meanwhile BVDV has 2 biotypes and several genotypes, so manufacturer has to formulate the vaccine in monovalent and multivalent form. However, occurrence of non-cytopathic biotype is more as compared to cytopathic biotype. Cytopathic biotype has not as much role in persistent infection. Thus NCP biotype is integral part of vaccine while cytopathic biotype is also included in the vaccine just for wide-ranging safety (Newcomer et al. 2017).

Killed Vaccine

It is also known as inactivated vaccine. As its name indicates, antigen is present in dead form. Antigen death is result of chemical or heat treatment. Prime motive behind this type of vaccine is to enhance the protection in contrast to Modified live attenuated vaccine. Moreover it doesn't suppress the immune system of the vaccinated individual as well as no pathological effects on fetal health (Kelling 2004). The main disadvantage of killed vaccine is that its dose has to be repeated to achieve the protective titer and immune response is late. Moreover 4 to 6 weeks are required for the activation of immune system of the vaccinated individual from the time of vaccination.

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CHAPTER 24

MYCOTOXINS PREVALENCE IN POULTRY INDUSTRY AND ITS PREVENTIVE STRATEGIES

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INTRODUCTION

Mycotoxins are the secondary fungal metabolites, as a worldwide concern. Fungal growth occurs in aerobic conditions in numerous raw feed ingredients its growth is unavoidable. There are almost 200 species of mycotoxins that are capable of producing mycotoxins. The fungi that produce the mycotoxins are mainly associated with 3 genera; *Penicillium*, *Aspergillus,* and *Fusarium*. While more than 500 known mycotoxins are formed through these fungi, only few of these mycotoxins have pathogenic features. The effects of mycotoxin-contaminated food and feed on people and animals ranges from minor symptom to death (Becer and Filazi 2010; Vikram Patial et al. 2018). Environmental factors such as grain, season, drought, and harvest time influenced the fungus growth and mycotoxins formation in the field during storage and transit. Long-term study indicates that feed and feedstuff may be mycotoxin pollutant materials; these pollutant feed materials typically include several mycotoxin species (Streit et al. 2012). These cereals and oilseeds existing in poultry feeds are the vegetable substance found during vegetation in cropland, storage, and transportation in various climatic conditions. It has been estimated by FAO (Food and Agriculture Organization) mycotoxins contaminate almost 25% of crops worldwide per year, resulting in wastage of roughly 1 billion metric tons of feed and food products annually. Mycotoxins are given special attention because of their adverse health effects, lower output owing to food spoilage, and economic consequences shown in international food and food product. As a result, feed and animal producers must avoid fungal development and the formation of mycotoxin (Kaya 2014). Feed quality, organoleptic characteristics, and nutritional quality are all affected by the presence of microscopic fungus. Generally, concerning animals and humans, mycotoxins show the toxic effects that are categorized through mutagenic, teratogenic, estrogenic, and carcinogenic properties (Cegielska-Radziejewska et al. 2013). *Aspergillus, Alternaria*, *Fusarium*, and *Penicillium* are the genera with the most poisonous species. The studies revealed that mycotoxins include ochratoxin, aflatoxin, T-2 toxin, fumonisin, zearalenone, patulin, and deoxynivalenol mycotoxins in food and feed (Pitt and Hocking 2009; Adeyeye 2020). Poultry mycotoxicosis is caused by eating a low quantity of adulterant over a long period of time. When a high concentration of mycotoxins is consumed, it produces immediate clinical signs and symptoms associated to particular important organs, the immune system, and other areas of avian physiology, as well as death (Abidin et al 2011). The maximum level of AFB1 in poultry feed has been established in the European Union. So, the maximum level of AFB1 in poultry feed has been set at 0.02-0.05 ppm (EU 2011). The health and production of poultry species are dramatically impacted via certain mycotoxins such as ochratoxin A (OTA), aflatoxin B1 (AFB1), fumonisin (FUM), T-2 toxin, and deoxynivalenol (DON) (Figure 1) (Murugesan et al. 2015). The goal of this study is to go through the importance of mycotoxins for poultry and their negative effects, as well as current developments in mycotoxins preventive measures.

Type of Mycotoxins

Aflatoxins

Aflatoxins are the detrimental secondary metabolites categorized as polyketide-derived furanocoumarins primarily formed by *Aspergillus* species, i.e., *A. parasiticus* and *A. flavus*. Feeds are putrefied spontaneously by AFB1, AFB2, AFG1 and AFG2. AFB1, AFB2, AFM1, and AFM2 are found in tissues, milk and biological fluids interrelated with polluted feed intake. These toxic chemicals have been classified as very dangerous to humans by the International Agency for Research on Cancer (IARC) (Dohnal et al. 2014). AFB1 was identified as the causal agent of Turkey X disease in England in 1960s, which caused young turkeys to die after eating infected peanuts (Rawal et al. 2010; Adeyeye 2020). Worldwide, AFB1 has a primary public

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health concern due to its carcinogenic and hepatotoxic effects. Aflatoxins are commonly found in feed constituents that are mainly used for poultry ration. Among aflatoxins, the most extensive and biologically active form is AFB1 (Zhao et al. 2011; Kim et al. 2013). AFB1 is a pro-carcinogen converted to a reactive form by the enzyme CYP450; nevertheless, AFBO (AFB -8,9 epoxide) is necessary for hazardous and carcinogenic activities (Rawal et al. 2010). This AFBO, which is unstable and changes into formamide-pyrimidine DNA adducts, forms an AFB-N7 guanine adduct with DNA, and its repaired activities are assessed as key indicators in carcinogenesis vulnerability. AFB-N7 guanine adducts are a potential biomarker in urine that may be used to measure exposure and probable risk in those who eat AFB1 (Dohnal et al. 2014).

In mammal primary detoxification route of AFB1 is by conjugation of AFBO to endogenous glutathione catalyzed through traditional decontamination enzyme glutathione-S-transferase. Xenobiotics include environmental contaminants and a chemical carcinogen absorbed in phase-II metabolism through the proteins' decontamination process (Hayes et al. 2005).

Reduced weight gain, decreased feed efficacy, decreased egg weight and production, enhanced liver fat, altered organ weight, and reduced serum protein level, carcass bruising, liver damage, poor pigmentation, and the decline in the action of various enzymes involved in the digestion of lipid, protein, starch, and nucleic acid. The data showed that aflatoxins cause immunosuppression, which leads to disease outbreaks, low antibody titers, and vaccination failure (Murugesan et al. 2015; Manafi et al. 2014). Furthermore, due to aflatoxicosis at the time of necropsy liver are enlarged and pale. Microscopically liver lesions include hepatic sinusoid congestion, focal hemorrhages, nodular lymphoid infiltration, biliary hyperplasia, necrosis, and centrilobular fatty cytoplasmic vacuolation. In addition, aflatoxin produces a malabsorption syndrome categorized through hypocarotenoidemia reduced concentration of pancreatic lipase, amylase, trypsin, bile salt, and RNase (Fig. 2) (Bbosa et al. 2013; Murugesan et al. 2015). Furthermore, when the broiler was fed AFB1/kg feed, the hepatic gene expression of superoxide dismutase epoxide hydrolase, GST, was reduced, whereas the gene expression of interleukin 6, 2HI, and CYPIAI was increased at the cellular level (Yarru et al. 2009).

Due to residues of AFB1 in food and feed, mycotoxins lead the health problems and economic losses in the poultry, as well as public health concerns. However, in eggs of layer aflatoxin B1 is accumulated. For example, it has been observed that when AfB1 was taken with feed, then 1/1200 was found to be accumulated in poultry meat; however, AFB1 was found 1/2200 to be accumulated in eggs (Hossain et al. 2012).

Ochratoxins

Ochratoxins are formed through *Penicillium* and *Aspergillus* species such as *A. niger*, *A. verrucosum,* and *A. ochraceus*. Most common and important form of ochratoxin is ochratoxin A (OTA), followed by OTB and OTC (Khatoon and Abidin 2018b). IARC categorized OTA as carcinogenic to humans (Pfohl-Leszkowicz and Mandervile 2012). *Aspergillus* species may produce OTA and OTB. Typically, the OTB level was lesser as compared to OTA. It has been determined that ochratoxin A is nephrotoxic, hepatotoxic, neurotoxic, immunotoxic and teratogenic by in vivo research in many animal species and multiple in vitro techniques. Molecular research on OTA exposed a non-DNA-reactive Genotoxic process that comprises of numerous epigenetic pathways largely linked with oxidative stress, cell division, cell proliferation and signaling disruption (Vettorazzi et al. 2013). In general, the mechanism of action of ochratoxin A action in kidney is carcinogenesis. In poultry, ochratoxin leads to major economic losses and health issues leading to porcine mycotoxin nephropathy (Stoev and Denev 2013). Ochratoxin-related illnesses are classified according to the severity of kidney damage caused by ochratoxin exposure alone or in combination with other mycotoxins (Stoev and Denev 2013). Similarly, tubular atrophy mainly in proximal convoluted tubules was recognized in human endemic nephropathy classified via cellular interstitial fibrosis, karyomegaly. Aristolochic acid from plants and mycotoxins such OTA and citrinin are the etiological agents of endemic nephropathy (Pepeljnjak and Klaric 2010). In poultry, ochratoxin signs and symptoms include anemia, weakness, reduced feed consumption, decreased egg production and growth rate, excessive mortality, and poor feathering (Khatoon and Abidin 2021). Pathophysiological alteration includes diminished glomerular filtration rate and urine concentration, injury of proximal tubular function, ultrastructural changes in renal integrity, and deterioration. It has also been described that poultry fed ochratoxin-A leads to enhance the relative weight of the pancreas, spleen, liver, gizzard, proventriculus, and testes. It has also been observed that the appearance of *Eimeria tenella* and its effect is greater in combination with OTA than the occurrence of coccidiosis alone in broiler chicks (Manafi et al. 2011). It has been analyzed that OTC oral LD50 values were 216mg/animal and OTA 166mg/animal in day-old chick. Other ochratoxin methyl or ethyl ester exposed lesser toxicity than OTA. In day-old chicks, OTA methyl ester was less hazardous than OTA; however, OTB ethyl and methyl esters were less toxic to orally exposed day-old ducklings (Heussner and Bingle 2015).

Fumonisins (FUM) are mycotoxins discovered in the culture of *Fusarium moniliforme* and chemically classified by Gelderblom and colleagues in 1988. It has been discovered the 6 distinct forms of fumonisins and their structures, including fumonisin (FA1), FA2, FB1, FB2, FB3, and FB4. In contrast, fumonisin B1 (FB1) has been recognized to be the major form generated through *Fusarium moniliforme.* Fumonisin B1 is categorized through IARC as probably carcinogenic to humans (Domijan 2012). In the gastrointestinal tract (GIT) and liver, fumonisin B1 was absorbed into incompletely hydrolyzed fumonisin B1 and then to be hydrolyzed form (HFB1) (Fodor et al. 2008). In piglets, fumonisin B1 has been found to be more hazardous than HFB1 (Grenier et al. 2012). In rat observed fumonisin B1 N-acylation and the development of HFB1 in the human cell lines (Guerre 2015). The toxicity of fumonisin in animals appears to be caused by a disruption in sphingolipid absorption. Fumonisin is a selective inhibitor of ceramide synthases like sphinganine/sphingosine N-acyltransferase, a strong enzyme necessary for ceramide synthesis and complex sphingolipids, according to current evidence. The sphingolipids sphingosine (SO) and sphinganine (SA) are increased in tissues when this enzyme system inhibited, and the SO: SA ratio changes. Enhanced SO: SA ratio has been described in the broiler, duckling, and turkey tissues fed fumonisin B1 (Tran et al. 2005). In chicks, turkeys, and ducks, mild to moderate toxicity was

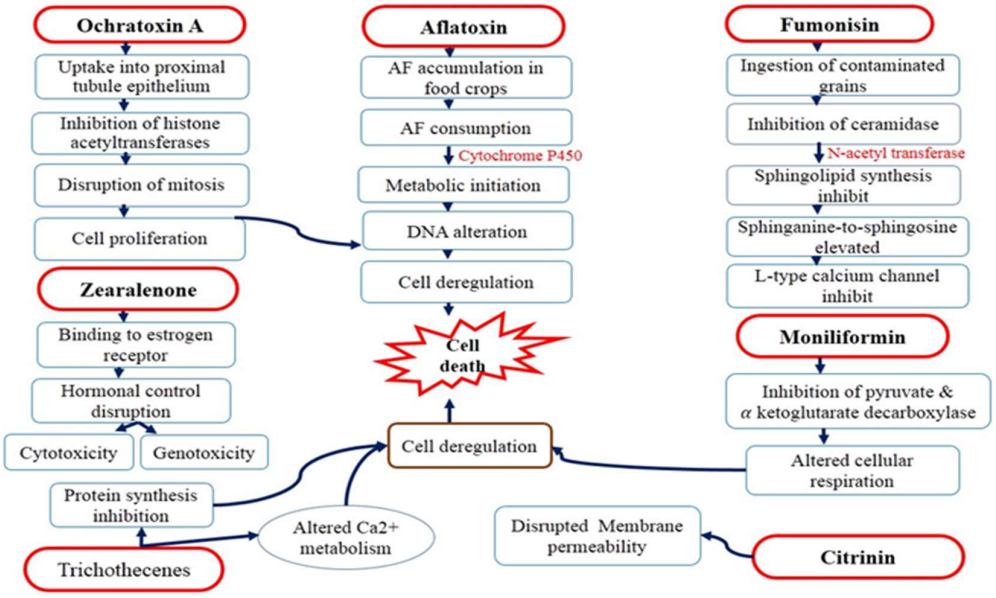

Fig. 1: In toxic cellular injury, cellular events due to mycotoxins.

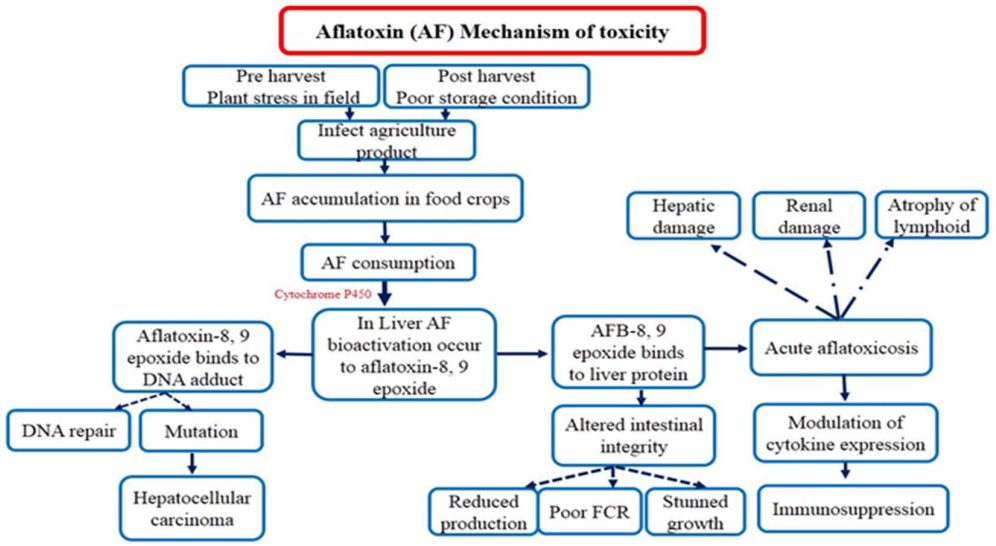

Fig. 2: Aflatoxins disease pathway.

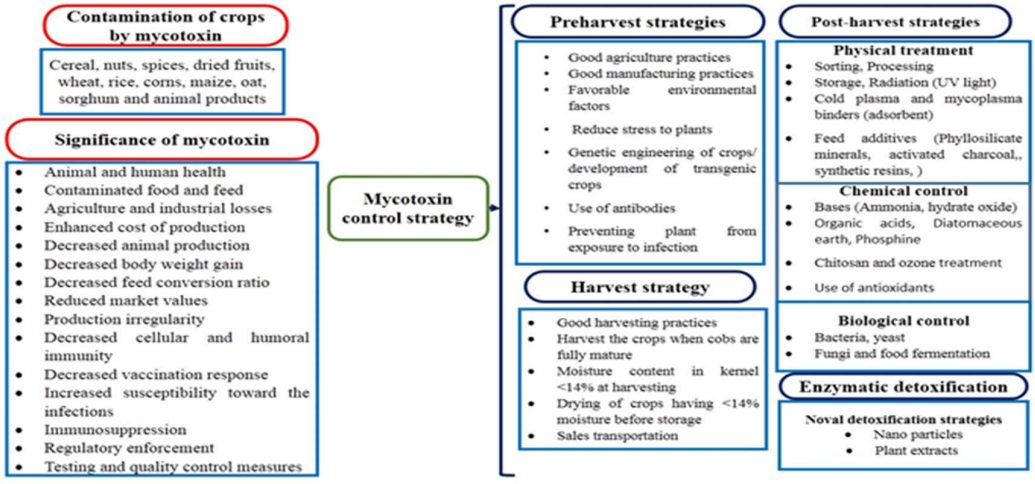

Fig. 3: Mycotoxin Control and decontamination in the food chain through pre- and post-harvest strategies.

Table 1: Aflatoxin contamination observed worldwide in food

Country	Food Ingredient	Level (Range)	Reference
Nigeria	Cereals	17-48.6 µg/kg	Odoemelam and Osu 2009
	Chili	0.1-96.2 µg/kg	Paterson 2007
Japan	Peanut, butter	2.59 µg/kg	Kumagal et al. 2008
Irish	Spices	18.5-27.5 µg/kg	Riordan and Wilkinson 2008
Bahrain	Spices	27.7-69.2 µg/kg	Musaiger et al. 2008
Kenya	Wheat	2-7 µg/kg	Muthomi et al. 2008
Italy	Spices	0.57-26.9 µg/kg	Romagnoli et al. 2007
Argentina	Maize	0-3.19 µg/kg	Broggi et al. 2007
Turkey	Red pepper spices	-97.5 µg/kg	Aydin et al. 2008
Vietnam	Rice	3.31-29.8 ng/g	Nguyen et al. 2007
	Maize	0-126.5 µg/kg	Trung et al. 2008
China	Rice	0.99-3.87 µg/kg	Liu et al. 2006
Pakistan	Poultry Meat & Feeds	3.27-6.97 ng/g	Hussain et al. 2010
Pakistan	Broiler meat	0.49-2 ng/g	Khan et al. 2013
Pakistan	Poultry feed and ingredients	0-149.2ng/g	Abidin et al. 2013 a&b

Table 2: Ochratoxin A contamination detected worldwide in food commodities

Country	Food Commodity	Level (range)	Reference
Tunisia	Cereals	55-117 µg/kg	Zaied et al. 2009
Turkey	Wheat flour	0.025-10.5 µg/kg	Aydin et al. 2008
Morrocco	Rice	0.15-47 µg/g	Juan et al. 2008
Nigeria	Cocoa beans	1.0-277 µg/kg	Dongo et al. 2008
India	Cereal products	39 µg/kg	Kumagal et al. 2008
Argentina	Peanut	5.6-130 µg/g	Magnoli et al. 2007
Vietnam	Rice	0.75-2.78 ng/g	Nguyen et al. 2007
Nigeria	Rice	24-1164 µg/Kg	Makun et al. 2007
Belgium	Wheat	39-832 µg/g	Tangni and Pussemier 2006
Ethopia	Cereals	54.1-2106 µg/Kg	Ayalew et al. 2006
Pakistan	Hatched chicks	0.01-1µg/Kg	Zahoor-Ul-Hassan et al. 2012
Pakistan	Poultry feed and ingredients	July & October Maximum positive sample	Abidin et al. 2017

Table 3: Fumonisin contamination detected worldwide in food

Country	Food Commodity	Level (Range)	Reference
Tunisia	Cereals	70-2130 µg/kg	Ghali et al. 2009
Vietnam	Maize	0.4-3.3 mg/kg	Trung et al. 2008
Southern Africa	Maize bread	142-550 µg/kg	Lino et al. 2007
Portugal	Maize	142-550 µg/kg	Lino et al. 2007
Brazil	Corn flakes	0.127-2.04 mg/kg	Caldas and Silva 2007
	Corn	0.06-17.60 µg/g	Almeida et al. 2002
Argentina	Maize	0-10.2 mg/kg	Broggi et al. 2007
Ethiopia	Sorghum	217 µg/kg	Ayalew et al. 2006
Iran	Corn	71-10674 µg/kg	Ghiasian et al. 2006
United States	Maize	23-79 ppm	Abbas et al. 2006
		0.4-3.3 mg/kg	Trung et al. 2008
Benin	Maize	0.6-2.4 mg/kg	Fandohan et al. 2005

seen by given the fumonisin B1 at 75-400 mg/kg for 21 days. Reduced body weight and liver disease were seen in chicks, ducks, and major turkeys (Bermudez et al. 1995).

Trichothecenes

Trichothecenes are fungal metabolites with an identical basic structure that includes HT-2, T-2, diacetoxyscirpenol (DAS), Neosolaniol, monoacetoxyscripenol (MAS), nivalenol, 4-diacetoxyscirpenol, 8-acetoxyneosolaniol, 4-acetoxynivalenol, DON (vomitoxin), 3-acetyldeoxxynivalenol. Trichothecenes are recognized as the most effective small-molecule inhibitors of protein production, and their primary harmful effects at the cellular level appear to be critical suppression of protein formation, followed by secondary interruption of RNA and DNA formation (Murugesan et al. 2015). The general assumption by IARC (International agency for research on cancer) was toxins resultant from *Fusarium sporotrichioides* categorized as carcinogenic to humans. The most significant mycotoxins are DON for livestock, normally the contaminant of wheat, corn, and other grains. In similar sources, low quantities of DAS and T-2 toxins are found occasionally. Trichothecenes are more tolerable in poultry and cattle than in pigs. In crops, T-2 toxin is less common as compared to DON (Sokolovij et al. 2008). In poultry, trichothecenes lead to chronic and acute poisoning. Acute poisoning has a standard

clinical presentation that can be easily evaluated; however, chronic poisoning includes non-specific clinical symptoms that are difficult to diagnose (Resanovic et al. 2009). Trichothecenes toxicity includes reduced growth, oral lesion, abnormal feathering, reduced egg production and quality of the shell, deterioration of bursa of fabricius, proteinemia, leucopenia, abnormal blood coagulation and immunosuppression (Danicke 2002).

Moniliformin

Moniliformin is a *Fusarium* species' water-soluble secondary metabolite. Moniliformin mycotoxin has been identified as contamination of various food components, including wheat, maize, barley, and other crops used in the production of chicken feed, all over the world. In addition, it is linked with cardiotoxicity in poults, chicks, and Japanese quail categorized through hypertrophic cardiomyopathy. Furthermore, in the kidneys and liver of poultry species, moniliformin has been found to have damaging effects (Sharma et al. 2012).

Citrinin

Citrinin (CIT) is a nephrotoxic mycotoxin that is produced when penicillin is used. Citrinum is commonly found in conjunction with ochratoxin in a variety of chicken feed additives (Ostry et al. 2013). Citrinin has been worried about the role of poultry species in endemic nephropathy (Stoev et al. 2010). Citrinin has been shown to be an immunotoxic, neurotoxic, embryotoxic, and teratogenic mycotoxin in a variety of animal species (Singh et al. 2014). Citrinin appears to operate primarily through the kidney, causing necrotic and degenerative changes in renal tubular epithelial cells.

Prevention and Control of Mycotoxins

The support of prebiotics and probiotics are expanding the intestinal barrier integrity and binding to mycotoxins that control the mycotoxins absorption in the bloodstream (Khatoon and Abidin 2020). Probiotic species i.e., *Bacillus* improve serum and liver antioxidant parameters such as glutathione peroxidase and total superoxide dismutase, as well as lowering the aflatoxin deposits in the liver and changing the chemical structure of mycotoxins with a methyl group and lactone ring (Zhang et al. 2016b; Hathout et al. 2011). The yeast cell wall from the gut may absorb the mycotoxins due to a weak van der Waal and hydrogen link that enhancing their contact (Jouany et al. 2005; Agriopoulou et al. 2017). Clay has non-nutritional advantages in terms of reducing mycotoxins effects by absorbing or binding the mycotoxins, therefore controlling their bioavailability and tissue residues. Bentonite clay enhances interlayer gaps, cation exchange capacity, and suitable pore sites by adsorbing and binding mycotoxins. This changes the structure of the phyllosilicate to enhance the efficiency and cation exchange capability (Onal and Sarikaya 2008; Agriopoulou et al. 2017). Because of the potential difference between them, another additive might adhere to mycotoxins by strong electrostatic contact.

Table 4: Generalized lesion produced by numerous mycotoxins in Poultry

Mycotoxins	Animal species	Lesions	References
Diacetoxyscirpenol (DAS)	Guinea pig	Inflammatory response	Ueno et al. 1970
T-2 toxin	Broiler chicks	Enhanced prothrombin time, inhibited factor VIII, and fibrinogen activities	Doerr et al. 1974
Trichothecenes	Geese, chick	Potent emetic and irritant activity	Palyusik and Koplik-Kovacs 1975
Trichothecenes	Poultry, swine	Stomatitis, dermatitis, proventriculus, gastritis, vomiting, and feed refusal	Wyatt et al. 1972
Moniliformin	Ducklings	Cardiotoxicosis leads to death	Burmeister et al. 1979
Zearalenone	Poultry	Salpingeal cysts, inflammation of edema, vent, and neck	Gedek 1980
Ochratoxin, aflatoxin, patulin, trichothecenes	Animal, human, poultry	Teratogenic and Embryotoxic effects	Lorenzana et al. 1985
Zearalenone	Boars, guinea cocks	Spermatogenesis disorder	Vanyi and Szeky et al. 1980
Deoxynivalenol (DON)	Dairy and poultry	Fatty liver, cirrhosis, and diarrhea	Huff et al. 1988
Diacetoxyscirpenol (DAS)	Poultry	Lesion on the tongue, jowl, upper and lower beak	Ademoyero and Hamilton 1991
T-2 toxins	Laying hen	Decreased egg production and feed intake, oral lesion	Diaz et al. 1994
Moniliformin	Poultry	Dyspnea, depression, cardiac dysfunction leads to death	Ledoux et al. 1995
Nivalenol	Chicken	Stomach mucosa erosion	Hedman et al. 1995
Fumonisin	Broiler	Enhanced activities of antithrombin III, reduced prothrombin time	Espada et al. 1997
Aflatoxin and Ochratoxin (Pakistan)	Chicken embryo	Embryo mortality was 50-83.3% by receiving AFB1 78.83-84.64 ng/egg	Saleemi et al. 2015
Aflatoxin B1 (Pakistan)	Broiler birds	Ruffled feather, depression, decreased feed intake, increased water intake, nervous sign (torticollis)	Hussain et al. 2016
Ochratoxin A (Pakistan)	Broiler chicks	OTA at the dietary level of 1.6, 3.2 and 6.4mg/kg induced oxidative stress in broilers	Hameed et al. 2017
Ochratoxin A	White Leghorn cockerels and broiler chicks	Immunosuppression by varied doses of OTA	Khatoon et al. 2013; 2017 and 2018a

Table 5: Additive with mycotoxin mitigation effects in Poultry

Additive	Doses	In-vitro/in-vivo	Mycotoxin	Effect	Reference
Dietary carbon supplementation	2.5 and 5.0 g/kg	Broiler chickens	Dietary contamination of 0.1, 0.2 and 0.6 mg/kg	Combating the immunotoxicity of 0.1 and 0.2 mg/kg dietary AFB1	Bhatti et al. 2021
Indigenous mycotoxins binder	1 and 2 g/kg	Broilers	Dietary intake of 300 µg/kg AFB1	Ameliorate the toxicopathological effects of AFB1 in a dose dependent manner	Saleemi et al. 2020
Dietary activated carbon	2.5, 5.0 and 10 g/kg	Broilers	Dietary intake of 0.15 and 0.3 mg/kg OTA	Partial reduction in intensity of OTA induced toxic effects also having limited adsorptive potential	Bhatti et al. 2020
Bentonite and montmorillonite	20g/kg dry matter of concentrate	Poultry and Goat	56.7 µg/kg AFB1 and 112.5 µg/kg ZEA	Reduced rumen level of AFB1 and ZEA, declined AFM1 in milk and ZEA in feces	Gouda et al. 2019
Allyl isothiocyanates (AITC)	Gel dispositive 5ml of AITC for 60 days	100 small scale silo system in vitro	A. *flavus* and P. *verrucosum*	Declined OTA formation by *P. verrucosum* and declined AF formation	Quiles et al. 2019
Yeast cell wall extract (1-3) β-d-glucan (βG)	3 g βG/day	Poultry and Dairy goat	25 µg AFB1/kg dry matter intake	Decreased milk AFM1 level, milk AFM1 extraction, and carry-over of milk AFM1	Aazami et al. 2019
Bentonite clay	3.7 and 7.5 g/kg	Broiler	0.1-0.6 mg/kg AFB1	Declined liver AFB1 residues by 41-87%	Bhatti et al. 2017
Distillery sludge	5, 10 and 20g/kg	Broiler	150, 300 and 1000µg/kg	Distillery sludge ameliorated 150 and 300µg/kg OTA induced toxicity	Khatoon et al. 2017
Bentonite	5, 10 and 20g/kg	Broiler	0.15, 0.3 and 1.0mg/kg	Few to no effects of feeding bentonite clay upon OTA- induced alterations in different immune parameters.	Khatoon et al. 2018a
Zeolite	2%	Laying duck	70 ppb AFB1	Numerically declined AFB1 residues in egg and liver while markedly declined AFB1 residues in duck meat by 65%	Sumantri et al. 2018
Difructose anhydride III	40 g DFA III/day	Poultry and Dairy	0.22-0.27 mg/kg ZEA	Reduced urinary extraction of total ZEA by 51-69.71%	Toda et al. 2018
Bentonite Clay	3.7 and 7.5 g/kg	Broiler chicks	Dietary adulteration of 0.1-0.2 mg/kg of AFB1, 0.15-0.3 mg/kg OTA	Ameliorate the toxic effects of AFB1 and OTA in various parameters	Bhatti et al. 2016
Thai bentonite (having montmorillonite)	Heating treatment of 25C-700C	In vitro	NS	Adsorption of mycotoxin between 49-97%	Wongtangtintan et al. 2014
Vitamin E	100mg/Kg	White leghorn breeder hen	0.5-2.5 mg/kg AFB1/kg dry matter intake	AFB1 immunotoxic effects in progeny chicks potentially mitigated by Vitamin E	Khan et al. 2013
Sodium bentonite	0.3%	Broiler	Diet having 50 µg/kg AFB1	When broiler was fed AFB1 in the diet leads to declined liver residues by 62.5%	Magnoli et al. 2010

Mitigation Strategies

For food product safety, food consumers' safety, and acceptance of international market, reduction of mycotoxins residues is necessary in animal products. Furthermore, mycotoxins reduction techniques must be safe and ecologically acceptable for animal products i.e., milk, meat, eggs, and tissues to be suitable and environmentally sustainable. As a result, we must create techniques to avoid the formation of toxigenic fungi and mycotoxins in the field and during storage. Thus, it is difficult during pre-and post-harvest the entire eradication of fungus and their metabolites. As a result, discovering a way to filter or eliminate mycotoxins from food or the food chain is critical. Microorganism, clays, botanicals, and various other harmless biosynthesized materials in animal products may be used to address such mycotoxins residues, and these anti-mycotoxigenic products have been used for this resolution (Khatoon and Abidin 2020).

Poultry

Legumes and grains are key components of the poultry diet. They've been recognized as a potential source of mycotoxins in animal feed. In Nigeria, cereals such as millets, sorghum, and maize, as well as their derivatives acquired from diverse agro-ecological zones, were polluted by *Fusarium* mycotoxins in

Table 6: Several microbes used as probiotic against numerous mycotoxins

Bacteria/fungi	Mycotoxins/fungi strain	Type of study	References
Pichia Kudriavzevii	AFB1	In vivo	Ali et al. 2021
R. erythropolis, Pichia guilliermondii, Metschnikowia pulcherrima	OTA	In vitro	Patharajan et al. 2011
Acinetobacter calcoaceticus	Ochra	In vitro	Halasz et al. 2009
B. subtilis, Trichoderma virens	AFB1	In vitro	Reddy et al. 2009
L. acidophilus, Bifidobacterium animalis	OTA, patulin	In vitro	Fuchs et al. 2008
Nocardia corynebacteroides	Afla	In vitro (chick)	Tejada-Castaneda et al. 2008
S. cerevisiae	AFB1	In vitro	Shetty et al. 2007
B. mojavensis	*Fusarium* species	In vitro	Bacon and Hinton 2007
B. subtilis, P. fluorescens	F. graminearum	In vitro	Nourozian et al. 2006
L. casei, L. rhamnosus	*A. flavus* strain	In vitro	Bueno et al. 2006
R. erythropolis, M. fluoranthenivorans	AFB1	In vitro	Teniola et al. 2005
S. cerevisiae	OTA	In vitro	Bejaoui et al. 2004
B. Glucans	Zearalenone	In vitro	Yiannikouris et al. 2004
S. cerevisiae (cell wall)	AFB1	In vitro	Santin et al. 2003

Table 7: Various antioxidants used against several mycotoxins

Antioxidant	Mycotoxin/fungi	Type of study	Reference
L. carnitine and Vitamin E	OTA	In vivo	Bhatti et al. 2018; Abidin et al. 2013
L. carnitine	OTA	In vivo	Abidin et al. 2013 & 2016
Silymarine	OTA	In vivo (Leghorn cockerels)	Khatoon et al. 2013; Ahmad et al. 2012
Zinc	OTA	In vitro	Zheng et al. 2013
Vitamin E	AFB1	In vivo	Khan et al. 2013
NAC BSO	OTA	In vitro	Bosch-Saadatmandi et al. 2012
PECE	OTA, AFB1	In vitro	Corcuera et al. 2012
Coffee beans	OTA	In vitro	Santini et al. 2011
Ocimum gratissimum L essential oil	AFB1	In vitro	Prakash et al. 2011
Piper betie L essential oil	*A. flavus*	In vitro	Prakash et al. 2010
BHA, PP	OTA (Peanut)	In vitro	Barberis et al. 2010
Vitamin E	OTA	In vitro	Fusi et al. 2010
Turmeric	AFB1	In vitro (Broilers)	Yarru et al. 2009
Vitamin A, C &E	AFB1	In vitro	Alpsoy et al. 2009

normal ranges or over the European Union's upper limit (Chilaka et al. 2016). Likewise, the mycotoxigenic impact seen in the liver of birds fed groundnut cake-based diets resulted in its use being limited by feed manufacturers. During the wet season, poultry farmers used fresh corn to reduce feed production costs. Involuntarily, maize grains retain more moisture, providing a suitable environment for the development of mycotoxins and fungal growth, as well as fatal effects on animals and residues deposit in their products. When aflatoxin enters the body, it causes mycotoxins to malformed in the liver via cytochrome P-450 related enzymes, resulting in the production of AFM1 and AFB1 (Biehl and Buck 1987). Aflatoxin-adulterated feed is purified predominantly in the kidney and liver, which are the primary sites for residue buildup (Hussain et al. 2010). Due to contamination with aflatoxin residues, the quality of eggs and meat has deteriorated, and these items are sold without being inspected. Anti-mycotoxigenic additives must be used to reduce mycotoxin residues in poultry-based products.

Conclusion

The frequency and incidence of mycotoxins in chicken, as well as its unique and cumulative amin deleterious effects, have become critical. Based on biodegradation adsorption of non-mycotoxin discovered in diverse microbial groups, a fresh perspective on real microbial decontamination methods is required in the field. Different harmful unpleasant toxic compounds in other study disciplines, as well as various mycotoxins, have structural similarities and are indicated to be successfully decontaminated by microbes. The accuracy of measuring the amount of various mycotoxins in agricultural supplies will be improved by using contemporary analytical technique such as liquid chromatography mass spectrometry LC/MS. The present enzymatic degradation method aids in the elimination of mycotoxins that are not bound by binder products. In general, mycotoxins continue to represent a greater hazard to poultry, and experts throughout the world are continuously looking for new management techniques.

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CHAPTER 25

ANTIMICROBIAL RESIDUES IN MILK AND DAIRY PRODUCTS

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INTRODUCTION

Milk is a nutrients-rich liquid produced in mammals and being known to humanity for thousands of years. Milk is one of the most commonly consumed food items globally owing to its excellent nutritional value for human health. Milk contains valuable proteins, diverse fats, and vitamins such as vitamin B12, riboflavin, and pantothenic acid. Milk industry also supplements their milk and dairy products with other vitamins, especially vitamin D. Important minerals are present in milk including calcium, magnesium, selenium, and phosphorus. Moreover, raw milk is processed for industrial purposes to produce a diverse dairy product such as cream, butter, cheese, and yoghurt (Scrafford et al. 2020; Almashhadany 2021a).

In the last decades, massive animal production has been aided by medicinal products; especially, anti-infective drugs. Antimicrobial agents became a cornerstone in the field of animal industry, especially for three primary purposes: therapeutic, prophylactic, and growth enhancement for weight gain (Arikan 2008). Antibiotics are usually administered to milk-producing animals in the drinking water or feed. Sub-therapeutic doses are commonly used as a prophylaxis to healthy animals considered at risk of certain diseases. Additionally, administration at minimum doses of antimicrobials, usually as a feed additive, may be practiced to suppress gut bacteria leaving more nutrients for animals to be absorbed (Jammoul and Darra 2019; Rasschaert et al. 2020).

Chemically, antimicrobial drugs are heterogeneous groups of bioactive small organic molecules naturally produced, at low concentrations, as secondary metabolites in microorganisms such as fungi and bacteria. These groups of drugs comprise antibiotics, antifungals and antiprotozoal that either kill or suppress the microbial pathogens (Singh et al. 2019; Savarino et al. 2020). It is estimated that the quantities of antibiotics used for animal industry worldwide are two-fold the quantities used for human medication and clinical settings. Nearly all the antimicrobials used for humans are also used for food-producing animals, including the most modern classes of antibiotics (Aarestrup 2012; Al-Mashhadany 2020). Antibiotics, also known as antibacterial agents, are a subset of antimicrobial drugs that are active against bacterial infections. Antibiotics either kill or inhibit bacterial growth by disrupting bacterial cell wall or protein biosynthesis in their cytoplasm (Al-Mashhadany 2020). The global use of antibiotics in livestock is 63,151 ± 1,560 tons/year (Van Boeckel et al. 2015). Unfortunately, remnants of such drugs remain in animals' tissues or eliminated in their milk or eggs (Al-mashhadany 2020).

Regulatory authorities affiliated with the European Union (EU) and Food and Drug Administration (FDA) have defined the antimicrobial residues (AMRs) as substances (or their metabolites) associated with impurities of pharmacologically active compounds in foodstuffs derived from treated animals (Aidara-Kane et al. 2018; Stella et al. 2020). Based on this criterion, even well-controlled administration of antimicrobial drugs will leave AMRs in animal tissues or products for a specific period, called withdrawal period, before AMRs become undetectable (Tang et al. 2017; Gholami-Ahangaran et al. 2019). Lack of awareness among breeders and farmers regarding the withdrawal periods and health risks associated with residues contamination, especially in developing countries, is globally recognized (Rana et al. 2019). Additionally, failure to follow the instructions of antibiotics manufacturers also accounts for residues occurrence in milk (Bacanlı and Başaran 2019).

In veterinary medicine, the withdrawal period (WDP) of a drug is defined as the minimum time between the administration of last dose and the production of meat or milk from the treated animal after which the AMRs are lower than the maximum residue limit (MRL) (Abo El-Makarem et al. 2020). WDP varies widely (ranges from hours to weeks) between different drugs as it depends on the chemical nature of the drug, dose, route of administered and species of animals to which drug is administered (Al-mashhadany et al. 2018; Okocha et al. 2018). Most public health authorities consider the presence of AMRs as an illegal practice in animal-derived foods above the MRL (Jayalakshmi et al. 2017). In turn, the MRL is defined by regulatory authorities as the highest concentration of a compound in food materials to be safe for animal or human consumption at a specific time (Almashhadany 2009; Kebede et al. 2014). The MRL of various antimicrobials, pesticides, and metals are established after rigorous studies by world-class regulatory authorities affiliated with the European Union (EU), the World Health Organization (WHO), and the American Food and Drug Administration (FDA).

The presence of AMRs in milk and dairy products have been a major concern for public health officials (Olatoye et al. 2016; Al-Mashhadany 2019; Alaboudi et al. 2021). Quality control systems require accurate and cost-effective quantification approaches to detect AMRs in milk and dairy products (Sachi et al. 2019; Almashhadany et al. 2020). There is an increasing

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trend in research on antimicrobial residues in milk and dairy products since their first detection in 1960s. The PubMed search of AMRs in milk and dairy products retrieved 463 articles published since 1960 up to 2000. Interestingly, additional 1006 articles were published since 2000 to date (29-Jan-2022).

Sources of AMRs in Milk and Dairy Products

Irrational use of antimicrobial drugs during treatment of infections is the main source of AMRs in milk (Zhang et al. 2009). Furthermore, indiscriminate addition of antimicrobial agents as feed additives is another important source (Na Lampang et al. 2007). Contamination of milk starts right from the intake of contaminated pasture or/and water by the animal (Akhtar and Ahad 2017). Furthermore, incorrect use of veterinary drugs in dairy animals without considering the WDP results of AMRs in milk and dairy products. The contaminants may also be introduced, with lesser extent, during milk collection, preservation, transport, processing, and packaging. Additionally, improper milking or milk collection, insufficient cleaning, poor hygienic conditions, i.e., inappropriate management practices, also contribute to milk contamination by drugs and chemicals (Sachi et al. 2019). Besides antimicrobials, other compounds like pesticides, insecticides, herbicides, detergents, disinfectants, mycotoxins, nitrates, nitrites, and heavy metals have also been detected (Shaikh and Patil 2020). The possible reasons for presence of residues in milk and milk products will be discussed in detailed as follows:

Therapeutical use of Antibiotics

The major source of AMRs in milk is the use of antibiotics in treating infectious diseases, such as clinical mastitis and other diseases. Diseased animals receive antimicrobial treatments through different routes, such as parenteral, oral (through food and water), topical, and by I/M or I/U infusions. Antibiotics as feed additives are administrated orally, while the intra-mammary route is considered as the most effective for mastitis. Theoretically, all these routes may lead to appearance of AMRs in the milk, but intra-mammary infusions cause a higher incidence of drug residues. The notation of "Unintentional Drug Residues" refers to the drug residues that occur in food due to circumstances not intended to treat or protect animals against diseases (Nisha 2008).

