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ANIMAL HEALTH PERSPECTIVES

Editors:

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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

POTENTIAL IMPACT OF REFORESTATION ON THE EMERGENCE AND RE-EMERGENCE OF PARASITIC DISEASES

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INTRODUCTION

Climate change has the potential to influence the proliferation and emergence of different types of vectors and pathogens, which ultimately affects the host-parasite relationships and their distribution (ESAP 2009). Climate change can affect animal health directly and indirectly by the transmission of diseases that are humidity or temperature dependent along with vector-borne and rodent-associated diseases (Chauhan and Ghosh 2014; Grace et al. 2015). Various species of animals including sheep, goats, cattle and horses are influenced by a variety of nematode infections and these are primarily affected by climatic conditions and specifically temperature changes (Kimaro and Chibinga 2013). A strong relationship has been observed between the epidemiological conditions of disease causing agents and climate conditions. The abundance and distribution of disease vectors are known to be influenced by climate factors like temperature, humidity and precipitation and they ultimately affect the pathogen carrying capacity, arthropod vectors, population dynamics and behavior of helminths (ESAP 2009).

Climate change has the oldest history with seven cycles of glacial retreat and advance over the last 650,000 years (Shaftel et al. 2019). Current warming trends and climate changes are in progress at an unprecedented rate due to increased human activities (Shaftel et al. 2019; IPCC 2021). According to an indication of the intergovernmental panel on climate change (IPCC), there was a 0.99°C increase in the mean surface temperature of the globe during the period 2001-2020 and a 1.09°C increase during 2011-2020 as compared to the preindustrial period of 1850-1900. This increase in the rate of temperature has been doubled during the last 50 years. The IPCC has further predicted a 0.2°C increase in temperature per decade and this is expected that by the end of the 21st century; the mean temperature will increase between 1.4°C and 4.4°C (IPCC 2021).

According to an estimate by United Nations, 55% of the world's population lives in urban areas with the lowest percentage (43%) in Africa and the highest percentage (83%) in North America. Urban areas have become double since 1992 (IPBES 2019), thus there are increasing problems associated with climate change affecting the health of humans and domestic animals in urban and peri-urban areas.

Climate Change as a Result of Deforestation

Rainforests of the world are found in South and North America, Australia, New Zealand, Asia and Europe (Moreno-Mayar et al. 2018; Qin et al. 2019). These rainforests have an impact on the global weather system, so their deforestation threatens the world's biodiversity by changing the climate (Esquivel-Muelbert et al. 2019). However, the burning of rainforests and their deforestation is a continuous process for decades (Anonymous 2019). From January to August 2019, Amazon forests in Brazil have experienced 74,155 fires which were 85% more than the previous year and ultimately destroyed 9700km^2 of rainforests (Richard et al. 2019). Recently, these fires were intentionally introduced into the Amazon forests for the sake of getting more land for increased agricultural activities (Ribeiro et al. 2018). Deforestation in Indonesia has also gained significant global attention because of the emission of massive pollutants and particulate matter (Tacconi and Muttaqin 2019; Tacconi et al. 2019). If we talk about India, the mining of minerals like coal, iron and limestone is the main factor involved in deforestation in many areas (Ranjan 2019). Additionally, the wildfire in Australia caused huge damage to the habitats and biodiversity, resulting in the risk of extinction of koalas (Lam et al. 2020).

Reforestation to Mitigate the Climate Change

Forests play an important role to maintain a sustainable environment, biosphere's functions and world's biodiversity. Approximately 30% of the land is covered by forests globally and one third of these forests have not been disturbed by human activities. However, the past 30 years have seen deforestation at a large scale and ultimately 178 million hectares of forests have been lost (FAO and UNEP 2020). To curb the effects of climate change, different countries have taken initiatives for preserving the existing forests along with reforestation. China has launched a large scale forest plantation campaign to reduce deforestation and develop

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national parks (Tollefson 2010). They have invested more than one billion US dollars in The Natural Forest Conservation Program and achieved brilliant results during 1997-1999 (Zhang et al. 2000). China has an advanced urban forest in the city of Wuhan which was called as the garden city of China in 2006. Wuhan reached the greening rate of 39.6% at the end of 2019 (Ye and Qiu 2021). Canberra is called the 'bush capital' because its urban forest is full of different species of eucalyptus (Brack 2002). Approximately, 0.7 million trees were under the management of the government by 2011 (both park trees and street trees), excluding the trees in national reserves and unmown parks (Cooper 2011).