Prophylactics use of Antibiotics

Sometimes, antibiotics are used to prevent disease establishment or to manage post-surgical risks of developing new infection. In both cases, AMRs are likely to reach milk and dairy products prepared from these animals (Nisha 2008).

Irrational use of Antibiotics

The misuse and overuse of antimicrobials in veterinary practices lead to accumulation of large amounts of residues that pose serious threats to human health and economy after consuming contaminated foods. Situation gets even worse by the lack or shortage of information among animal breeders about withdrawal periods of antibiotics (Nisha 2008; Cepurnieks et al. 2015).

Extra-label Drug usage (ELU)

ELU refers to the use of approved drugs but without strict adherence to the labelled directions. ELU can occur in many forms such as using a drug only approved for human to treat infections in animals or when a drug approved for one species of animal is used in another (Ali et al. 2020). The following examples illustrate different cases of ELU: -

A. Administration via non-recommended route, (e.g. flunixin intramuscularly).
B. Increase or decrease of specified dose on the label.
C. Administering a drug for any disease not listed on the label.
D. Administering a drug to different animal species.
E. Violation of treatment duration.
F. Shifting the amount of drug per injection site.

Inadequate Withdrawal Period

Violation of withdrawal period (WDP) for the administered antibiotics leads to elevated levels of AMRs in milk or milk products (Kebede et al. 2014).

Use of Drug-contaminated Equipment

Any equipment used for milking process should be clean and sterile. Contamination of equipment during maintenance can retain residues inside the machine and subsequently released into the milk during production. Additionally, milking items should not be placed in or near the medication preparation area. Similarly, inadequate cleaning of equipment may lead to the carryover of trace amounts of antimicrobials from one batch to another.

Improper Discarding of Empty Antibiotics Containers

Inappropriate discarding of empty containers of antibiotics in the farm premises plays an important role in contaminating feeds of animals. Animals may even lick those containers or accidentally are exposed to contaminated feeds.

Contaminated Feed and Water

Antimicrobials can also enter the milk through contaminated feed and water. Improper storage of feeds or water next to antimicrobial containers or preparation area may accidentally introduce those chemical into feed or water. The interaction of any drug with nutritional components is an important clinical phenomenon that significantly influences the therapeutic prognosis of diseased animals. Drug-Nutrient Interactions (DNIs) occur either directly between drug and nutrient or indirectly between drug and nutritional status or vice-versa.

Disease Status

The disease status of an animal negatively affects the metabolic system and consequently the pharmacokinetics of administered drugs. For instance, it has been found that cattle with acute mastitis accumulate apramycin in their udders ten times higher than the levels healthy cows (Ture et al. 2019). Such concentrations may last for longer periods even after apparent recovery of cattle (Anika et al. 2019).

Improper Cleaning of Equipment

Equipment pollution may come from any of the materials that have been in contact with the instrument surfaces, so cleaning is one of the significant techniques in pharmaceutical manufacturing. In the pharmaceutical industry, Good Manufacturing Practices (GMP) requires that the cleaning of drug manufacturing equipment be validated.

Impact of ARMs on Human Health and Dairy Industry

Impact of ARMs on Human Health

Ideally, milk should not contain any constituents or any exogenous chemicals that may result in toxicity or other health issues in consumers. Milk safety regulations were set to protect consumers from potential harms associated with AMRs in milk and dairy products. Not only direct consumers are affected by AMRs, but also the starter cultures in the dairy industry are affected negatively leading to economic losses (Beyene 2015). The presence of AMRs in food has been associated with hazardous threats such as toxicity, drug allergy, hypersensitive reaction, and development of antibiotic-resistant bacteria.

Drug Allergy and Hypersensitive Reaction

A range of allergic reactions to drugs have been linked to AMRs which can take the form of life-threatening toxicity, mild cutaneous reactions (rashes and itches), serum sickness, anaphylaxis, and delayed hypersensitivity.

Effects on Human Gut Microbiota

Massive numbers of microorganisms and viruses, collectively called gut microbiota, colonize healthy human intestinal tract. The colon harbors the majority of microbial communities that include bacteria, fungi, protozoa, archaea, and viruses. The gut microbiota is estimated to be ~1013–1014 microbial cells (Sender et al. 2016). Gut microbiota varies from person to person but they play similar roles such as competition with pathogens and providing vitamins (LeBlanc et al. 2013). Disruption of balanced microbial population in gut can trigger diseases of different nature at different ages ranging from allergies at an early age to inflammatory bowel disease (IBD) in young adults. AMRs of broad-spectrum antibiotics may inhibit a wide range of gut microbiota, which provide chances for pathogenic species to expand their populations and initiate diseases.

Development of Antimicrobial Resistance (AMR)

Development of antimicrobial resistance by pathogens has been a global concern since early 1960s. Misuse of antibiotics has been the main contributing factor for emergence of resistant strains that were naturally susceptible to different antibiotics. A recent alarming report estimated that up to 10 million individuals would be victims of drug resistant pathogens by 2050 unless the problem of resistance is mitigated (de Kraker et al. 2016). Continuous exposure of bacterial species to sub-inhibitory concentrations of antimicrobial agents is a selective pressure for resistant phenotypes. The genes responsible for drug resistance can be transferred horizontally between bacteria associated with milk and dairy products and the pathogenic species.

Effect of AMRs on Bone Marrow

Before being banned in numerous countries, chloramphenicol had been used for decades to treat various infections in cattle. However, later studies revealed that chloramphenicol toxicity in susceptible individuals might result in aplastic anemia. Residues of chloramphenicol may remain in milk and dairy products or edible tissues of treated animals and trigger dysfunctions of bone marrow in susceptible humans.

Carcinogenic and Mutagenic Effects

Many substances with abilities to induce cancers are called carcinogens. Residues of certain antibiotics have carcinogenic properties and interact with intracellular components such as proteins, DNA, and ribonucleic acid, which alter their normal functions. The term mutagen describes any chemical or physical agent that can damage the genetic material or cause inheritable changes in the sequence of DNA. The mutagenic effect is another dangerous impact of ARs, which can cause mutation of DNA molecule or damage of chromosomes.

Teratogenicity

Chemical substances that induce congenital abnormalities in embryos are called teratogens. Most of their damaging effect is exerted during the gestational periods. Long-term exposure of mothers to AMRs at the early stage of pregnancy has been linked to handful of congenital anomalies.

Other Harmful Effects

Consumption of milk or dairy products with residues of tetracycline or azithromycin has been found to cause lifelong discoloration of nails, teeth in children or impairments of the immune and cardiovascular system (Kurjogi et al. 2019). These drugs are usually reabsorbed through entero-hepatic circulation and deposited in teeth and bones for long periods.

Impact of ARMs on Dairy Industry

Effects on the Dairy Industry

Microbial fermentation had been exploited to increase shelf life and safety dairy products such as cheese and yoghurt. The presence of AMRs in raw milk, even at low concentrations, is a significant threat to the fermentation process. Impairment of starter cultures or total inhibition of microbial growth may result in a low-quality products or total waste of resources and customer confidence (Kumar and Chordia 2017).

Inhibits Lactic Acid Fermentation

Fermented dairy products, such as fermented milk, whey beverages, and yoghurt include most of the food containing probiotic cultures. The fermentation of milk offers a simple way to increase its shelf-life, improving its safety. Various strains of fungi and bacteria are used to ferment milk to produce a wide variety of dairy products, viz. cheese, curd, kumis, yoghurt, and kefir. The main bacteria are Lactic Acid Bacteria (LAB), used for clotting milk. The nature of all these products depends on the pre-treatment of the milk, the temperature (climate), the type of milk used, conditions of fermentation, and the subsequent industrial management (Kumar and Chordia 2017).

Effects Flavor Development

Lactic acid bacteria (LAB) change lactose to lactic acid. Production of lactic acid, acetaldehyde and diacetyl are their main assistances to the flavor of fresh cheeses and cultivated milk. However, cheese flavor cannot be produced without starter bacteria. Rennet alone is mainly responsible for forming large, medium and small peptides but, without interaction with other enzymes, can produce only methionine, histidine, glycine, serine and glutamic acid at quantifiable levels. Free amino acids in Cheddar cheese are mainly the result of microbial peptidase activity. Together with the products of glycolysis, these amino acids form substrates for secondary flora, the nature of which, in many cases, determines the cheese variety. In matured cheeses, the starter bacteria die out quickly, and the rate at which they lyse and release their enzymes into the system influences the rate at which free amino acids are formed.

Techniques used for Detection of AMRs in Milk

Different assessment tests have been developed to detect AMRs in milk and dairy products (Table 1). Laboratory detection of AMRs in milk and dairy products is performed in two phases; (i) screening for the presence of different antimicrobial groups and (ii) identification of the specific antimicrobial in the sample (Almashhadany 2020, 2021c). Screening tests should be inexpensive, rapid, and easy to perform with good sensitivity and specificity. The most widely used tests are those based on the inhibition of specific bacterial species in response to the presence of AMRs in milk samples.

Prevention and Control of AMRs in Milk and Dairy Products

The overall public health impact of drug residues in milk and dairy products can be reduced by cooperation between and support for researchers, veterinarians, farmers, producers, manufacturers, consumers, and legislative and food safety authorities. Various international organizations such as the WHO, FAO and the European Commission have confirmed the significance of wisdom, prudent, and rational use of antimicrobials in food-producing animals to reduce the negative impacts of AMRs on public and animal health. Indeed, avoidance or complete removal of AMRs from milk and dairy products is not an easy task. However, approval of confirmed safety techniques and guidelines may help in reducing the residues to non-toxic levels (Khaniki 2007; NMPF 2016; Rama et al. 2017). The guidelines are summarized as follow:

Regulatory Laws and Good Practice

Farms are the first and most important point where control strategies can successfully limit the AMRs in milk and dairy products. Livestock health and raw milk quality are responsibilities of farmers. Adoption of efficient animal health programs to reduce mastitis and other infections among lactating animals is necessary (Sarrazin et al. 2014). On-farm biosecurity and implementation of effective management practice for infected animals are critical practices to prevent further spread of infections and further needs for more antimicrobial usage (Nöremark and Sternberg-Lewerin 2014). Various authorities concerned with food safety measures have emphasized the paramount role of testing raw milk before selling or processing for new dairy products. For instance, USDA specifications require that all processing plants must evaluate the AMRs in raw milk before initiating any food processing attempts. Moreover, the American Occupational Health and Safety (OHS) legislations as well as other similar authorities in various countries have urged the hygiene standards and health safety of farms and milk collection workplaces (Reed et al. 2013).

Development of Public Awareness

Farmers and feed dealers are the most important occupations that need to be aware of rational use of antimicrobial agents. Understanding the risks and benefits of these chemical is expected to play crucial roles in their practices in farms and cooperation with surveillance campaigns. Awareness programs and educational training of farmers and feed dealers are highly encouraged. Dissemination of health awareness through the media (audio, visual media, and newspapers) to highlights the hazards of AMRs would reduce the burden of milk contamination.

Table 1: Techniques used for detection of AMRs in milk

Technique	Features/types	Accuracy	Note
Microbiological	1- Low cost 2- Ability to analyze a large number of samples 3- Ability to detect a broad spectrum of antibiotics 4- Need short time for sample preparation 5- Easy to use 6- Can be efficiently adopted by laboratory staff 7- Do not require expensive equipment 8- Has two types: A- Direct method B- Extraction method	+	Based on bacterial models that should meet the criteria of being: 1- Highly sensitive to antibiotics 2- Fast-growing Bacteria 3- Resistant to environmental conditions 4- The incidence of genetic mutations is rare 5- Heat resistant Examples: *Bacillus subtilus*, *B. cereus*, *B. stearothermophilus*, *Lactobacillus bulgaricus*, and *Streptococcus thermophiles*
Immunochemical	1-Radio Immuno Assay (RIA) 2-Enzyme Linked Immunosorbent Assay (ELISA) 3- Electrophoresis method	++	
Chemical	1- High-Performance Liquid Chromatography (HPLC) 2-Thin layer Chromatography (TLC) 3-Liquid Chromatography (LC) 4- Gas – Chromatography (GC)	+++	

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Monitoring

Periodic monitoring of AMRs in milk and nationwide surveillance are needed to track down any sources of milk contamination. Improper detention centers and absence of monitoring systems may facilitate the presence of AMRs in levels exceeding the MRLs since the contamination goes unnoticed under such circumstances. Several monitoring methods are well-established to evaluate the levels of AMRs and their identity (Kebede et al. 2014). These methods vary greatly in terms of ease applicability, sensitivity, and cost. Rapid testing of the presence of antibiotics in raw milk to grant its quality has become a major task for farmers and dairy industry. The conventional analytical methods are either too slow or do not enable quantitative detection of antibiotic residues, so alternative methods that are rapid, cost effective, and easy to perform should be considered. The recent applications of biosensors generate qualitative and quantitative data regarding the AMRs in tested milk (Beltrán et al. 2015). Inadequate detection facilities of AMRs and shortage of monitoring system of residues may be considered as significant drawback in establishment of control strategies especially in developing countries.

Milk Processing

In addition to their role in preparation of different dairy products, milk-processing techniques that involve pasteurization have also desirable effects against AMRs. Indeed, pasteurization heat is sufficient to deactivate many AMRs that may exist in raw milk. The toxic activities of AMRs may disappear if they are subjected to chemical alterations. Still other processing techniques use UV radiation that also deactivate certain AMRs (Rahman et al. 2021).

Drugs used for Lactating and Non-lactating Animals

Farmers should be aware of those certain drugs are authorized to be used for dry animals only and vice versa. Strict adherence to labeled instructions is necessary. Regarding the lifelong additives, the Acceptable Daily Intake (mg per kg of body weight) should also be considered and only permitted quantities are to be administered (Almashhadany 2021b).

On-Farm Biosecurity

On-farm biosecurity is an essential part of disease prevention and control; this applies to live animal contacts and indirect contacts, e.g., via professionals visiting farms in their work (Nöremark and Sternberg-Lewerin 2014). In the 21st century and late 20th century, there has been a shift from treating individuals toward disease prevention, which has led to an increasing emphasis on the implementation of biosecurity (Sarrazin et al. 2014).

Dairy Producers

Worthy milk has high fat and protein, low number of bacteria per ml, low number of somatic cells per ml, no dirt, no adulterations, no AMRs, produced by healthy cows, and handled by healthy people. So the dairy farmers should apply Good Agricultural Practices (GAP) when using antimicrobial agents in different sectors, such as milking techniques, milking hygiene, animal health, animal welfare, nutrition, and environment.

Prevention from Diseases

Farmers must follow the hygienic and proper management practices at animal farms and dairy units. Farmers are responsible for the health of their livestock. They should implement a preventive animal health program to reduce the incidence of disease, maintain milk quality, and implement an effective mastitis management program to reduce the use of antibiotics. Herbal sources of medicines may be considered an alternative option for treating diseases.

Tools

Routine testing of milk to detect residues of different antibiotics to grant the quality and safety of milk has become a significant task for farmers and the dairy industry. Beltrán et al. (2015) confirmed the use of biosensors. These biosensors are classified into five separate groups according to the bio-recognition employed. However, the use poor detection facilities as well as lack of proper monitoring system of residues in foods considering the maximum residue limits (MRLs) might be taken as vital causes for higher risk of milk derived antibiotic residues (Kebede et al. 2014).

Methods

Several procedures have been mentioned in the literature to monitor AMRs in milk. These methods vary in application, sensitivity, and cost. The amount of antibiotic residues in milk can be confirmed using qualitative techniques such as immunological and microbiological screening tests; and quantitative tests such as the analytical way (Pericas et al. 2010, Alves et al. 2020). Rapid testing of the presence of antibiotics in raw milk to grant its quality has become a major task for farmers and dairy industry. The conventional analytical methods are either too slow or do not enable quantitative detection of antibiotic residues while alternative methods that are rapid, cost effective, and easy to perform should be considered.

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CHAPTER 26

GENETICS OF METABOLIC DISORDERS IN ANIMALS

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METABOLIC DISORDERS

Metabolic diseases specify a list of conditions due to deficiency of vital nutrients which results in disruption of animal's normal metabolic processes. These disorders are multifactorial and happen commonly at periods of high environmental stress such as during late pregnancy and early lactation times (de Bem et al. 2021). Basically, metabolic syndromes are characterized by group of physiological, genetic and metabolic abnormalities in farm animals. Epigenetic factors play an important role in pathogenesis of metabolic disorders (Fan et al. 2020). The etiology of metabolic disorders persists of both genetic as well as environmental factors. These mechanisms include DNA modification and alteration in expressing noncoding of RNAs (Oliveira Junior et al. 2021). Disturbance of metabolic processes in dairy animals are due to hormonal changes. In cattle, main metabolic disorders include milk fever, ketosis, hypomagnesaemia or grass tetany, fat cow syndrome and ruminal acidosis. These conditions can cause acute, or potentially fatal deficiency (Stepanov et al. 2021). Different genetic parameters which cause inherited metabolic disorders are also called inborn errors of metabolism. Due to various nutrient gaps between its supply and demand which cause complexities for farm animals to adapt the environmental changes. In result of these potential feed deficiencies in dairy animals, serious metabolic health problems occurred (Arora et al. 2019). To prevent these diseases, proper nutritional diet is important during late pregnancy and lactation periods of animals (Chebel 2021).

Dysfunction of metabolic processes in animals is highly related to body malfunctioning which results in different diseases. Specifically in cattle, group of metabolic disorders affect dairy cows promptly after parturition (Pryce et al. 2016). Animal scientists commonly use the term metabolic disorders to specify a group of diseases which are identified by disturbance of plasma metabolites (Sundrum 2015). Fact that, these are called metabolic disorders due to reason of deprivation of essential metabolites which are crucial for the metabolism of body in animals (Stančáková and Laakso 2014). For instance, ketosis is related with increased ketone bodies (beta-hydroxybutyric acid) in bloods, milk fever is linked to decreased blood calcium, acidosis is associated with high production of organic acid (acetic, butyric acid) in rumen, fatty liver is accompanied with enhanced level of non-esterified fatty acids in liver (Brito et al. 2021). Transition period that consists of three weeks before and three weeks after parturition is very crucial for dairy cows. In this time period, various hormonal imbalances, major drop in feed intake and moving of non-lactating to lactating state occur, due to which different changes develop metabolically in farm animals (Ho et al. 2021).

High genetic merits vary in dairy cows due to which they adapt different nutritional and environmental challenges. Disturbances in normal metabolism of animal's body result in physiological changes that may occur slowly or abruptly (Wallis and Raffan 2020). To understand the mechanisms of adaption between nutritional demands of dairy animals, various metabolic, immunological and environmental changes in response to their genetic processes should be understand carefully to combat the diseases (Brito et al. 2020). Heritability of metabolic disorders in farm animals is highly relevant to bioavailability of corticosteroid hormone imbalances and its relation to receptor functioning (Chebel 2021). Veterinary genetics of companion animals is associated with metabolic dysfunction, premature morbidity and mortality rate and various health conditions within species across the globe (Wallis and Raffan 2020). Variations in genetic spectrum of host species and environmental conditions are the reasons due to which animals develop metabolic disorders on basis of different genetic parameters (Stančáková and Laakso 2014).

With the advent of modern techniques in veterinary science, multiple associations have been specified between single nucleotide polymorphisms which are located on special genes and their relation to host specific metabolic disorders are studied on these genetic interactions (Oliveira Junior et al. 2021). Genome wide association study is used to determine the gene variants which are responsible for causing multiple metabolic health problems in animals (Kalugniy et al. 2021). Developments in new strategies like "omics" may help to overcome the problems of breeding genetics for combatting the heritably transferred metabolic diseases. Proportion of phenotypical variants and differences between cohort of animals attributed towards genetic variations of metabolic issues among farm and dairy animals (Berry et al. 2011). Metabolic diseases can be detected at the early stages in animals by using different veterinary diagnostic tools such as quantification of fat protein ratios, level of metabolites and body scoring (Donadeu et al. 2020). Contributions to control animal metabolic diseases with recent technologies including significant genetic selection makeup on exposure to environmental stressors is the need of era (Brito et al. 2020).

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With the help of advanced techniques and practices in branch of veterinary science, genetics of metabolic disorders can be examined fully in order to prevent them and to develop new ways of treatment.

Prevalence of Metabolic Disorders

Incidence of metabolic disorders in dairy cows is specified by fat to protein ratio present in feed and daily milk production. Mostly, high prevalence rate is occurred in early lactation period due to increased physiological demands of animals (Gantner et al. 2016). Specifically, prevalence of ketosis is indicated by various factors such as breed, season, herd related factors and parity. Ketosis in farm animals can be influenced by non-esterified fatty acids, plasma glucose levels, ketone concentration in urine and blood (Fukushima et al. 2020). Subclinical ketosis is 80 percent prevalent in dairy animals due to production of chemical byproducts such as ketone bodies (Raja et al. 2021). Occurrence of hypocalcemia in dairy animals is highly associated with prevalence of other transition period disorders in animals. Risk of developing mastitis during their lactation period is eight times higher in cows having hypocalcemia. Risk factors including supplements addition in feed, monitoring of metabolite profile test and level of minerals is reported in affected animals. Good quality hay and grains should be supplied in feed in order to prevent calcium deficiency (Saed et al. 2020).

Subclinical mastitis in dairy cows is 15 to 40 times more prevalent than clinical mastitis. Associated factors which influence the prevalence are breed, age, stage of lactation, body scoring, parity in lactating cows developing mastitis (Islam et al. 2020). Acidosis in farm animals is associated with fat to protein ratio in feed at lactation stage. High prevalence is indicated by intervals in different lactation stages. As a result, yield of daily milk is evaluated by each class of parity in cows (Magrin et al. 2020). Grass tetany, also called hypomagnesaemia is more prevalent in cattle and grazing animals. It is characterized by low levels of magnesium in digestive system of cattle. Major risk factors are muscle incoordination and paralysis of muscles which can be prevented by using adequate amount of magnesium in feed (Sadiq et al. 2021). Fat cow syndrome mainly present in cows is associated with feed management practices. Manifestation of this syndrome is related to extensive metamorphosis of liver. Clinical signs include obesity in periparturient cow and cure is to replace the high energy diet with balanced diet (Fiorentin et al. 2018). Ruminant tympany or bloat is defined as indigestion influenced by excessive accumulation of gas in rumen and it is common cause of sudden death in cattle. Pasture bloat is more prevalent in both dairy and beef cattle. Prevalence of pasture bloat is high in ruminants animals due to consumption of various forages such as grazing alfalfa, white, red clover and winter wheat at early growth stages (Abdisa 2018).

Pathogenesis of Metabolic Disorders

Metabolism is group of all chemical, physical and metabolic processes which occurs in animal's body associated with absorbance and synthesis of vital organic metabolites. In farm animals, these diseases affect body's metabolic pathways (Wallis and Raffan 2020). Acute and subacute ruminal acidosis is more prevalent in dairy herds. Poor forage quality and poor bunk management are the reasons due to which cows develop these disorders (Pinotti et al. 2021). Reduced or inconsistent feed intake manifest cows with subclinical acidosis. Progression of ruminal acidosis include low appetite, low fat content, salivation, lethargy and ultimate death. Laminitis is inflammatory and non-infectious disease of the foot. Diet rich in fermented carbohydrates which induces an acidotic state is main reason for the pathogenesis of laminitis. Other infections such as mastitis and foot rot also contribute towards the etiology of this disease (Islam et al. 2020). Environmental factors of hard surfaces, poor bedding, lack of exercise also cause cattle to develop the laminitis (Arora et al. 2019).

Milk fever or hypocalcemia is due to presence of low levels of blood calcium in dairy cows. Affected animals can show signs of muscle tremors, weakness, depression and bloating (Saed et al. 2020). Calcium supplements should be given in diet for calcium deficiency as proper nutrition helps in prevention of metabolic disorders (Wallis and Raffan 2020). When animals have increased energy demands in late pregnancy and early lactation, a condition called ketosis occur in dairy animals. Due to higher energy demand, blood glucose level decreases and body undergoes stress which results in production of ketone bodies which are toxic if excess in number (Fukushima et al. 2020). Pathogenesis includes neurological signs and symptoms which may progress to death in farm animals (Mann et al. 2019). Hypomagnesaemia indicates decrease levels of blood magnesium also referred to grass tetany in dairy cattle. Reduced feed intake which is deficit in magnesium leads to twitching of muscles, shoulder and face in cows making them restless and irritable (Stepanov et al. 2021). Early detection of metabolic disorders in transition cows is difficult to manage but there are different parameters which are helpful in diagnosis in order to control and treatment of metabolic disorders (Magrin et al. 2020). Different factors are responsible for the pathogenesis of metabolic disorders in animals as demonstrated in Figure 1.

KETOSIS

Ketosis or pregnancy toxemia is defined as deficiency of blood glucose level when animal has high energy demands during late pregnancy or early lactation. If animal is unable to eat enough food and does not meet the energy demands, this results in consumption of body fats which leads to production of ketone bodies, the chemical byproducts that are toxic in nature if produced in large quantity (Lei and Simões 2021). Occurrence of ketosis has high genetic correlation with milk

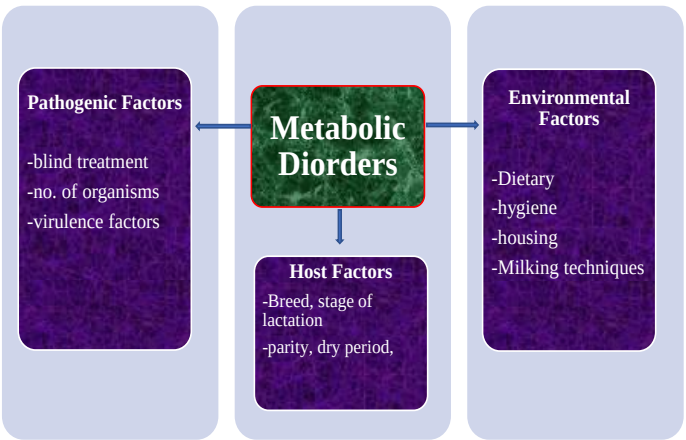

Fig. 1: Pathogenesis of metabolic disorders in animals (Zigo et al. 2021).

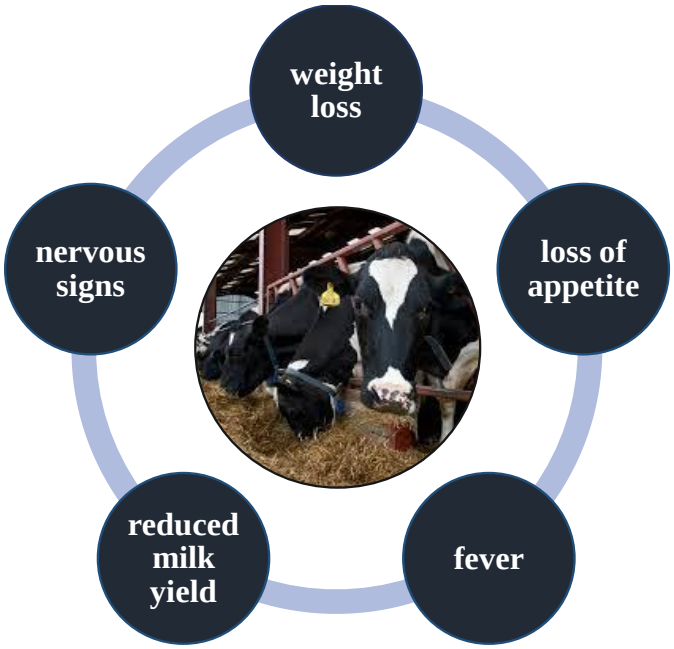

Fig. 2: Clinical signs and symptoms of ketosis in dairy cows (Nazeer et al. 2021).

yield in lactation period of cows. However, phenotypic relationship to milk production and ketosis cannot be measured easily. Different linear models of genetic heritability and phenotypic relationship for epidemiological studies of ketosis are studied which elaborate the milk production in dairy cows (Sundrum 2015). Symptoms can be decrease in milk yield, weight loss, loss of appetite, fever and may develop nerve disorders specially in dairy cattle as described in Figure 2 (Nazeer et al. 2021). Diagnosis is based on commercially available kits which are used to check the prevalence of ketone bodies in urine and milk in given sample. Ketosis can occur with other metabolic diseases as well as other infections such as mastitis in cows and buffaloes and become riskier if not properly treated (Raja et al. 2021).

Treatment

Ketosis is treated with oral drenching of propylene glycol (KETOL) to lactating cows. Glucose and glucocorticoid therapy are administered repeatedly for rapid recovery. Supply of adequate dietary supplements is important factor for the cure of ketosis. Gastric stimulants to increase the appetite should be added in the feed of cows (Pryce et al. 2016). Corticosteroids can be used as additional supplements for supportive therapy in farm animals. Intravenous injection of dextrose is standard treatment for ketosis in livestock animals (Raja et al. 2021)

Prevention and Control

Good quality feed with addition of supplements is key preventive measure for ketosis in cows. Monitoring of nutritional demands in pregnant and lactating cows is important in order to control the ketosis (Nazeer et al. 2021). According to Cocco et al. (2021), assessment of body condition at early stages will help farmers to prevent the loss of farm animals due to ketosis. Thus, this condition can be avoided by adopting preventative measures to control ketosis in dairy cows.

MILK FEVER

Milk fever or hypocalcemia is most common mineral related metabolic disorder in which blood calcium level is decreased. It usually occurs in high productive cows around calving (Oliveira Junior et al. 2021). Cows with parturient paresis show general symptoms of illness such as loss of appetite, low body temperature, looks groggy, complications in production of manure and urine as explained in Table 1 (Rajadurai et al. 2021). Milk fever is influenced by many genetic factors. Incidence of milk fever is heritable trait in dairy cattle and varies depending upon breed. Certain breeds of cows are more susceptible to milk fever such as Swedish red & white, Jersey and Channel Island (Cariappa et al. 2021b). Older cows are more prone to milk fever. Deficiency of calcium in their diet may result in tremors which also stimulates osteoclasts i.e., breakdown of bone cells eventually collapse of animals (Ibrahim and Kirmani 2021).

Treatment

Milk fever can be treated with use of calcium borogluconate solution by subcutaneous injection. General nursing such as keeping animals at warm and dry place is also important (Priya et al. 2021). Calcium supplements should be given in the diet to avoid the deficiency. Fluids are administered as supportive therapy to rehydrate in case of milk fever. Good feed and clean water are also important in order to avoid this condition in dairy cows. Treatment on time can save animal otherwise cow may die due to circulatory failure or respiratory collapse (Baqir et al. 2021).

Prevention and Control

Feed management practices should be adopted by farmers as a preventive measure for control of hypocalcemia in farm animals. DCAD (Diet Cation-Anion Difference) strategy must be applied to dairy cows (Ibrahim and Kirmani 2021). Supplementation with vitamin D should be used for the intestinal adsorption and bone mobilization of calcium before calving. Oral calcium drenching around calving is also recommended as a supplement to intravenous therapy (Cariappa et al. 2021a). By adopting preventive measures on farms, milk fever can be controlled and animals can be saved.

Milk Fever Stages	Description	Signs and Symptoms
• Stage I • Stage II • Stage III	• Early stage (subclinical) • Subclinical • Clinical	• loss of appetite, nervousness, hypersensitivity, weakness • fast heart rate, trembling muscles, lay down in flanking position • lying flat on ground, comatose and death

Table 1: Milk fever stages, signs and symptoms

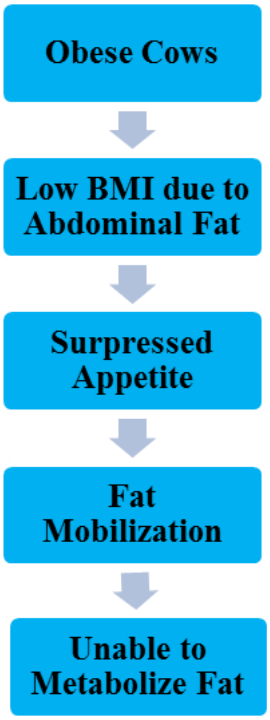

Fig. 3: Stages in fat cow syndrome.

GRASS TETANY

Grass tetany is defined as low blood levels of magnesium also referred to as hypomagnesaemia or grass staggers in dairy animals. Mostly, it is prevalent in late gestation or heavy lactation time period of cows (Rendig and Grunes 2015). Older cows during winter and spring are at more risk of developing Grass tetany. Animal loses magnesium in urine, milk and feces, so proper amount of magnesium is needed as supplement in their feed and diet. Early signs including muscle twitching, aggressive behavior, staggering gait and eventually death in severe cases (Ahmed et al. 2021). Any form of stress can also stimulate Grass tetany in dairy animals such as mustering, estrus, starvation and cold weather. Grass-dominant pastures account for more prevalent cases of Grass tetany (Berry et al. 2011). Due to different genetic parameters, dairy and farm animals adapted themselves to environmental changes and when they are unable to meet these challenges, abnormalities occur in their normal metabolism leading to highly prevalent diseases (Oliveira Junior et al. 2021).

Treatment

Treatment of Grass tetany includes increasing blood serum levels of magnesium. Solution of magnesium sulfate is injected simultaneously with intravenous injection of calcium-magnesium gluconate (Cantón et al. 2021). Grass tetany is always an emergency requiring medical attention in cattle and farm animals. Veterinarians suggested that magnesium salts play a vital role in treating grass tetany. Care is necessary as after treating with magnesium supplement, animals should be left to respond without stimulation and then moved off the tetany-prone pasture. Oral sources of magnesium should be administrated in order to avoid the relapses which help to restore the blood magnesium levels in body of farm animals (Zelal 2017).

Prevention and Control

Doncel et al. (2021) recommended increased magnesium intake with good quality forage as effective measures in prevention of grass tetany in cattle. Maximizing the adequate amount of essential mineral is important in feed of animal as a precautionary measure. Fertilizers rich in nitrogen and phosphorus should be avoided. Prevention to grass tetany mainly involves raising the levels of magnesium in forages which leads to higher absorption of dietary magnesium. With the help of good management practices at farm and feed testing, grass tetany can be prevented in dairy cows (Pinotti et al. 2021).

FAT COW SYNDROME

Fat cow syndrome occurs sporadically depending on feed management practices near parturition. It is also called fatty liver disease. Metabolic, infectious, digestive and reproductive imbalance are simultaneously faced during fat cow syndrome which affects the obese cows during parturition. Different stages of fat cow syndrome have been shown in Figure 3 which explains how fat cow syndrome develop in dairy cows (Ho et al. 2021). Poor feed quality and consumption of unbalanced diet are the main reasons due to which farm animals developed this syndrome. Disease develops with change in endocrine profile during calving and with the onset of milk production (Stepanov et al. 2021). Clinical signs include presence of inappetence, progressive debilitation, elevated temperature, weakness and loss of body functions (Arora et al. 2019). Timing of observing off feed cows is important in diagnosis of fat cow syndrome. Manifestation of this disease is due to excessive fatty metamorphosis of liver which leads to increased hepatic lipogenesis (Kalugniy et al. 2021). Management factors that are related to nutrition are also crucial in the incidence of fatty liver in dairy cows. Genetics of fat cow syndrome increase the probability mutations that affect feed intake, lipid metabolism and secretion in liver. Physiological tests that examine the susceptibility of cows to fatty liver determine the genetic basis of this disease (Oliveira Junior et al. 2021).

Treatment

Treatment of fat cow syndrome includes feeding a balanced diet, symptomatic treatment and good supportive care according to nutritional demands of cows (Cocco et al. 2021). High quality hay should be given in feed to farm and dairy animals. Vitamin and mineral supplements must be added to fulfill the nutritional requirements of dairy cows during calving (Abdoul-Aziz et al. 2021). Better management practices at farms can help farmers to treat the disease. Minimizing the stress risks is important in treating fat cow syndrome specially after parturition in farm animals (Cariappa et al. 2021b). Choline chloride is administrated orally in order to treat this condition. Furthermore, broad spectrum antibiotics can be used as a therapeutic agents (Agus et al. 2021).

Prevention and Control

Prevention of fatty liver can be achieved by supplying dairy animals with adequate amount of essential nutrients and healthy environment with fresh air. Glucose supplements can be given to fat animals as a precautionary measure. Balanced diet with additional supplements should be provided to herd

animals so that fat cow syndrome can be prevented. Deep and soft bedding should be used in order to maintain a good environment for dairy cows (Bobe et al. 2004). Good farm practices including over-crowding of cattle, unpalatable feeds, abrupt changes in diet and environmental stress should be avoided for the control and prevention of fat cow syndrome (Sundrum 2015).

RUMINAL ACIDOSIS

Ruminal acidosis is defined as bovine metabolic disorder which affects feedlot and dairy cattle. It is associated with ingestion of large amount of carbohydrate-rich feed which results in accumulation of acids in rumen (Donadeu et al. 2020). Early lactating cows and cows with high intake of grains in their diet are at special risk for acidosis. Decreased milk production, low fat content in milk, diarrhea, laminitis, high culling rate and body scoring are some clinical signs of ruminal acidosis in animals (Abdoul-Aziz et al. 2021). Most common diagnostic techniques for acidosis is based on determination of pH of rumen, also milk fat test is used to diagnose the acidosis. Prevalence rate of acidosis ranges between 11% to 13% in lactating cows (Chen et al. 2021). Liver abscesses may occur in severe condition in dairy cows. The main forms of ruminal acidosis are acute ruminal acidosis and subacute ruminal acidosis (Hu et al. 2021). Fluid balance is disturbed in this disease. Normal pH of rumen ranges from 6.5 to 7.0. Drop in pH of rumen results in cessation of movement in rumen impacting appetite and feed conversion (Zhang et al. 2021). Relationship between ruminal acidosis and expression of genes regulates intracellular pH and ruminal epithelial cells metabolically (Komisarek et al. 2021).

Treatment

Selection of treatment for acidosis depends on severity of clinical symptoms. Cows with more severe condition such as depressed state, off feed and severe diarrhea should be avoided from feeding grain diet (Ma et al. 2021). Orally about 120g of sodium bicarbonate and an electrolyte replacer should be used as treatment therapy to diseased animals. Most important is feeding management practices to minimize the risk of acidosis in cattle (Mensching et al. 2021). Thiamine injection is recommended for cattle with acute acidosis. Baking soda can also help as primary treatment therapy (Srivastava et al. 2021). Removal of rumen contents is important and replacement with ingesta taken from healthy cows can save the animals. Procaine penicillin G should be administrated to all affected animals to minimize the bacterial rumenitis and liver abscesses (Komisarek et al. 2021).

Prevention and Control

Commonly used management practices for control of ruminal acidosis are increased proportion of roughage in diet of animals and decrease the starch intake. Maintaining good rumen health is essential as a preventive measure. Consistent supply of dry forage and use of prebiotics in addition to supplements is necessary for maintaining the pH balance of rumen (Chen et al. 2021). Supplementing the diet with direct-fed microbials that enhance lactate utilizers in rumen may reduce the risk of acidosis in cattle and dairy cows. Ensure the cattle to receive adequate fiber which promotes healthy movement in rumen and enhance saliva production for lower rumen acidity (Ma et al. 2021). Thus, by adopting preventive measures and good farm practices, ruminal acidosis can be controlled in cattle and dairy cows.

Conclusion

Veterinary genetics of farm animals is associated with metabolic dysfunction, premature mortality rate and various health conditions across the globe. Variations in genetic spectrum of host species and environmental conditions are the reasons due to which animals develop metabolic disorders on basis of different genetic parameters. Single nucleotide polymorphism is among the advanced technique in veterinary science which can be used to examine the gene nutrient interaction. Genome wide association study is used to determine the gene variants which are responsible for causing multiple metabolic health problems in animals. New strategies like "omics" may help to overcome the problems of breeding genetics for combatting the heritably transferred metabolic diseases. Proportion of phenotypical variants and differences between cohorts of animals attributed towards genetic variations of metabolic issues among farm and dairy animals. Contributions to control animal metabolic diseases with recent technologies including significant genetic selection makeup on exposure to environmental stressors are need of era.

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CHAPTER 27

RESPIRATORY AND REPRODUCTIVE TRACT DISORDERS CAUSING HERPESVIRUSES IN ANIMALS

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INTRODUCTION

The word herpes derived from the Greek word "herpein" which means "to creep". Herpesviruses are diverse, widely successful viruses, able to infect a wide range of animals from mussels to humans, with most animal species having at least one of the type of herpesvirus. It is likely that every vertebrate species is infected with several herpesvirus species (Fig. 1). Most of domestic animal species are infected with diseases caused by herpesvirus, including infectious bovine rhinotracheitis, pseudorabies and malignanat catarrhal fever etc. Many of these viruses will be discussed later in this chapter. Herpesviruses co-evolve with their individual hosts in order to adapt with them (Carter et al. 2007).

Almost two hundred species had been identified up to 2007 but it is expected that many more exist and are yet to be discovered (Pellet 2007) and few of them are associated with mammals, birds, reptiles, amphibian, fishes, and mollusks. Most of the herpesviruses can cause latent and lifelong persistent infection in host. During latency virus shows very limited gene expression but recurrent virus replication occurs during stress. Upon stress, virus can reactivate and initiate active replication which leads to shedding, transmission, and the development of detectable antiviral immune responses. Therefore, latent infections in subclinically infected hosts allow herpesviruses to maintain themselves in susceptible hosts (Roizman and Knipe 2001).

General Properties

Herpesviruses are traditionally classified by their structure (Fig. 2). Herpesviruses are double stranded DNA with an icosahedral capsid that is coated by a structure named "tegument" (Thiry et al. 2006). The whole virus is surrounded in an envelope studded with glycoproteins, which are important for cell-cell spread and cell entry (Mettenleiter 1994). DNA in the core has been reported to be arranged in the form of a toroid, possibly supported by a spindle made up of proteinaceous fibres embedded in the capsid, although the precise arrangement of the DNA is not known (Baines 2011; Roizman and Knipe 2001). The capsid consists of 162 capsomeres of 9.5x12.5nm with a total diameter of ~100nm. During a cell infection non-enveloped capsids exist in 3 forms: A-capsids have no core structure, B-capsids contain a scaffold but no genome and C-capsids contain the DNA genome that replaces the scaffold (Pellet 2007).

The tegument refers to the structure between the capsid and envelope that has varying thickness depending on the infection stage and was reported to have no distinctive features (Baines 2011). One of the important roles of the tegument is to provide pre-synthesized proteins that can influence the host environment on cell entry to suit the virus, including shutting down host protein synthesis and inhibiting infection defences (Pellet 2007).

The envelope is made up of lipids and virus-specific glycoproteins in greater numbers than other enveloped viruses. It has a trilaminar appearance and is thought to be derived from altered cell membranes.

Although highly divergent, all the herpesviruses have four common characteristics:

1) They are involved in enzymes coding, metabolism of nucleic acid, synthesized DNA and involved in the processing of proteins.
2) Initially viral DNA is synthesized than nucleus is responsible for assembly of capsid, whereas cell cytoplasm is responsible for the final processing of the virion.
3) When infectious progeny are produced by the parent virus the host cell lyses occurs.
4) The most well-known property of the herpesviruses is their ability to persist in latent form for the host's lifetime, from this latent state the virus can be reactivated and the animal becomes infectious again (Fig. 3) (Roizmann et al. 1992; Pellet 2007).

The variation of the Herpesviridae is represented by the sites where they attain latency; their host cell ranges and the length of replication cycles which leads to a wide range of clinical signs and diseases; individual to each virus and its preferred host.

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Subfamilies of Herpesviridae	Alpha		Beta		Gamma	
Cytopathology	Cytolytic		Cytomegalic	Lymphoproliferative	Lymphoproliferative	
Growth Cycle	Short		Long	Long	Variable	
Latent infections	Neurons		Gland, Kidneys	Lymphoid Tissue	Lymphoid Tissue	
Genus	Simplexvirus	Variocellovirus	Cytomegalovirus	Roselovirus	Lymphocryptovirus	Macavirus
Examples	BHV-2 HHV-1 HHV-2	BHV-1 BHV-5 CHV-1 EHV-1 EHV-3 EHV-4 PHV-1	HHV-5 PnHV-2 PnHV-3 PnHV-4	HHV-6 HHV-7 McHV-9	HHV-4 CalHV-3 PoHV-2 GoHV-1	AHV-1 AHV-2 OHV-2 BHV-6

Fig. 1: Classification of Herpesviruses of Veterinary Importance (MacLachlan and Dubovi 2017): AlHV1: Alcelaphine Herpesvirus, BHV: Bovine Herpesvirus, CalHV: Callitrichine Herpesvirus, CHV-1: Canine Herpesvirus, EHV: Equine Herpesvirus, GoHV: Gorilline Herpesvirus, HHV: Human Herpesvirus, McHV: Macacine Herpesvirus, OvHV2: Ovine Herpesvirus, PHV: Porcine Herpesvirus, PnHV: Panine Herpesvirus, PoHV: Pongine Herpesvirus.

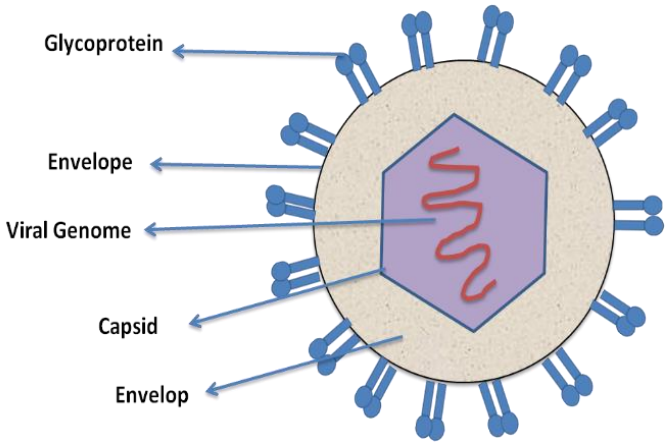

Fig. 2: A typical herpesvirus virion. Diagrammatical representation showing the major structural components of Herpesvirus.

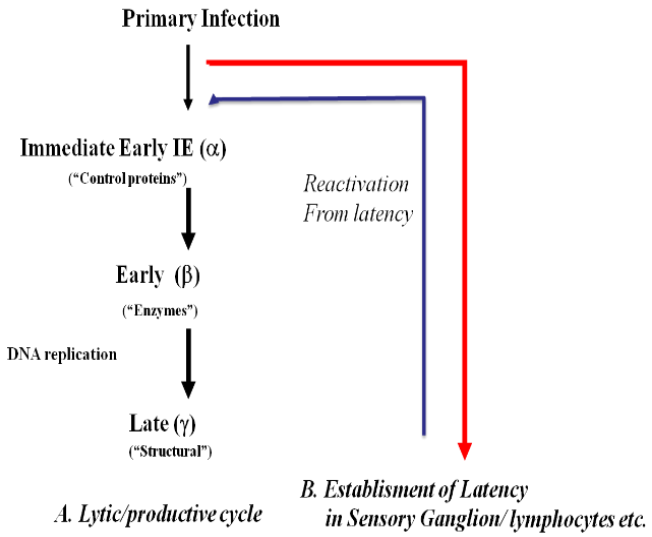

Fig: 3: Schematic Diagram of Herpesvirus Lifecycle.

Herpesvirus Replication

For the replication of herpesviruses, key proteins include those involved in cell entry, regulation of viral gene expression, metabolism nucleotide, viral DNA synthesis, structural proteins and assembly of virions. It is also necessary for the virus to avoid the host response to viral gene expression. This will usually involve programmed cell death, which the virus needs to delay long enough to allow viral replication (Pellet 2007). Upon the infection of a cell and release of viral DNA into the nucleus, viral replication depends on 'temporal' phasic gene expression of 4 key groups of genes. The α (or immediate-early gene) expression is stimulated by tegument proteins such as the α-TIF (alpha gene trans inducing factor).

The resulting proteins initiate viral gene replication by stimulating expression of β (or early genes) including genes important in DNA replication such as DNA polymerase. γ (or late genes) are then expressed, sometimes as two groups, 'leakylate' genes that just require the onset of viral DNA synthesis and 'true' late genes that are entirely dependent on prior viral DNA synthesis. This final phase of gene expression includes the synthesis of many of the structural proteins such as the glycoproteins, allowing the formation and assembly of progeny virions as capsids containing the viral genome core. Progeny virion are released from the nucleus into the cytoplasm where final processing takes place and the tegument and envelope is formed before release from the cell (Nanbo et al. 2018).

Bovine Herpesviruses

Bovine Herpesvirus-1

Bovine herpes virus-1 belongs to family Herpesviridae, subfamily AlphaHerpesvirinae and genus Varicellovirus. BoHV-1 causes infectious bovine rhinotrachitious, infectious balanopothitis and infectious pustular vulvovaginitis, conjunctivitis, abortion, encephalitis and generalized systematic infection in bovines. It has the tendency to affect both younger and older cattle as well as buffaloes (Jones et al. 2006).

Subtypes

BoHV-1 can be further divided into subtype which are 1.1, 1.2a and 1.2b (Table 1). Subtype 1.1 is related to the respiratory disease and abortion in infected animals. This

virus is usually included in BHV-1 vaccine. Subtype 1.2a causes infectious pustular vulvovaginitis, infectious balanoposthitis in male animals and mild respiratory disease and abortion in female animals. Subtype 1.2b is associated with infectious balanoposthitis and infectious pustular valvovaginitis syndrome but not implicated in abortion (Biswas et al. 2013).

Epidemiology

BHV-1 is widely spread worldwide and reported in many countries including Pakistan, USA, Canada, India, turkey, Zaire, Italy, Belgium (Castrucci et al. 1997; Boelaert et al. 2000; Yesilbag et al. 2003; Rajkhowa et al. 2004; Riaz et al. 2021). BHV-1 causes the infection in ruminants, domestic and wild cattle. In UK prevalence of BHV-1 was recorded about 83% in unvaccinated herds (Woodbine 2009). The sero-prevalence of this virus has been determined from 36 to 48% in Central and South America and 14 to 60% in Africa (Straub 1990), 63 to 86% in Egypt (Mahmoud et al. 2009), 36% in China (Yan et al. 2008), 60.1% in India (Nandi and Kumar 2010), 10.7% in Iran (Erfani et al. 2019), 69% in Pakistan (Ur Rehman et al. 2021). Riaz et al. (2021) first time reported the molecular detection and characterization of BHV-1 from clinical case of IBR in Pakistan. IBR infection has not yet been reported in some countries like Switzerland, Sweden, Austria, Denmark and Finland (Ackermann and Engels 2006).

Table 1: Different subtypes of BoHV-1

Subtype	Infection
1.1	Respiratory disease and abortion
1.2a	Infectious pustular vulvovaginitis, infectious balanoposthitis, cause mild respiratory disease and abortion
1.2b	Infectious balanoposthitis and infectious pustular valvovaginitis

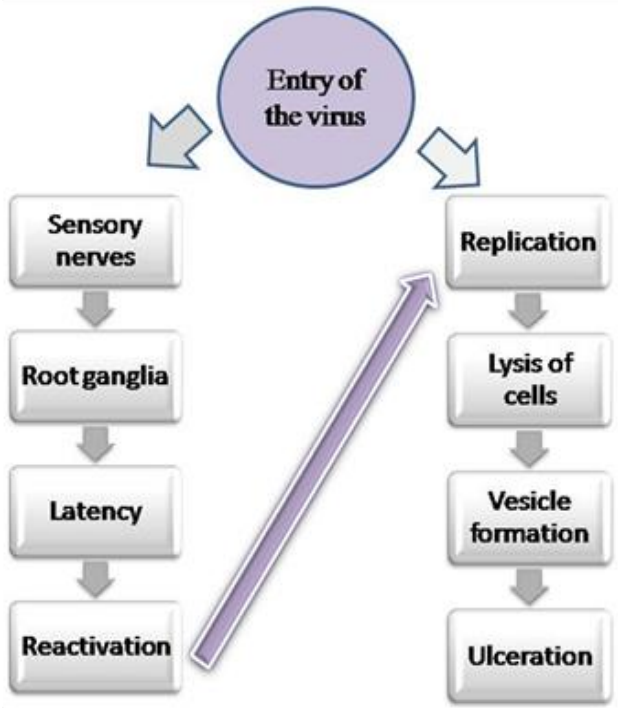

Fig. 4: Schematic diagram of Lifecycle of BHV-1 in susceptible host.

Pathogenesis and Pathology

Entry of BHV-1 occurs through the aerosol route, direct contact with nasal secretions, vaginal secretions or semen from infected animals. Subsequent to the virus entry into the body, replication of this virus takes place in the mucosae of respiratory or genital tract. Monocytes and white blood cells are responsible for the transport of the virus to the target organs within the body (Fig. 4). The virus may also enter into the axons of local nerve cells during the primary infection. Later virus is transported to the regional ganglions of peripheral nerves and attain latency in terminal ganglion (in respiratory infection) and sacral ganglion (in genital infection) (Nandi et al. 2009). Several viral glycoproteins and two cellular receptors are involved in the entry of the virus into the cell. Initially glycoprotein C (gC) of the virus binds to the heparan sulphate proteoglycan receptors of the cell (Mettenleiter 1994). Tissue damage and necrosis of epithelial cells take place in both respiratory and genital infection due to virus infection. Foetal infection and abortion may also occur in pregnant cows due to viraemia. Immunohistochemistry demonstrated the presence of necrotic foci in the peripheral tissues. An intense inflammatory reaction can also be developed in the respiratory and reproductive tract like shipping fever and endometritis (Majumder et al. 2015).

Clinical Signs

The clinical sign and symptoms of IBR include fever, nasal discharge, coughing, difficulty in breathing, conjunctivitis, loss of appetite, depression, drop in milk production, vesicles, ulcer, and abortion (Engels and Ackermann 1996). The nasal mucosa is usually hyperemic, hemorrhagic, ulcerated and covered by a cream-colored diphtheritic membrane. The breath may be fetid. Abortion may occur at second or third trimester of gestation, and the virus has also been reported to cause mastitis. Infectious pustular vulvovaginitis affected cows develop fever, depression and anorexia. Micturition is frequent and painful due to inflammation of vulva and vagina. Lesions of infectious balanoposthitis in bulls and the clinical course of disease are similar to their equivalents in affected cows. Genital and respiratory diseases are rarely diagnosed simultaneously in the same herd.

Diagnosis

Diagnosis of the infection of BHV-1 is performed by taking history of animals, observing sign, symptoms and gross lesions. Virus can be isolated from different organs (kidney, lungs, nasal swabs, vaginal swabs, spleen, fetus) and can be grown on cell culture and cytopathic effect can be indicative of the presence of the virus (Turin and Russo 2003). In the early stage of infection, electron microscopy can be used to check the virus particle in samples (Nandi et al. 2009). Nested PCR and Real-time PCR are widely used to detect the BoHV-1 DNA in samples (Moore et al. 2000). Immune responses of this virus can be characterized by the IgG and IgM antibody formation. Virus neutralization test and ELISA test particularly indirect and competitive ELISA are widely use to detect the antibodies (Biswas et al. 2013).

Prevention and Control

Marker vaccines, inactivated vaccines, subunit vaccines and modified live vaccine are available to control the infection of BHV-1. Due to latent nature of the virus, vaccination cannot control the infection completely but reduces the severity of clinical signs. This virus has ability to go into latency and reactivate from the latency in future so re-vaccination after 6-months interval should be done regularly. Modified live vaccine should not give to the pregnant animals because it causes abortion (van Oirschot 1995).

Bovine Herpesvirus 2

Bovine herpesvirus 2 (BHV-2) also belongs to the subfamily Alphaherpesviridae. BHV-2 causes two types of infection; Pseudo-Lumpy Skin disease (generalized benign skin infection) and Bovine Mammillitis (localized ulcerative mammillitis) (Radostits 2014).

Epidemiology

The infection caused by BHV-2 has been reported in most of the countries worldwide. Prevalence of this virus is higher in temperate regions of the world. First time, Bovine mammillitis was reported in Ireland, UK, USA, Canada, Australia, France, Italy, Brazil, Rwanda (Martin and Scot 1979; O'Connor 1985). Serological study of infection of the BHV-2 has been obtained in Namibia, south Africa, New Zealand, Belgium (Horner and Raynel 1988; Barnard 1997). Pseudo-Lumpy skin disease was first time diagnosed south Africa, Kenya, USA, and Australia (Woods et al. 1996).

Pathogenesis and Pathology

BHV-2 can be transmitted through air, semen, biting insect, milking equipment, and milker's hand. This virus reaches the dermis through skin lesion and may cause short viraemia and establishes latent infection in skin and neurons (Martin and Scott 1979). Re-activation of BHV-2 and development of clinical signs take place due to the stress with udder edema and hormonal changes during calving (Kemp at al. 2008). This virus causes the sporadic condition and affects lactating animals (especially first calvers and first 2 month of lactation) (Sharma et al. 1998). Within the 3 weeks infection can spread in the herd and incubation period is about 4-10 days. Blue or purple lesions develop on the udder, teats and perinea which look like ulcers and resolve within four weeks without complications. Gulwaddee (Punjabi term literally mean 'neck cutter') may be used for the Bovine herpes mammillitis due to deep non heeling ulcerative lesion on teat. The incubation period ranges from 5 to 9 days in Pseudo-lumpy skin disease. Lesions may also develop on lips, nose, and mouth of the suckling calves Pseudo-lumpy skin disease is febrile and generalized disease which appear on the skin of complete body and show depression in the center. Ulcers like nodules may occur after few days (Gourreau and Pauluzzi 1988).

Clinical Signs

Clinical signs of the pseudo-lumpy skin disease includes mild fever, followed by the sudden appearance of skin nodules on the face, neck, back, and perineum. In Mammillitis lesions can be seen of mostly on teats but sometimes a generalized skin disease can be seen. Other lesions and signs include ulcers, vesicles, pustules on mucosa, edema and sloughing of skin of mammary glands (Scott and Holliman 1984). Milk yield may be reduced by as much as 10% as a result of difficulty in milking the affected cows, and concurrent mastitis.

Diagnosis, Prevention and Control

Diagnosis can be performed by mean of PCR and seroprevalence can be found out through ELISA. Virus can be isolated and detected from infected tissues, ganglion and milk samples.

Commercial vaccine against BoHV-2 is not available yet but thymidine kinase deficient strains have been used to develop live-attenuated vaccines. Proper management practices are required in the herds to prevent infections. Insect repellent sprays should be used to stop biting flies. Healthy cow should not be kept with the affected cows. Disinfection of the milking machine with iodophores before taking milk from the healthy animals is necessary to stop the spread of the virus (Smee and Leonhardt 1994).

Bovine Herpesvirus-4 (BoHV-4)

Bovine Herpesvirus Type 4 belongs to subfamily Gammaherpesvirinae and genus Rhadinovirus. The natural host of the virus is primarily cattle, but several ruminant and non-ruminant species like Felis catus (Cat) Panthera leo (Lion) are susceptible to BoHV-4 infection. BoHV-4 cannot be associated with specific disease, usually results in a subclinical infection in respiratory and reproductive track (Macchi et al. 2018).

Epidemiology

BoHV-4 is a ubiquitous virus. Its presence has been reported from many countries, worldwide. Almost more than 40 strains of BoHV-4 have been identified across the world. These strains are divided into three categories: European strain (Movar33/63-like strain), American Strain (DN 599-like strain) and African buffalo strain (Gagnon et al. 2017). The global Seroprevalence of cattle is estimated to be between 4 to 30 %. In some herds, the prevalence may be as high as 80% (Chastant-Maillard 2015).

Pathology and Pathogenesis

BoHV-4 has very complicated and ambiguous method of pathogenicity. It has been isolated from diseased animals as well as from healthy animals. It is mainly transmitted via nasal and uterine exudate. The BoHV-4 virus replicates primarily within epithelial cells in the mucosa following the propagation within blood lymphocytes. It shows special propensity towards endometrial (uterus lining) and endothelial cell (blood vessels lining) leading towards disease development by stimulating the inflammatory response.

Primary infection begins with lytic cycle marked by entry via skin and mucus followed by multiplication through cell lysis. Lytic cycle ultimately ends at formation of ulcer. Following primary infection, BoHV-4 establishs a latent infection by residing in peripheral blood leukocytes, the nervous system and lymphoid organs. The latent virus reactivates in response

to a variety of stimuli such as cellular stress e.g. calving, immunosuppression or dexamethasone treatment (Chastant-Maillard 2015).

Clinical Signs

Pathogenic potential of BoHV-4 is low until and unless it combined with some other infectious organisms such as Escherichia coli and Trueperella pyogenes. Thus, designating BoHV-4 as cofactor or secondary pathogen (Szenci et al. 2016). BoHV-4 especially stimulates infection in postpartum period causing abortion, vulvovaginitis, endometritis, mastitis, infertility and stillbirth. It has also been isolated from respiratory tract infection, pneumonia, keratoconjunctivitis, mammilititis, pyrexia and from skin lesion etc (Gagnon et al. 2017).

Diagnosis, Prevention and Control

To date, no vaccination and specific medication are available against BoHV-4. Diagnosis can be performed by means of PCR and seroprevalence can be found out through ELISA. The only treatment is in the form of general health care. Recovered animals are frequently latent carriers of infection and will shed virus sporadically, thus serving as a source of infection for others (Chastant-Maillard 2015).

Bovine Herpesvirus 5

Bovine herpesvirus 5 (BHV-5) is species belongs to the genus Varicellovirus, subfamily alphaherpesvirinae. This virus is also called as bovine encephalitis herpesvirus because it causes meningoencephalitis in bovine. Firstly, BHV-5 was taken under the subtype of BoHV-1 as BoHV-1.3, but later it was considered separate type due to its neuropathogenicity. Bovine herpesvirus 5 replicate in CNS and causes neurological disease (Studdert 1989).

Epidemiology

Bovine meningoencephalitis infection caused by BHV-5 was first time reported in Australia in 1962 (Johnston et al. 1962). Now this virus is reported in most of the countries i.e., USA, Europe, Brazil, and Argentina (Abdelmagid et al. 1995; Ely et al. 1996; Vogel et al. 2004). The mortality and morbidity rate are 15-50% and 100% respectively (Johnston et al. 1962). Due to the serological cross-reactivity with the bovine herpesvirus 1, the prevalence of the this is unknown (Vogel et al. 2003).

Pathogenesis

Main portal of entry of the bovine herpes virus into the animal is respiratory tract. In case of BHV-5, primary replication occurs in mucosa lining of respiratory tract and virus also replicate in the central nervous system which leads to the neurological damage (Perez et al. 2002; Vogel et al. 2003). There are two routes of this virus after entering into the body, first is olfactory tract (through axons terminals which innervate olfactory mucosa) and second is trigeminal ganglia (site where latency occur) (Nakazato et al. 2000). After reactivation from the latency, mild neurological signs could be seen. BoHV-5 was also be discovered from the central nervous system of the aborted fetus (Perez et al. 2002; Vogel et al. 2003; Kirkland et al. 2009)

Clinical signs

BHV-5 causes infection in upper respiratory tract with hyperthermia in initial stage. Neurological signs occur after 9-10 days of infection which involve circling, depression, nystagmus, teeth grinding, opistothonus, and tremors, anorexia, hypersalivation and recumbency (Carrillo 1982; Meyer et al. 2001).

Prevention and Control

No vaccines are available against the BoHV-5 but sometime vaccines of BoHV-1 could be used against the infection of BoHV-5 due to close antigenic relationship (Cascio 1999).

Malignant Catarrhal Fever Causing Herpesviruses

Malignant catarrhal fever (MCF) is a sporadic, mostly fatal disease of cattle and other cloven-hoofed species including deer, water buffalo, bison and swine (Schultheiss et al. 2000). MCF is characterized by acute lymphoproliferation and marked necrosis in susceptible species. MCF is mainly caused by one of the two gammaherpes viruses from genus Macavirus; ovine herpesvirus 2 (OvHV-2) and alcelaphine herpesvirus 1 (AlHV-1). OvHV-2 is naturally present in sheep and causes sheep associated MCF (SA-MCF) while AlHV-1 is naturally present in wildebeest and causes wildebeest associated MCF (WA-MCF). These viruses do not produce any clinical disease in their natural hosts but cause MCF in susceptible species some of which are closely phylogenetically related to the reservoir hosts (Bridgen and Reid 1991).

The MCF Virus (MCFV) group include ten members which belong to the genus macavirus of the Gammaherpesvirinae; AlHV-1, OvHV-2, AlHV-2 caprine herpesvirus 2 (CpHV-2) (naturally infects goat), Ibex-MCFV (naturally infects Nubian ibex), hippotragine herpesvirus 1 (HipHV-1) (naturally infects roan antelopes), Muskox-MCFV (naturally infects musk ox), Aoudad-MCFV (naturally infects aoudad) and MCFV-WTD causing the classic MCF in white-tailed deer (may infect domestic goats naturally) (Davison et al. 2009; Russell et al. 2014; Li et al. 2014; O'Toole and Li 2014).

Epidemiology

WA-MCF was initially observed in Africa and SA-MCF was initially observed in Europe however these are found worldwide wherever sheep or cattle or other MCF susceptible species are kept together (Russell et al. 2014). SA-MCF has been reported in Europe (Collery and Foley 1996; Yus et al. 1999), America (Reid and Robinson, 1987), Africa (Rossiter 1981), the Middle East (Abu Elzein et al. 2003), and Southeast Asia (Wiyono et al. 1994) and Pakistan (Riaz et al. 2021).

The transmission of both AlHV-1 and OvHV-2 from wildebeest calves and lambs, respectively, appears to occur by contact or aerosol transmission routes, under 1-year of age (Russell et al. 2014). Shedding of the virus from the natural host is associated with lambing or calving (wildebeest) (Li et al. 2014). There have been reports of transmission over distances of five kilometres between lambs and bison without any physical contact. Infected lambs can transmit OvHV-2, through nasal secretions to susceptible species without direct contact. Similarly, the oral and nasal discharges by wildebeest

calves living in a closed area may initiate the transmission (O'Toole and Li 2014).

Pathology and Pathogenesis

The pathogenesis of MCF is poorly understood. Studies have shown different models of pathogenesis of MCF viruses in natural and susceptible hosts. It is assumed that the virus takes entry into host's body through the upper respiratory tract and causes primary infection. Later with the progression of infection, a generalized cell-associated viraemia develops. However, studies have reported that virus is not present in the tissues showing lesions and it is thought that tissue damages in MCF are due to immunopathological basis. Cell-mediated/cytotoxic reactions have been implicated in the development of lesions (Quinn et al. 2011). Widespread post-mortem (PM) lesions can be recognized in MCF affected cases however the severity of PM findings can vary between animals. PM lesions also include extensive inflammation, ulcerations and petechial haemorrhages of the systemic mucosal membranes, necrotic lesions of mucosa, enlarged lymph nodes, papules and erosive lesions on the tongue (Russell et al. 2014). Lesions in the upper respiratory track involve hyperaemia of the nasal passage, nasal turbinates, larynx and trachea with the presence of mucopurulent exudates. Ulcers causing corneal perforations result in the entrapment of the iris in the cornea (Schultheiss et al. 2000). Lymphocytic infiltration is found as major histopathological finding in the affected tissues.

Clinical forms of MCF

MCF-susceptible species are considered as dead-end hosts. This disease is characterized by the sudden onset of fever as well as ocular and nasal discharge, corneal opacity, generalized lymphadenopathy, lymphoid cell infiltration, degenerative lesions in the mucosa of the upper respiratory tract and the gastrointestinal tract. The natural incubation period for cattle is generally 2 to 10 weeks but can be as long as 9 months. There can be five overlapping clinical forms of MCF; head and eye form, peracute, alimentary, neurological, and coetaneous (Russell et al. 2014; O'Toole and Li 2014).

The head and eye form of the disease is the most common and characteristic. In this form, symptoms are predominantly seen in head and neck regions of the animal. Discharge from eyes and nose is a classic feature of this form (Fig. 5). Other signs include fever, inappetence, lesions from buccal cavity and muzzle, diarrhoea and depression. Initially the nasal discharge is serous but may progress to mucopurulent and purulent. Ocular swelling, corneal opacity, photophobia, enlargement of lymph nodes in the head and neck region, hyperventilation, and/or death are the additional presentations which can be seen in affected animals (Costa et al. 2009).

Peracute MCF is the most severe form of the disease. It is characterized by pyrexia, depression, diarrhoea, dysentery, occasional oral and nasal mucosa inflammation and death within 24-72 hours. Severe hemorrhagic gastroenteritis is prominent in all affected animals. Intestinal MCF is similar to the peracute form (Davison et al. 2009).

Cutaneous MCF and Neurologic MCF are rare forms of disease and appear more often in wild ruminants than in cattle. Cutaneous lesions may present in the form of circular alopecia that secreted clear yellow exudates in the regions sometimes at the base of the horns, the dewclaws and the interdigital space (Davison et al. 2009). Nervous signs such as hyperaesthesia, incoordination, head shaking and pressing, nystagmus and muscle tremors may be present in the absence of other clinical signs.

Fig. 5: A cow exhibiting clinical signs of head and eye form malignant catarrhal fever.

Diagnosis

Diagnosis is based on history and clinical presentations, like corneal opacity and extensive vasculitis characterized by fibrinoid degeneration and significant lymphoid infiltration. AlHV-1 can be isolated from the peripheral blood leukocyte in calf thyroid cells. Cell culture system has not yet been developed to propagate OvHV-2. However, presence of this virus or its antigens/DNA can be detected by viral gene specific PCR tests from the nasal and blood samples. Although for the detection of serum antibodies efforts have been made and a competition inhibition ELISA was developed (Li et al. 2014) but it was not found reliable enough as compared to PCR or histopathological tests. Competition Inhibition ELISA to some extent can be used to detect increasing or decreasing levels of antibodies in a herd.

Control and Prevention

Due to short clinical course of the disease, MCF affected animals can die within a few days after the presentation of initial symptoms and the treatment is not viable. Serological testing has been used as an important diagnostic tool for both natural and susceptible species. AlHV-1 and OvHV-2 are serologically related (Schultheiss et al. 2000) and antibodies from carrier sheep and MCF affected cattle are able to recognize AlHV-1 antigen (Rossiter 1981). Due to the lack of an OvHV-2 permissive cell culture system, attenuated AlHV-1 vaccines were used to try and provide protection against OvHV-2, however all the efforts proved unsuccessful. Efforts are underway to develop systems which could help in the production of OvHV-2 vaccines. The other reliable control measure for MCF is to keep susceptible species separated from potential natural hosts (Lankester et al. 2016).

Equine Herpesviruses

Equine herpesvirus 1 (EHV-1) and equine herpesvirus 4 (EHV-4) are two antigenically different alpha heprpesviruses and responsible of important febrile respiratory diseases in horses (Poelaert et al. 2019). Equine herpesvirus 2 (EHV-2) is also a commonly found EHV and causes upper respiratory tracts infections and conjunctivitis in horses of all ages. Equine herpesvirus 3 (EHV-3) mostly causes diseases related to reproductive tract like equine coital exanthema, a benign, progenital exanthematous disease. Equine herpesvirus 5 (EHV-5), a gamma Herpesvirus, has been found to be associated with equine multinodular pulmonary fibrosis (EMPF) however the exact mechanism describing the role of EHV-5 in disease is unclear (Negussie et al. 2017). Most important of the equine herpesviruses are EHV-1 and EHV-4 (discussed below).

Epidemiology

Both EHV-1 and EHV-4 are distributed worldwide wherever horse populations are present. These viruses cause acute respiratory disorders, characterized by rhinopharyngitis and tracheobronchitis. It has been reported that outbreaks due to EHV-4 occur in foals annually (Garvey et al. 2019). Abortion is common in pregnant mares due to EHV-1. Abortions can occur at any time during pregnancy without the demonstration of clinical signs and symptoms. EHV-1 also has a neurologic form in which a higher rate of morbidity and mortality is seen. Transmission of EHV-1 and EHV-4 occurs by direct or indirect contact with infectious nasal secretions, aborted fetuses, placentas, or placental fluids (El Brini et al. 2021).

Pathogenesis

These viruses begin to multiply in the upper respiratory tract. Later it spreads to through lymph nodes, lower respiratory tract and lungs. Like other alpha herpesviruses EHV-1 and EHV-2 also attain latency in trigeminal ganglia. Infection with EHV-4 appears to be limited to the respiratory tract without obvious viraemia. EHV-1 replicates in female reproductive tract and causes cell-associated viraemia that can lead to abortion. EHV-1 also causes vasculitis and thrombosis in the placenta and transplacental tissues of the foetus which also contribute in abortion (Bryant et al. 2018; El Brini et al. 2021).

Clinical Findings

The incubation periods of EHV-1 and EHV-4 are 2–10 days. Clinical signs are fever, serous nasal discharge, malaise, pharyngitis, cough, indigestion, and enlargement of regional lymphnodes. Secondary bacterial infections are not uncommon which worsen the respiratory symptoms leading to pulmonary disease (Pavulraj et al. 2021). Horses which already have encountered the virus previously develop antibodies which give some protection and only mild infection occurs in subsequent attacks. Neutropenia and lymphopenia is also common in all the infected horses. Other lesions include lung edema, thymic and hepatic necrosis; haemorrhages on various organs (myocardium, kidneys and spleen). Histologic pictures revealed also the presence of Intranuclear inclusion bodies in above mentioned tissues (Holz et al. 2019).

In neurologic form of EHV-1, less apparent infection occurs, with clinical symptoms ranging from in-coordination and posterior partial or complete paralysis and recumbency (El Brini et al. 2021). In neurologic form less severe ulcers or bleeding occurs in meninges, brain, and spinal cord parenchyma has also been reported. A few weeks after infection, abortion can occur between 7 and 11 months of gestation (Bryant et al. 2018).

Diagnosis

Animals with equine viral rhinopneumonitis usually show similar clinical signs and symptoms as of influenza, viral arteritis, or other respiratory diseases and difficult to diagnose clinically. Virus isolation from nasal cavity and upper respiratory tract during early course of infection and molecular tests like PCR / real-time PCR can help in confirmatory diagnosis. In cases of abortion, a similar pattern of diagnostic tests can be performed on infected mares and aborted foetus. Active and productive virus can also be isolated from other tissues including lungs, liver, adrenal gland, and lymphoreticular tissues. Serologic testing of mares after abortion has little diagnostic value. Recently, peptide-based ELISA has been developed that not only confirms the increasing levels of EHV-1 antibodies in mares but also allow differentiation of EHV-1 and EHV-4 specific antibodies (Pavulraj et al. 2021).

Treatment, Prevention and Control

Like other viral infections, there is no specific treatment for EHV infection. For prevention and control of EHV-1 and EHV-4 related diseases, regular vaccination with inactivated or live attenuated vaccines and proper management practices are recommended. New horses should not be mixed in old herds and should be isolated for 2-3 weeks before mixing in other horses, especially pregnant mares. To prevent respiratory disease caused by EHV-1 modified-live EHV-1 vaccine is available. Vaccination for both EHV-1 and/or EHV-4 should begin from 4–6 months of age and then at 4–6 wk after first dose. A third dose is also recommended in virus prevalent areas at the age of 10–12 months. In pregnant mares, vaccination should be performed more frequently at 3rd, 5th, 7th, and 9th month of pregnancy with inactivated EHV-1/EHV-4 ((Bryant et al. 2018; Pavulraj et al. 2021).

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CHAPTER 28

VACCINATION AND IMMUNOLOGY IN LARGE ANIMALS

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INTRODUCTION

Pathogenic microbes pour major threat to both humans and animals while the protection against these pathogens is essential for their survival (Balloux and van Dorp 2017). Environmental opportunistic microbescan induce serious infections in them if no external protection is provided (Lanzas et al. 2020). However, to counter these diseases, both humans and animals are equipped with several defense mechanisms which offer instant protection against the assault by these pathogens (Hart 2011).

Innate immunity, a non-specific type, is the first resistance against any infection and its response is always prompt, dependent on macrophages and their secreted proteins *i.e.* cytokines that identify the conserved features of pathogens (Carrillo et al. 2017). It can be explained in better way when right after the birth, the gastrointestinal tract, skin and mucous membranes are inhabited by normal microbial flora in a symbiotic way (Belkaid and Hand 2014). These microorganisms are tolerated in these body parts and in this way provide a natural antibacterial defense mechanism by preventing the harmful microbial invasion (Belkaid and Hand 2014). In contrary, adaptive immunity, a specific type, is the most sophisticated form which is trained in learning any previous contact(s) with the pathogens and based on that it thrashes them on their re-infection (Deets and Vance 2021). So in other words, the immune system's learning from the previous exposureand responding the pathogen in a calculated way is known as 'effector memory' and its memorizing the contact for future exposure is known as 'central memory' (Deets and Vance 2021). Among the immune cells, lymphocytes harbor the immunological memory which is produced during delivering a response against infectious agent (Nicholson 2016). Moreover, the immunological responses are not merely restricted to infectious agents but also against harmless pollens and some therapeutic drugs and hence, induce allergic reaction (Marshall et al. 2018). Immune system is also responsible for immunological surveillance (scrutiny) by which it can detectthe neoplastic tissue changes and have potential to eliminate it (Ribatti 2017). Vaccine is the best external source that primes and boosts the adaptive immunity in host (Laupèze et al. 2021). The Fig. 1 below describes various features of an ideal vaccine. Any vaccine which is available currently or in future will be more reliable whose characters will be close to the features of ideal vaccine (Kamel et al. 2019).

History of Vaccines

In 1798, Edward Jenner published- *An Inquiry into the Causes and Effects of the VariolaeVaccinae*-in a booklet, a disease discovered in some of the western counties of England, particularly Gloucestershire, known by the name of Cow Pox (Jenner 1800). Strictly speaking, vaccination was not discovered by Jenner, instead, he was the first one who scientifically proved that immunization from disease can be overcome by targeted interference (Boylston 2013). It was actually the'Benjamin Jesty (1737–1816)' a dairy farmerfrom England who was vaccinated against smallpox after so many exposures (Boylston 2013).

The concept and the term *vaccination* was coined into the light spot about hundred years ago by Louis Pasteur (Gomes 2021). Pasteur inoculated in chickens with "stale" cultures of *Pasteurella multocida,* in 1878 (Guzman and Montoya 2018). At first, chickens developed sickness and later recovered so he re-inoculated them with *fresh* bacterial culture. This time, the chickens received the "stale" culture were recovered whereas, those that were not exposed to the stale culture were died. Pasteur's studies were in quite concordance with Jenner's published studies on smallpox and then in the honor of Jenner, he coined the term "vaccine" (Guzman and Montoya 2018).

William Smith Greenfield in the Great Britain and Pasteur along with Henri Thullier, Charles Chamberl as well as Pierre Paul Émile Roux in France had started developing vaccine against anthrax bacteria *i.e. Bacillus anthracis* in cow and sheep, in the early 1880s (Sternbach 2003). Following 10 years, the German analysts Friedrich Loffler and Paul Frosch identified the first filterable infectious agent of mammals *i.e.* Foot and Mouth Disease Virus (FMDV) (Wang and Liu 2020). Later, a heat-inactivated vaccine was developed which provided long-term immunity against this virus.

Immune sera were great discovery in the field of biological science and horses have a great contribution in understanding the basic immunological tools behind it (Kaufmann 2019). In a chain of experiments, Emile Roux and Alexandre Yersin, trailed by Emil von Behring (Nobel Prize winner in Medicine, 1901) and Shibasaburo Kitasato immunized horses for immune sera production against the diphtheria toxin (Cavaillon 2018). One morebreakthrough in the development of vaccine was the Marek's disease (MD) vaccine generation in 1970's. MD was the herpes-virus induced cancerous disease in chickens (Reddy et al. 2017).

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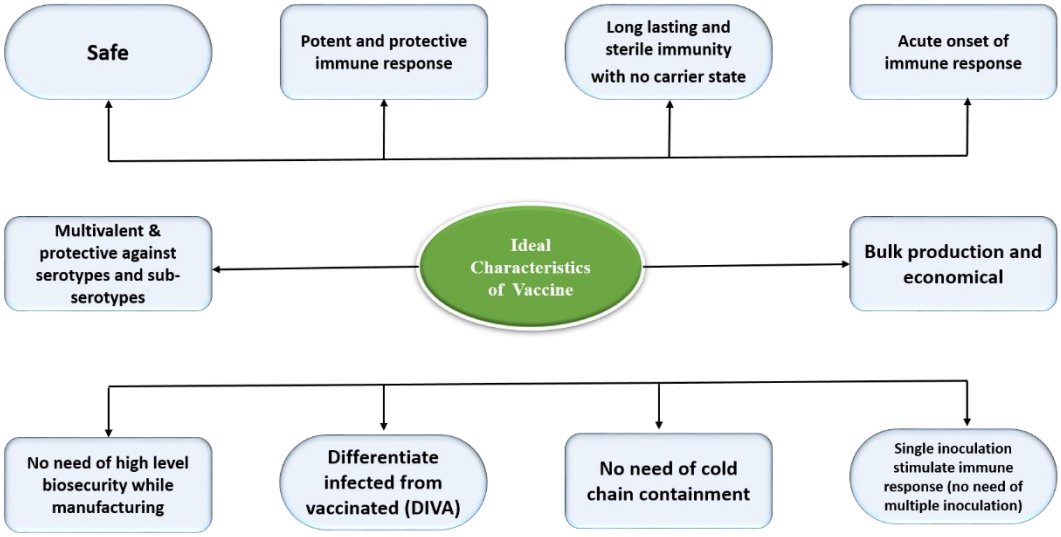


Fig. 1: Characteristic features of an ideal vaccine regarding its production, provision of protection and containment (Kamel et al. 2019).

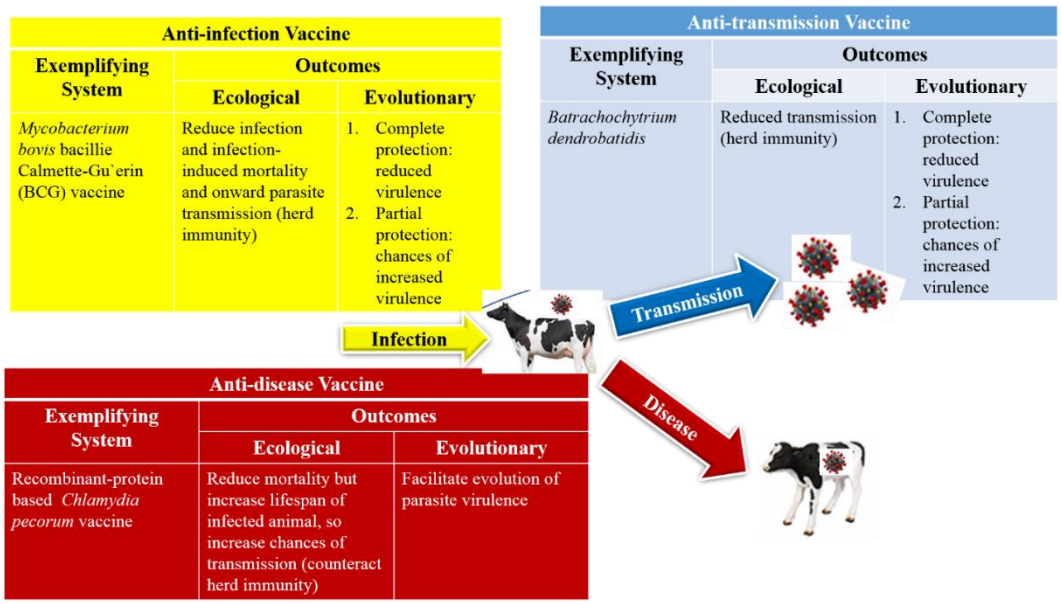


Fig. 2: Ecological and Evolutionary Outcomes of the Exemplifying System (Barnett and Civitello 2020): Categories of defective vaccines, such as anti-infective, anti-transmission and anti-disease lead to the production of phenotypic resistance strains in the vaccine species and ultimately impact the evolution of pathogen and epidemiology.

In between 1960 and 70, with the advent of various techniques in molecular biology, the establishment of recombinant vaccines' competition was at the top (Plotkin 2014). In 1981, a polypeptide vaccine synthesized biologically was considered the first published report (Skwarczynski and Toth 2016). FMD virus VP3 structural protein was cloned in prokaryotic plasmid and then transformed into *E. coli* protein expression strain. Six cattle and 2 swine were vaccinated with this purified protein. Neutralizing antibodies were produced and protected them against FMD virus challenge. This strategy helped in understanding the utilization of farm animals in the vaccine development (Guo et al. 2013). Molecular dynamic ranking was used by Kotecha and colleagues to calculatestability of FMD virus capsid. These calculations were then confirmed by X-ray crystallography (XRD) which showed improvement in *in vivo* (Vaccinating cattle) immunogenicity (Li et al. 2021). The Fig. 2 demonstrated below represents the impacts of flawed vaccines in pathogen's resistant phenotype emergence, transmission and epidemiology (Barnett and Civitello 2020).

Conventional Veterinary Vaccines

Historically, approach of empirical trial-and-error was used for veterinary vaccines development and its immunity mimics with immunity produced by natural infection (Doolan et al., 2014). Protection against a broad range of viral and bacterial pathogens was induced by traditional inactivated or killed vaccines. Inactivated, killed, toxoid and live-attenuated are the main conventional vaccines which are licensed to use. For the animal and public health improvement, these vaccines and their extensive use has contributed a lot. A disadvantage of conventional vaccine was its cost ineffective production and multiple booster doses for getting optimum immunity (Meeusen et al. 2007; Delany et al. 2014).

Moreover, *in vitro* cultured pathogen was used to induce whole-organism based vaccination. However, this strategy is suitable for pathogens with low immunogenic variability and not for those having high immunogenic variability. As highly immunogenic variability equips the pathogens for hijacking the

Table 1: Livestock vaccination schedule

Month	Large Animals especially Bovines	
	Up to 1 year	Adult
January	FMDV & ETV	HSV
February	-	FMDV & Anthrax
March	-	Theileria
April	-	BQ
May	FMDV & ETV	-
June	HSV	HSV
July	-	-
August	FMDV & ETV	HSV
September	-	FMDV & Anthrax
October	-	RP
November	-	-
December	HSV	HSV

*FMDV= Foot and Mouth Disease Virus, ETV= Enterotoxaemia, HSV= Bovine herpes virus: BQ= Black Quarter, RP= Rinder Pest

immune response (Doolan et al. 2014). Another lacking point in traditional vaccine is that its scheme to work is like immunity induced by natural infection. This is not as appropriate for many pathogens as this type of vaccine provides only suboptimal protection and with so many adverse effects of stimulated inflammatory reactions (Zepp 2010). For examples, a pathogen causing chronic disease can co-sustain with host for a longer period of time despite the presence of host immunity (Doolan et al. 2014).

Table 1 below shows vaccination schedule that is commonly followed throughout the year in large animal practice in the India and Pakistan (Singh and Borkotoky 2018).

Live-Attenuated Veterinary Vaccines

The passage of pathogen in an atypical host or cell weakens it and in this way, live attenuated vaccines are produced. This vaccine is then inoculated in its actual host expecting that the pathogen has lost its pathogenicity but retained its immunogenicity (Meeusen et al. 2007). However, practically such vaccines are not safe for so many pathogens and many get activated and induce the disease. Moreover, they cause many side effects, local inflammatory reactions and autoimmune disorders consequently may get back their virulence. Possible reasons could be their improper culture, passage and refrigerated preservation (Babiuk et al. 2003; Meeusen et al. 2007). Their benefit is that they don't need any adjuvant that can enhance their immunogenic potential as they retain their infecting and replicating ability in host cells.

The strategy associated with developing vaccines is quite intricate as it uses live cells and hence, difficult to optimize. These live vaccinations are hard to design due to the multifaceted macromolecular nature of pathogens. Live-attenuated vaccines are comparatively less hard to make than inactivated vaccines as they don't need adjuvant in their formulation with least downstream handling (van Gelder and Makoschey 2012). Attenuated vaccines were used by considering them as older technique; now-a-days, specified mutagenesis is employed to produce vaccine virus strains.

Immunologically characterized and safe vaccines are recombinant ones prepared by reverse vaccinology technique (Delany et al. 2014). The desired regions can be inserted or deleted (Indels) by using molecular techniques. Therefore, this approach can overcome the drawbacks of live-attenuated vaccines, representing a doable strategy.

Inactivated Veterinary Vaccines

Currently, killed viral strain or bacterial serotype are used and adjuvant them in aluminum hydroxide or oil to formulate inactivated vaccines (Meeusen et al. 2007). These are cost effective in production and stable in environmental conditions. Vaccine pathogens are normally cultured in bioreactors or roller bottles type cell culture and then killed/inactivated by chemical or physical treatments which can denature their proteins and damage their nucleic acids. As they lost their infective and replicative ability, so adjuvant(s) is added for improving their immunogenic potential (van Gelder and Makoschey 2012). Their safety profile is quite improved; however, their ability to provide long-term immunity is compromised (Cho et al. 2002). Many vaccines are unable to handle with the prevalent pathogenic strain of virus or bacteria, thus circulating field strain sample collection, pathogen isolation and culturing for vaccine production are necessary to combat new outbreaks (Meeusen et al. 2007).

Toxoids

These vaccines have bacterial toxins which are responsible for inducing disease. So, commercially formed inactivated/killed toxins (toxoids) are pooled with traditional adjuvants. The drawbacks of producing toxoids are; the amount produced of the toxin *in vitro* is erratic and for some toxins high level biosafety measures are required (Arimitsu et al. 2004). Recombinant toxoids can overcome such restrictions, by producing them in ample quantity with low reactive potential. For example, the production of recombinant *E. coli* toxins takes only 2–3 days using simple growth media. Formaldehyde is used for inactivation and requires least biosafety precautions because of the removal of the toxic domain of the protein (Moreira et al. 2016).

Conventional Subunit Vaccines

A specific part (genetic material or protein) of pathogen that can stimulate host's immune system is present in subunit vaccines (SV). If long chain carbohydrates which are present in the bacterial capsule are used as SV, then these are known as polysaccharide vaccines. However, these SVs are incapable to recruit T helper cells as this kind of immunity can only be recruited by some antigenic protein part which provides a protective immune response. Protein-polysaccharide-conjugate (covalently linked to a carrier protein) technology has been used to overcome the disadvantages of inactivated toxin (toxoid), such as; tetanus or diphtheria toxoids. By using a conjugate vaccine, the immune responses to the polysaccharides are dramatically improved (Dintzis et al., 1992). Virus-like particles (VLP) vaccines do not hold any replicative genetic material but permit antigenic presentation in a repetitive, ordered array just like the virion structure that only increase the immunogenicity (Jennings and Bachmann, 2008). VLPs can efficiently provoke both cell-mediated and humoral immune responses without necessitating an adjuvant. This is due to the close resemblance of molecular scaffolds of VLPs to the native viruses and the absence of genetic material. However, for commercially producing these vaccines, such approaches have yet to be employed (Liu et al. 2012).

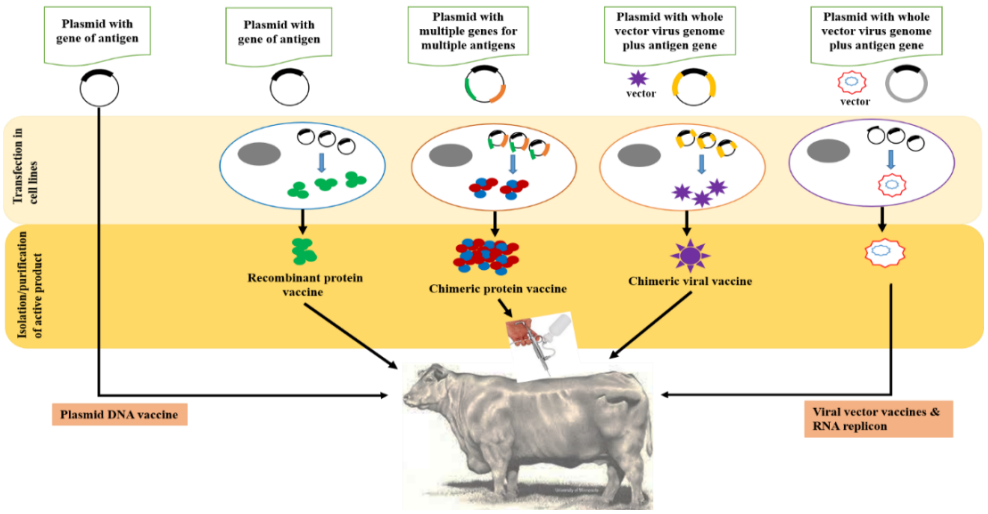


Fig. 3: Schematic diagram of novel recombinant vaccine technologies from their production to vaccination (Aida et al. 2021)

Biotechnology Applied to Next Generation Vaccine Development

Genomic analyses of pathogens and enhanced understanding of the mechanisms of pathogenesis have resulted in new antigen discovery and the development of recombinant veterinary vaccines. A large amount of draft and whole-genome sequencing of viruses, prokaryotes and eukaryotes pathogens have been performed (Pizza et al. 2000; Tettelin et al. 2000; Vasconcelos et al. 2005; Kremer et al. 2016). These advancements have also improved antigen discovery and the characterization of variability between viral pathogens, which typically contain fewer than ten genes, and eukaryotic pathogens, which often encode >10 000 genes (Cho et al. 2002; Aurrecoechea et al. 2007). The genome sequencing technologies and the approaches used to screen the genome and proteome of a pathogen have greatly improved the efficiency of antigen discovery (Seib et al. 2012) because relevant antigenic structures can identify and produce recombinant vaccines that contain only the antigen necessary to elicit protective immunity.

Genomic databases generally contain whole genome sequences and the complete repertoire of encoded proteins from which vaccine screening is possible (Bagnoli et al. 2011). Surface-exposed antigens, secreted proteins, and toxins are commonly viable vaccine candidates against bacterial infections (Ravipaty and Reilly 2010). However, further *in vivo* investigation of antigens is still necessary and desirable. Comparative genomic analysis software can be used to perform gene comparative analysis by basic sequence similarity searches. Sequence similarity algorithms facilitate the comparison of predicted coding sequences (ORFs) with known genes/proteins in public databases, and are commonly used to predict the degree of gene conservation among a bacterial population.

In silico analysis may also result in enhanced protein antigen qualities such as expression and solubility. As native gene sequences retain their own specific codon usage that reflects the composition of their respective genomic tRNA pools, gene sequences may be optimized for higher expression levels in any heterologous system (Bagnoli et al. 2011). One drawback of reverse vaccinology is that it cannot be used to predict polysaccharides or lipids, which are often included in vaccines as active compounds. Fig. 1 has shown a scheme of recombinant vaccine development strategies.

One negative aspect of reverse vaccinology is that it can't be helpful in anticipating lipids, which are usually associated with vaccinations as unique compounds. Fig. 3 represents the 6 unique techniques of various recombinant vaccines right from their generation/isolation or purification of antigens to the application of vaccination on animal (Aida et al. 2021). Starting from plasmid-DNA vaccines, the gene of interest (GOI) of the desired immunogen is incorporated inside the plasmid. This is used as an active ingredient to vaccinate the animal. Vaccine recipient's cells, the plasmid-DNA vaccine occupy the DNA encoding for the target immunogen is translated into protein of interest. The immunogen is then appeared from the cell, resultantly inciting an immune response (Aida et al. 2021). Recombinant chimeric and protein vaccines employ the similar methodology. Nevertheless, transfection of these plasmids is done in appropriate cell-lines for antigen protein expression. Then immunogen(s) is/are collected, filtered, and made into the final vaccine form (Aida et al. 2021). In case of chimeric viral vaccines, they use a plasmid with complete viral genome that is used as a carrier to transfer the gene of interest for the desired antigen. Like above the plasmid transfected in an appropriate cell-line for whole virus expression along with the integration of antigen. The virus is then collected and filtered, and formulated into a final vaccine form (Aida et al. 2021). Viral vectors use a virus that had been specifically made to express the required gene of interest. The vaccine will release the recombinant genes into the host cells. Genes of interest will be transcribed into the target antigen which will then be expressed and induce an immune response (Aida et al. 2021). Ribonucleic replicon vaccines use a RNA segment that codes the desired immunogens encapsulated in a vesicle carrier. Upon entering in the cell of host, the RNA is translated directly, which results in the expression of the antigen which is being targeted (Aida et al. 2021).

Vectored Vaccines

The usage of immunogen/quality movement system had worked with the improvement of new preventive and curative immunizer contender. To pass on guarded protein(s) to the immune plan, vector counter acting agent development uses a vector. These vectors are for the most part antigenic and can show various immunogenic effects. Recombinant vector inoculations are named live vector antibodies and naked

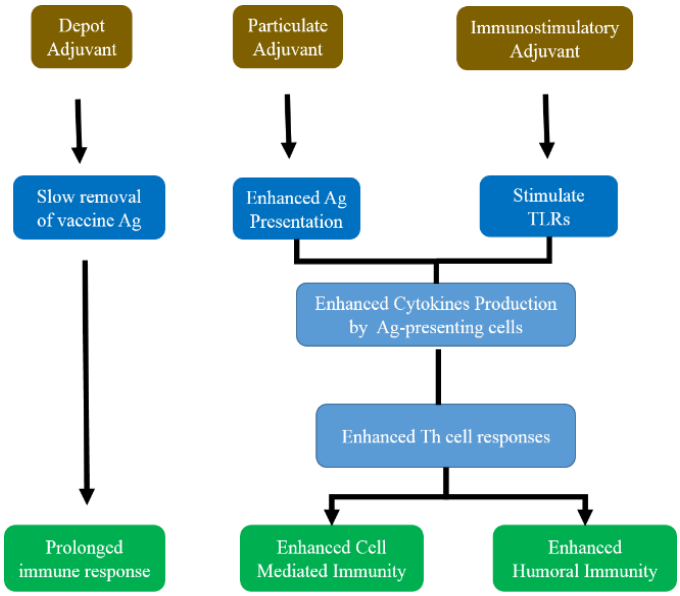
Deoxyribonucleic acid antibodies. Plant vaccinations are also vector antibodies that have basic capability in animal drugs.
Conventional live vectors are reduced organisms or contaminations that, just as prompting their own innate opposition, can similarly be used as for conveying the immunogens of other pathogens. For external characteristics, Poxviruses which join the fowl pox, canary pox and vaccinia diseases have been used as vectors successfully. Poxviruses can oblige a great deal of new characteristics and can pollute mammalian cells, achieving the announcement of tremendous measures of encoded protein. At the present time, the canary pox disease vector system has been applied as a phase for an extent of animal antibodies in opposition to FLV, WNV, EIV, Rabies contamination. BCG, which is the bacterial reduced vector, has been perused up for quite a while. Recombinant BCG provides gigantic ability for conveying a huge numeral of heterologic immunogens and is able to affect solid safety (Rizzi et al. 2012).
Using plants for assembling and passing on immunogen through sources of food is uncommonly profitable. The usage of transgenic plants shows an imaginative progression that has revealed novel streets in the inoculation ventures. In animal vaccinology, genetically modified plants can make and pass on immunogen through feed of animal (Shams 2005).

Nucleic Acid Vaccines

DNA vaccinations produce antigens in the genuine host. We can describe Deoxyribonucleic acid (or Ribonucleic acid) vaccination as "a plasmid consisting of a bacterial, viral or parasite which may be conveyed in cells of mammal or a quality inscribing a mammalian protein (non-microbial ailments). This nature is implanted into a plasmid close by reasonable genetic parts, for instance, powerful eukaryotic publicists for transcriptional control, a polyadenylation signal plan for consistent and convincing translation. The mRNA is translated after plasmid is transferred inside cells. The mRNA is consequently deciphered, achieving the host cell contraption making an immunogenic protein. The host safe structure sees the imparted proteins as new, and this can incite the headway of a humoral and cellular safe response.
Vaccination of veterinary side with stripped DNA which codes immunogens for virus' protection in various methods addresses an optimum framework for viral antibodies. Since it does not simply beat the effects produced with vector immunity and live vaccines yet also propel the acknowledgment of cytotoxic T cells after enunciation of the immunogens intracellularly (Meeusen et al. 2007).

Adjuvants for Recombinant Animal Vaccines

The development and use of adjuvants to inoculation (immunogens) pass on a couple of advantages, for instance, segment saving, extended sufficiency in the more established, and enlarging of the humoral or/and cell safe respond. Subdivisions of recombinant units are routinely favored over inactivated or live microorganisms; regardless, they usually are not so much capable of provoking immune response and need the extension of an adjuvant to accomplish cautious invulnerable responses (Soema et al. 2015). The impacts of immune modulation depend on the specific adjuvant used related with particular antigens. Types of adjuvants are presented below in the Fig 4 and designed by taking help from online source https://veteriankey.com/vaccines-and-their-production/#s0075.
A couple of adjuvants have been listed for use in animal inoculations, similar to mineral salts (aluminum) (Li and Cui 2014); emulsions (Montanide) (Peter et al. 2001; Miles et al. 2005) biodegradable polymeric micro-particles, and nanoparticles. Furthermore, the elective extent of adjuvants is depicted as "safe potentiators" as they apply ramifications for safe introduction (Ott et al. 2000). A couple of adjuvants act by confining antigens in locales, called as terminals, to give an extensive time frame of antigenic inclination. Along these lines, a couple of veterinary inoculations are as emulsions in oil. This for the most part more seasoned style advancement is, regardless, a solid philosophy that accomplishes a powerful combustible respond and slow antigen opportunity, this is what was not recombinant subunit vaccines. Rather than the solidly safe authorizing emulsion-type adjuvants, aluminum salt adjuvants are not good for prompting Th1 or cell-mediated invulnerable actions take place to any enormous degree; in any case, they are useful Th2 inducers, prompting elevated antibody titers in the vaccinated organism.



Depending upon size either, endocytosis or phagocytosis masks the particles. The foreign particles are either typified in nano particle's framework core or adsorbed upon the surface layer of the nanoparticles (Slütter et al. 2009). As of now, micro-particles from polymer have still not been fruitfully made as an immune response thing. Micro-particles overall redesign the acknowledgment of Th2-type, humoral obstruction, while nanoparticles advance Th1-based, cell-mediated safe responses (Li and Cui 2014).

Role of Reverse Vaccinology In animal Health Perspectives

The improvement of animal antibodies is a troublesome endeavor; however, reverse vaccinology is significantly reassuring as a part of animal vaccination progression. Basic breakthrough has been made in the space of vaccinology during the hour of genomics, and state of the art vaccinations are set to dynamically influence animal prosperity. We can assume significantly more progress in vaccinology and the improvement of new fruitful animal antibodies that safe the

host from compelling ailments just as against various diseases or continuous issues. Believe it or not, switch vaccinology is as of now being used in various bacterial, viral, and eukaryotic microorganisms and has been powerful in giving new antigens to the arrangement of novel vaccinations (Bagnoli et al. 2011; Buonaguro and Pulendran 2011). Furthermore, the limit of sensible arrangement to additionally foster contender immunogens can give extended confirmation against immunogenically factor organisms (Seib et al. 2012).

Now a day, a new breakthrough in the field of vaccinology is mRNA vaccines which are obvious by dozens of publications on pre-clinical and clinical data in the last 2 years. These vaccines are mostly used for cancer protection but they have also been designed for various infectious organisms *e.g.* Zika virus, influenza virus, Ebola virus, *Toxoplasma gondii* and *Streptococcus* spp. Thus, the coming time is the era of mRNA engineering in the field of vaccinology and all the basic and clinical research trial findings will be used in designing various transcriptional drugs.

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CHAPTER 29

BIOCHEMICAL IMPLICATIONS OF TOXIC INSULTS AND CURRENT REGIMENS FOR DETOXIFICATION

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INTRODUCTION

Brief Overview of Types of Toxic Insults

In routine life, humans and animals are exposed to a number of environmental and household toxicants. These compounds alter the normal biochemical processes such as activate or inhibit the enzymes involved in metabolic processes, production of free radicals thus producing a state of oxidative stress as well as may cause the mutation, cytotoxicity, epigenetic changes and abnormal autophagy. Any of such biochemical implication will lead to toxic manifestations in the living system. There are numerous ways for detoxification of such toxic insults such as antidote administration, chelation therapy, use of nutraceuticals, phytochemicals as well as nanomedicines. We shall highlight the above-mentioned topics with particular reference to the pesticides and heavy metals in this chapter.

Pesticide's term is used generally for agrochemicals identification such as fungicides, bactericides, insecticides, herbicides or rodenticides (Sule et al. 2022). Pesticides may be grouped into diverse chemical classes like organophosphates, carbamates, organofluorines, pyrethroids, triazoles and bipyridyl herbicides (Georgiadis et al. 2018). About 2 million tons of pesticides are being utilized globally per annum (Sharma et al. 2019). The World Health Organization (WHO) has appraised that, in developing countries, every year around 3 million workers undergo severe poisoning from pesticides, of which approximately 18,000 of them ultimately die (Min et al. 2017). The exposure mode to pesticide includes the gastrointestinal, dermal and inhalation one (Yurumez et al. 2007).

Metals have numerous commercial and occupational applications as well as diversity of uses in medicines. There are many examples which can be used in this context like chromium and nickel are used widely for stainless steel production that is primarily important for surgical and prosthetic equipment. Arsenic, on other hand, is administered for acute promyelocytic leukemia. Numerous studies have shown that these metals exposure is linked to carcinogenic and toxic effects on human as well as animals (Valko et al. 2005). Drinking water contamination with high levels of chromium and arsenic has been related with lung, liver and skin cancer hence representing a grave threat in several countries. Ineffective product recycling with high metals concentration and continuously increasing consumption of toxic metals anticipates worsening of such issues in future. That's why explication of molecular and biochemical pathways regarding metal induced carcinogenesis is of great attention regarding upgrading of drug designing for anti-cancer moieties as well as risk assessment (Galanis et al. 2009).

Exposure of Toxic Agents with Biological Systems

Interaction of an organism with toxic agent is dependent on the dose reaching at certain site as well xenobiotic's affinity for that site. In case when xenobiotic has interaction with multiple sites, each site will have its own affinity which is measured as dissociation constant Kd (or Km for enzymatic site). With increase in dose, the xenobiotic interacts with an increasing number of diverse sites, with decreasing affinities. In cell culture, and to a reduced degree in whole animals, it is likely to reach concentrations rarely achieved in environment. Thus, a biological ligand interaction with high xenobiotic concentrations may be a valuable tool for mechanistic studies, it does not essentially depict the toxicity site of that same agent at lower concentrations (Ross 2010).

BIOCHEMICAL IMPLICATIONS OF TOXIC INSULTS

Alterations and/or Inhibition of Enzymatic Activity

Xenobiotic interaction with enzymes at sites other than substrate-binding sites may decrease or increase the activity of enzyme. Enzyme activation, increase in maximum enzyme velocity without increasing enzyme amount, may be instigated by allosteric accumulation of co-factor which may help in carrying substrate to juxtaposition at enzymatic site of action. Removal and chelation of co-factor, in contrast, might inhibit enzyme (Ross 2010). There are several examples which can be narrated in this context.

The primary target of organophosphates is acetylcholinesterase (AChE) which hydrolyzes a chief neurotransmitter, acetylcholine (Sharma et al. 2005). Although

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experimental evidences show little correlation between degree of organophosphate produced AChE inhibition. However, organophosphates may result in numerous human body disorders. The organs which might be affected are muscles, kidneys, liver, hematological, immunological systems etc. (Possamai et al. 2007). The prime clinical signs of acute intoxication by organophosphates are irreversible inhibition of enzymatic activity in blood and nervous system which results in acetylcholine accumulation and activation of nicotinic and muscarinic receptors which consequently result to death (Aygun et al. 2007). The swiftness of ACh accumulation mainly depends upon organophosphate exposure level. Acute toxicity is demonstrated as cholinergic crisis and accompanied with miosis, weakness, excessive secretion by glands and muscle fasciculation (Lukaszewicz-Hussain 2010).

Enzyme activity may be altered by heavy metal exposure. Lead poisoning may lead to anemia by inhibition of two important enzymes i.e. δ-aminolevulinic acid dehydratase (ALAD) and ferrochelatase tangled in heme biosynthesis (Mense and Zhang 2006).

Oxidative Stress

Oxidative stress is imbalance between the free radical production and the defense system of body's antioxidants which may be enzymatic or non-enzymatic. Augmented reactive oxygen species (ROS) production and diminution of organism's antioxidant system may result in induction of oxidative stress. ROS are highly unstable as they have one or greater unpaired electrons. That's why, they damage the molecular function and structure in order to get stability by attacking nearby molecules to get another electron. ROS are products of usual metabolic process as well as in cell signal transduction hence show significant role in pathogenesis (Morgan et al. 2007).

All the biological macromolecules such as nucleic acid, protein and lipids may be attacked by free radicals. Lipids, however, are highly vulnerable. The metabolic pathway of cytochrome P450, mitochondrial respiratory chain and inflammation are the main sources for the endogenous production of ROS. ROS may be formed by numerous exogenous sources like metal ions, radiation and chlorinated compounds (Valko et al. 2006).

In chronic or sub-chronic exposures of organophosphates, oxidative stress is considered as prime toxicity mechanism (Ranjbar et al. 2005). Oxidative stress induced by pesticides is instigated by both reactive nitrogen species (RNS) and reactive oxygen species which are linked to numerous diseases including inflammation, neurodegenerative, cardiovascular diseases and cancer. Both RNS and ROS may activate at least five independent signaling pathways including mitochondrial induced apoptosis (Sule et al. 2022). Reactive species are generated in this process. The organophosphate (OP) alter normal homeostasis of antioxidant resulting in depletion of antioxidant, if the prerequisite of constant antioxidants is not sustained (Vidyasagar et al. 2004). In OP toxicity, another way of ROS generation is oxidative phosphorylation inhibition coupled with consumption of high energy as well as increased glucose and ATP release in order to meet energy requirements of body (Milatovic et al. 2006; Rahimi and Abdollahi 2007).

Oxidative stress diminishes the pool of GSH (Zasadowski et al. 2004). GSH levels may also be decreased owing to its partaking in conjugation reactions or reduced cellular ability for regenerating GSH. Intoxication by OP results in oxidative stress which is demonstrated by alterations in activity and/or levels of anti-oxidant enzymes and non-enzymatic antioxidants in different organs respectively. The studies have also demonstrated oxidative stress as augmented concentration of lipid peroxidation marker, malondialdehyde (MDA) and ROS as well (Akhgari et al. 2003). Organophosphate exposure induces hyperglycemia which lead to enhance non-enzymatic glycation by requisite glucose binding or by-products to protein and form complex compounds i.e. advanced glycation end products (AGEs), ultimately leads to functional and structural alteration of protein. Glycated proteins through AGEs activate specific membrane receptors leading to induction of intracellular oxidative stress. Hyperglycemia, thus, is among one of the mechanisms of oxidative stress due to OP intoxication (Rahimi and Abdollahi 2007). A list of different studies regarding *in vitro* and *in vivo* evidences of oxidative stress induced by different pesticides and effect on markers has been shown in Table 1.

Heavy metals also manifest toxicity by production of ROS. Lead (Pb) has great affinity to the reactive –SH group of GSH and may decrease the level of GSH. The antioxidant function of GPx, SOD, CAT, metalloproteins, regarding free radical detoxification might be affected due to Pb exposure. Lead can persuade oxidative damage in diverse organs via directly affecting on membrane lipid peroxidation and hence reducing antioxidant parameters (Kasperczyk et al. 2005; Balali-Mood et al. 2021).

Cellular Deaths, Mutagenicity and Genotoxicity

Dysregulation of cellular proliferation, epigenetic and genetic changes and abnormal activation of pathways of cellular transduction epitomize the key mechanisms of carcinogenesis induced by metals. Base modifications of nucleotide, crosslinking of DNA proteins as well as single and double strand breaks are common genetic effects. Epigenetic effects are primarily linked with histone and DNA methylation leading to inapt gene silencing which ultimately cause alteration in gene expression and tumor development (Salnikow and Zhitkovich 2008; Akhtar et al. 2021). Dysregulation of cellular differentiation and growth is typical feature of cancer phenotype. Various transcription factors, which control prime cellular responses like cell cycle progression and cellular apoptosis, are activated due to dysregulation of cellular proliferation by metals. These transcription factors include tumor suppressor protein p53, AP-1, NFAT and nuclear factors NF-κB (Leonard et al. 2004). Metals, thus, interfere with signal transduction pathways and hence modulate gene expression. The classical PI3K/Akt/mTOR cascade and MAP kinase pathways are targets of various metals.

The main regulator for cellular adaptation to hypoxia is hypoxia inducible factor-1 (HIF-1) (Ke and Costa 2006). It controls many processes perilous for cellular survival like angiogenesis, erythropoiesis, apoptosis, pH regulation and iron metabolism. Raised HIF-1 expression, transcriptional activation and stabilization are related to different cancers like ovarian, lung, prostate, breast cancer etc. (Galanis et al. 2008). HIF-1 is the main integrator of cell signaling pathways which induce tumor angiogenesis. HIF-1, in response to hypoxia activates the vascular endothelial growth factor (VEGF). VEGF scores angiogenesis and tumor progression. Thus, HIF-1 pathway induction is essential for carcinogenesis. Metals can hinder the regulation of HIF-1 by intermingling with HIF-1 hydroxylases or

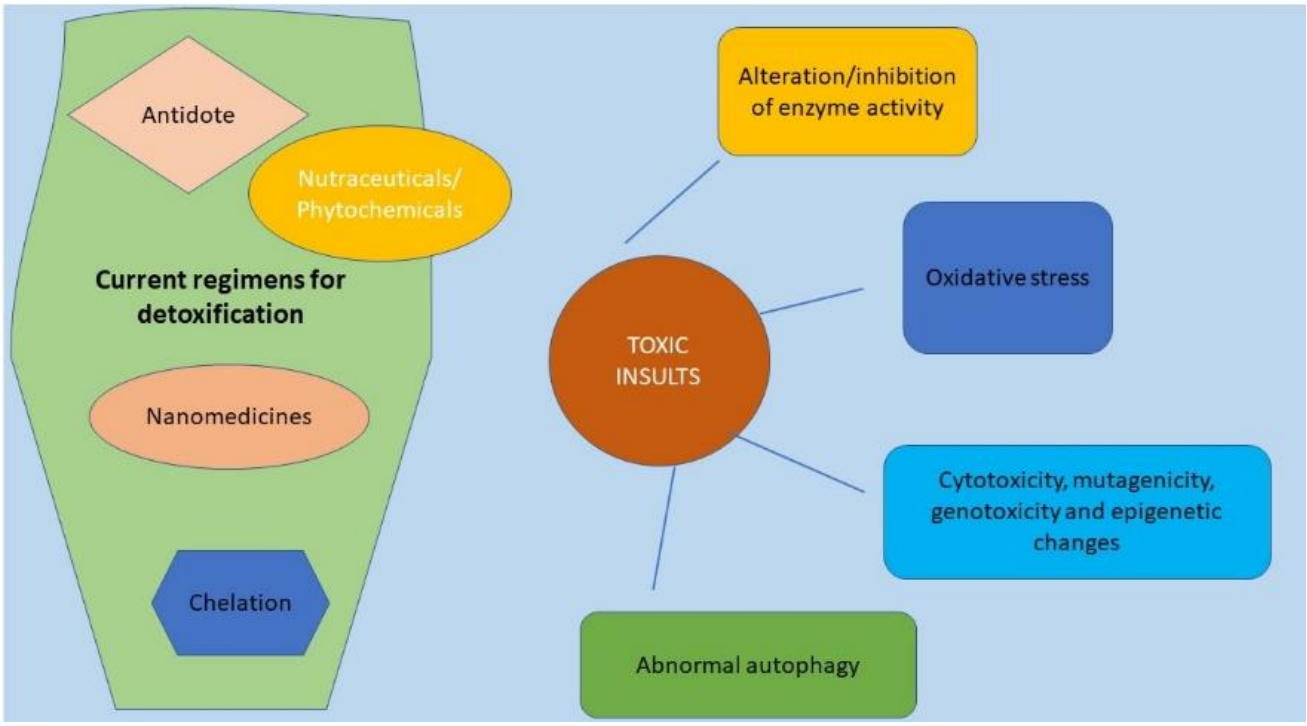

Fig. 1: Schematic diagram showing the possible biochemical implications following toxic insult and current regimens used for detoxification.

Table 1: *In vitro* and *in vivo* evidences regarding involvement of oxidative stress induced by different pesticides

Pesticide name	Mode of study*in vitro*/*in vivo*)	Underlying mechanism	Reference
Chlorfenvinphos	*In vivo* (rats)	Enhancement of lipid peroxidation,	(Lukaszewicz-Hussain 2001)
Diazinon (acute intoxication)		Lipid peroxidation enhancement, altered GPx activity in liver	(Teimouri et al. 2006)
Fenthion	Mice, rats	decreased GSH level and increased MDA in RBCs	(Buyukokuroglu et al. 2008)
Dichlorphos	Fish	Increased GSH, decreased MDA	(Varga and Matkovics 1997)
Quinalphos	Rats	Increase activity of GPx, SOD, GR and CAT in the liver. While GSH and lipid peroxides remained unchanged	(Dwivedi et al. 1998)
Profenofos, λ cyhalothrin, azadirachtin	*Tubifex tubifex*	Initial induction and then reduction in GST and GPx, Throughout induction in CAT and MDA	(Chatterjee et al. 2021)
Cypermethrin	*Oncorhynchus mykiss*	SOD, CAT, GPx activities were increased	(Atamanalp et al. 2021)

Table 2: An insight to heavy metal induced epigenetic changes and carcinogenesis

Name of heavy metal	Carcinogenic class by International Agency for Research on Cancer (IARC)	Underlying mechanisms of cancinogenesis	Reference
Nickel	Possible carcinogen to humans (Group 2B)	Epigenetic alterations, ROS generation and hypoxia signaling pathway activation, disruption of cellular iron homeostasis DNA hypermethylation, alterations of histones modifications, increased heterochromatin condensation	(Ke et al. 2006; Salnikow and Zhitkovich 2008; Zhao et al. 2009)
Vanadium pentoxide	Possible carcinogen to humans (Group-2B)	Vanadate-induced oxidative stress caused expression of HIF-1α and VEGF in human prostate carcinoma cells	(Livage 1991; Gao et al. 2002)
Cobalt	At high doses possible carcinogen (Group-2B)	Inhibition of DNA repair mechanisms, induction of damage of DNA, Aneuploidy and DNA exchange b/w sister-chromatids	(Cancer 1991; Valko et al. 2005)
Arsenic	Carcinogenic to humans (Group1)	Greater cellular proliferation, ROS generation affecting the hypoxia signaling pathway	(Cancer 1987; Simeonova et al. 2000; Huang et al. 2004; Shi et al. 2004)
Chromium	carcinogenic to humans (Group1)	Oxidative stress leading to Cr-DNA adducts, DNA-protein cross-links formation, single and double stranded DNA breaks	(Cancer 1990; Salnikow and Zhitkovich 2008)

by induction of ROS generation which may also activate HIF-1. The underlying carcinogenic mechanism of different metals has been given in Table 2.

Autophagy

An essential catabolic process, mammalian autophagy, involves the degradation of lysosomes and recycling of organelles and cellular proteins. Autophagy is upregulated in metabolic stress conditions such as deprivation of nutrients and greater availability of metabolic intermediate products (Chavez-Dominguez et al. 2020; Saran et al. 2021). Autophagy has shown to escort programmed cell death type II as considered by large intracellular vesicles and the assignation of the autophagy machinery. However, its function, as an active cellular death mechanism remnant controversial (Green and Llambi 2015). Autophagy is introduced by phagophore formation which slowly closes to produce autophagosome, a

double membrane vesicle. Later, it fuses with lysosome to produce autolysosome which permits its contents to be recycled and degraded. Conditions of cellular stress like hypoxia, oxidative stress and ER stress trigger autophagy in order to enable cells for adopting unfavorable conditions (Saran et al. 2021). Endoplasmic reticulum, in mammalian cell, is prime compartment which enables folding of newly synthesized proteins and initiation of vesicle movement. A potent stimulus of autophagy is unfolded protein response (UPR) which is a major ER stress pathway. ER stress sponsors autophagic responses in variety of ways such as UPR downstream effectors RNA-dependent protein kinase-like ER kinase (PERK), induction of Ca+2 and the inositol-requiring protein-1α (IRE1α) pathways (Song et al. 2018). Autophagy is induced by released calcium from ER via activation of the CaMKKβ-AMPK pathways (Borodkina et al. 2016). Carcinogenic metals have been shown to be strong inducers of autophagy. Cancer cells, moreover, take advantage of the autophagic progression to endure aggressive environments. It has been established that heavy metals like Cd and As impair autophagy and hence promote tumorigenesis (Pal et al. 2017), while the successive inhibition of autophagy blocked metal-induced carcinogenesis.

CURRENT REGIMENS FOR DETOXIFICATION

There are various regimens for detoxification. We have discussed few of the currently used such as antidote therapy, chelation therapy, nutraceuticals and medicinal plants for amelioration of toxic insults as well as nanomedicine based novel regimens in this context. A schematic diagram has shown the various biochemical implications and current regimens for detoxification (Figure 1)

Antidotes for Detoxification

Antidotes alter the toxic substance kinetics or change its effect at receptor binding site (Garbino et al. 1997). Antidotes have 4 mechanisms:

A) By neutralizing the effects: OP toxicity is managed with antidotal treatments which consist of two regimens. In pre-treatment, pyridostigmine bromide (acetylcolinestrase inhibitor) is used while in post-treatment, atropine sulphate (anticholinergic) and 2PAM-chloride are used (Gray 1984).

B) By direct action on the involved toxin: Specific binding which is obtained by the chelation, bio-scavenger and immunotherapy for heavy metal, organophosphorus and digoxin respectively (Peter et al. 2007; Pichamuthu et al. 2010). Non-specific binding is obtained by the use of intra-lipid and activated charcoal therapy. The activated charcoal therapy is mostly used for the decontamination of the gastrointestinal toxicity (Merigian and Blaho 2002; Chyka et al. 2005). Salicylate is acidic in nature and it is eliminated by the help of urinary alkalization (Weinberg et al. 1998; Proudfoot et al. 2004; Eddleston et al. 2008; Pillay 2008). C) By decreasing the level of toxic metabolites: Toxic metabolites decrease with the help of binding and convert it to less toxic metabolites.

D) Antidotes acting on the toxin binding site: It can be accomplished by competitive receptor and enzymes inhibition, on the GABA receptor complex e.g. fomepizole and ethyle alcohol are competitive antagonist of ethylene glycol toxicity (Barceloux et al. 2002) and the naloxone is the competitive antagonist for the opioid toxicity by displacing the opioid compound from opioid receptor (Lavonas et al. 2015; Lynn and Galinkin 2018).

Chelation Therapy for Detoxification

Ideal chelator must have low toxicity, same distribution properties just like the heavy metals, highly water soluble, make rapid elimination of toxic substances and have great ability to penetrate the cell membrane (Flora and Pachauri 2010). Different chelators are used for the detoxification of the lead toxicity which bind to the toxin in blood and make them inactive. Edetate Calcium Disodium is given intravenously while succimer/Unithiol is used as oral chelator for the detoxification of lead toxicity (Lowry 2010). Combine therapy is used to attain the synergistic effect of two chelating agents. The combine therapy of DMSA and CaNa2EDTA against the lead poisoning increases the elimination of lead and make a faster recovery (Mishra et al. 2008).

Nutraceuticals and Phytochemicals for Detoxification

Nutraceuticals and phytochemicals which are rich in antioxidants are used for detoxification and amelioration of organ function following toxic insults. Literature has provided many examples in this context.

Quercetin, a flavonoid (Mao et al. 2018) is present in high concentration in onions, soybean potatoes and many fruits, has potential to reduce the oxidation stress and the toxicity which is produced by the cadmium (Wang et al. 2020). Proanthocyanidins, a flavanol, is obtained from the grape seeds and also used for cadmium toxicity (Hemingway and Karchesy 2012). Glufimet, a derivative of glutamic acid, is obtained from the eggs and protein-based food and has ability to detoxify the alcohol insult (Perfilova et al. 2021). P-coumaric acid, an isomer of the coumaric acid, is abundantly present in the fruits and vegetables and has antioxidant activity (Zang et al. 2000; Kong et al. 2013; Pragasam et al. 2013). Naringenin, another flavonoid, present in citrus fruits, grape fruits and tomatoes, has antioxidant property (Pietta 2000). Naringenin decreased the toxic effect of arsenic induce hepato- toxicity in the rat (Mershiba et al. 2013). A carotenoid pigmentlike lycopene, present in excessive amount in the red grape fruit, tomato and watermelon, has ability to reduce the atrazine (herbicide) induced hepatotoxicity (Xia et al. 2016). Turmeric is routinely used nutraceutical and is used to remove heavy metals from the body (Rafati-Rahimzadeh et al. 2014).

In the animal study, the corriander seeds extract also detoxifies the lead toxicity in rat (Sharma et al. 2010). *Terminalia arjuna*, a medicinal herb, has antioxidant activity (Nammi et al. 2003). This medicinal plant also produces cardio protection effect in rat against arsenic toxicity (Manna et al. 2008). *Chenopodium album*, an important medicinal plant, detoxifies the CCL4 induced toxicity (Baldi and Choudhary 2013). *Casuarina equisetifolia* was used against the gentamicin induced nephrotoxicity in the rat and it was found to reduce the toxicity of gentamicin (El-Tantawy et al. 2013).

Calendula officinalis flowers have the neuro-protective property which is evaluated by the monosodium glutamate induced neuro-toxicity in rat. Rats treated with this plant produced less sign of the toxicity induced by monosodium glutamate (Shivasharan et al. 2013). Alpinia galangal has potential to reduce the toxic effect of the anti-cancerous drug like cyclophosphamide (Qureshi et al. 1994).

Few heavy metals like nickel and chromium produces the lipid alteration which is detoxified by the administration of the *Allium sativum* (Gupta et al. 2008). It also contains the potential to reduce the lead induced toxicity in goat, rat and chicken (Hanafy et al. 1994; Senapati et al. 2001; Badiei et al. 2006).

Nanomedicine for Detoxification

Nanomedicine is an emerging field for drug delivery (Akhtar et al. 2020; Akhtar et al. 2020), diagnosis and detoxification now-a-days. Developments in nanotechnology may offer new ways in intoxication support by using nanostructured biomaterials, such as nanoparticles, liposomes, liquid crystalline nano-assemblies, micellar nanocarriers and ligand-based NPs (Muhammad et al. 2017).

Curcumin loaded chitosan nanoparticles are used to detoxify the effect of arsenic toxicity (Yadav et al. 2012). Nanoparticles interact with ozone have great potential to reduce the Aflatoxin B1 produced toxicity (Puzyr et al. 2010). The quercetin, when it is encapsulated in the chitosan/alginate nanoparticles, resulted in increased antioxidant potential (Tzankova et al. 2017). Similarly, curcumin loaded chitosan nanoparticles also improved the oxidative stress status of experimental animals following cypermethrin induced toxicity (Anwar et al. 2020). Cerium oxide (CeO2) nanoparticles have great ability to scavenge the free radical (Rzigalinski et al. 2003; Chen et al. 2006). Another current regimen is use of biomimetic nanosponge for detoxification of toxic substances. These nanosponges are made up of polymeric nanoparticles and coated with biomimetic membranes (Hu et al. 2011).

Conclusion

There are many environmental and household toxic insults which alter the routine biochemical processes. There are many underlying biochemical mechanisms of these toxic reactions. Numerous detoxification strategies are used currently including antidote, chelation therapy, phytochemicals, nutraceuticals and nanomedicines. However, much more working is required in order to have better control on toxic insults.

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CHAPTER 30

IMMOBILIZATION AND RECYCLABILITY OF Β-GLUCOSIDASE FROM THERMATOGA MARITIMA ON BIOPOLYMER-COATED MAGNETIC NANOPARTICLES

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INTRODUCTION

Today's most industrially used enzymes are derived from mesophilic organisms, and their use is limited in harsh conditions (Sarmiento et al. 2015). Therefore, there is a need for improved enzymes which can stand against extremes of pH, temperatures, salinity, and substrate specificity (Akanbi et al. 2020). Enzymes derived from extremophile organisms that are well adapted to extreme conditions might be a better alternative than mesophilic enzymes (Elleuche et al. 2014; Li et al. 2018). The commonly used methods are divided into rational designs and irrational designs. At present, more fixed-point mutation techniques are being used in rational designs. The recent interest in nanotechnology has provided a wide variety of nano scaffolds, which may support enzyme immobilization due to its potential applications in biotechnology and biomedicine (Ansari and Husain 2012). Immobilization of enzymes is useful for commercial applications owing to the simplicity of handling, ease of separating enzymes from reaction mixtures and reusing, a lower transfer resistance to solve the diffusion problem, lower operating cost, and a potential increase in heat and pH stability (Tischer and Wedekind 1999; Ansari and Husain 2012; Verma et al. 2013; Vaghari et al. 2016; Alnadari et al. 2020; Alnadari et al. 2021; Abdalmegeed et al. 2022).
Biological systems and their components have a significant impact because biotechnology involves nano-sized molecules such as proteins and nucleic acids. Researchers use existing technology to develop modern applications that use nanoparticles to immobilize enzymes (Dyal et al. 2003). Several nanoparticles have been widely used to produce nanostructures, such as nanorings, nanowires, nanotubes, nanorods, etc. (Ni et al. 2007; Ali and Winterer 2010). Several bio-nano processes have been developed using nanostructures with biomolecules as nano-blocks (Ansari and Husain 2012).
Improving the efficiency of enzymatic hydrolysis has become a hot spot in current research. The operation stability of free enzyme is poor, it is difficult to reuse, and the production cost is high, limiting its large-scale industrial application. Enzyme immobilization is considered one of the methods to overcome the above shortcomings in applying free enzymes (Rodrigues et al. 2013; Rueda et al. 2016; Arana-Peña et al. 2021). Previous research involved optimizing enzymes immobilized on a few cells and further improving immobilization efficiency. β-glucosidase is immobilized on various forms of magnetic nanoparticles (MNPs), such as chitin-coated MNPs and chitosan-based MNPs. Finally, the economic development and application prospects of MNPs are discussed, and a summary and outlook are given.

β-Glucosidases

β-Glucosidases (E.C. 3.2.1.21) are the major glycoside hydrolases ubiquitous in bacteria, archaea, and eukaryotes. β-Glucosidases play a crucial role in biological processes such as metabolizing cellulose and other carbohydrates, developmental regulation, and defense mechanisms against

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pathogen invasion and other industrial applications (Cairns and Esen 2010). Based on the amino acid sequence similarity, which reflects structural and catalytic mechanisms, β-glucosidases are grouped by CAZy into glycoside hydrolase (GH) families 1 and 3 (Aspeborg et al. 2012). GH3 family members have aspartic acids as nucleophile/base in the active center, whereas GH1 β-glucosidases have glutamic acids as nucleophile/base in the active center (McCarter and Withers 1994). β-Glucosidases show activity with natural substrates, as well as with synthetic aryl-glycosides and some aglycons. According to the substrate specificity, β-glucosidases are classified into three groups: (1) aryl-β-glucosidases with high specificity for substrates such as pNPG, (2) true cellobiases with high specificity towards cellobiose, and (3) broad substrate specificity enzymes. Most β-glucosidases are members of the broad substrate specificity family with diverse catalytic mechanisms, including cleavage of β-1,4, β-1,6, β-1,2, α-1,3, α-1,4, and α-1,6 glycosidic bonds (Cairns et al. 2015; Tokpohozin et al. 2016).

Structure of β-glucosidase

Since the discovery of β-glucosidase from bitter almond juice by Wohler et al. in (1837), researchers have conducted intensive research on β-glucosidase over the past 100 years. With the development of computational biology, scientists have used X-ray crystal derivation technology to study complex three-dimensional structures of proteins (Gloster et al. 2007). The type of β-glucosidase is enormous, and its molecular structure is also different. There are various types of β-glucosidases, such as monomers, dimers, trimers, tetramers, pentamers, and hexamers (Alnadari et al. 2020). The structure of the enzyme is highly similar. Figure 1. shows the three-dimensional structure of the Thermotoga Maritima β-glucosidase A (Tm-BglA) in the form of a tetramer and the three-dimensional structure of a single subunit. T.Maritima is a thermophilic and halophilic gram-negative bacterium. It grows at temperature up to 90°C., It is the only bacterium known to grow at this high temperature and NaCl concentrations of 5-10% (Robert Huber). Tm-BglA is an unspecific β-glycosidase with diverse substrates and principal activity with various galactosides such as lactose. The enzyme is highly active and stable within a broad pH range (Xue et al. 2015). The T. Maritima genome contains genes for β-glucosidases (strain ATCC 43589 / MSB8 / DSM 3109 / JCM 10099) with a length of 446 amino acids. β-Glucosidase A gene (ATCC-43589) was previously crystallized and biochemically characterized. The purified recombinant protein over-expressed in E. coli appears as 52 KDa when analyzed by SDS-PAGE (Xue et al. 2015; Guo et al. 2018). E351 acts as a nucleophile, E166 as acid/base catalyst, and E408 as a substrate-binding site in Tm-BglA (Figure 1). The main transgalactosylation products from Tm-BglA are 6'-galactosyl lactose [β-D-Gal-(1→6)-D-Lac] and 3'-galactosyl lactose [β-D-Gal-(6→1)-D-Lac] (Sun et al. 2014).

Physicochemical Properties of β-glucosidase

The group of β-glucosidase is comprised of two types of intracellular and extracellular enzymes. Most organisms contain only intracellular or extracellular enzymes, but very few microorganisms contain/ produce both intracellular and extracellular β-glucosides. Physicochemical properties of β-glucosidase like amino acid sequence, molecular weight, isoelectric point, specific enzyme activity, optimum pH, pH stability, optimum reaction temperature, and thermal stability are greatly influenced by the source of enzyme. Table 1 enlists the differences in relative molecular mass, isoelectric point, optimum pH, and optimum temperature for different sources of beta-glucosidase.

Studies have shown few isoelectric points (pI) of β-glucosidases, which are not much different. Their pI generally lies in the acidic range ranging from 3.5 to 5.5 (Bauer et al. 1996). The isoelectric point of a few β-glucosidases is in the alkaline range (Hósel and Barz 1975). In most cases, β-glucosidase is suitable for reactions under acidic conditions, and some are suitable for reactions under neutral conditions. Generally, β-glucosidases are stable at pH 4.0 to 9.0 (Cairns and Esen 2010). The enzyme's optimum pH and pH stability are influenced by its source and structure. These are among the factors needed to be considered when characterizing the physicochemical properties of the enzyme.

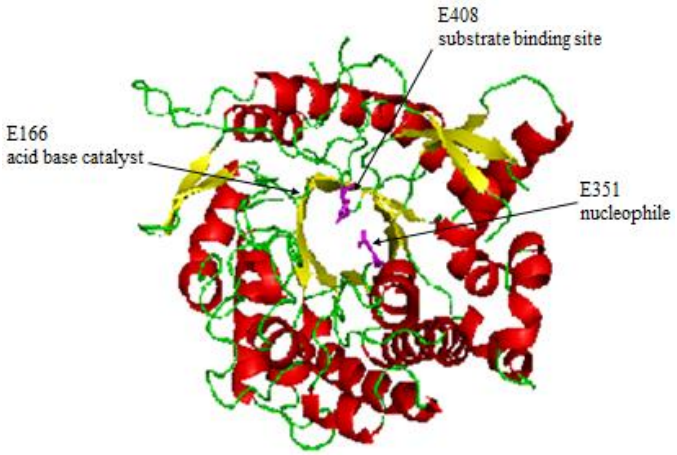

Figure 1: Three-dimensional structure of Tm-BglA: In this crystal structure of monomeric Tm-BglA, the red part represents the α-helix; the yellow part represents the β-fold; the green part represents the loop; the rose-red stick represents the acid-base group E166 and the nucleophilic group E351 (this figure was generated by Pymol software).

The activity of β-glucosidase is primarily affected by the temperature. The difference in the enzyme source will lead to a significant difference in optimal reaction temperature and thermal stability. The optimum reaction temperature for β-glucosidase ranges from 40 to 110 °C. Most of the psychrophilic β-glucosidase exist in the deep sea and are purified from the cold-adapted Micrococcus antarcticus (Fan et al. 2011), and the optimum reaction temperature is 25 °C. Mesophilic β-glucosidase is more common. Generally, the optimum reaction temperature of plant-derived β-glucosidase is about 40 °C, and the optimum reaction temperature of β-glucosidase derived from fungi is deviates from 50 to 60 °C. Once the temperature exceeds 60 °C, the enzyme activity rapidly decreases within a short time (Nagano et al. 2005). The optimum reaction temperature for thermophilic β-glucosidase generally varies from 70 to 90 °C. The optimal reaction temperature of the thermophilic T. Maritima-derived β-glucosidase is in the range of 75-80 °C (Nagano et al. 2005). However, the optimum reaction temperature and thermal

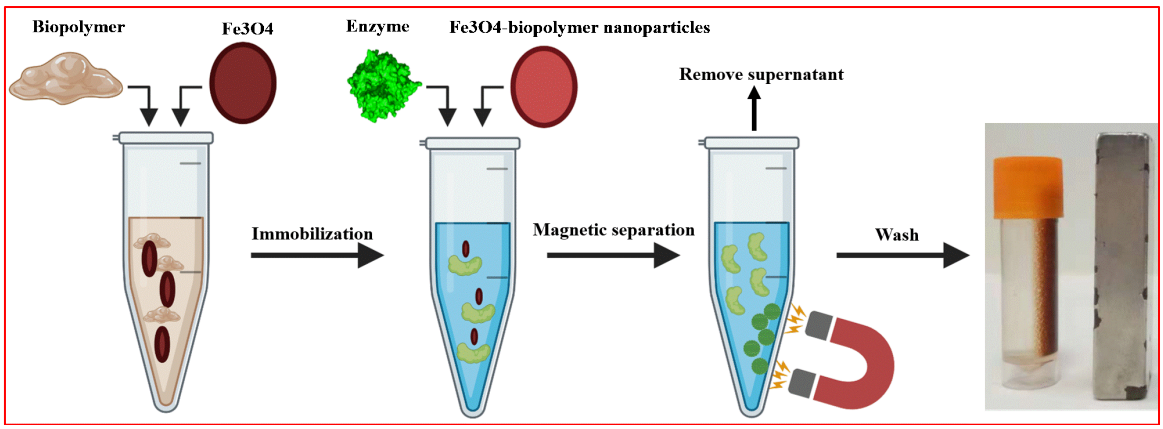

Fig. 2: Schematic representation of the immobilization of enzyme with the biopolymer-coated Fe_3O_4 MNPs.

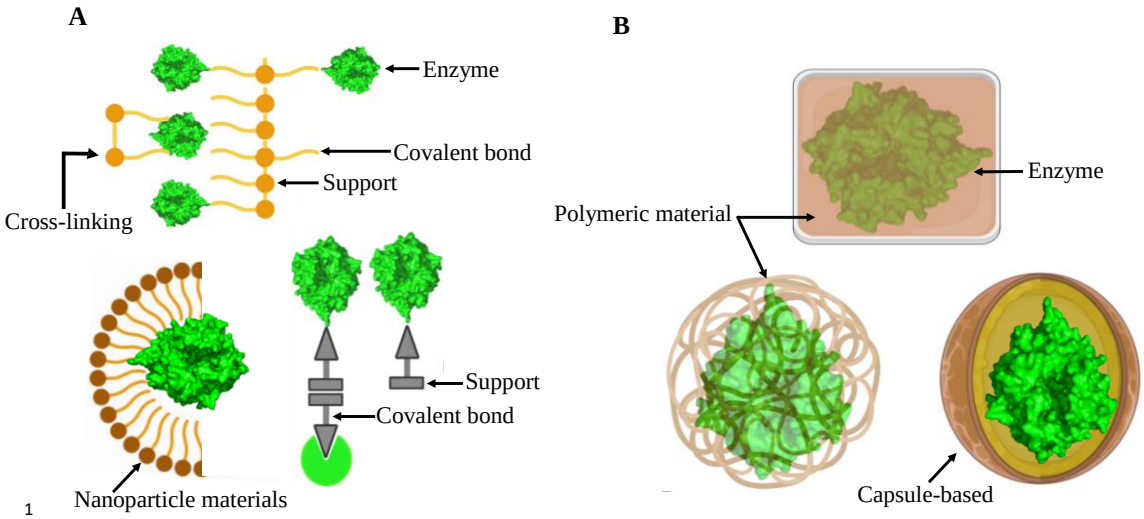

Fig. 3: Schematics of the two most common enzyme immobilization techniques: (A) Covalent attachment/cross-linking, (B) Entrapment and adsorption.

Table 1: The physicochemical properties of β- glucosidase from different organisms

Strain	Relative molecular mass (kDa)	Isoelectric point	Optimum pH	Optimum temperature (°C)	Reference
Micrococcus antarcticus	48		6.5	25	(Fan et al. 2011)
Paecilomyces thermophila	56.7		6.0	55	(Yang et al. 2013)
Thermoanaerobacter ethanolicus	48		7.0	75 ~ 80	(Song et al. 2011)
Thermotoga Maritima	48		6.2	90	(Gabelsberger et al. 1993)
Pyrococcus furious	230	4.4	5.0	102 ~ 105	(Kengen et al. 1993)
Pichia etchellsii	186		6.0	50	(Wallecha and Mishra 2003)
Cicer arietinum	13.5	5.9 ~ 7.1	7.0	30	(Hósel and Barz 1975)
Apis mellifera	72	4.5 ~ 4.8	5.0	20 ~ 50	(Pontoh and Low 2002)

stability of β-glucosidase derived from common archaea are higher than those of β-glucosidase derived from hyperthermophilic bacteria. The optimal reaction temperature of β-glucosidase released by Pyrococcus furious is 102-105 °C, and its half-life can reach 85h at 100°C. Both thermophilic and hyperthermophilic bacteria have good industrial application value and are often the focus of development and transformation.

Advancements in Techniques for Preparation of Magnetic Nanoparticles

MNPs have garnered extensive interest in recent decades as potential carriers for enzyme immobilization due to their large surface area and presence of surface hydroxyl groups, making it easier to functionalize and attach to enzyme molecules (Bilal et al. 2018). Reduced steric hindrance is characterized by low porosity and excellent mechanical stability, which are critical for constructing a stable enzyme matrix catalytic system (Li et al. 2013; Al-Dherasi et al. 2021). These qualities result in a significant improvement in enzyme or biomolecule loading capacity. The magnetic features of these carrier materials allow the encapsulated enzyme to be readily isolated from the reaction media, allowing the enzyme reaction to be promptly terminated and the enzyme to be recovered for further use. This eliminates the time-consuming centrifugation step, considerably simplifying the enzyme immobilization and recovery process (Liu et al. 2018). Figure

2 shows a list of magnetic nanoparticles that may be used as carrier materials to immobilize various industrially relevant enzymes and their biotechnological uses.

Because of the benefits of superior solubility in many solvents, high surface area, and strong magnetic properties, the synthesis of magnetic nanoparticles has been aggressively explored for diverse applications in recent years. Many uses of magnetic nanoparticles rely on manipulating their characteristics using magnetic fields, which is dependent on the efficacy of the particle's magnetic moment and magnetic field gradients (Xie et al. 2014). Multiple magnetic domains with evenly magnetized patches exist in bulk ferromagnets. A non-uniform magnetization distribution (magnetic domain wall) with a distinct magnetization vector separates each magnetic domain (Usov and Nesmeyanov 2020). Since the vector of each magnetic domain is not aligned, the net magnetization is low. The interaction between the magnetic dipole-dipole and the dipole field may cause aggregation and formation of relatively large micrometer-sized linear aggregates; a sharp decrease in specific surface area may cause capillary clogging (Mohammed et al. 2017). As a result, MNPs may be efficiently functionalized with thioester molecules and carboxyl groups, then conjugated to amino groups. Therefore, magnetic nanoparticles give a high surface-to-volume ratio, which aids in the enzyme's high binding capacity and catalytic selectivity. Due to the small diameter and magnetic moment of single-core superparamagnetic nanoparticles, the effect of the force applied to them is poor (Ansari and Husain 2012).

Although there are many pure iron oxide phases in nature, the most popular manganese oxides are nanoscale zero-valent iron (nZVI), Fe_3O_4, and γ-Fe_2O_3 (Chekli et al. 2016). They have different physical and chemical properties because of their iron oxidation state and ability to remove pollutants. Nevertheless, in the case of multi-core composites, the induced magnetic field is high enough to allow medium field strengths and gradient values for magnetic targeting.

Enzyme Immobilization Methods

Choosing the proper immobilization technique is a crucial aspect of the immobilization process as it determines the enzyme activity and properties in a particular reaction (Chiou and Wu 2004). The synchronization of two general classes of models describes complex networks. There is a general method for immobilizing enzymes, namely, the physical-chemical method (Mohamad et al. 2015). The physical-chemical method is characterized by weaker, monovalent interactions such as hydrogen bonds, covalent bonds, affinity binding, van der Waals forces, hydrophobic interactions, ionic bonding of the enzyme with the support material, mechanical containment of enzyme within the support (Costa et al. 2005; Guisan 2006; Pan et al. 2022). There are also three principal techniques for immobilization of enzymes, namely, entrapment (encapsulation), binding to a support (matrix or carrier), and cross-linking (Figure 3).

However, due to the fundamentally complicated nature of the enzyme structure, no single approach is optimal for all compounds or goals. The formulae may be used to determine the predicted immobilization yield (Alnadari et al. 2021).

$$\text{Immobilization yield} = \neg((Pt-(Ps+Pw)))/Pt\times 100 \quad (1)$$

Where Pt is the total protein content of the enzyme preparation, Ps and Pw are the supernatants after immobilization and protein concentrations of washed fractions, respectively.

Applications of Enzyme Immobilization on MNPs

Immobilization of β-glucosidases on Magnetic Particles

Currently, many enzymes used in biotechnology, including β-glucosidases, have been covalently immobilized to MNPs using various ligands (Alnadari et al. 2021). According to kinetic tests, the stability and activity of the enzyme and the nanoparticles are greatly enhanced under pH, temperature, and substrate concentration when compared to free enzymes (Abraham et al. 2014). Furthermore, magnetic field capabilities showed an enzyme complex recovery efficiency, preventing enzyme contamination of the final product. (Gencic and Grahame 2003). Similarly, a new and efficient approach for immobilizing β-galactosidase of Aspergillus oryzae has been devised employing magnetic Fe_3O_4-chitosan nanoparticles as a carrier (Pan et al. 2009). Using lactose as a substrate and immobilized enzyme as a biocatalyst to prepare galacto-oligosaccharides, the maximum yield of galacto-oligosaccharides is 15.5% (w/v) at a 50% hydrolysis rate of lactose (Pan et al. 2009). As a result, this approach might offer a novel and economically viable strategy for immobilizing thermostable enzymes to produce nanoparticle enzyme complexes (Atacan et al. 2016).

Immobilization of β-glucosidases on Chitin

Chitin is a natural polysaccharide. It is the second most abundant renewable natural polysaccharide after cellulose. Chemically, chitin is connected by β (1→4) to 2-acetylamino-2-deoxy-β-D-glucose units (or N-acetyl-D-glucosamine) to form a long-chain linear polymer. It is insoluble in most solvents. Alnadari et al. (2020) reported using magnetic nano Fe_3O_4 to immobilize the marine thermoform β-glucosidase on three different biopolymers, such as chitin and chitosan, and sodium alginate. Chitin shows the strongest binding affinity by fusing the target protein with a new thermostable chitin-binding domain. Chitin is also the highest enzyme that restricts space binding through chitin. In addition, the results showed that the production of galacto-oligosaccharides increased significantly, and the maximum production of galacto-oligosaccharides was 31.23% after the second delivery cycle. Compared with free enzymes, the immobilized β-glucosidase on chitin nanoparticles maintains 79% activity and produces 31.23% galacto-oligosaccharides after 10 repeated cycles, showing high efficiency and reusability (Alnadari et al. 2020). It is worth noting that magnetic separation technology has been successfully used to repeatedly use immobilized β-glucosidase to obtain repeated batches of galacto-oligosaccharides without significant activity loss.

Immobilization of β-glucosidases on Chitosan

Chitosan is a copolymer of N-acetyl-glucosamine and D-glucosamine. N-deacetylation generates it to varying degrees, distinguished by the degree of deacetylation. Chitosan is insoluble in water and acidic solutions below pH 6.5 due to

amino groups (Krajewska 2004). Chitosan possesses a wide range of biological and chemical properties (Sha et al. 2011). It is frequently utilized as enzyme immobilization supports in powders, flakes, and gels of various geometrical configurations. Alnadari et al. (2021) reported effective immobilization and enhanced stability of β-glucosidases on chitosan surfaces. By combining target proteins with MNPs, Chitosan displayed the highest binding affinity. Chitosan was also the highest enzyme linking throughout the chitosan-restricting space, in addition to the enzyme kinetics. Alnadari et al. (2020) reported considerably higher output (i.e., 26%) of galacto-oligosaccharides after passing the fifth cycle. Compared with free enzyme, which produced 24 percent galacto-oligosaccharides after 12 hours, the immobilized-glucosidases maintained 40 percent activity. They manufactured a maximum of 28.67 percent galacto-oligosaccharides after six repeated cycles demonstrating great effectiveness and reusability. Compared to free enzymes, immobilized β-glucosidases processing using magnetic separation technique for monotonous batch-wise galacto-oligosaccharides exhibited excellent qualities as it was highly efficient with minimum activity loss.

Immobilization of β-glucosidases on Sodium Alginate

Sodium alginate is a polysaccharide product derived from brown seaweed that thrives in colder climates. Due to its biocompatibility and processability, a polysaccharide containing glucuronic acid and mannuronic acid groups is a priority matrix (Chan et al. 2002; Abdin et al. 2021). In the presence of calcium, it is a reversibly insoluble polymer that changes solubility. (Smidsrød and Skja 1990). Many biological compounds have been successfully encapsulated using cross-linked alginate to date. (Mittal et al. 2005; Chi et al. 2008). β-Glucosidase was immobilized by using sodium alginate as a carrier. The immobilized enzyme was used to hydrolyze cellobiose and cellulose further. The best immobilization efficiency was obtained using the crosslinking-embedding method when sodium alginate concentration was 3.5%, the β-glucosidase dosage was 100 U/g carrier, glutaraldehyde concentration was 1%, CaCl2 concentration was 2%, and immobilized time was 2 hr. In the repeated batch process of cellobiose hydrolysis, the yield was maintained higher than 90% during 20 batches` (Zhao et al. 2007).

Conclusions and Future Outlook

Immobilization is one of the most frequent strategies for enhancing the utilization of enzyme catalysts in various industrial applications. This technology is widely used in biotechnology to recover the reproducibility of catalysts and products and develop more durable and stable natural biocatalysts. Immobilization is influenced by the support material, biocatalyst, and immobilization process. Because of its ease of separation, recycling from magnetic fields, greater specific surface area, and high mass transfer capacity, MNP is particularly appealing for the immobilization of enzymes among the known supports. In enzyme catalysis, the right mix of biocatalysts and MNP opens up a world of possibilities. However, success is contingent on their interaction, and an optimized scheme is required to boost the enzyme's catalytic activity, stability, and recovery. After being optimized for immobilization, magnetic enzyme bioconjugates can be used in a range of applications, including detection of environmental pollutants, monitoring glucose and cholesterol levels, and generating different metabolites of economically important medications and biodegradation of hazardous substances. The magnetic-enzymes bio-conjugates can be used in a range of sectors, including environmental pollutant detection and bio-sensing, glucose and cholesterol monitoring, pharmaceutical metabolite synthesis, and hazardous substance biodegradation. Despite various attempts in recent years to collect comprehensive information on immobilization, joint efforts are still needed to examine the surface-function link, the nanoparticle-enzyme binding site, and the significance of conformational changes in the immobilization process. The usage of MNP provides a highly effective approach for enzymatic immobilization and opens new horizons.

Author Statements

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CHAPTER 31

APPLICATIONS OF NANOTECHNOLOGY IN FISH HEALTH

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INTRODUCTION

Nanotechnology

Nanotechnology is a highly favorable field that extends to many zones of scientific and technological applications. Nanoparticles (NPs) are defined as particles that have a dimension in the range between 1–100 nm (Nowack and Bucheli 2007), that are one hundred to thousand times smaller in diameter than the paper thickness or human hair. A remarkable rise in applications of NPs have been indicated by many researchers over the last decades due to their unique properties (specifically particle surface area, size, surface charge, reactivity and shape) relative to their dissolved counterparts or bulk forms (Maurer-Jones et al. 2013) Additionally, their unique features have raised concerns due to their physiological responses to biological systems after quick interactions with surrounding materials. The emergence of NPs into aquatic ecosystems related to overuse has led investigators to study their various characteristics, behavior, sources, and ecotoxicological effects (Bundschuh et al. 2018). Although, many studies have informed the impacts of nano-based materials on human health, their ecological impacts, comprising fate, mechanism of toxicity, have been measured in recent years to encourage the sustainable use of these nanomaterials.

Main Sources of Nanoparticles

Nanoparticles can enter the surrounding environment from different natural and anthropogenic activities. Natural sources include soil erosion, forest fires, dust storms, and volcanic activities and engineered nanoparticles from anthropogenic sources can enter the ecosystem during their life cycle through three main emission strategies,

(i) release during manufacturing of nano-enabled materials
(ii) release during their excessive use and
(iii) release after direct disposal of products having NPs (waste handling).
They can be entered either directly into the environment or indirectly through a mechanical system such as landfills or wastewater treatment plants.

Bioavailability and Transformational Processes of Nanoparticles

Aquatic media can change the fate and performance of toxicants and their subsequent e bioavailability to fish, is well recognized. For example, metal speciation models reported that water chemistry such as calcium contents, pH, and hardness affect the concentration of metallic ions and their bioavailability to aquatic animals (Leeuwen and Town 2005). Abiotic factors (e.g., pH, temperature and water hardness), occurrence of natural organic matter (NOM) and characteristics of colloid chemistry eventually regulate the fate and behavior of synthetic NPs in water and this phenomenon has been studied (Klaine et al. 2008) and various significant ideas have arisen which are closely related to fish ecology: (1) NPs have ability to build suspensions in fluids, they don't take solution forms regarding the traditional water chemistry; (2) NPs take the form of agglomerates (weak interaction between particles), or aggregates (e.g. combinations of particles) and this colloidal nature strongly influence their bioavailability; (3) colloidal nature is greatly affected by few abiotic parameters of aquatic environment, and the utmost significant fact here are water pH, existence of divalent ions; though temperature of water and occurrence of natural organic substances in aquatic environment may also be vital.
Nanoparticles have undergone various modifications depending on them inherit properties and characteristics of aquatic media. Biological, chemical, and physical alterations are the key procedures that clearly explain the activities of NPs in aquatic environments (Lowry et al. 2012). Physical practices encompass different agglomeration, accumulation, and deposition. Chemical practices cover photochemical reactions, suspension, and redox reactions like oxidation, while biological applications include the bacterial breakdown and bacteriological modifications (Lead et al. 2018).

Chemical Transformation

Redox reactions include the transmission of electrons between chemical entities in natural ecosystems. Metals such as iron and silver repeatedly experience redox processes (Liu et al. 2005). Three kinds of ecosystems can be described on the basis of redox-state: oxidizing ecosystem having rich oxygen level like

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aerated soils and natural aquatic water, reductive ecosystem that face depletion of oxygen e.g., water of ground and rocks rich in carbon and dynamic redox ecosystem such as tidal zone in which various oxido-reduction reaction can occur. Photo-oxidation and photo-reduction are the reactions that are catalyzed by sunlight and change the oxidation state of nanoparticles and the tenacity of reactive oxygen species. For example, TiO2 and CNTs are activated by light and are capable of producing ROS (Chen and Jafvert 2011). Activities and performance of nanoparticles can change by large molecules and when organic and inorganic ligands are adsorbed on nanoparticles.

Physical transformations

Physical transformations occur at all phases of the life history of nanoparticles, and it take the form of aggregation and agglomeration. Aggregation and agglomeration are the same processes and can be transformed into each other. Aggregation is defined as close bonding between particles via electrostatic forces of attraction that may result in a reduction in surface area. Two types of aggregation are homoaggregation that occurs between the same type of NPs and heteroaggregation that occurs between NPs and other materials of the environment. Agglomeration increases the size of nanoparticles that affect their fate, behavior, and toxicity. Less surface area of nanoparticles decreases toxicity, which affects in turn ROS generation or suspension (Lowry et al. 2012). Spongy aggregates are capable of forming sediments than the compact form which remains suspended in aquatic media and undergoes corrosion processes producing smaller particles that absorb natural organic matter (Chekli et al. 2015).

Biological Transformations

Biological transformation is the third type of transformation that particularly affects nanoparticles. In aquatic environments where nanoparticles are directly associated with living creature's biological transformation is unavoidable. The exposure of nanoparticles to aquatic organisms occurs via many ways such as redox reactions, alterations in nanoparticles surface by coating or contact with other materials. These alterations will affect the reactivity, behavior, and surface chemistry of NPs. It has been proven that occurrence of redox responses in bacterial species (Shewanella and Geobacter) may lead to Ag+ reduction and the formation of silver nanoparticles. Moreover, it was described that transformation of PEG (polyethylene glycol) coverings on nanoparticles indicates their aggregation (Kirschling et al. 2011). Finally, the fate and performance of nanoparticles are different in different aquatic environments. The behavior of suspended organic matter and colloidal particles in sea water would be entirely different due to estuarine systems (Guzman et al. 2006). It was found that ENPs transformational processes directly correlate with ecological conditions of aquatic media (Ju-Nam and Lead 2008).

Applications of Nanotechnology on Fish Health

Fisheries and aquaculture sectors can be developed with the help of modern nanotechnology like quick disease recognition, increasing the fish power to absorb chemicals, hormones and injections quickly. Present prediction from National Science Foundation (USA), computes the worth of the global nanotechnology industry at USD one trillion by 2015. This could be possible due to extensive use of nanomaterials, not only in electrical and materials sectors but also in domestic, agriculture divisions involving fish culture and its services in biological sciences for detection of biomolecules, treatment of cancer, expansion of non-viral vectors for genetic engineering, transmission of foreign DNA, targeting medicine supply, clinical surgeries etc. although more knowledge is needed to increase probable use of nanotechnology in fish culture. Fish is considered main constituent for diet of poor people, basically relies on indispensable food and fish meat provide too much protein content. Almost 150 g of protein from fish meat provides 50-60% of an individual's daily requirement (Mohanty 2015). Solid confirmation data highlights the fact that intake of fish meat, especially fatty fish, decreases the chance of coronary heart disease (CHD) deaths. Although, fish culture is still under insecurity in terms of tapping interrogation on its sustainability, where large number of pollutants from aquaculture exert harmful effects on their productivity and the aquatic ecosystems. In this context, nanotechnology is emerging as an innovative field for skill and technology for agriculture, development and revolution (Rodrigues et al. 2017). Table 1 shows the applications of different nanoparticles in fish health. Nanotechnology in fish has various direct and indirect applications:

Direct Application

i. Fish growth
ii. Fish feed
iii. Reproduction and gonadal maturation
iv. Fish disease management

Nanoparticles play a central role in the effective supply of nutrients, vitamins and trace minerals as nano-feed additives e.g., selenium, iron and vitamin C (Jimenez-Fernandez et al. 2014). Fish feed comprising nanosized nutrients help in their assimilation and passage in the intestine, to increase their development, reproduction and immunity of fish (Chris et al. 2018). The services of nanotechnology in controlling fish illnesses are more effective as compared to chemicals that exert numerous harmful effects like aquatic pollution, resistant bacterial strains and deposition of chemical remains.
Nanomaterials are known as antiviral, antibacterial, anti-parasitic and antifungal. Furthermore, poly-lactic-glycolic acid and chitosan NPs play a significant role in medicine and hormone supply, and immunization (Bhat et al. 2019). Definitely, nano-vaccination has many benefits over traditional methods, as it increases constancy, bioavailability, and residual period (Kitiyodom et al. 2019). Moreover, the utmost prominent service of nanotechnology in fisheries is its use in quick and real identification of fish pathogens (Elsheshtawy et al. 2019).

i. Fish Growth

Proper growth rate of desired species defines the profitability of the aquaculture industry. Aquaculturists have always desired for a new element that can promote growth of the cultured fish species. Nanotechnology has extensive potential for enhancement of fish as immunomodulators (Kumar et al. 2019) and growth promoters (Zhou et al. 2009) and when provided with fish diet as supplement. Researchers from the Russian

Table 1: Applications of frequently used nanoparticles in fish health

NPs	Test Organism	Target Organs/systems	Results	References
Chitosan	*Danio rerio*	Biomarkers and whole organism	overexpression of HSP70, opaque yolk, decreased hatching, increased mortality	Hu et al. (2011)
Cerium Oxide (CeO_2)	*Paracentrotus. lividus*	Survival, bioaccumulation, nervous system, gene expression	Ce bioaccumulation in digestive, the reproductive and immune systems	Falugi et al. (2012)
Silicon Oxide (SiO_2)	*Danio rerio*	Survival, development, behavior	Decreased hatching rate of embryos, increased mortality, decreased total swimming distance	Duan et al. (2013)
Gold (Au)	*Hediste diversicolor*	Behavior, oxidative stress	Impaired borrowing behavior and feeding, significantly increased stress-related biomarkers	Mouneyrac et al. (2014)
Silver (Ag)	*Synanceia verrucosa*	Breathing behavior, reproduction, ROS, antioxidant enzymes	Decrease breathing, increased production of ROS and antioxidant enzymes	Volker et al. (2015)
Selenium (Se)	*Tor putitora*	Digestive System	Positive effects on the physiological aspects	Khan et al. (2016)
Copper Oxide (CuO)	*Daphnia magna*	Growth, reproduction, Genotoxic effects in hemocytes	Low impact on the growth and reproduction	Adam et al. (2015)
Iron (Fe)	*Oryzias latipes*	Breathing system, oxidative stress	Exposure to NPs led to a combination of hypoxia and production of ROS	Chen et al. (2013)
Aluminum Oxide (Al_2O_3)	*Bacterial activity*	Antibacterial activity of Al-NPs	No inhibition exhibited by Al NPs against different bacterial isolates	Swain et al. (2014)
Tin Oxide (SnO_2)	*Paracentrotus lividus*	Survival, bioaccumulation, nervous system, gene expression	Tin bioaccumulation in digestive, reproductive, and immune systems,	Falugi et al. (2012)
Lanthanum (La)	*Daphnia magna*	Survival and motility	Immobilization of *D. magna*	Balusamy et al. (2015)
Titanium Oxide (TiO_2)	*Hediste diversicolor*	Oxidative stress	Lipid peroxidation and nitric oxide production increased, GSH activity decreased	Zhu et al. (2011)
Zinc Oxide (ZnO)	Crassostrea *gigas*	Zn bioaccumulation, pathological destruction, oxidative stress	Induction of oxidative stress, zinc bioaccumulation in gill and digestive glands	Trevisan et al. (2014)
Zinc Oxide (ZnO)	*Labeo rohita*	Oxidative stress	Lipid peroxidation, Superoxide dismutase and catalase decreased	Aziz et al. (2020)

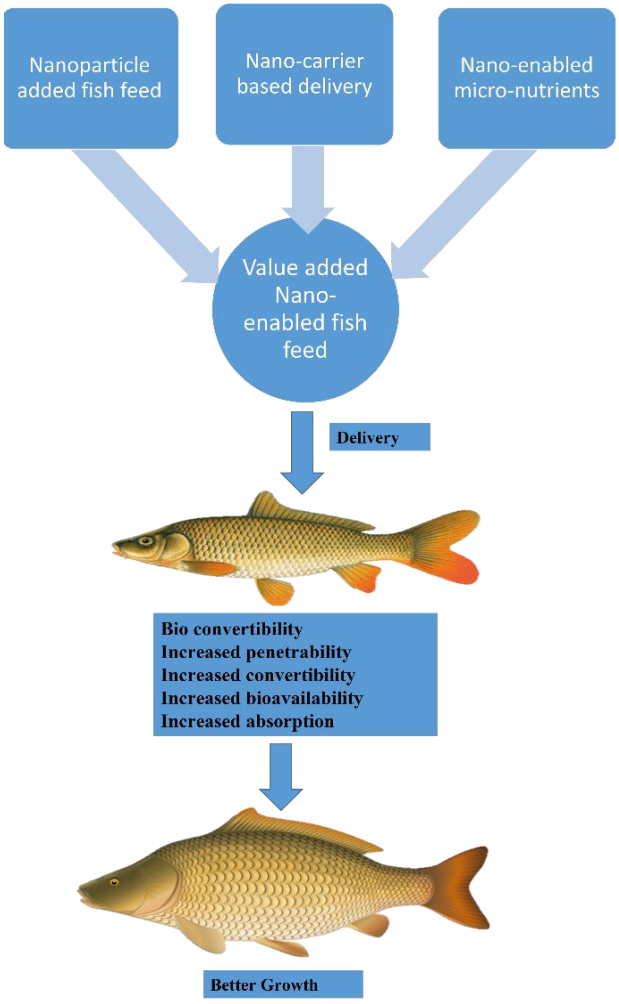

Fig. 1: Applications of nanomaterials in fish feed.

Academy of Sciences have described that both sturgeons and young carp displayed a higher growth rate (24% and 30% respectively) after exposure to an iron nanoparticles-based diet. Studies have reported that nano-selenium as diet supplement could improve the weight, muscle Se concentration and antioxidant response of crucian carp (Carassius auratus gibelio) than other selenium- based sources (Ashouri et al. 2015). It can be indicated that fish health conditions can be influenced by nano-techniques in fisheries and aquaculture, and pond-ecosystems have shown great potency for nanomaterials. Various nanotechnological processes can provide high quality preserved food. Silver NPs have been reported to enhance growth, and metalloprotease level in zebrafish (*D. rerio).* Similarly, increased growth patterns were found in common carp (*C. carpio*) and grass carp (*C. idella*) and after exposure to selenium and zinc oxide nanoparticle-supplemented diets, respectively (Saffari et al. 2017). Magnesium oxide NPs are found to stimulate development of *M. rosenbergii* (Srinivasan et al. 2016). Moreover, Cu-NPs have been reported to promote growth and immunity of sea beams (El-Basuini et al. 2016). *Ctenopharayngodon idella* (grass carp) and *Labeo rohita* fed with ZnO nanoparticle supplemented diets lead to higher rates of RBC and growth.

ii. Fish Feed

One of the most significant applications of nano-techniques in aquaculture and fish health is in feed preparation, where the NPs are effective directly for growth enhancement, nutrient delivery, and feed production per unit time. Apart from these three major areas, efforts are being made in the use of NMs to change the texture, structure, and quality of feed, as well as the preparation, processing, packaging, storage, transportation, and traceability of fish feed, which are also the major fields where nanotechnology is playing its role (Figure 1).

Chitosan NPs are gaining popularity in the animal feed industry because they are a polysaccharide "poly (1,4--D-glucopyranosamine)" with low toxicity, immunogenicity and antimicrobial potential (Vendramini et al. 2016). Different studies have shown the usage of chitosan NPs to increase the shelf life and availability of nutrients in fish. Nanoparticles have the ability to penetrate the intestinal fish epithelium and chitosan NPs have shown promising results in delivering ascorbic acid to the targeted organs of the fish when fed with NP-infused feed due to their reduced size (Jimenez-Fernandez et al. 2014).

Undoubtedly nutraceuticals are recognized to show an important role in immunological parameters and growth. however, their proper incorporations needed higher costs. That's why, deep care should be followed during their usage to avoid wastage and maximize their utilization. In fisheries, literature is available that supports nanotechnological techniques in the actual distribution of dietary nano-supplements and nutraceuticals. These systems are basically designed to increase the bioavailability, nutrients efficacy by enhancing their solubility and defense from extreme conditions of the gut. Addition of 1 mg of Selenium nanoparticles in per kg diet displayed important role in (*Cyprinus carpio*) growth and antioxidative system of common carp than control ones (Ashouri et al. 2015). Moreover, selenium (Se), manganese (Mn) and zinc (Zn) NP as a supplement in diets enhanced stress resistance and bones strength of gilthead seabream (Sparus aurata) (Izquierdo et al. 2017).

Diets having iron NPs as a supplement and and probiotic (*Lactobacillus casei*) significantly increased growth in rainbow trout (Mohammadi et al. 2015), whereas diets having MnO-NPs significantly improved growth and immunity of prawn (*M. rosenbergii*) (Asaikkutti et al. 2016). Likewise, copper NPs as supplement at 20 mg/kg significantly improved growth rate, specific and non-specific immunity of prawn, (*M. rosenbergii*) and red sea bream, (*Pagrus major*) (El Basuini et al. 2017). Gold nanoparticles significantly improved the metabolic enzymes, oxidative stress, and hepatotoxic markers, etc. Sharif Rohani and coworkers reported the beneficial effects of Aloe vera NPs-based diets on growth, body composition and survival rate of Siberian sturgeon (*Acipenser baerii*) than control ones. (Sharif et al. 2017). Common carp (*Cyprinus carpio*) fed with ginger nanoparticles per kg feed exhibited 100% relative percentage survival (Korni and Khalil 2017).

Neem (*Azadirachta indica*) built AgNPs showed improved immunomodulation and antibacterial activity in fingerlings of *Cirrhinus mrigala* confronted with *Aeromonas hydrophila* (Rather et al. 2017). Most recently Erdem and co-researchers prepared AgNPs from *Aeromonas sobria* to check their antibacterial properties against various fish pathogens (*E. hermannii, P. rettgeri, H. alvei, M. morganii subsp. Sibonii, C. braakii, A. hydrophila, E. coli* and *E. cloacae*). And for the management of fish health, NPs were approved as antimicrobial agents against *A. hydrophila* (Erdem et al. 2018). Nanoencapsulation technique reveals new assets like the adding of carbon nanotubes into trout fish diet causing in the preparation of hard pelleted feed.

iii. Reproduction and Gonadal Maturation

Fish breeding and reproduction is a vital segment in the fish industry as the brood stock management is a preliminary step in controlled breeding and to get a healthy broodstock, proper gonadal maturation is crucial. This gonadal maturation has been attained by injecting multiple hormones or by adding various supplements to feed during the prespawning phase. Both of these conventional ways have their issues, as injections cause handling stress and occupational pains while the hormonal therapy of testosterone and progesterone through feed supplements has a major drawback of hormones leaching in water during delivery. With the advancement of nanotechnology, this issue has been addressed by the implantation of hormonal pellets by nanocarriers at the prespawning stage under the fish skin (Kailasam et al. 2006). This technique would solve the issue of leaching by increasing the retention period of hormones in the fish body and triggering the maturation of the gonads by gradual and slow release of hormones as the results showed the higher retention time and longer life span of luteinizing hormone in the fish circulation. NPs of chitosan can be used to transfer endogenous hormones and release them in a precise way.

The use of chitosan nanoconjugates with reproductive hormones exhibited promising results as Rather et al. (2013) used chitosan-gold nanoconjugates with LHRH in the female *Cyprinus carpio* to solve the issue of the short life span of LHRH in fish blood. Their results showed that the concentration of reproductive hormone in blood circulation was consistent for a longer period in fish that solved the issue of multiple hormone injections and thus by increasing the relative number of mature eggs and significantly higher fertilization rate in the female fish. The application of nanoconjugates of chitosan and hormones also showed a significant increase in the expression levels of SoX9 (SRY-Box Transcription Factor 9) gene that is associated with the gonadal development of male and female *Clarias batrachus* (Bhat et al. 2016). The plant extract (Eurycomanone) used as nanoformulation of chitosan conjugate injected in the male *Claria smagur* showed a significant increase in the intracellular selenium and calcium level, the improved cellular structure of testes, gonado-somatic index (GSI), and overall better reproductive capacity of the fish (Bhat et al. 2019). The conventional way of injecting brood with the hormones causes another major issue that is the accumulation of hormone residues in the tissues after breeding. This problem was also solved by the minimal accumulating effect of nano-delivery of hormones thus by preventing the retardation of broodstock by hormonal residues that led to the cheaper sale of post-breeding broodstock.

In commercial aquaculture, another common problem is incomplete vitellogenesis in female fishes that leads to malfunctioning of oocyte maturation and ovulation. This issue can be solved by controlling the reproductive process of fish as the production of monosex Tilapia was a breakthrough to avoid premature spawning, small-sized, unmarketable fish, and pond overcrowding. The poly lactic-co-glycolic acid nanoparticles were loaded with fadrozole (an inhibitor of estrogen synthesis), incorporated in the feed of *Oreochromis niloticus*, fed through a nano-drug delivery system. These fadrozole loaded nano-carriers inhibited the production of estrogen hormone in the fish, thereby, producing 100% masculinization of tilapia that attained their marketable size without premature spawning (Joshi et al. 2019).

Another problem that occurs frequently in commercial aquaculture is incomplete vitellogenesis in female fishes, which results in the failure of oocyte maturation and ovulation in the female fishes. It is possible to solve this problem by controlling the reproductive process of fish, as demonstrated by the production of monosex tilapia, which was a breakthrough in

the effort to avoid premature spawning, small-sized, unmarketable fish, and pond overcrowding.

In fish culture, before focusing on breeding techniques, the health of the broodstock is more important. For this purpose, the feed enriched with all the necessary microminerals, and vitamins is fed to develop high fecundity in the broodstock. Among these minerals, selenium has shown successful results in improving the breeding, fertilization, and reproductive capacity of male fishes (Sarkar et al. 2015). Similarly, calcium plays a significant role in egg activation, whereas low levels of phosphorous reduce fecundity in female fishes. Vitellogenesis in the fish system can also be improved by the minerals and vitamins delivery because they play a significant role in fish breeding as Vitamin E improves the egg quality, ovulation rates, and gonado-somatic index, while vitamin C influence the vitellogenesis and production of the steroids in fish. Significant work on the nano-carrier of minerals and vitamins in the fish breeding program has shown results; for example, silver nanoparticles in the zebrafish diet triggered the hatching and breeding in zebrafish along with the overexpression of Oct4 protein (Sarkar et al. 2018).

iv. Fish Disease Management

With the advancements in aquaculture technology, various detrimental diseases to organisms are also emerging. A huge annual loss is happening in the fisheries industry due to the continuously increasing incidence of diseases caused by different pathogens. To control this loss, efficient detection methods and preventive measures from pathogenic infestation are considered key elements for enhancing overall productivity and healthy fish products. Different strategies have been developed viz. vaccines, immunostimulants, antibiotics, etc. to deal with the various pathogenic diseases encountered in fish culture. Nanotechnology can significantly contribute to these fields through various innovative methods, also restricting the use of conventional technology in medicine. The following schematic diagram is describing the applications of nanotechnology in fish disease management (Figure 2).

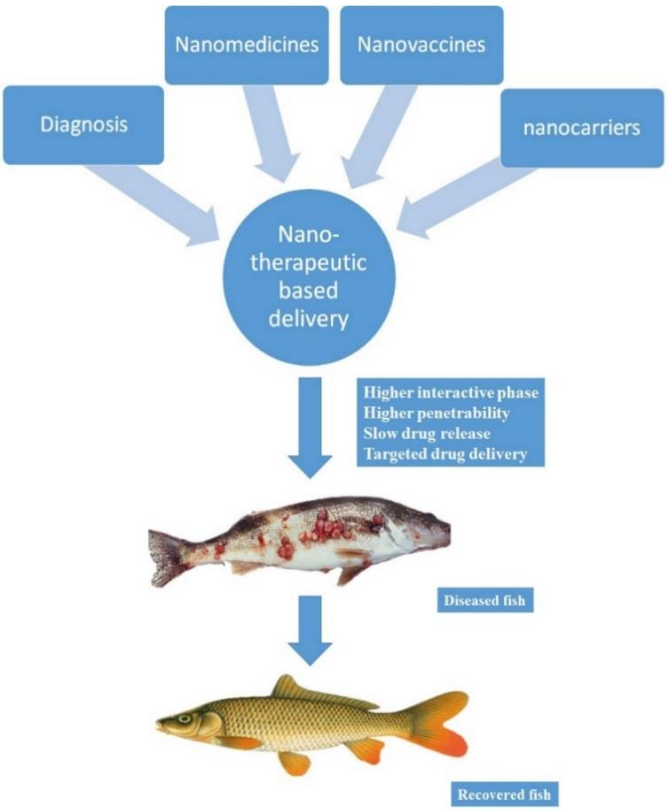

Fig. 2: Nanotechnology in fish disease management.

A. Diagnosis

Aquaculture scientists in the recent times are looking for a simple and effective technique for timely detection and diagnosis of main fish pathogens. Nanoparticles have been used to diagnose diseases in a quick and sensitive manner, and these detection methods are known as nanodiagnostics. A highly sensitive immunodiagnostic assay has been formed by loading gold NPs with alkaline phosphatase and antibodies for the treatment of viral white spot syndrome in shrimps (Thiruppathiraja et al. 2011). A similar approach using DNA-loaded AuNPs and LAMP was developed to detect viral white spot syndrome in fish. This method was sensitive, specific, and field-detectable. A similar approach using DNA-loaded AuNPs and LAMP was developed to detect viral white spot syndrome in fish. This method was specific, sensitive, and field-detectable. Yellow head virus in shrimps was visually detected at early stages by combining AuNPs colorimetric assay with LAMP.

Nanosensors are also effective and simple tools for detecting pathogens. Important fish viruses such as betanoda, aquabirna, and salmonid alpha can be detected using a variety of nanosensors (Crane and Hyatt 2011). In fish tissues, bacterial diseases are more common than fungal and viral diseases, and their early detection is crucial for disease management. Gold nanoparticles have been used in the form of *A. salmonicida* antibody-gold NPs conjugated for the diagnosis of furunculosis in fish (Saleh et al. 2011). Graphene oxide is currently being used in the development of electro-chemical biosensors due to its biocompatibility and specific chemical composition (Natarajan et al. 2017).

These immunosensors also demonstrated a modest, delicate, real-time, and speedy detection method for WSSV in shrimp samples. Biosensing is a modern, unique, and, alternatively, a better quantitative and qualitative method as compared to PCR amplification detection methods. Another electrochemical DNA biosensor was made by the conjugation of Au-NPs and a DNA reporter probe for the detection of fungi (*A. invadans*) in the fish at a lower rate than PCR (Kuan et al. 2013). In an immunomagnetic assay for NNV in grouper fish, Yang et al. (2012) used magnetic NPs covered with anti-NNV antibody from rabbits. A magnetic field applied to magnetic nanoparticles reduced their motility if they were bound to viral antigens, allowing for immunodiagnosis. The titer virus was determined by magnetic immuno-assay.

Unmodified Au-NPs were used to develop a colorimetric assay for detecting of carp virus. Gold nanoparticles were added after the probe, which was complementary for carp virus. If there was any target viral RNA existed, it directly hybridized with the probe, preventing the probe from the gold NPs. The Au-NPs could clump together, causing the solution to change red color into blue. Without the need for early amplification of viral DNA and RNAs, this technique was specific and fast. The same approach was used to develop a specific, sensitive and rapid assay for detecting the virus of DNA, cyprinid herpes virus-3 (Saleh and El-Matbouli 2015).

B. Nanomedicine

Nanomedicine is an emerging field of nanotechnology that offers a plethora of opportunities to improve fish health by employing the intrinsic properties of many types of nanoparticles. Nanomaterials with antimicrobial and therapeutic capabilities, such as nanosilver and zinc oxide, have previously been used to lower pathogens in aquaculture systems. The phenomenon of nanomedicinal is generalized, non-specific, and extensively applicable in animal health.

Researchers are testing nanoparticles' antibacterial properties, which could be valuable nano-medicines for fish species (Table 2). Graphene emerged as viable, low-cost commercial nanomaterial. The oxidized graphene is easily processed and soluble in water (Brisebois and Siaj 2019). Graphene oxide inhibited important pathogens of water such as *P. aeruginosa, S. aureus, E. coli*, and *V. harveyi*. When graphene oxide interacts with pathogens, it induces cell membrane impairment, lysis and mechanical enfolding (Kumar et al. 2019).

Application of metal oxide nanoparticle in aquaculture disease management has been extensively studied and found that nanoparticles such as ZnO-NPs, CuO-NPs, Au-NPs, Ag-NPs, and TiO2-NPs possess the principle of fighting against various disease-causing pathogens (Cheng et al. 2009). ZnO nanoparticles are reported to inhibit the growth of various pathogenic bacteria viz. *Staphylococcus aureus, Flavobacterium branchiophilum, Aeromonus hydrophila, Vibrio species, Pseudomonas aeruginosa, Edward seillatarda Bacillus cereus* and *Citrobacter spp.* (Swain et al. 2014), whereas CuO nanoparticles are effective against Saprolegnia sp. from the host white fish and also used as strong antifungal agent. Au-NPs have been tested against bacterial pathogens of fish and observed to be possess antibacterial properties. Silver nanoparticles are used as an effective antibacterial agent as it releases Ag+ ions which bind to bacterial cell membrane proteins and disrupt the membrane of the bacterial cells. Also, Ag-NPs are found to be effective against multidrug resistant bacteria among them methicillin resistant *Staphyloccus aureus* is one.

Various herbal and phyto-extracts are used to treat fish diseases as potential drugs. Different nanoparticles are prepared at optimized hydrodynamic conditions by using medicinal plant/herbal extracts, and a complex of the phyto-nanoformulation is then administered as a medicine with synergistic effects of both. Phyto-nanoformulation of plant extract and Ag-NPs composite has been observed to be work as antibacterial against *Aeromonas hydrophila*, causing Motile *Aeromonas Septicemia* in fish.

C. Nanovaccines

Disease outbreaks are one of the most significant issues to aquaculture's development and long-term viability. Among various approaches used to solve this major problem, vaccination is one that showed promising results in its utilization in both conventional and modern medicine. In this context, the use of nanoparticle carriers of vaccine antigens such as chitosan and poly-lactide-co-glycolide acid in combination with mild inflammatory inducers may deliver fish. with higher protection not only against bacterial pathogens, but also against certain viral diseases with vaccine-induced side effects.

Nano-delivery of drugs are attributed to some unique properties as controlled particle size, structure, shape, distribution, surface charge, sustained release, regulation, target-specific, multi-route delivery pathways, and degradation of nano-carriers. Nanoparticles are vastly used as drug carriers for multiple reasons including improved bioavailability, prolonged residence time and stability in stomach, highly efficient absorption, dispersion of vaccine antigens to gut-lymphoid cells, and controlled degradation. Nanoparticles as a drug delivery vehicle Chitosan and PLGA NPs have been examined for their effectiveness as a vehicle for delivery of drugs in fish.

Furthermore, nanocapsules can be used to vaccinate fish, which will be difficult to digest and degrade thus showing long-term effects. These nanocapsules contain short strand DNA, that is readily absorbed into fish cells when employed in living medium. The ultrasound method is utilized to burst the capsules, releasing DNA and inducing an immunological response in the fish as a result of the vaccine. This will reduce the cost and effort of disease management, drug and vaccine delivery, and other aspects of aquaculture while lowering the cost of feeding (Assefa and Abunna 2018).

Indirect Applications of Nanotechnology

Indirect applications of nanotechnological techniques are mainly concerned with quality of water:

i. Wastewater treatment
ii. Biofouling control
iii. Removal of heavy metals

Several carbon nanotubes, metal oxide nanomaterials, and natural nano-adsorbents have been used to remove contaminants from the water. Also, water purification from

Table 2: Applications of nanotechnology in fish disease management.

Nanoparticle	Test Organism	Pathogen	Nanomedicines / Nano-vaccines	References
PLGA	*Salmo salar*	IPNV	PLGA Nanoparticle-TA	Munang'andu et al. (2012)
Chitosan	*Danio rerio*	VHSV	NPrgpG, pICrgpG	Kavaliauskis et al. (2016)
PLGA	*Labeo rohita*	*Aeromonas hydrophila*	Np-rOmpW	Dubey et al. (2016)
Alginate	*Oncorhynchus mykiss*	*Ichthyophthirius multifiliis*	OCMCS-HA/aerA-NPs	Heidarieh et al. (2015)
Chitosan	*Saltator maximus*	TRBIV	pDNA-CS-NPs	Zheng et al. (2016)
Chitosan/TPP	*Lates calcarifer*	Nodavirus	pFNCPE42-CS/TPP	Vimal et al. (2014)
Calcium phosphate	*Labeo rohita*	*Aeromonas hydrophila*	SP-CaNP	Behera and Swain (2011)
PMMMA-PLGA	*Oreochromis niloticus*	*Streptococcus agalactiae*	PTRBL/Trx-SIP	Zhang et al. (2015)
PLGA	*Panaeolus olivaceus*	LCDV	pEGFP-N2-MCP	Tian and Yu (2011)
Chitosan	*Acanthopagrus schlegelii*	*Vibrio parahaemolyticus*	pEGFP-N2-OMPK	Li et al. (2013)
PLGA	*Oncorhynchus mykiss*	IHNV	PLGA-pCDNA-G 11 PLGA-pCDNA-G 22	Adomako et al. (2012)
Liposome	*Epinephelus bruneus*	*Vibrio harveyi*	Liposome-V. harveyi	Harikrishnan et al. (2012)
Carbon nanotubes	*Ctenopharyngodon idella*	GCRV	SWCNTs- pcDNA	Zhu et al. (2015)
OCMCS-hyaluronic acid	*Cyprinus carpio*	*Aeromonas hydrophila*	OCMCS/aerA-NPs	Liu et al. (2016)

variety of pathogens shows another preferred outcome of nano-techniques to limit contagious diseases (Tayel et al. 2019). Additionally, the anti-fouling impact of some nano-based materials, by reducing phosphate levels and lessening the growth of micro-organisms and algae, is an important nano technological application, to attain water quality in ponds of fish.

i. Wastewater Treatment

The accretion of food remnants and the presence of a large number of organisms is one of the known issues in fish culture. These pathogens cause many fish diseases by such as bacteria, viruses, fungus, and protozoa. Anti-pathogen drugs have significant risks on pathogen resistance, fish health, non-targeted animals, and the environment. Nanotechnology in water disinfection and sterilization may be a possible answer to all these issues (Tayel et al. 2019). The sterilization efficiency depends on the pollutant type, concentration, nano-material concentration, temperature, and light intensity as well. Nanoparticles of TiO2 were observed to sterilize 98% of E. coli, *V. anguillarum* and *A. hydrophilain* water during sunlight, whereas composite of nanomontmorillonite and Cu showed bactericidal properties to clean fishpond waters (Rati et al. 2014).

The composite of nano-Ag coated zeolite and activated carbon has significantly reduced the Saprolegnia infection in the eggs of rainbow trout. In fish farming, the water quality was also reported to get suitable after the application of chitosan-Ag nanoparticles. These nanoparticles also decreased the total count of fecal coliform, fecal streptococci*, P. aeruginosa*, and *S. aureus*, significantly (Hamad 2019). TiO2-NPs generate highly reactive hydroxyl (OH), superoxide (O) and peroxyl radicals (O2). Free radicals alter the structure of cell membranes, causing apoptosis and sterilization.

The nanodevices are useful to enhance the water quality in fisheries, reducing the water exchange rate, increasing rate of survival and yield of fishes. Aquaculture uses activated alumina or carbon nano-materials with zeolite and compounds having iron to hold aerobic and anaerobic biofilm to remove contaminants (nitrites, ammonia, and nitrate). Similarly, ultra-fine nanoscale iron powder can effectively clean up contaminants like polychlorinated biphenyls, trichloroethane, carbon tetrachloride, and dioxins paving the way for nano-aquaculture.

ii. Biofouling Control

Bio-fouling (buildup of microorganisms, algae, and plants on moist surfaces) is additional problem faced by fisheries. Bio-fouling reduces water flow, oxygen ranks, and food bioavailability, while increasing weight, deterioration and cage deformation. Nanotechnology can help enhance disease management, feed formulation, and biofouling control in aquaculture and shrimp cultivation. Invertebrates (mussels and barnacles) and algae (diatoms and seaweeds) can be monitored by covering or painting nanostructures with metal oxide nanoparticles such as CuO, ZnO, and SiO2. This can be attained by improving antifouling control and developing an effective antifouling surface.

Aquaculture antimicrobial compounds and new packaging materials for marine items could all benefit from this antifouling. Some nanomaterials (nano-ZnO and nano-CuO) can help remove fouling organisms from fish aquaculture. They act as a barrier to fouling agents due to their great surface area. CuO nanoparticles have been reported to show significant results in controlling bio-fouling. Lanthanide oxide nanoparticles can absorb phosphate from the surrounding water, reducing algal and other microbial growth. Nanotechnologies make NanoCheck, a pool and fishpond cleaner. It works by absorbing phosphates from water and preventing algae growth with 40 nm lanthanum-based particles. Moreover, nanoscale weedicide and soil-wetting agent delivery may help control aquatic weeds in large water bodies and reduce stress from climate change and pollution (Ashraf et al. 2011).

iii. Removal of Heavy Metals from the Water

Ligand-centered nano-coating can be utilized to remove heavy metals because it can be restored by handling the bi-functional self-assembling ligand with the nano-coating medium that was used before. This process can be done in the same place where the ligand was used (Farmen 2009). Nanoparticles of metal oxides, such as nanosized FeO, AlO2, MnO, CeO2, MgO and TiO, having increased surface area and affinity for metal adsorption are currently preferred nanotechnology for wastewater treatment.

For testing how well they can remove metals under different conditions, mathematical models and analytical techniques like XAS and NMM have become very important for developing new technologies and better applications for making metal oxide nanoparticles. For heavy metal removal, ligand-based nanocoating can be used due to its high absorption potential and low cost. Using crystal clear technology for water purification, several metal layers are bonded to one substrate (Farmen 2009). The high reactivity and large surface area of nanomaterials make them ideal for heavy metal removal from water and waste. Heavy metal adsorption is specific to metal oxide nanoparticles such as nanosized FeO, AlO2, MnO, CeO2, MgO and TiO with high surface area and affinity for aqueous systems.

A detailed study of the impact of humic and fulvic acids on heavy metal removal by nanotechnology from aqueous solutions such as photocatalytic, carbon-based, and iron-based nanomaterials was published by Tang et al. (2014). This study also looked into how humic and fulvic acids interact with each other and the environment. Chitosan nanoparticles are used as ligands and absorbents in the process of heavy metals removal. Recent research has focused on removing heavy metals from clays like montmorillonite, bentonite, and kaolinite using chitosan nanoparticles due to clays' inherent ability to remove heavy metals. For the removal of heavy metals from aquatic environment, chitosan-magnetite nano-composites and nano chitosan-clay composites were also used (Fang et al. 2017).

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CHAPTER 32

USE OF NANOTECHNOLOGY IN TREATING SOME IMPORTANT VIRAL ANIMAL DISEASES

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INTRODUCTION

Over the course of history, technology has been the best friend to all living creatures when it comes to their welfare as it has helped us find new ways to solve many problems that had never been solved before. When it comes to medicine and all related biological fields, technology has provided a gateway to solving hurdles that were in the way of solving health-related problems of all living beings. In this chapter we have reviewed the part that Nanotechnology has played in providing better answers to the questions regarding viral veterinary diseases over the course of years. There is no doubt in the fact that vaccination is the only solution to prevent viral diseases in living beings, but we can improve every technique and treatment with the aid of nanotechnology these days. Nanoparticles play the role of finer adjuvants with lesser cytotoxicity and even better results than any other alternatives. We will discuss different Virucidal Nanoparticles in this chapter along with the major work that has been done in successful trials to prepare nanoparticle-antigen conjugated vaccines.

Viruses are incredibly small pathogenic particles that can cause several kinds of diseases in both animals and humans. They cannot survive on their own. They are also called the "obligate intracellular parasites". They are only composed of coating proteins called the capsid and DNA or RNA. The simplicity of genetic material gives them a major property of rapid mutation. Some viruses also have an outer coating called an envelope. They cause significant mortality and morbidity in animals. Despite dumbfounding advancements in the pharmaceutical industry, no sure-fire antiviral drugs have been synthesized till now. Thus, viruses are a nuisance for both the sick and the doctors attempting to treat viral diseases (Ulmer et al. 2006).

Nanotechnology

The word "nano" here clearly exclaims the small size of the object that will be discussed here. Despite its small size, nanotechnology has found a wide range of uses in all kinds of industries and futuristic advancements. The United States' National Science and Technology Council has defined nanotechnology as the working being with particles of size ranging from 1-100nm. The term nanotechnology itself refers to the ability to measure, manipulate and rearrange matter at the nanoscale level. This nano scale level includes atomic, molecular and supra-molecular stages of matter where a man can perform experiments to achieve the desired reaction from the nanoparticles (Emerich and Thanos 2003).

Even the most primitive nanotechnology experiments have revealed that nanotechnology holds a promise of unmatched animal production, best animal welfare, long-lasting animal health, guaranteed animal safety and quickly effective animal medicine. This will serve as a booster for animal-related industries like the meat and milk industry. This will in turn also solve the issues of food shortage and balanced nutrients for humans (El-Sayed and Kamel 2020).

Nanotechnology will also help researchers to deal with minute quantities of materials, such as DNA and RNA, easily and precisely hence, making genetic material manipulation faster. This will quicken the genetic improvement programs by many folds. Problems like DNA loss during cloning or the inability to select proper genes within lesser generations of larger animals will be solved.

The main problem of losses in poultry is viral diseases that spread uncontrollably among flocks and kill hundreds of birds in a short time. The most disturbing factor here is that it cannot be controlled because we lack antiviral agents and have no hope of recovery once a viral outbreak begins. Thus, nanotechnology will be the almighty saviour for poultry farmers. Nanotechnology will defeat the arch-nemesis of the poultry industry for good.

Researchers all around the globe are also working on several types of other nanoparticles that instead of killing viruses deliver enzymes which can render viruses unable to reproduce. This will stop the progression of diseases even if the virus enters a host's body. Development of orally consumable drugs containing such nanoparticles is underway.

Nanobots or nanorobots are another attempt to begin a promising era in medicine related to cell repair technology. These bots will be programmed to help the natural healing system much like antibiotics.

In recent years, nanotechnologists have widely investigated the development of antiviral agents for the treatment of previously hard to fight viral diseases. Nanotechnology will not only improve the effectiveness of current medicinal options, but it will also provide the manufacturers with a whole new branch of remedies that will be a hundred times more effective than older ones.

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Nanotherapeutics

The use of nanoparticles for the sake of treating a disease is termed nanotherapeutics making it a separate branch of nanotechnology medicine. This refers to the use of nanoparticles combined with antiviral drugs (Wang et al. 2012). Antiviral drugs can be encapsulated with nanoparticles which will protect it against viruses when given systemically. If given locally, the nanoparticles will form a surface coating that prevents further spread of the drug from the desired location. The nanoparticles have a long half-life in circulation and good drug loading characteristics. Such properties make it a good companion for hydrophobic chemicals. These drugs cannot be given directly in their free form because of their hydrophobic nature (Cojocaru et al. 2020).

Virucidal Nanoparticles

Nanoparticle researchers have tried to check the virucidal effects of various metal and sheet-based nanoparticles to determine their use against several diseases (Szunerits et al. 2015). Metal nanoparticles and graphene-based nanosheets were discovered to be naturally virucidal due to their unique physicochemical properties. Their simple mechanism of action is interacting with the envelope or capsid of viral proteins to disrupt structural integrity and inhibit infective behaviour. Some of these particles can also stop viral replication (Liu et al. 2017). Here are discussed different types of nanoparticles which have a virucidal effect and come in handy while fighting off a viral disease:

Nano-metals

Silver nanoparticles are the most widely exploited metal nanoparticles in terms of medicinal research (Rai et al. 2014). Experiments have shown that silver nanoparticles can inhibit a broad range of viruses with the least chances of developing resistance even in the case of highly mutative viruses. Silver nanoparticles have worked astonishingly both extracellularly to block entry of viruses and intracellularly to prevent replication of the virus (Figure 1). It has been used successfully in several experiments for treating human immuno-deficiency virus and influenza virus diseases (Singh and Laura 2012). Similarly, gold particles synthesized from seaweed *Sargassum wightii* were also found to be effective against the herpes virus in an experimental trial.

Nano-emulsions

A category of nanoparticles that has simple and low-cost synthesis but is still active for the treatment and prevention of several viral infections is nano-emulsions. Nano-emulsions can be produced by mixing a lipid phase and an aqueous in presence of a surfactant material. Like several other types of nanoparticles, its mode of action is also dependent upon interaction with the viral particles (Hamouda et al. 2001). Nano-emulsions have shown good efficacy against enveloped viruses like herpes, influenza and vaccinia viruses. An old example of nano-emulsion is 8N8 made from 8 volumes of tributyl phosphate, 64 volumes of soybean oil and 8 volumes of triton X-100. It was used to disrupt bacterial membranes.

Graphene-based Nanosheets

Graphene is a material with excellent thermal and electrical conductivity properties. In the medical industry, graphene has appeared as a novel anti-viral material that is especially helpful in disinfecting viruses. The two unique two-dimensional structure of graphene is the physiochemical property that gives it antiviral characteristics. Graphene has an exceptionally high surface volume ratio allowing it to intercept viruses effectively. Graphene oxide is negatively charged and carries sufficient reactive oxygen species on its surface to destroy viruses through redox reactions and forces of electrostatic attractions (Geim and Novoselov 2007). These interactions are especially effective if virus capsid proteins are positively charged (Figure 2). Some virus capsids have high arginine protein content making them positively charged.

Nano-decoys

Initially developed to bind and neutralize bacterial toxins, the nanodecoys soon found their application against viruses too (Rao et al. 2020). They can be designed to neutralize specific viruses through ligand-receptor interactions (Angsantikul et al. 2018). This use of nanodecoys against viral infections was recently reported. Nanodecoys can be broadly classified into two main types. The first type is invader nanodecoys that are specially designed to bind with a specific viral receptor (Figure 3). Virions encountering such particles are also captured and blocked by nanodecoys to prevent infection (Hu and Zhang 2014). The second type of nanodecoys is formed by coating purified cell membrane contents from a target around the nanoparticles to form nanosponges. These nanosponges can then carry antiviral drugs or antigens for the safe delivery of a vaccine into the body without compromising vaccine effectiveness.

Ligand Functionalized Nanoparticles

Understanding of cellular pathways involved in the destruction of viruses by nanoparticles is important to help us add supporting materials with nano-metals or other nanoparticles that can increase their effectivity. These supporting particles are called ligand functionalized nanoparticles (Baram-Pinto et al. 2009). As an example, it was seen in lab animals that the use of mercapto-ethane sulfonate along with silver nanoparticles inhibits entry of the herpes virus by competitively binding to cellular heparan sulfate.

Cellular Nanosponges

Ligand-functionalized nanoparticles may serve as good potentiating agents and effective materials for blocking viral entry, but they have a crippling disadvantage, that is the requirement of full knowledge relevant to receptor-ligand interaction before they can be put to effective use. Hence, a thorough study of the cell and target virus is required to use the ligand functionalized nanoparticles effectively. The ligands are very specific in their interaction. On other hand, viruses are rapidly undergoing mutations and may change the shape of receptors over time. These disadvantages can be covered by broadening the use of nano decoys by application of cell-like nano-platforms (Rao et al. 2020). These nanoparticles will serve as the basis for displaying the same surface receptor as

Table 1: List of different nanoparticles used for different veterinary viral diseases for various purposes like diagnostics, vaccination and treatment:

No.	Disease	Virus	Family	Nanoparticle	Purpose
1.	Foot & Mouth Disease	FMDV	*Picornaviridae*	AuNPs	Vaccine
2.	Newcastle	NDV	*Paramyxoviridae*	Ag@SiO2	Vaccine
3.	Rabies	Rabies virus	*Rhabdoviridae*	AgNPs	Vaccine
4.	Canine Distemper	CDV	*Paramyxoviridae*	H-Nanoparticles	Vaccine
5.	Canine Parvo	Parvovirus	*Parvoviridae*	PLGA	Vaccine
6.	Bluetongue	BTV	*Reoviridae*	Glycan Gold NPs	Vaccine
7.	Bovine Viral Diarrhoea	BVDV	*Flaviviridae*	AuNPs	Serological detection
8.	Marek's Disease	MDV	*Herpesviridae*	TLR-Ls	Impedes tumor development
9.	Infectious Laryngotracheitis	GaHV-1	*Herpesviridae*	SN-TiO2-PSP	Serological detection
10.	Hydropericardium syndrome	FAV-4	*Adenoviridae*	AuNPs	Serological detection
11.	Infectious Bronchitis	IBSV	*Coronaviridae*	AgNPs	Virucidal activity
12.	Egg Drop Syndrome	EDSV	*Adenoviridae*	AgNO3-NPs	Antiviral activity
13.	Fowlpox	FPV	*Poxviridae*	AMS	Antiviral activity
14.	Feline Panleukopenia	Parvovirus	*Parvoviridae*	AuNPs	Serological detection
15.	Infectious Bursal Disease	IBDV	*Birnaviridae*	AgNPs	Virucidal activity

FMDV= Foot & mouth disease virus; AuNPs=Gold-Nanoparticle; NDV=Newcastle disease virus; Ag@SiO2=Silica coated Silver Nanoparticles; AgNPs= Silver Nanoparticles; CDV= Canine distemper virus; PLGA=Poly (lactic-co-glycolic acid); BTV=Bluetongue virus; BVDV=Bovine viral diarrhoea virus; MDV= Marek's disease virus; TLR-Ls=Toll-like receptor ligands; GaHV-1=Gallid alphaherpesvirus-1; SN-TiO2-PSP=Spiky titanium dioxide nanoparticle-loaded plantains semen polysaccharide; FAV-4=Fowl adenovirus-4; IBSV=Infectious bronchitis virus; EDSV=Egg drop syndrome virus; AgNO3-NPs=silver nitrate nanoparticles; FPV=Fowlpox virus; AMS=Aluminium Magnesium Silicate; IBDV=Infectious bursal disease virus.

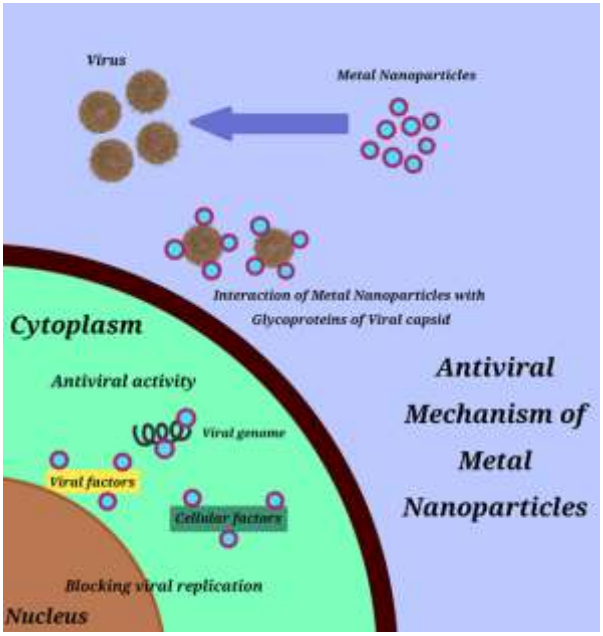

Fig. 1: Pathway adopted by metal nanoparticles to stop viral replication

the cell on the nanoparticles (Figure 4). Hence, cell membrane coating technology helped the researchers to broaden the use of nano decoys and ligand functionalized nanoparticles (Zhou et al. 2019).

An important use of cell membrane-derived nanoparticles is for detoxification. While displaying the same receptors as cells, the nano receptors will divert harmful toxins away from susceptible cells. The nanosponges will then react with the toxins and bind them rendering their toxicity harmless for other healthy cells of the host (Hu et al. 2013).

Nanoparticles for Different Veterinary Viral Diseases

Foot and Mouth Disease (FMD)

Foot and mouth disease (FMD) is a highly contagious viral disease of many domesticated and wild cloven-hoofed animals that is caused by foot and mouth disease virus (FMDV) which belongs to the family *Picornaviridae* (Martinez-Salas et al. 2008). FMDV is an RNA-containing and filterable *aphthovirus* of this family and it has seven different sero-types based on their antigenic structural characteristics which include about 80 total variants of this devious disease-causing virus. Vaccination has been the only tool to prevent this disease in domesticated cloven-hoofed animals for decades. Commercial vaccines are used for this purpose but many times over the course of the history of this disease, these vaccines have failed and caused enormous losses to the dairy business (Doel 1996).

Many times, the vaccination doesn't fail yet the disease appears in flocks because of the slow process of immunity development in the animals. But now a new hope has emerged regarding the prevention of this disease. Research studies have been held and experimental procedures have concluded that nanotechnology can not only provide the successful vaccination of FMD, but it can also make the immunity development process faster than the commercial vaccines available for this disease (Park 2013). Peptide antigen-nanoparticle combinations have been proposed to provide vaccinated-to-immuned status in flocks. For this purpose, gold nanoparticles (AuNPs) were conjugated with the antigen peptides in combination with CFA. This study suggested that vaccination with the conjugate of AuNPs and antigen peptides (VP1 of the native peptide) has shown a more immense immunity response in the animals against FMD as compared to the animals vaccinated with commercial FMD vaccines available in the market. Higher titre of the antibodies was observed in the test animals along with even more sensitivity shown against the antigen immunoassay. A considerable increase in the production of proinflammatory cytokines (especially IFN-γ) and the peritoneal macrophages was also observed. In short, this study laid the foundation to future research works regarding the vaccination of animals against FMD using gold nanoparticles (AuNPs) conjugated with antigen peptides (Moisa and Kolesanova 2010).

Newcastle Disease

A highly virulent disease of domestic and wild poultry birds caused by Newcastle disease virus (vNDV) which belongs to the family *Paramyxoviridae* (Davis 2001). The higher susceptibility and higher mortality rates caused by this disease mostly lead to epidemics in avian flocks and wild poultry populations. Just like any viral disease, vaccination is the only way to prevent enormous losses caused by NDV in the poultry business industry (Chen et al. 2007). DNA vaccines are a great option for vaccination but there are still some obstacles to delivering it into the bird's body through traditional ways (Steel et al. 2008). As the traditional methods of vaccination include injecting it via intramuscular route, this way doesn't help much when the antigen wouldn't be able to reach the target APCs which then leads to vaccination failure (Romer-Oberdorfer et al. 2003). This necessity raised many questions in mind which led to the suggestion of considering the use of better adjuvants for delivering ND vaccines to the APCs effectively. Previous studies (Wang et al. 2011) have shown that all the disadvantages brought to light using DNA vaccines can be prevented by using nanoparticles by the aid of nanotechnology by building a mucosa delivery system using metal nanoparticles. Silver and silica nanoparticles were used in combination to deliver the DNA vaccine right where it should've been delivered in the first place (to antigen presenting cells APCs). The Ag@SiO2 hollow nanoparticles were used for this purpose. The beneficial properties manifested by these Ag@SiO2 hollow nanoparticles included safest delivery of plasmid DNA, lower chances of cytotoxicity, uniformity in the structure along with higher stability even in fluctuating temperatures (Alexander 1990).

Rabies

Rabies virus belongs to the *Rhabdoviridae* family, and it causes acute encephalomyelitis in both animals as well as humans (Finke and Conzelmann 2005). Annually more than sixty thousand people die of Rabies worldwide, of which 99% of the cases are from Asia and Africa. No doubt that Rabies is a preventable disease with the aid of vaccination tools these days. The World Health Organisation (WHO) has recommended a control strategy for this disease through vaccination worldwide (Bahloul et al. 2005). Vaccinating humans, dogs and other domesticated animals as well can help in limiting the invading ways of this virus into living bodies. Commercially prepared vaccines are available in the market for this purpose, most of which are alum-based (Wang and Singh 2011). Alum is used in vaccine manufacturing against many diseases these days. Along with many advantages, there are certain disadvantages of using vaccines that are alum-based. Recently trials have been manifested in search of better adjuvants than alum in the industry of vaccine manufacture (Zhao et al. 2014).

Many have used metal-based nanoparticles for this purpose like silver and gold nanoparticles (AgNPs & AuNPs) (Dykman et al. 2010). Green synthesis of nanoparticles has made their production way easier and cheaper these days (Sivakumar 2011). AgNPs have been used as adjuvant to deliver DNA/RNA of many viruses to produce immunity in animals. A study (Lindblad 2004) was held to test green synthesized AgNPs to deliver the Rabies vaccine as adjuvant against the commercially prepared alum-based Rabies vaccines. The results showed immense improvement in the successful vaccination along with lesser time taken to build immunity against the disease (Asgary et al. 2016). An increased humoral response to the antigen of the Rabies virus was also observed. As the search for better adjuvants in aids of vaccination against viral diseases continues, we see promising assistance in vaccine formulation from nanotechnology and metal nanoparticles. Yet there are still some limitations in their clinical application because of the higher cost in their production and public's access. As the work goes on, we'll tackle these hurdles as well (Asgary et al. 2016).

Canine Distemper

Canine distemper is a highly contagious and fatal disease of dogs, ferrets and giant pandas, caused by Canine Distemper Virus (CDV) which is an enveloped and single stranded-RNA virus that belongs to the family *Paramyxoviridae* (Plattet et al. 2016). Available means of getting rid of this disease include inactivated vaccines, subunit vaccines, DNA vaccines along with attenuated vaccines of CDV (Avota et al. 2013). Some studies regarding CDV also suggested that utilizing fusion (F) proteins along with purified hemagglutinin (H) proteins have shown great immune responses in dogs. While other studies also found that DNA vaccines containing H protein of CDV also come in handy in immunizing minks against Morbillivirus infections (Ge et al. 2015). Vector vaccines also show great immune response in subjects as compared to that of attenuated vaccines (Jensen et al. 2015). While inactivated or killed vaccines show poor immunization response and require multiple doses to keep the animal immunized. Some studies reported that animals that got vaccinated with attenuated vaccines of CDV developed leukocytopenia and typical erythematous rash of distemper. Some studies have shown that nanoparticle-based antigens are better adjuvants for immunizing animals against CDV as these vaccines show multiple advantages over inactivated, attenuated and subunit vaccines of CDV (Wang et al. 2013). These advantages include higher immunogenicity, more stimulation to antigen presenting cells (APCs) and better adjuvant effects to express better innate and adaptive immune responses (Cheng et al. 2010).

Parvovirus

Parvovirus causes enteric and myocardial diseases in dogs worldwide. Canine parvovirus is a single stranded DNA virus. It is a small self-replicating virus. Parvovirus infection has a high mortality rate of about 90% of cases ending in the fatality of the animal (Derman et al. 2014). Absence of any kind of treatment leads to a mortality rate going even above 90%. The use of a vaccine against parvovirus has not been very successful. The attenuated virus or dead viral particle used as a vaccine is phagocytized by the protease enzymes present in the cell, rendering it useless for production of immunity (Prittie et al. 2004). Due to the failure of the traditionally used vaccines, a synthetically prepared peptide-based vaccine for the treatment of canine parvovirus soon emerged. To overcome the problem of the phagocytosis of live or attenuated vaccine antigens by the protease enzymes, the synthetically prepared peptide-based vaccine was introduced into the cell in a nanoparticle-based delivery system (Casal et al. 1995). Poly (DL,lactic-co-glycolic acid) (PLGA) is a widely

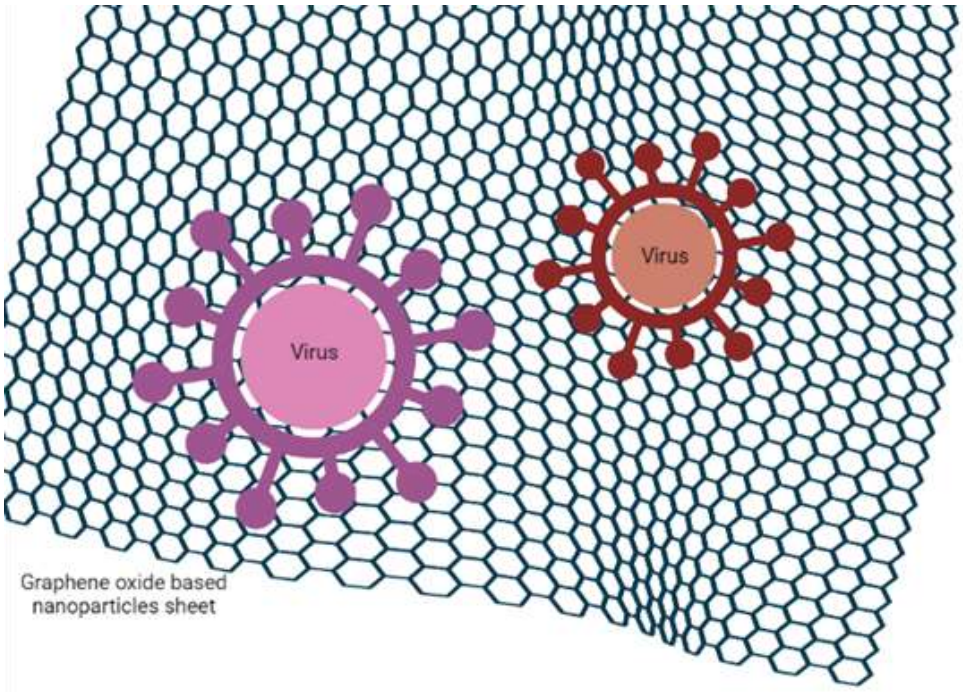

Fig. 2: Negatively charged Graphene oxide-based sheet attracts and entraps viral particles with positively charged protein coating. This entrapment renders the viral particles disabled preventing their attachment with healthy cell membranes.

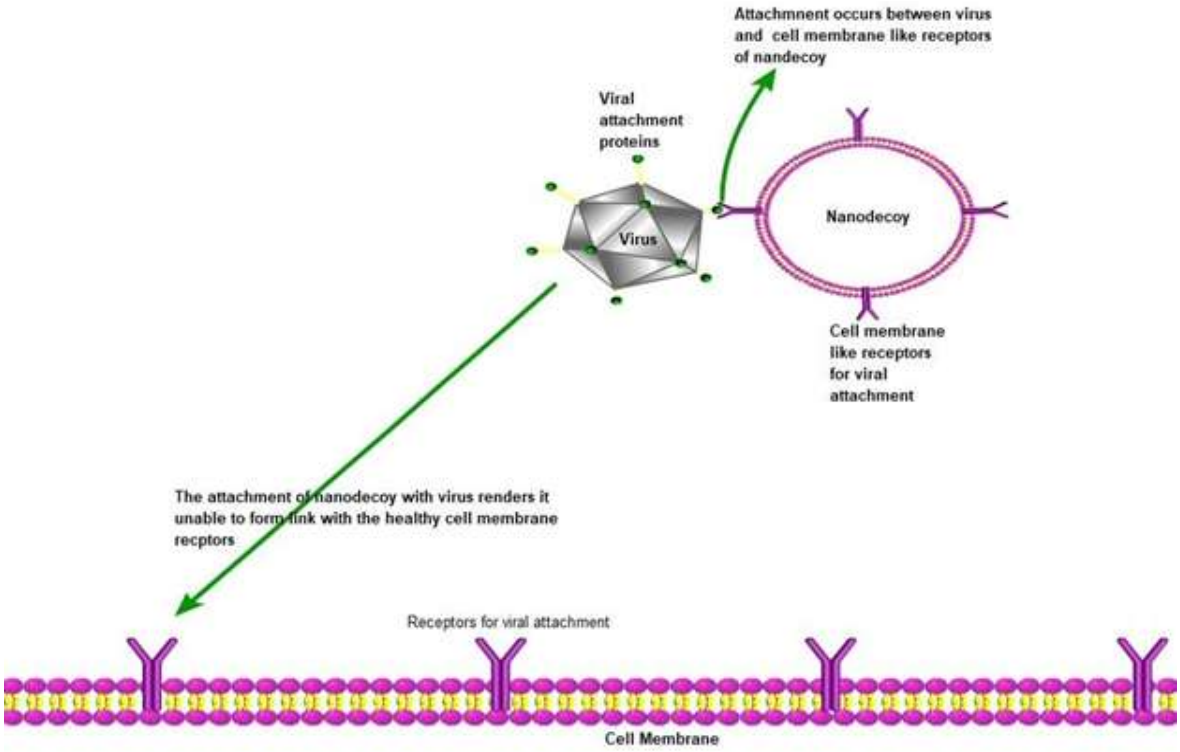

Fig. 3: A nanodecoy attaches itself to a viral particle to prevent it from linking with the receptors of a healthy cell membrane.

used nanoparticle-based carrier system. The nanoparticle delivery system might be a protein or a polymer. It can also be a nanoparticle (Langeveld et al. 1994). During the preparation of the carrier system, the following steps were followed as standard protocol. The W-1 L19 peptide of the canine parvovirus is encapsulated in the PLGA delivery system in the presence of oil or double water emulsion as a solvent. The uniformity of the size and smooth, spherical surface of the nanoparticles play an important role in the functioning of the carrier systems. The application of the peptide-based vaccine in a nanoparticle delivery system proved to be more advantageous as compared to the introduction of the vaccine directly into the cell. For instance, the release of antigen (vaccine) can be controlled over time if required. The nanoparticle delivery system also provides protection against the cell proteases. The surface area for absorption is increased. Use of nanoparticles eliminates the requirement for a booster dose. It also plays a vital role in enhancing the availability of vaccine antigen in the cell.

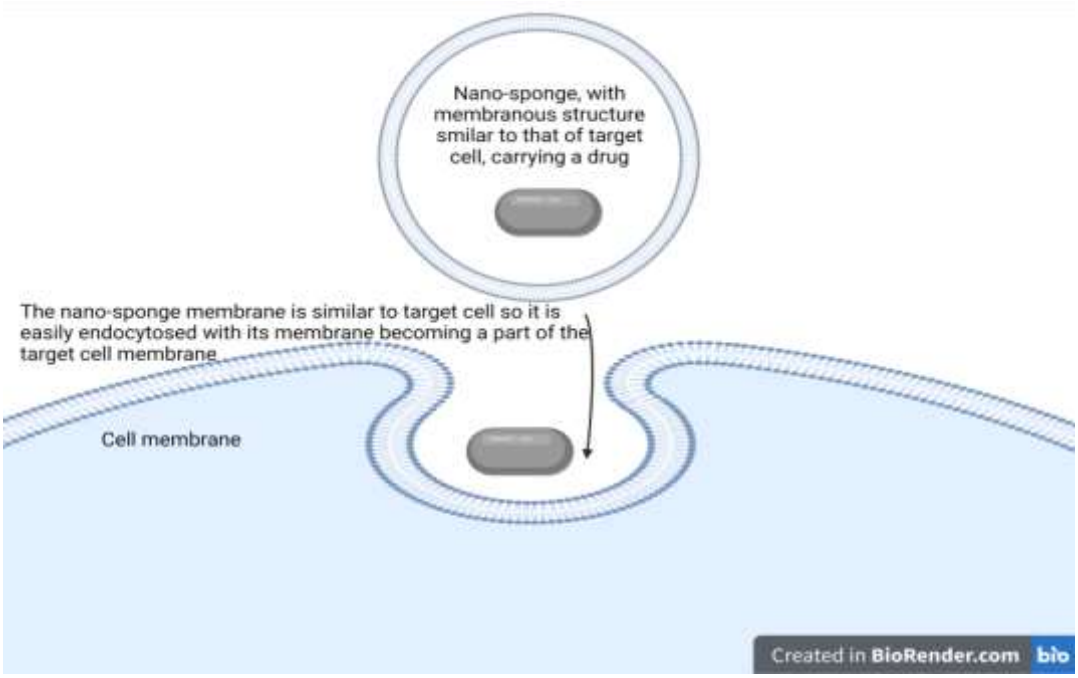

Fig. 4: The mechanism of carrying a drug safely to its target by use of nano-sponges.

Blue Tongue

Bluetongue is a non-enveloped virus with a double capsid protein coating. The capsid of this virus is composed of seven different types of proteins (Zhang et al. 2016). The complex structure of this virus is the reason for making it nearly incurable. Its infection manifests as a serious disease in sheep. It also infects cattle, horses and deer (White and Eaton 1990). The genome of this virus appears to be uniquely made up of 10-double stranded RNA segments. Bluetongue virus has several serotypes but the outermost spike-like VP2 protein has maximum importance. At least 28 different serotypes of the bluetongue virus have been discovered by researchers until now (Wu et al. 2019). The vector responsible for the spread of this virus is the Culicoides species, gnats (biting midges). It is a widely distributed disease in sheep. There are seven structural proteins (VP1-VP7) but VP2 is the most important and causes disease (Zhang et al. 2016). VP2 is the main protein of attachment to the cell for the virus once it gains entry into the body. Experimental data has suggested that VP2 protein interacts with the Sialic acid (SA). The VP2 protein interacts with alpha 2, 3 linked and alpha 2,6 linked SA. To inhibit bluetongue virus infection, lectin inhibitors are used as nanoparticles. As a nanoparticle, lectin inhibitors target SA linkages. The main competitor of lectin inhibitors is alpha 2,3, linked to SA. The main binding sites for SA on VP2 are determined by using mass spectrometry. The higher affinity of VP2 for alpha 2,3 linked SA is revealed by the glycan array (Baker et al. 2019).

Summary

Viruses have been responsible for causing several kinds of diseases in all living beings. Previously viruses have been very hard to fight off because of their rapid mutation along with our inability to develop more and more antiviral drugs. The only option that still stands is vaccination as a prophylactic agent. Vaccination wasn't still a bullet-proof plan as it had some holes like vaccination failure, disease outbreak before the immunity response even showed up, vaccination routes and the slow delivery of antigen to the APCs. Then some recent studies showed that the solution to all of these problems faced by the vaccination schedules lay in nanotechnology. Nanoparticles have several modes to fight off a virus and they can also deliver the vaccine to the right receptors at the APCs. Gold nanoparticles (AuNPs) have been proven helpful to vaccinate animals against foot and mouth disease virus. Silica coated silver nanoparticles (Ag@SiO2) have done the same for Newcastle disease virus in the poultry sector. Likewise, some animals have shown immune response against rabies as well when they were vaccinated with green synthesized silver nanoparticles conjugated with the rabies virus. These research studies have laid a foundation for future achievements in this direction where vaccination and nanotechnology go on fighting all the viral diseases as a prophylactic step.

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CHAPTER 33

METABOLISM OF SPERMATOZOA

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INTRODUCTION

All living cells, including spermatozoa, need energy to grow and function. Spermatozoa require adenosine triphosphate (ATP) for successful fertilization and hyperactivation, acrosome reactions, motility, and capacitation. ATP is generated from adenosine diphosphate (ADP), so the adding and removing phosphate groups from the ADP molecule is the main activity of all life duration (Mukai and Travis 2012; Mannowetz et al. 2012).

Spermatozoa consist of special structures (du Plessis et al. 2015). Major metabolic pathways taking place in spermatozoa are adenosine triphosphate (ATP), oxidative phosphorylation (OXPHOS), and glycolysis, which provide spermatozoa with the necessary energy to perform their basic functions (Sengupta et al. 2020). ATP is produced by glycolysis in the tail and head. ATP is also produced by OXPHOS in mitochondria. In proportion to glycolysis, mitochondrial respiration is thought to be a more effective ATP-building process. Although many researchers have reported that ATP is formed by glycolysis along the flagellum, it is believed that the energy necessary for sperm motility is primarily created by respiration (Ferramosca et al. 2008).

Cellular Respiration

ATP production can be aerobic or anaerobic. The enzymes needed for glycolysis are mainly found in the sheet in the main part of the tail. OXPHOS, conversely, takes place in the mitochondria situated in the middle part. OXPHOS is a more effectual ATP production method than glycolysis (Storey 2008). During spermatogenesis, deoxyribonucleic acid (DNA) condenses into a crystalline structure that provides mechanical protection against damage from reactive oxygen species (ROS) and allows sperm to be organized for easy motility (Miller et al. 2010).

Activation of sperm flagellar motility requires energy metabolism and activation of motor organs. Flagellar motion is commenced by the motor activity of axonal dynein arms to stabilize microtubule pairs (Tash 1989). The initiation of flagellar movement depends on the phosphorylation of axonal dynein. Dynein ATPase is then enabled. After hydrolysis, ATP is transformed into a force that causes the microtubules to slide against each other (Shingyoji et al. 1977). The calmodulin-dependent protein phosphatase calcineurin phosphorylates dynein then reverses the process (Smith 2002). Phosphorylation, dephosphorylation, corresponding inactivation and activation of dynein arms take place asynchronously along the circumference and length of the axoneme (Wargo & Smith 2003). The axoneme folds radially in two directions and rearranges where the dynein arms are active. The changes in result are regulated by intracellular calcium (Ca^{+2}) (Nakano et al. 2003).

Axonemes require a constant supply of ATP to keep going the functions of the female and male reproductive systems. Spermatozoa can utilize an assortment of monosaccharides such as fructose, glucose, mannose and can metabolize pyruvate, lactate, glycerol and acetate via the glycolytic pathway. Enormous polar molecules like glucose cannot cross the membrane and their transportation is made easier by membrane-bound proteins (Kasahara and Hinkle 1977). These proteins are generally divided into two groups. The first is the sodium-linked glucose transporter (SGLT), which is bound to ATP, and the second is the facilitating sugar transporter (GLUT, glucose transporter) (Mueckler et al. 1985; Scheepers et al. 2004), which provides passive transmembrane channels for sugars. GLUTs are classified according to their ability to transport vitamins, amino sugars, and hexose sugars (such as fructose, mannitol, glucose) (Angulo 1998). During glycolysis, monosaccharides with six carbons are transformed into two pyruvate molecules (du Plessis et al. 2015).

In the next step, the acetyl group is obtained from acetyl-CoA, the carboxyl group is lost in the form of CO_2, and the pyruvate is further oxidized. The acetyl group is then oxidized to CO_2 through the citric acid cycle. Electron donors, such as NADH and $FADH_2$, are formed because of metabolic processes such as glycolysis, fatty acid oxidation and the citric acid cycle. These molecules with high electron transfer potentials are high-energy molecules. Electron flow then passes from $FADH_2$ or NADH to O_2 through protein complexes positioned in the internal mitochondrial membrane. The proton motivating force is due to the pumping of protons, which results in an uneven distribution of protons in the mitochondrial matrix due to electron transfer. ATP is synthesized by ATP synthase as the proton is returned to the substrate (Erecińska and Wilson 1978; Gnaiger 2001).

Glycolysis

The mitochondria responsible for sperm respiration and oxidative phosphorylation are positioned in the middle of sperm. The tail and head of the sperm do not have respiratory

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enzymes but do have glycolytic enzymes, that's why they are called glycolytic active sites (Travis and Kopf 2002). Therefore, glycolysis is a crucial road for ATP generation in spermatozoa. These enzymes are mainly positioned in the fibrous sheet of most of the tail, which is required for glycolysis. Several glycolytic enzymes specific to these cells are found in a fibrous sheet of spermatozoa. These are hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase (GAPD), phosphoglucokinase isomerase, and LDH (Cárdenas et al. 1998). After hexose metabolism is initiated, the major control steps of glycolysis are sugar isomerization and phosphorylation to produce glucose-6-phosphate, one of the major intermediate metabolites (Rigau et al. 2002). This step of phosphorylation of monosaccharides is controlled by a family of hexokinase proteins with high affinity for glucose. It has been reported to trigger a maximum rhythm of glucose usage in canine and porcine spermatozoa even at glucose levels as small as 1-5 mM. In this way, mammalian spermatozoa produce very quick and concentrated metabolic responses to use glucose, even at very little concentrations (Storey 2008).

Glucose molecules are overthrown to pyruvate molecules by an enzyme-catalyzed reaction. Some of the free energy released by the successive glycolytic reactions is conserved in the creation of NADH and ATP. The fortune of pyruvate changes depends upon environmental status. Pyruvate gets into the citric acid cycle; under anaerobic conditions, it turns into lactic acid under aerobic conditions (Pasupuleti 2007).

Oxidative Phosphorylation

However, after glucose is turned into pyruvate by glycolysis, just an exiguous part of the entire free energy of glucose is released. The molecules of oxidized glucose are 30 molecules of ATP. Glycolysis releases only 2 ATP molecules. Therefore, glucose metabolism in mitochondria can generate 15 times more ATP (Kim et al. 2007).

OXPHOS is an extra complex procedure that occurs in an organized manner and contains two constituents of the inner mitochondrial membrane, ATP synthase, and respiratory chain. The most important factor in determining the OXPHOS ratio is the availability of ADP. Although sperm can keep alive solely on glycolytic energy, they require OXPHOS to mature and differentiate. Mitochondria play key role in ATP production through OXPHOS. This is why they are called the "power plants" of the cell. Mitochondria make up the majority (~15-22%) of the total cell volume and an adult sperm contains approximately 72-80 mitochondria (Rajender et al. 2010). Although mitochondria consist of four distinct parts (outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and a matrix), sperm mitochondria are functionally and morphologically diverse from somatic mitochondria (Piomboni et al. 2012). These differentiations can be based on the hard packing of mitochondria around the sperm axofilament. This tight junction is formed by selenoproteins and disulfide bonds that confer mitochondrial stability (Calvin 1981). The inner mitochondrial membrane bends into formations called cristae, which are the major places for the creation of ATP and OXPHOS. Mitochondria in spermatozoa have isoenzyme isoforms and specific proteins not detected in somatic mitochondria, such as VIb, cytochrome C, lactate dehydrogenase (LDH), and the hexokinase subunit of cytochrome C oxidase (Hess et al. 1993; Hüttemann et al. 2003; Goldberg et al. 1977).

Mammalian spermatozoa can also generate energy with anaerobic glycolysis or pyruvate oxidation. Mitochondrial lactate dehydrogenase X (LDH-X) allows NADH to oxidize pyruvate to lactate (Blanco and Zinkham 1963). Concerning sperm motility, changes in enzymatic activity in the mitochondrial respiratory chain may have an effect (Luft 1995). The electron transport chain is obtained of four multimeric complexes (complexes I, II, III, and IV) and two mobile couriers (cytochrome C and coenzyme Q) (Luft 1994).

The first largest element of the mitochondrial respiratory chain is NADH dehydrogenase or complex I. Complex I catalyzes the oxidation of NADH within mitochondria, firstly from the shuttle mentioned above and secondly from a few dehydrogenase reactions that occur within mitochondria (Koopman et al. 2010). A substrate named succinate is for succinate dehydrogenase or complex II, which is an intermediary in a citric acid cycle. Complex II does not carry protons into the intermembrane area and thus does not conduce to the production of the proton motivating power (Rutter et al. 2010). Cytochrome BC1 or complex III is larger than complex II. Complex IIIs functions are as a homodimer in the inner mitochondrial membrane. Complex III can be inhibited by antimycin A and methimazole, whose reduced equivalents target small molecules of cytochrome C (Zara et al. 2009). The final componentry of the mitochondrial respiratory chain is the IV complex or the other name is cytochrome C oxidase. The complex IV receives reducing equivalents from cytochrome C and releases them into the O_2 molecule to form H_2O. The complex IV is specifically blocked by azide, cyanide and CO (Fontanesi et al. 2008).

ATP synthase is physiologically linked with the mitochondrial respiratory chain (Devenish et al. 2008). This complex consists of two major components, F1-ATPase and Fo-ATPase, located in the inner membrane. The Fo-ATPase includes proton channels from the intermembrane gap to the substrate, while the F1-ATPase catalyzes ATP synthesis. Thus, ATP synthase utilizes the physiologically distributed free energy in the inner mitochondrial membrane to synthesize ATP (Boyer 2001). Oligomycin is a potent and private inhibitor of Fo-ATPase that ties up ATP synthesis, thereby blocking mitochondrial respiration (Wittig and Schägger 2009).

Some researchers reported that the gene SLC22A14, a biological cation carrier-like protein, is one of the genes essential in the male fertility. They indicated that SLC22A14 is positioned in the inner mitochondrial membrane in the middle of sperm and is a riboflavin transporter. It has been reported that when the SLC22A14 gene is damaged, male motility is suppressed, leading to infertility. In addition, removal of this gene has been reported to impair fatty acid beta-oxidation and flavinase activity. Disruption of the riboflavin transporter has also been shown to inhibit oxidative phosphorylation (Kuang et al. 2021).

Medium with high glucose concentrations has been reported to inhibit mitochondrial activity in naturally derived sperm as compared with sperm in medium with low glucose concentrations. Under these conditions, oxygen consumption in spermatozoa should decrease and the ability to respire should decrease. However, mitochondrial function was more pronounced in thawed sperm than in natural sperm ($p<0.05$). This increase is thought to be due to "nonproductive" oxygen

consumption to maintain the mitochondrial proton gradient (Moraes et al. 2021).

Melatonin has been reported to inhibit mitochondrial permeability transition pore (MPTP) inauguration and lactate concentration and to improve ATP production and acetyl-CoA concentration, as well as sperm motility and viability. Melatonin was reported to inhibit the permeability of FOF1-ATP synthase, isocitrate dehydrogenase, citrate synthase, and oxoglutarate dehydrogenase complexes from mitochondria to the cytoplasmic fraction induced by MPTP inauguration. They also indicated that melatonin increases the activity of complexes I, II, III, IV, and the oxygen utilization capability of frozen-thawed spermatozoa by increasing mRNA expression of respiratory chain complex components. They showed that melatonin enhances OXPHOS in frozen-thawed ram sperm by inhibiting cryopreservation-induced MPTP inauguration (Fang et al. 2020).

Moderate addition of glucose to porcine spermatozoa was found to increase ATP and GSH/GSSG ratios and linear motility, and to reduce reactive oxygen species values compared to spermatozoa without glucose. In addition, sperm with added glucose were reported to have higher NADPH levels, higher glucose-6-phosphate dehydrogenase (G6PD) activity, higher mitochondrial activity, and a higher NADPH/NADP+ ratio. However, they found that 1,6-bisphosphate fructose aldolase (ALDOA) was unchanged. It has been reported that 6-aminonicotinamide, a G6PD inhibitor, decreases linear sperm motility. Itaconic acid is a cellular regulator of metabolic reprogramming (Wei et al. 2019). It is a metabolite resulting from the transformation of cis-aconitic acid, an intermediate in the tricarboxylic acid (TCA) cycle (Michelucci et al. 2013). They suggested that moderate glucose increases sperm itaconic acid content and both endogenous and exogenous itaconic acid increases total itaconic acid-modified and itaconic acid-modified ALDOA content. Also, addition of itaconic acid inhibits glycolytic enzymes to activate glycolysis and increase oxidative phosphorylation (OXPHOS) to improve linear sperm motility patterns. Thus, it has been reported that itaconic acid produced by OXPHOS arranges the glycolytic/PPP passing to maintain redox homeostasis. An itaconic acid-dependent mechanism acts a momentous role in keeping going linear sperm motility (Zhu et al. 2020).

Phosphoglycerate kinase 2 (PGK2), testis-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDHS) and lactate dehydrogenase (LDHC) are expressed only during post-meiotic spermatogenesis. Although expression levels were higher in youngs, lower expression rates of these glycolytic enzymes were detected in olds. The expression of these enzymes was also found to be low in weak and immature spermatozoa. The expression rates of PGK2, LDHC, and GAPDHS in spermatozoa were related to sperm linear motility. Studies have shown that the expression of PGK2, LDHC, and GAPDHS in spermatozoa may be related to sperm qualification and that there may be the same molecular mechanisms in immature and weak spermatozoa that account for sperm quality (Liu et al. 2019).

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CHAPTER 34

FUNGI ASSOCIATED WITH SHEEP SKIN

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INTRODUCTION

The kingdom "Fungi" is a large, diverse group of organisms ranging from simple yeast cells (e.g., Candida spp.) to complex fruiting bodies producers (e.g., mushrooms and puffballs). Fungi found worldwide grow in a diverse environment and develop in several habitats where humidity, carbon, and nitrogen are available (Naranjo-Ortiz1 and Gabald 2019).
Fungi are eukaryotic microorganisms that digest food via extracellular enzymes and absorb simple nutrients through their cell walls. Most fungi reproduce by various spore types and have a body composed of microscopic hyphae. They are septated or non-septated tubes. Fungi are heterotrophs and gain their carbon and energy from external sources (Webester and Weber 2007).

Fungal Animal Relation- A General View Fungal Animal Relation -A General View

Fungal-invertebrate Relation

Fungi can grow on and in both invertebrate and vertebrate animals. Several fungi can attack insects, and others destroy nematodes, and in nature, they play an essential role in keeping populations of these animals within the expected size. Entomopathogens, are fungi that infect insects. Mainly, they belong to phyla Ascomycota, Zygomycota and Chytridiomycota. These fungi attack and consume several types of insects. They can change the behavior of infected ants after brain infection to produce "Zombie-ant" (Evans et al. 2011; Hughes et al. 2011). Entomopathogens such as *Beauveria bassiana* and *Metarhizium anisopliae* are so effective in killing insects that they are used as biological control agents for insect pests (Erler and Ates 2015). The order Entomophthorales includes many very specialized entomopathogens. A known example is *Entomophthora musae*, the fungus proliferating a mass of white conidia on dead flies (Carris et al. 2012).
Some fungi develop structures to capture nematodes. A common nematode predatois *Arthrobotrys oligospora,* a fungus with sticky networks of hyphae. When the worm in the soil touches the adhesive mycelia, it cannot escape. The predator fungus invades the worm cuticle and utilizes the interior body contents (Niu and Zhang 2011).

Fungal-vertebrate Relation

There are about 1.5 million fungi, and fortunately, a few are pathogens of vertebrates; they are about 300 species, some of these fungi can cause severe infections (Carris et al. 2012). Pathogenic fungi of vertebrates follow various pathogenic mechanisms. Some of them cause death even the fungus does not invade the animal body (Voyles et al. 2009).
There are common fungal diseases, "mycoses" for both humans and animals. The most common mycoses are the skin infections" dermatophytosis". They are restricted in dermis and related structures i.e., hairs, nails, claws, hooves, Wool, fur and feathers etc. (Ridzuan et al. 2021).
Several fungi use animal skin as a reservoir host that allows them to live and reproduce. The resident fungi of healthy animal skin may become pathogenic agents with predisposing conditions. *Candida* sp., for example, is the causative agent of superficial candidiasis in animals with weak immunity (Dworecka-Kaszak et al. 2020).

Fungal Lifestyle

Most fungi inhabit terrestrial environments, where they have been divided into three groups based on their modes of life. Saprotoph that feed on dead organic matters. Parasitic fungi, if they grow on living organisms and cause harm, and mutualistic if the living organism benefits.

Saprophytic Fungi "Decomposers"

Most members are terrestrial, but they live in most habitats on earth. Soil is the main reservoir of saprophytic fungi, where they grow on the upper layer of the damp part. The fungal population thrives in soil rich in decaying debris from plants and animals. The group play a critical role in the natural balance of ecosystems (Frąc et al. 2018).
The decomposer fungi group is active biodegradable. They hydrolyze complex plant tissues -cellulose and lignin- via cellulase and phenoloxidase.
In the soil, the role of fungal decomposers, and in fewer degrees bacteria and microphones, making it possible for members of the other kingdoms to live. Decomposer fungi reside in animal's skin and their appendages. And they play various roles here (Wilson 2005).

Mutualistic

Mutualistic fungi are less in numbers than the other two groups The symbiotic relationship between plants and fungi attracted attention before that with animals. Studies have extensively and deeply referred to mycorrhiza and its

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applications (Figueiredo et al. 2021; Li et al. 2021). Endophytic fungi are used as a successful biological control agent in phytopathology. Mutualism with large animals needs high caution to assign authentic and allochthonous fungi associated with the animal bodies (Lavrinienko et al. 2021)

Mycobiota of animals either reside in the gut or on the coat. The gut mycobiota has a further health significance than normal skin flora (Nilsson et al. 2019; Richard and Sokol 2019).

Most studies of skin mycobiota were conducted for small animals and pets due to the widespread of home pets and their relation with human infections. The health impact of normal fungal flora on dogs and cat skin or domestic farm animals is their conversion to infectious agents (Lund et al. 1999; Meason-Smith et al. 2016; Dworecka-Kaszak et al. 2020).

Parasitism

The fungus lives on the plant or animal hosts. Parasites impair hosts and affect their functions. Most pathogenic (disease-causing) fungi are parasites. The fungal phytopathology is more harmful than those caused by other pathogens (Hussain and Usman, 2019). Fungal infections (Mycoses) are less epidemic than bacterial in animals except among immunocompromised cases, where are the main reason for mortality (Fisher et al. 2012).

Animal fungal diseases are classified as superficial, cutaneous, subcutaneous and systemic mycoses according to the level of the infected tissue. The harms of infection include suppressing animal activities, reducing product quantity and quality, and causing death in some systemic mycoses cases (De Hoog et al. 2000). Dermatophytes are skin fungal pathogens. They benefit from surface skin secretions and cause several skin diseases (Sindha et al. 2015). They may also penetrate the skin through a wound of insect bites or various accidents that damage the dermis. Dermatophytes are distributed worldwide and have attracted particular attention in public health (Rashidian et al. 2015).

Animal Skin Mycobiom (non-pathogenic)

Skin is the largest organ in the animal body. It acts as a barrier against the entry of microbes from the outside environment. The majority of microorganisms that live on it are harmless and even beneficial to the hosts. Bacteria, fungi, and viruses are commonly recorded from human and animal skin. They are variable in number and type depending on endogenous and exogenous factors. Previous studies showed that the two main factors determining the interaction status between fungi and animals are animal immunity level and the fungus biological activity (Mańkowska-Wierzbicka et al. 2015). Fungi can evade animal defense via various virulence factors. Ultimately the host response is the factor that determines if a microbe is a commensal or a pathogen (Cogen et al. 2008)

Skin mycobiome "mycobiota" is a part of microbiota. The term refers to a fungal community that inhabits animal skin. Mycobiome members utilize keratin, the essential protein of skin and its appendages, hairs, wool, fur etc. (Aho 1983; Dworecka-Kaszak et al. 2020). Mycobiomes favor the saprophytic living style on skin habitat and may transfer to parasitic where they are infectious factors. Either environmental effects or weak animal immunity predisposes change of fungal life mode (Casadevall and Pirofski 2000).

The different animals have their specific normal fungal flora and seem more diverse than humans. The mycobiome of dog's skin, for example, created dominant filamentous saprophytes *Cladosporium*, *Alternaria*, and *Epicoccum* (Meason-Smith et al. 2015).

Aho (1983) examined 246 dog hair samples. Thirty-two genera of filamentous fungi were recorded. *Cladosporium, Penicillium, Aspergillus*, and *Alternaria* were the highest number. In Egypt, Bagy (1986) recognized 16 genera from 98 samples of dog healthy hairpieces. In another study carried out in Serbia, *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Mucor* sp., and *Fusarium* sp. were isolated from 67 dog hairpieces. In Nigeria, Moses and Sunday (2001) isolated *Aspergillus* (5sp.), *Chrysosporium* (4sp.), *Fusarium* (2sp.), *Penicillium* sp., *Pithomyces* sp., *Geotricum* sp., *Alternaria* sp., and *Cladosporium* sp. from dog samples.

In addition to filamentous fungi, several yeasts inhabit animal skin. *Malassezia pachydermatis* is a common cutaneous lipophilic inhabitant of numerous warm-blooded animals. It can cause several cutaneous infections, especially in dogs (Charles et al. 2020). *Candida* sp also has a high prevalence on dog skin and can cause severe infections for animals or owners (Yurayart et al. 2011).

Studies showed that the properties of animal skin sites affect fungal communities. Meason-Smith et al. (2015) explained that canines have different mycobiota across the hairy and non-hairy body surface. Skin mycobiota surveys of several animals have been done during the previous years. The fungal flora of golden hamsters showed *Rhizopus* spp., *Penicillium* spp., *Cladosporium* spp., *A. fumigatus*, *A. flavus* and *Mucor* spp. were the most frequently isolated. These saprophytic moulds, besides the dominant yeasts *Malassezia* spp., *Rhodotorula* and *Candida* spp. showed no relation with age and gender of tested animals (Mahnaz et al. 2014). They also concluded that the fugal community does not depend upon animal age and gender.

The "normal" fungal flora of pet cats was diverse. Fifteen genera were isolated, including13 saprophytes (86.6%) and two dermatophytes. *Aspergillus, Alternaria, Penicillium* and *Cladosporium* spp. were the most frequently isolated saprophytes (Philpot and Berry 1984). Sixteen saprobic genera were isolated from goat hairs in Taif/KSA (Awad 2017). The study highlights the high diversity of fungal isolates of goatskin habitat. *Aspergillus* spp. and *Penicillium* spp. are predominant. In addition to the following keratinophilic (non-dermatophytes) isolates *Acremonium, Alternria, Boteryatricum, Chaetomium, Cochliobolus, Cladosporium, Curvularia, Geotrichum, Paecilomyces, Phoma, Rhizopus* and *Scopulariopsis* (Awad 2017). The same results were obtained several wrkors aroun the world (Ogawa et al. 2008; Nichita and Marcu 2010; Sallam and Alkolaibe 2010; Emenuga and Oyeka 2013; Luján-Roca et al. 2016).

From dromedary hair samples, 15 filamentous fungal genera were isolated. The predominant are *Aspergillus* followed by *Penicillium, Mucor, Alternaria, Rhizopus, Chrysosporium, Acremonium, Scoupolariopsis, Cladosporium Fusarium, Psuedallescheria* and *Stachybotrys.* The isolated yeasts were *Candida*, *Geotrichum* and *Malassezia* (Shokri and Khosravi 2011)

Christiane et al. (2011) collected 56 hair samples from healthy coat cattle. The samples harboured 30 different genera, among which the most frequent were: *Nigrospora, Fusarium, Curvularia, Alternaria, Epicoccum, Paecilomyces and Trichoderma*.

Animal Dermatophytes and human health

Dermatophytosis is a fungal infection of the dermis. It is caused by *Microsporum, Trichophyton* and *Epidermophyton. Microsporum canis, M. gypseum* and *Trichophyton mentagrophytes* are the most common dermatophytes of animals (Indarjulianto et al. 2017; Moriello et al. 2017).

Dermatophytes have attracted the attention of mycologists since the med of the twentieth century (Cooke 1952; Kaplan et al. 1957; Menges and Georg 1957). There has been a significant increase in dermatophytosis prevalence in humans and animals during the previous years. Etiologic fungi were isolated from many mammals worldwide, and they are the most frequent human mycoses (Hay et al. 2014). Studies of animal dermatophytosis covered the wild and domestic types, pets, experimental animals etc. (Aho 1983; Lopez-Martinez et al. 1984; Shatyha et al. 1988; Ahdy et al. 2016; Haggag et al. 2017; Dalis et al. 2019).

The incidence of *Microsporum* and *Trichophyton* is generally more than *Epidermophyton*. They were isolated from pigs, rabbits, dogs, goats, sheep, and cows (Efuntoye and Fashanu 2001). A study of dermatophytes in dogs, cast, horses, parrots, and calf demonstrated that *Trichphyton* occurred in the five types, *Microsporum* in three, and *Epidermstophyton* in cat samples only (Sever et al. 2021). The ecological type of dermatophytes (geophilic, anthropophilic, and zoophilic) determines the most influential factor. Khosravi and Mahmoudi (2003) collected 790 suspected dermatophytoses from 9 mammals and one bird type. The suspected samples showed 248 dermatophytes. The highest prevalent fungi were *Microsporum canis*, *Trichophyton verrucosum*, *T. mentagrophytes* and *M. gypseum*.

Nine dermatophytes were isolated from dog and cat hair and skin samples (Bernardo et al. 2005). They were *Microsporum gypseum, M. cookie, M. persicolor, M. canis, M. nanum, M. vangreuseghemii, Trichophyton mentagrophytes, T. Terrestre terrestre* and *T. ajelloi*. Murmu et al. (2015) reported that *M. canis* was the highest incidence of dermatophytes in dogs, cats, and humans compared to other species.

Animal skin mycobiota can be a source of human infections. Dermatophytes may be transmitted from wild or domestic animals to humans. The transmission depends on the degree of direct contact or through contaminated fomites or breeding cages and soil beds of infected animals. The environmental changes and human living style also affect fungal transmission. *Microsporum cains* is the most etiologic dermatophyte of cats. It is transmitted after direct contact or fallen hairpieces (Frymus et al. 2013). The agent *T. equinum* transmutes from the infected horse by direct contact or fomites (Overy et al. 2015).

The mouse favus disease is caused by *T. quinckeanum,* and it is transmitted to humans by cats that prey on the infected mice (Szathmary 1966). Several dermatophytes transmitted to humans from wild animals, the hunting and truffle dogs are the common transmitters from infected Preis or soil (Moretti et al. 2013). Previous studies conducted in Europe, America and Africa recorded dermatophytes from several animal types such as boars, Florida panthers, wild felines, Grant's gazelles, marmots, Eastern cottontail and free-living red foxes (Mancianti et al. 1997; Rotstein et al. 1999; Albano et al. 2013; Gallo et al. 2005a, b). The undomesticated hunting cats were more infected than domestic, which indicates that fungus is contracted from rodents or soil (Drouot et al. 2009).

Fungi on Sheep wool/a view of Fungal Community

Sheep infections were studied intensively worldwide, especially the zoonoses and influential economic groups. Parasites have been studied more than fungi (Swar and Shnawa 2021). Sheep wool has diverse mycobiota, including dermatophytes, opportunistic fungi, and keratinophilic/saprophytic. It seems that sheepskin provides a suitable environment for fungal growth and multiplication. The keratinous fibers are soft, flexible and keep heat. These properties enhance the growth of dermatophytes and keratinophilic fungi. In the subtropical region and middle east countries, the free grazing of sheep is commonly followed (Fig. 1). Roaming of sheep herd causes increasing in total expected count and diversity of sheep wool fungal contamination.

S Several studies were conducted on wool fungi and the most related works were selected for the current review. These include Shtayeh et al. (1989), Abdel-Gawad (1997), Al-Bader et al. (2000), El-Said et al. (2009), and Sallam and Al-Kolaibe (2010). The studies have been carried in Meddle East Region include Jordan, KSA, Libya, Iraq, and Yemen respectively. A total of 70 genera were recorded (Table 1). Although studies were temporally and spatially diverse; eight genera were found prevalent in all studies with prevalence of 8.75%. These genera include *Acremonium Alternaria*, *Aspergillus*, *Chaetomium*, *Chrysosporium*, *Emericella*, *Penicillium* and *Chaetomium*.

Alternaria, Aspergillus, Chrysosporium, Emericella, Penicillium and Chaetomium were recorded as predominant isolates (Al-Bader et al. 2000; El-Said et al, 2009; Sallam and Al-Kolaibe, 2010).

In recent work, Al-Bader (2018) explained that (*Chaetomium* and Penicillium beside *Cladosporium*) were the highest occurrence% among 16 genera isolated from north of Iraq (Fig. 2).

Acremonium, *Alternaria*, *Aspergillus*, *Chaetomium*, *Chrysosporium*, *Emericella*, *Penicillium*, *Scopulariopsis*.

The results of close related works on fungi associated with wool (Table-1) recorded (70) fungal genera. Shtayeh et al.1989; Abdel-Gawad 1997; Al-Bader et al. 2000; El-Said et al. 2009; Sallam and AL-Kolaibe 2010). Although studies were temporally and spatially diverse; eight genera (8.75%) were found prevalent in all studies. Acremonium (1), Alternaria (2), Aspergillus (3), Chaetomium (4), Chrysosporium (5), Emericella (6), Penicillium (7), Scopulariopsis (8).

El-Said et al. (2009) mentioned that Aspergillus, Chaetomium, Emericella, Alternaria and Chochiobolus were predominant, while Sallam and AL-Kolaibe (2010) recorded (Chrysosporium, Aspergillus, and Scopulariopsis) as the highest occurrence genera. The genera (Aspergillus, Penicillium and Alternaria) showed a high prevalence (Al-Bader et al. 2000). In recent work, Al-Bader (2018) explained that Aspergillus and Penicillium beside Cladosporium were the highest occurrence% among 16 genera isolated from wool (Fig. 2).

Through an intensive work related to sheep and fungi interaction, the researchers mentioned remarkable results, such as recording a new species from animal's skin including *Microsporum cookie and Microsporum distortum* (Ajello 1959; di Menna and Marples 1952). Abdullah et al. (2000) isolated and identify a new species of Cephaliophora from shhep wool. A rare case of fungal growth inside sheep hydatid cyst was observed by Al-Bader et al. (2000).

The primary origin habitat of sheep wool mycobiota is soil. They can utilize various waste keratinous materials such as nails, hairs, horns, feathers, hooves, wool hairs, and stratum corneum.

Fungal genera (Table-1) showed a keratinophilic affinity, they are commonly isolated from soil by baiting method. Kumar and Kuaswaha (2021) used five types of keratinized substrate, human hair, cattle hair, human nail, horn, and feather. They recorded 32 fungal genera and *Chrysosporium* inhabited all types of baits.

Kumawa et al. (2019) recorded sixteen genera from semi-arid soil in Rajasthan, India, *Chrysosporium, Fusarium*, and *Aspergillus* showed the highest frequency%. While, Abdulla and Osman (2018) isolated ten genera, Aspergillus had the predominant frequency of 86.8%.

Nineteen species belonging to 11 genera were isolated. *Chrysosporium* and *Aspergillus* had high frequency (Eze et al. 2019). Six genera were diagnosed from soil samples in Libya, and *Aspergillus* was the highest frequency (Altayyar et al. 2016).

Fungi on sheep skin: an industrial impact view

Sheep wool is one of the most significant textile raw materials used for human commodities. The physical properties of wool fibers and their keratin content make them a target for keratinolytic and keratinophilic fungi. *Chrysosporium, Aspergillus*, and *Penicillium*, for example, can readily colonize wool hairs and destroy the structure of the fibers Soomro 2000). Moreover, fungi on sheep wool may be the reason for developing respiratory infections in tannery workers.

Also, biodeterioration is globally considered as one of the critical factors that can impair the properties of leather and the products made from them. During the industrial

Table 1: Fungal genera of sheep wool recorded in five studies (Middle-east countries)

Ref. No.	Shtayeh et al (1989)	Abdel-Gawad (1997)	Al-bader et al. (2000)	El-Said et al. (2009)	Sallam & ALKoliabe (2010)
1	*Acremonium*	*Acremonium*	*Acremonium*	*Acremonium*	*Acremonium*
2	*Alternaria*	*Alternaria*	*Alternaria*	*Alternaria*	*Alternaria*
3	*Arthroderma*	-	*Arthroderma*	-	-
4	*Aspergillus*	*Aspergillus*	*Aspergillus.*	*Aspergillus*	*Aspergillus*
5	-	-	*Botryotrichum*	*Botyotricum*	-
6	*Chaetomium*	*Chaetomium*	*Chaetomium*	*Chaetomium*	*Chaetomium*
7	*Cladosporium*	-	*Cladosporium*	*Cladosporium*	*.Cladosporiu*
8	*Chrysosporium*	*Chrysosporium*	*Chrysosporium.*	*Chrysosporium*	*Chrysoporium.*
9	-	*Chociobolus*	-	*Chociobolus*	-
10	*Drechslera*	-	*Drechslera*	-	-
11	*Emericella*	*Emericella*	*Emericella*	*Emericella*	*Emericella*
12	-	-	*Fusarium*	*Fusarium*	*Fusarium*
13	-	-	*Geotrichum*	-	*Geotrichum*
14	-	-	*Humicola*	*Humicola*	-
15	-	-	*Microascus*	*Microascus*	-
16	*Mortierella*	-	*Mortierella*	-	-
17	-	*Mucor*	*Mucor*	*Mucor*	-
18	-	*Nectaria*	-	*Nectaria*	-
19	*Paecilomyces*	-	*Paecilomyces*	*Paecilomyces*	-
20	*Penicillium*	*Penicillium*	*Penicillium*	*Penicillium*	*Penicillium*
21	-	*Rhizopus*	*Rhizopus*	*Rhizopus sp.*	*Rhizopus*
22	*Socpulariopsis*	*Scopulariopsis*	*Scopulariopsis*	*Scopulariopsis*	*Scopulariopsis*
23	-	*Stachybotrys*	*Stachybotrys*	-	-
24	*Trichoderma*	-	*Trichoderma*	*Tricoderma*	-
25	-	*Tritirchium*	-	-	*Tritirchium*
26	-	*Ulocladium*	*Ulocladium*	*Ulocladium*	-
27	*Sterile hyphae*	-	*Sterile mycelia*	*Sterile mycelia*	*Sterile mycelium*
28	*Candida*	-	*Candida*	-	*Candida*
The isolated dermatophytes and the rare genera					
29	*Aphanoascus*	*Monodicytes*	*Cephaliophora*	*Circinella*	*Eurotium*
30	*Botrytis*	*Myrothecium*	*Exserohilum*	*Curvelaria*	*Thermoascus*
	Ctenmyces	*Pleospora*	*Papulospora*	*Gilmanella*	*Thermomyces*
31	*Doratomyces*	*Setosphaeria*	*Phoma*	*Mycospherella*	*Trichophyton*
32	*Exophiala*	*Trichophyton*	*Preussia*	*Nigrospora*	-
33	*Giliocladium*	-	*Spedonium*	*Setosphaeria*	-
34	*Harosporum*	-	*Thielavia*	*Torula sp.*	-
35	*Memnoniella*	-	-	*Trichophyton*	-
36	*Microsporum*	-	-	-	-
37	*Monilia*	-	-	-	-
38	*Monoascus*	-	-	-	-
39	*Phialophora*	-	-	-	-
40	*Staphylotricum*	-	-	-	-
41	*Trichocladium*	-	-	-	-
42	*Trichophyton*	-	.-	-	-
43	*Trichosporrella*	-	-	-	-
44	*Trichothecium*	-	-	-	-
45	*Verticillium*	-	-	-	-
46	*Wallemia*	-	-	-	-

Fig. 1: Free grazing of sheep-Iraq.

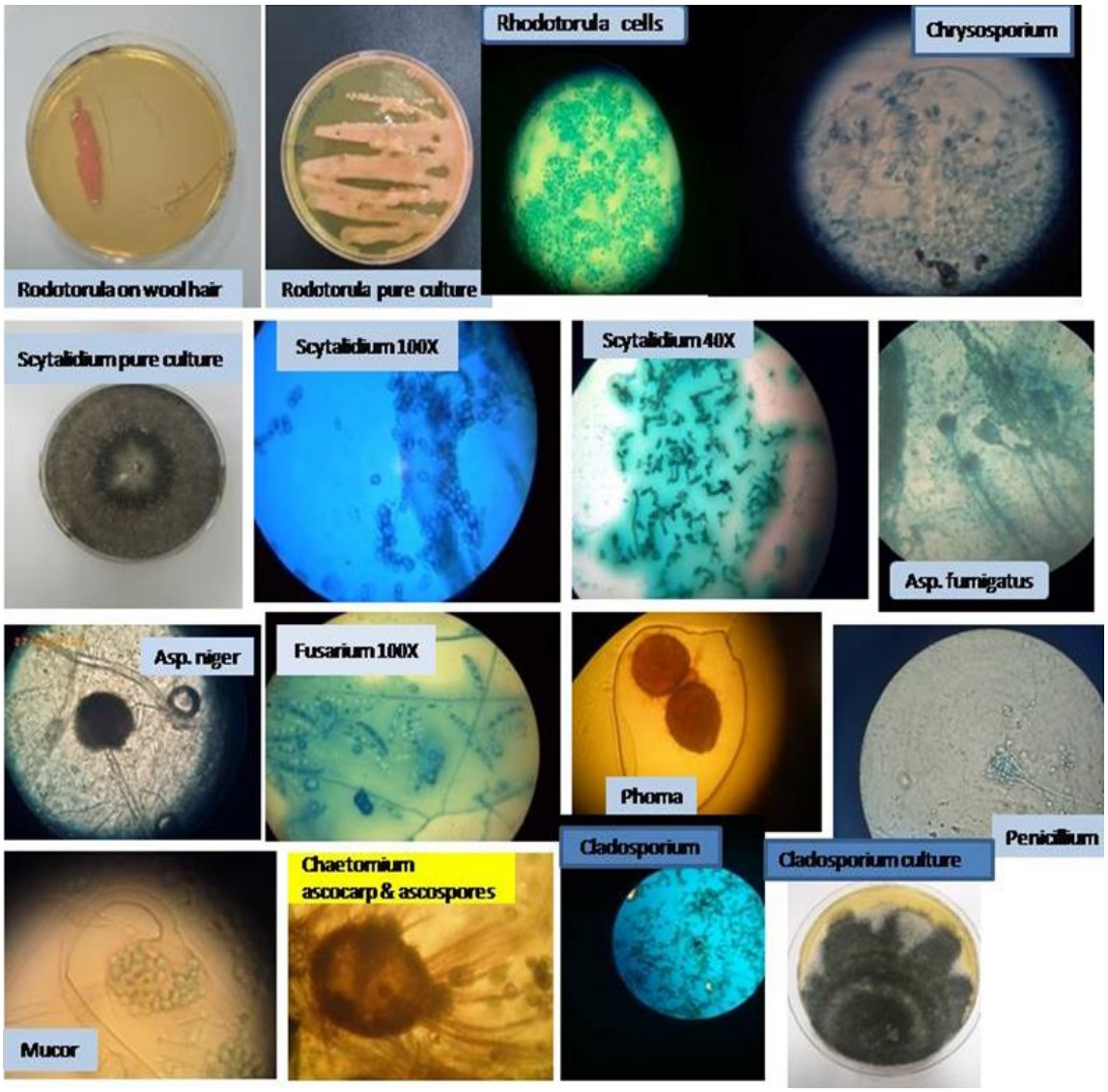

Fig. 2: A number of fungi isolated from wool hair (Al-Bader 2018).

processing of sheepskin, several fungi tolerate the high NaCl concentrations (20–30%) w/v. *Aspergillus*, *Penicillium* and *Alternaria* were isolated from salted sheep skin (Ozdilli et al. 2007). Fungi were isolated during all processing stages. From an Indian factory of the leather industry, fourteen genera were isolated with *Aspergillus* and *Penicillium* as predominant fungi (Srinath et al. 2002). The active protease producer fungi deteriorate processed leathers. Penicillium sp., for example, can grow and multiply during processing stages and cause significant effects on leather quality (Wilson 2005). During the drying stage of leathers, fungi may also develop because of the favorable temperature and humidity (Ozgunay et al. 2010). Generally, fungi are the most causative agents for industrially treated leather damage. They include *Penicillium, Aspergillus,*

Mucor, Rhizopus, Paecilomyces, and *Trichoderma* which survive during tanning conditions. They utilize the tanning agent depending on tanninase secretion. *Candida albicans* and *Staphylococcus aureus* showed poor and moderate growth on finished leather samples (Orlita 2001). Depending on the previous information, the leather products (e.g., footwear) will be the source of fungal infection as tinea pedis. To prevent or reduce mycoses from a different leather product, they should be treated with a suitable fungicide.

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CHAPTER 35

PATHOGENESIS AND PREVENTION OF ASCITES SYNDROME IN BROILERS

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INTRODUCTION

The main consequences of this type of cardiopulmonary failure are systemic arterial hypoxia (deficient arterial oxygen saturation) and pulmonary hypertension and the terms pulmonary hypertension, pulmonary hypertension syndrome, and ascites syndrome are often used interchangeably. Systemic hypertension increases important hematological parameters such as red blood cell count, hemoglobin level, and mean red blood cell count. Pulmonary hypertension increases filling cell (PCV) volume and blood viscosity, resulting in right ventricular (RV)-specific hypertrophy, which is a stress response to elevated pulmonary arterial pressure, and blood relative increases in RV mass as a percentage of PC or ventricular occupancy. Percentage calculation of total mass (TV). The RV:TV ratio for conventional broilers is typically less than 0.25. The main factors contributing to ascites in commercial broilers are early exposure to low temperatures (low-neutral temperatures) and rapid growth to maximize the genetic potential of the broiler through an energy and protein-rich diet. pulmonary hypertension syndrome, broiler ascites, heart failure syndromed and altitude sickness (Liu 2016), it is characterized by increased pulmonary artery pressure, increased pulmonary vascular resistance, right ventricular hypertrophy (HVR), and ascites (Liu 2016). It is a metabolic disease caused by the joint action of multiple pathogenic factors. The main pathological changes of broiler chicken ascites syndrome are accumulation of a large amount of pale yellow serous fluid in the abdominal cavity and pericardial cavity, with the right ventricular thickening and hypertrophy. The disease was first reported in the United States in 1946, and also began to be reported in North America in 1958. Since then, the disease has quickly become a common phenomenon in the chicken industry in high altitude and cold areas, and since 1896, the disease has been reported in many provinces in China(Liu 2016). In the process of livestock and poultry breeding, the disease is most often manifested in groups, and it is most common in 2-3 week old chicks, which shows its incidence rate is as high as 5% or more. The peak of death is more common at 4-8 weeks of age, and the recovery period is still accompanied by death, which can bring huge economic losses to the global breeding industry every year. Therefore, AS is considered to be one of the important factors restricting the rapid development of the chicken industry (Hormozi et al. 2017; Shi et al. 2017; Parveen et al. 2020). Therefore, in view of the huge impact of this disease on the global stockbreeding, this article will discuss this disease from the aspects of the causes of the disease, clinical symptoms, pathological changes, related research results, prevention and control measures, and aims to provide theoretical guidance for the prevention and treatment of ascites syndrome in broilers.

Since ambient temperature, energy level, and growth rate directly affect the metabolism of bases, which in turn affects the oxygen needed by the animal, it can reduce oxygen demand and ascites, reduce the growth rate of chickens by cutting, and create them under thermoneutral conditions.

Contributing Factors for Ascites Syndrome

Genetic Factors

Grow Too Fast

A large number of studies have shown that broiler ascites syndrome mostly occurs in fast-growing broiler strains, such as Avian broiler, AA broiler, Ross broiler, and Sanhuang chicken. In the long-term genetic selection process, the growth rate of broilers is accelerated and the metabolism is vigorous, but their cardiopulmonary function has not been improved synchronously (Hormozi et al. 2017). Therefore, in the process of its metabolism, the consumption of oxygen can easily reach the critical point of cardiopulmonary oxygen supply function, and the body is easily in a state of hypoxia. In addition, the anterior vena cava and pulmonary capillaries of broilers are underdeveloped, which is easy to cause pulmonary vein congestion. Therefore, it is very prone to pulmonary hypertension, right heart failure and even ascites syndrome.

Limited Lung Capacity

Since the lungs of poultry are close to the back of the rib cage, the dorsal side of the lungs is embedded in the sternum ribs, which limits its expansion, and in the long-term breeding process, the ratio of heart, lung and body weight of broilers is getting smaller and smaller. When the blood flow increases, it is restricted by the lungs, and there are few spare capillaries to cope with the increased blood flow. The capillary filling degree is high, and the pulmonary vascular resistance increases, so it is easy to cause pulmonary hypertension.

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Environmental Factor

High Altitude

Ascites syndrome in broilers was first discovered in high altitude areas, and altitude is an important cause of ascites in broilers. In high-altitude areas, the oxygen is thin, the partial pressure of oxygen is low, and the oxygen concentration in the air is low, which leads to the decrease of blood oxygen saturation in the blood of broilers, the increase of red blood cells, and the increase of blood viscosity, which can easily lead to the occurrence of ascites syndrome in broilers.

Poor Ventilation

In the cold spring and winter seasons, some chicken houses use coal stoves for heating and heat preservation, which increases the consumption of oxygen in the chicken houses. In the state of oxygen and harmful gas stimulation, it is easy to cause damage to the lung function, increase the burden on the heart, cause heart failure, and cause lesions to other organs such as the liver, thereby causing ascites.

The Cold Weather

The cold weather can increase body heat production by enhancing metabolism, resulting in increased oxygen demand and compensatory increase in cardiac output in broilers; in addition, cold can also lead to increased hematocrit and blood viscosity, as well as the formation of pulmonary hypertension.

Poor hygiene

If the manure in the chicken house is not cleaned in time, it will ferment and spoil for a long time, increase the concentration of toxic and harmful gases, and increase the probability of broiler chickens suffering from ascites syndrome.

Feed Factor

Feed factors mainly include the following aspects: 1) Broilers eat high-energy, high-protein or pelleted feed, which increases growth rate (Kalmar et al. 2013), boosts the body demand for oxygen and promotes the occurrence of ascites syndrome in broilers (Liu et al. 2017). 2) High levels of sodium ions in feed or drinking water can lead to increased blood volume and increased incidence of ascites syndrome in broilers (Shi et al. 2014). 3) Deficiency of vitamin E or lack of trace elements such as phosphorus and selenium can also cause ascites syndrome in broilers. 4) Excessive daily feed intake or excess nutrition of broilers can cause excessive growth of broilers, relative hypoxia of the body, and increase the incidence of ascites (Hasanpur et al. 2016).

Disease Factor

Respiratory Disease

Early respiratory tract injury caused by hatching factors of respiratory diseases, acute respiratory tract injury caused by virus, damage to lungs and air sacs caused by climate or management conditions, etc., all directly or indirectly affect the oxygen absorption capacity of broilers, causing tissue hypoxia, which in turn leads to blood and blood in broilers. Decreased oxygen saturation induces ascites syndrome in broilers.

Toxic Disease

When broilers eat moldy feed or encounter aflatoxin-contaminated feed, it will cause damage to the heart, lungs, liver and other parenchymal organs, and then the disease will occur (Liu et al. 2017).

Nutritional Metabolic Disease

Broiler chickens suffering from rickets due to early nutritional deficiencies have a narrow thorax and limited space for lung expansion. They cannot fully utilize the function of the lungs when breathing, resulting in hypoxia, which is easy to cause ascites syndrome.

Other Factors

Improper medication or long-term use of sulfonamides can cause liver and kidney damage in broilers (Tisljar et al. 2011; Varmaghany et al. 2015), increase vascular permeability, increase the burden on the heart, and cause ascites syndrome (Hassanzadeh et al. 2008).

Pathogenesis

Many scholars at home and abroad have conducted in-depth exploration and research on the pathogenesis of AS, and a more consistent view is that the occurrence and development of this disease is closely related to the formation of pulmonary hypertension (PH). That is, PH is the main reason for the occurrence and development of AS. The increase of blood flow through the lungs and the increase of pulmonary vascular blood flow resistance are the main factors leading to PH. Hypoxia is the most important trigger for ascites syndrome in broilers, as well as reduced blood capacity in the capillaries of the lungs. Any factor that can increase the body oxygen demand can promote the occurrence of PH. Oxygen is a vital component in the energy metabolism process and is required for thermoregulation, activity, growth and any form of energy use in broilers. Studies have shown that chickens prone to ascites syndrome require more oxygen than chickens not prone to ascites syndrome due to their high metabolic activity and low venous partial pressure of oxygen, and the oxygen partial pressure in arterial blood is higher than that of chickens. There was no significant difference in carbon dioxide partial pressure between the two chickens. Higher partial pressure of carbon dioxide in the venous blood of broilers can increase pulmonary arterial pressure. And studies have shown that the content of carbon dioxide in venous blood is related to genetic background. Elevated carbon dioxide in venous blood in domestic chicks is a predisposing factor for right ventricular hypertrophy and ascites syndrome. Chickens with low FCR have lower metabolic heat production and therefore require less oxygen. Low oxygen requirements and low feed conversion ratios can result in reduced thyroid hormone activity and reduced susceptibility to ascites syndrome, so an imbalance in oxygen demand can result in insufficient oxygen supply (hypoxia). This imbalance may be caused by endogenous and exogenous factors, and the two factors have a synergistic enhancement effect, resulting in additional or even synergistic

effects leading to increased sensitivity to ascites syndrome. In the case of absolute and relative hypoxia, the body's blood oxygen saturation decreases, and the cardiac output compensatory increases. At the same time, in order to increase the oxygen supply to the tissue, the kidney secretes a hormone that stimulates the production of red blood cells and hemoglobin, which promotes the production of red blood cells and hemoglobin. A large number of red blood cells can enhance the oxygen transport capacity of the blood, so that the hematocrit, the number of red blood cells and the value of hemoglobin increase, the specific viscosity of whole blood increases, and the deformability of red blood cells decreases, and all of these are compensatory physiological effects of the body to adapt to hypoxia.

With the increase of PCV and BRV, the cardiac load increases, the right ventricular wall is hypertrophied, and the cardiac chamber volume increases, which leads to right heart failure, chronic hypoxia, and stimulates pulmonary vascular endothelial cells to produce and release mediators such as endothelin and endothelin, etc., which trigger the proliferation of smooth muscle cells, resulting in thickening of the vessel wall and the formation of PH; and further cause excessive right ventricular afterload, and the body further exacerbates the vicious circle of relative hypoxia. With the development of the disease, heart failure, serious obstruction of venous blood return, congestion and swelling of internal organs, increasing venous pressure, and fluid exudation to form ascites.

It has long been found that high dietary salt content can cause ascites syndrome and edema in chickens and turkeys, especially young chickens are more sensitive than adult chickens. Toxic components in poisoning, Na^+ in any source of diet can enhance the toxicity of Na^+, due to low serum osmolarity and underdeveloped kidneys in chicks, it is difficult for them to regulate their own serum Na^+ levels. High sodium causes increased PCV, decreased red blood cell deformability, and increased blood volume, resulting in increased blood viscosity, pulmonary vascular remodeling, and increased pulmonary capillary resistance to blood flow, resulting in PH. The same is true for high cobalt loads, which can affect blood viscosity in broilers and increase the incidence of AS. Studies have shown that the weight ratio of the right ventricle to the whole ventricle is significantly increased in chickens with ascites syndrome. In the occurrence and development of broiler ascites syndrome, pulmonary hypertension precedes right ventricular hypertrophy, and the two promote each other and strengthen together. Many scholars believe that the pathological basis of ascites syndrome is hepatic lymphatic circulation disorder. Increased production and obstruction of hepatic venous return are one of the most important factors for the formation of ascites in broilers. Due to changes in lymphatic circulation, further changes in the venous circulatory system are caused, resulting in ascites syndrome in broilers. The rapid growth rate of modern broilers makes them very sensitive to various stresses in the internal and external environment. The rapid growth of chickens and excessive metabolic heat production may be one of the important reasons for the lesions of the thoracic duct.

Clinical Symptoms

Sick chickens are lethargic, have a loss of appetite, and have disheveled feathers; the abdomen is obviously enlarged and drooping, the skin of the abdomen becomes thinner and brighter, and there is a sense of fluctuation when touched by hand (Luger et al. 2003), and the pale yellow liquid can be drawn out with a syringe; difficult breathing, unwilling to stand, slow movement, and drooping wings. In some chickens, cyanosis of combs and beards can also be seen, accompanied by diarrhea; sick chickens often die 1 to 3 days after the occurrence of ascites, and sometimes the sick chickens suddenly fall to the ground and die, especially when subjected to strong external stimulation and stress.

Pathological Changes

Necropsy revealed a large amount of fluid containing fibrinous translucent jelly in the abdominal cavity, which was clear yellowish brown; pericardial effusion increased, the heart volume increased significantly, the right heart was dilated and hypertrophied, filled with blood, and the myocardium was thinned; severe lung stasis blood, edema, blood-red fluid with small bubbles can be seen after incision; liver edema, blood stasis, hard texture, sometimes brittle and easy to rupture; kidney enlargement, blood stasis, often white urate deposition; intestinal wall thickening, blood stasis, chest muscle, leg muscle blood stasis, and systemic organs are accompanied by different degrees of blood stasis (Hassanzadeh et al. 2004).

Preventive and Therapeutic Measures

Precaution

Selective Cultivation

The chicks hatched from the eggs produced by breeders around 28 weeks of age are relatively small in terms of individuals and organs at 1 day of age, and different breeds of broilers have different susceptibility to ascites syndrome. The varieties with better development of lungs and other organs have a certain role in preventing the occurrence of ascites syndrome (Luger et al. 2001).

Environment

During the breeding process, attention should be paid to the ventilation of the chicken house, so that harmful gases such as carbon dioxide and ammonia and dust can be discharged in time to ensure sufficient oxygen, reduce environmental stimulation, and reduce the load on the heart and lungs; in the cold season, pay attention to heat preservation, reduce the probability of hypothermia-induced ascites syndrome in broilers; control the light time to slow down the growth rate of broilers within 4 weeks (Hasanpur et al. 2016). Try to keep males and females separately to meet different metabolic needs; adjust the stocking density reasonably, clean up excrement in time, establish a strict disinfection and epidemic prevention system, reduce the occurrence of respiratory diseases and other diseases, and reduce stress (Hasanpur et al. 2016).

Feed

Reasonable feeding restriction should be carried out in the early stage and to control the growth rate of broilers, feed low-energy and low-protein diets to reduce oxygen demand; prevent excessive sodium intake, sodium bicarbonate can be

Table1: Drug treatment of ascites syndrome in broilers

Drug classification	Medicine	Dosage	Treatment effect
Diuretics	1% furosemide injection	intraperitoneal injection 0.3ml/time	Symptomatic treatment to relieve
	Hydrochlorothiazide	mix feeding bid 4-5ml/chick	symptoms of chicken flocks
Antibacterials	0.05%penicillin procaine	Injection after aspiration of peritoneal fluid	
	Gentamicin	0.2ml-0.3ml/time	
Hepatoprotective drugs	vitamin C	Drink	
Cardiotonic	10% Sodium coffee	Im 0.3ml/time	
Antidote	Rhubarb Soda Chips	Po 1piece/chick	
Other	urease inhibitor	mix feeding	
	Sodium Selenite.	Ih	

Table 2: Chinese medicine prescription for broiler ascites syndrome (g)

Prescription one		Prescription two		Prescription three	
Drug name	Dose	Drug name	Dose	Drug name	Dose
Atractylode	20	Atractylodes	20	Porcine	20
Mutong	30	Poria	20	Guizhi	20
Porcine	30	Alisma	20	Mulberry bark	30
betel nut	30	pawpaw	20	Alisma	30
Citrus aurantium	30	jujube	20	tangerine peel	30
rhubarb	40	Atsushi	20	psyllium	30
Alisma	40	Muxiang	20	big belly	30
tangerine peel	45	ginger peel	20	Mutong	30
Poria	45	dried ginger	20	Poria	60
Atractylodes	45	Mulberry bark	20	Astragalus	60
Bacteria	45	big belly	20	raisins	60
green leather	45	gentian	40		

used instead of sodium chloride as a sodium source; phosphorus levels should not be too low, and selenium and vitamin E content should be sufficient and vitamin C can also be added to the feed to improve the anti-stress and disease resistance of broilers. Reduce the use of drugs that are harmful to the heart and lungs, and do not feed spoiled and moldy feeds; in addition, adding tanshinone IIA to the feed can effectively prevent the occurrence of ascites syndrome in broilers (Hormozi et al. 2017).

Treatment

Physiotherapy: Use a sterile syringe to extract the peritoneal effusion of sick chickens.

Medical treatment: See Table 1 and 2. Once broiler ascites syndrome occurs, the cure rate is low, and the treatment principles of symptomatic treatment, diuresis and detoxification, liver protection and kidney protection are mainly adopted, and the effect of traditional Chinese medicine treatment is relatively good.

Chicks were vaccinated against Newcastle disease and infectious bronchitis by intranasal instillation at 7 days of age, and a booster immunization was given at 21 days of age to reduce the incidence of ascites caused by respiratory diseases. Bacterial pathogens such as *Escherichia coli*, Mycoplasma, Streptococcus and other bacterial pathogens can be prevented by broad-spectrum antibiotics. It is recommended to use 2.5% cefquinome sulfate injection. Due to the short time to market, the drug resistance rate is extremely low, and the effect is very ideal. Before 15 days of age, intramuscular injection is carried out according to the dosage of 4000 chickens/100ml, and 15 to 30 days old according to the dosage of 2000 chickens/100ml. It can be doubled on this basis. After 30 days of age, due to the risk of drug residue exceeding the standard, it is not recommended to use chemical drugs. Traditional Chinese medicines that clear heat and benefit the lungs can be used for conditioning to prevent drug residues. Montmorillonite was added to the feed at a dose of 0.1% to reduce the damage of mold. For chicken farms with frequent occurrence of this disease, VC can be added to the drinking water according to the actual dosage of the drug at a concentration of 10g/100kg of water, and drink continuously for 3 days to quickly improve the symptoms. For high mortality flocks, an additional 1% linseed oil can be added to the feed to control mortality quickly.

Summary

Broiler chicken ascites syndrome is caused by the interaction of internal and external environmental factors. There is no particularly effective treatment for the disease, focusing on prevention. Therefore, in the breeding process, it is necessary to strengthen feeding management, pay attention to environmental hygiene, reasonably cooperate with the diet, reduce the stress on the flock, and provide an excellent growth environment for the flock.

Research Results

Related Polyclonal Antibody Preparation

The available literature shows that the theoretical circles conduct research from the following aspects. For example, studies have shown that the RPS14 gene (ribosomal protein S14) maintains normal physiological functions *in vivo* by regulating ribosome biosynthesis and translation of essential proteins, so some scholars focused on the preparation of RPS14 polyclonal antibody, and carried out further research on the application of this polyclonal antibody in broiler ascites syndrome, the author aimed to investigate the possible role of RPS14 in chicken ascites syndrome (BAS). They were able to generate polyclonal antibodies against RPS14 and studied the localization and expression of the RPS14 protein in key tissues of various animals. Researchers were able to generate polyclonal antibodies against RPS14 and study the localization

and expression of RPS14 protein in key tissues of various animals (Wideman et al. 1999). In addition, some scholars focus on the role of some important genes in diseases. Some scholars focus on the role of MEOX2 gene in the development of ascites syndrome in broilers. The starting point is that MEOX2, as a transcription factor with important regulatory functions in the proliferation and differentiation of vascular smooth muscle cells and vascular endothelial cells, may inhibit the occurrence of AS by controlling the angiogenic phenotype, which has important research significance (Buys et al. 1999). In this experiment, they used PCR amplification method to obtain MEOX2 gene, used TA cloning technology to obtain pUCm-T-MEOX2 recombinant plasmid, and carried out bioinformatics analysis on it, aiming to provide a reference for the efficient prokaryotic expression of MEOX2 gene and protein purification, and provide a strong basis for the connection with pulmonary hypertension, and provide a reference for the regulation and prevention mechanism of AS.

Peroxisome proliferator-activated receptor alpha (PPARα) plays a key role in regulating metabolic homeostasis, inflammation, cell growth and differentiation, while PPAR (peroxisome proliferator-activated receptor) functions as lipid metabolism and important regulators of glucose metabolism have been extensively studied. To this end, the scholar reported the prokaryotic expression and purification of chicken PPARα protein, and successfully produced polyclonal antibodies against recombinant PPARα protein. PPAR is a member of the nuclear receptor superfamily consisting of PPARα, PPARβ/δ and PPARγ. PPARs regulate many metabolic pathways by activating endogenous ligands (such as fatty acids and their derivatives) or synthetic agonists that bind to regulatory elements of the PPAR response, heterodimerize the retinol X receptor, and control many genes involved in adipogenesis and lipids Metabolism, maintenance of inflammation and metabolic homeostasis. PPARα, the first member of the PPAR family to be discovered, is a transcription factor mainly expressed in energy-consuming tissues such as liver, kidney, heart, muscle and some inflammatory and immune cells, among which PPARα is used to control the core of FS. Numerous experiments have shown that activation of PPARα may enhance the protective effects of fatty liver, inflammation and fibrosis.Some scholars showed that PPARα deletion increases fatty liver and inflammation in PPARα null mice when fed a high-fat diet (McGovern et al. 1999; Luger et al. 2003). This mechanism is based on the physical interaction between the transcription factors PPARα, NF-κB, and AP-1, resulting in the inhibition of their transcriptional activity, thereby reducing the expression of these target genes. Regarding energy metabolism, PPARα is a therapeutic target for energy metabolism disorders. In type 2 diabetes, PPARα agonists are used as oral hypoglycemic agents. Other researchers have also reported severe and persistent hypoglycemia in mice after PPARα was knocked out on an empty stomach. Furthermore, after liver-specific knockout of the PPARα gene, mice developed hepatic lipid accumulation, resulting in fatty liver.

However, the use of prokaryotic expression system to clone and express PPARα protein in the production of chicken polyclonal antibody has not been reported yet. Furthermore, the potential role of PPARα in chicken pathogenesis has been poorly studied. In this study, a large number of PPARα polyclonal antibodies were successfully prepared, and the generated antibodies were used to localize the endogenous PPARα protein to the liver, kidney and hypothalamus. The relationship between PPARα and lipid metabolism has been investigated, leading to future research into the pathogenesis of dyslipidemia.

Judging from the data collected in this subject, many scholars have done a lot of research on the clinical application of polyclonal antibody to broiler ascites syndrome. As to Gu (Gu et al. 2021), she successfully developed RPS14 polyclonal antibody, and verified the efficacy of polyclonal antibody on broiler chicken ascites syndrome in clinical practice, which laid a solid foundation for conquering the disease. As to Huang (Huang et al. 2020), she carried out gene cloning and corresponding biological information analysis of MEOX2, which laid a solid foundation for the preparation of subsequent related antibodies, and then provided a strong basis for the pathological detection of pulmonary artery remodeling and gene drug therapy for broiler ascites syndrome.

Tanshinone IIA

Tanshinone is a kind of traditional Chinese medicine with wide clinical application, belonging to Lamiaceae, and it was first recorded in the classic Chinese medical book "Shen Nong Baicao Jing", and it was called "abandoned horse grass"; in "Compendium of Materia Medica" (Hu et al. 2017). The active substances in Salvia miltiorrhiza are mainly divided into two categories: fat-soluble and water-soluble. The fat-soluble substances are mainly o-quinone-type diterpene quinone compounds, and most of their skeletons have three- or four-membered carbocyclic o-quinone or p-quinone structure. The water-soluble ones mainly include polyphenols, danshensu, protocatechuic aldehydes, acids, etc. TIIA is a diterpene quinone lipid-soluble active ingredient extracted from its roots or stems. After long-term pharmacological and clinical treatment observation and research, it has been proved that TIIA has the functions of scavenging oxygen free radicals, anti-tumor, anti-oxidation, antibacterial and anti-inflammatory, calcium antagonism, improvement of microcirculation, protection of vascular endothelial cells, anti-atherosclerosis, prevention of angina pectoris and myocardial infarction, and anti-cancer pharmacological effects. Especially in the role of cardiovascular system, it has the ability to dilate arteries, increase myocardial contractility, etc., and is a commonly used traditional medicine for clinical treatment of heart disease. Several studies have shown that Tanshinone IIA plays an extremely important role in anti-tumor, anti-oxidation, and protection of the heart. In some studies, scholars took AA broilers as research objects, and explored the risk of ascites syndrome by detecting the production performance of broilers, analyzing the bioinformatics of pulmonary artery gallus transcriptome, pathological changes of various tissues and organs, and measuring blood-related biochemical indicators. The differentially expressed genes of broiler pulmonary artery and the changes of the metabolic pathways involved, monitored the prevention and treatment effect of TIIA on broiler ascites syndrome, and provided a certain basis for further research on the pathogenesis and prevention of broiler ascites syndrome (Wang et al. 2013). In addition, in pulmonary hypertension, mild inflammation often occurs, and mild inflammation is related to polycyclic aromatic hydrocarbons, and infiltrating inflammatory leukocytes are found in the internal organs of polycyclic aromatic hydrocarbons, and some inflammatory cytokines are associated with polycyclic aromatic hydrocarbons, such as IL-6、IL-1β、NF-κB and P38.

Therefore, some scholars used high-salt drinking water to simulate PAHs and evaluated the relationship between tanshinone IIA (TIIA) and PAHs.

The available literature shows that the theoretical circles have mainly carried out related research on the following aspects:1). To study the effect of Tanshinone IIA (TIIA) on pulmonary arterial hypertension (PAH) in broilers. 2) The prevention and treatment effect of TIIA on ascites syndrome in broilers was monitored, which provided a basis for further research on the pathogenesis and prevention and treatment of ascites syndrome in broilers. These studies have achieved excellent results, and laid a solid foundation for the subsequent conquest of broiler chicken ascites syndrome. Scholars also have a very positive attitude towards the clinical application of tanshinone in this disease (Luger et al. 2001; Wang et al. 2011; Tan et al. 2011; Wang et al. 2012).

Pulmonary Artery Remodeling

Ascites syndrome (AS), also known as pulmonary hypertension, is a global metabolic disease. The transformation of pulmonary artery is a key link in the formation and development of AS. The exact relationship of pulmonary artery mRNAs and SNPs in regulating AS progression is unclear. Therefore, the scholars obtained pulmonary artery tissue from pathological sections and pathological anatomical observations of diseased chickens (Yang et al. 2016). SNP, inDel, and mRNA data were analyzed using GATK and ANNOVAR software to examine previously reported SNP loci for 985 genes (437 elevated, 458 elevated). Pathological examination showed that there was a lot of yellow fluid in the abdominal cavity and pericardium in this group, the cardiac index and hematocrit of ascites were significantly changed, and the pulmonary artery was changed and thickened. Heart sections show vacuolar degeneration of myocytes and disintegration of myofibers. In addition, ALDH7A1, IRG1, GGT5, IGSF1, DHX58, USP36, TREML2, SPAG1, CD34 and PLEKHA7 are closely related to the pathogenesis of AS progression to pulmonary artery remodeling. In conclusion, our study further elucidates the molecular mechanism of pulmonary artery remodeling in AS progression (Yang et al. 2016). Recent studies have demonstrated that the impact of pulmonary vascular remodeling on PAH development involves a complex multifactorial process in which endothelin-derived vasoactive molecules, such as endothelin-1, vascular endothelial growth factor, and insulin-like growth factor-II, altered the growth (Hassanpour et al. 2009; Hamal et al. 2012; Rabinovitch et al. 2014). These molecules are increasingly recognized as key elements and potential therapeutic targets for the treatment of PAH. Furthermore, these protein-coding genes contain at least one conserved microRNA-binding site (miRNA) and a large number of unreserved sites; most protein-coding genes are likely to be under the control of miRNAs. However, SNPs of target genes may alter miRNA expression due to altered mRNA levels. Therefore, it has also been suggested that SNPs of disease risk-related miRNA target genes (mRNAs) may play a role through their effects on miRNAs.

This study now provides a unique place to assess whether SNP loci and miRNA target genes affect miRNA expression in PAH broilers, leading to AS. To determine whether specific SNPs alter miRNA expression levels, we used PAH and tissues other than PAH to assess expression differences between genotypes (Yang et al. 2016). However, since SNPs can alter miRNA expression levels in PAH and non-PAH tissues alike, this association does not necessarily lead to the potential risk of PAHs in broiler AS. To determine whether SNPs are associated with PAH through miRNA regulation, we sought to understand the regulatory mechanisms by which miRNAs and SNPs are associated with target genes that alter the pulmonary vasculature remodeling phenotype. Finally, we assessed the SNPs involved in miRNA expression and the potential risk of AS in PAH broilers.

In the process of disease development, the issue of remodeling and thickening of pulmonary arteries has also attracted the attention of scholars at home and abroad. Several researchers have investigated the aberrant expression of microRNAs and mRNAs in pulmonary arterial transformation in ascites syndrome. Previous studies have shown that miRNAs play important roles in the biology of developmental timing, differentiation, cell death, proliferation, and metabolism (Liu et al. 2017). Recently, dysregulation of miRNAs has been shown to be associated with many cardiovascular diseases, including pulmonary hypertension (Zhou et al. 2015). In this study, researchers obtained pulmonary artery tissue from AE chickens and chickens without AE and performed miRNA sequence analysis, miRNA-mRNA association analysis, and pathological examination. Among known and novel miRNAs, 29 miRNAs were found to be significantly differentially expressed, and the regulation of miR-155, miR-23b-3β, miR-146b-5β, and miR146b-3β was closely related to arterial pathogenesis. transformation. in the process of AS. The miRNA-mRNA association analysis showed that these 29 differentially expressed miRNAs regulated 162 differentially expressed target genes. Of these, 20 miRNAs were associated with 18 predicted target genes that appear to be involved in pulmonary artery remodeling, mainly involved in three broad physiological processes: the hypoxia sensing response (HIF1α, NHE1, STAT5 and STAT3), endothelial permeability dysfunction (CD44, TRAF2, CDK2AP1, LZTFL1, JAZF1, PEBP1, LRP1B, RPS14 and THBS2) and inflammation (MEOX2, STAT5, STAT3, IRF8, MAP3K8, IL-1BETA and TNFRSF1B). In this study, the pathological transformation of the pulmonary artery in AS broilers continued. In conclusion, this analysis further elucidates the molecular mechanism of lung remodeling in AS progression (Liu et al. 2017). In addition, some researchers have investigated the issue of abnormal miRNA and mRNA expression during pulmonary artery remodeling in ascites syndrome. They believe that the transformation of the pulmonary artery is a critical step in the development of AS. To further elucidate the molecular mechanism of pulmonary artery remodeling, they obtained pulmonary artery tissues with and without AS from chickens, and performed miRNA sequence analysis, miRNA-mRNA association analysis, and pathological examination. Twenty-nine significantly differentiated miRNAs were found among known and novel miRNAs, of which 18 were up-regulated and 11 were down-regulated. Through GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analyses, their predicted potential targets included a wide range of functional clusters. The increase of miR-155, miR-23b-3β, miR-146b-5β and miR146b-3β is closely related to the progression of AS in the pathogenesis of lung remodeling.

Conclusions

Although broiler chicken ascites syndrome is a disease that is difficult to overcome and has a huge impact, according to the

research results collected so far, different scholars have studied the disease from different angles and approaches to overcome the disease. Overall, the trend is positive, it's only a matter of time, and the academic community is quite positive about the disease's prospects.

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