Urban Forests for Ecosystem Restoration

An urban forest is defined as the combination of all the shrubs and trees which make the green covers in urban areas and this is the most important element of nature in these areas (Jones and Davis 2017). The plantation of street trees is considered an adaptation and mitigation strategy to reduce the climate change effects in several areas of the world. Urban forests provide multiple benefits to the environment by effectively regulating functions, referred to as ecosystem services (Seamans 2013). Many studies have concluded that urban forests help modify the microclimate by reducing extreme weather conditions (Gill et al. 2007). Urban vegetation has been known to reduce the land surface temperature and give a cooling effect in cities (Park et al. 2017). Studies on the dynamic changes and landscape patterns of urban forests can help understand the functions of urban forests and maximize the ecosystem services (Wang et al. 2017).

Urban forests have a key role in green infrastructure, which provide great aesthetic and social services such as mental and physical health, recreation, landscape variety, seasonal dynamics, historical and cultural values, along with ecological, physical and climatic benefits (Tyrv¨ainen et al. 2005). Thus, restoration of an ecosystem is in highlights globally because this seems to be a cheap solution to resolve the issue of climate change and biodiversity loss (Lewis et al. 2019). The importance of restoration of the ecosystem has been recently announced by the United Nations by declaring the decade of 2021-2030 as 'The Decade on Ecosystem Restoration' (Chazdon and Uriarte 2016).

The Possible Impact of Urban Forests on Parasitic Diseases

Although forest restoration has positive impacts on climate change and sustainable biodiversity, it can also pose some threats to the local ecosystem (Veldman et al. 2015, 2017). Landscape pattern is considered to be an important factor in landscape epidemiology to understand the spread of infectious diseases in urban areas (Webster 2020). Similar studies have confirmed the close association between infectious disease risk and landscape structure and composition (Messier et al. 2015). Recent studies in China analyzed that plantation of mixed species in reforestation increased the number of various species of arthropods (Hua et al. 2016). In various systems of host-parasite relationships, parasite diversity is mainly influenced by host diversity irrespective of any spatial scale (Kamiya et al. 2014). Ecological attributes of the host diversity have the potential to influence parasite richness. For example, a diversified population of parasites can be found where greater numbers of hosts are available, as found in primates and hoofed mammals (Vitone et al. 2004; Ezenwa et al. 2006). Likewise, urban forests can be the reason for the availability of a variety of hosts for parasites where they can complete their life cycle stages and cause the transmission of various diseases in humans and livestock.

Transmission of Vector-borne Pathogens

It has been well studied that most dangerous diseases originate from biodiversity-rich areas like tropical rain forests in Africa and Asia (Vidal 2020). Arthropod species such as ticks, mosquitos, triatomine bugs, black flies and sand flies transmit various vector-borne diseases. The temperature has a direct effect on vector reproduction and survival rates because of their ectothermic nature, therefore, it influences the vector activity patterns (biting rates), intensity, habitat suitability, abundance and distribution (Martin et al. 2008). The temperature can influence the development of parasites, their abundance and vector distribution, it can be the most vital factor related to the transmission of vector-borne pathogens (Lafferty 2009; Cable et al. 2017). Dust and wind can also play a major in the transportation of pathogens to distant areas and the dispersal of pathogens depends on the wind speed and air temperature (Boxall et al. 2009). It has been documented in several studies that a positive correlation exists between the climate drivers and the disease transmission capacity of vectors. These climate drivers include humidity, rainfall, duration of sunshine and temperature (Li et al. 2014; Cheke et al. 2015) which can be provided by the rainforests. Changing climate can modify the intensity and distribution of vector-borne diseases and possibly threaten the success of disease control programs (Tesla et al. 2018; Mordecai et al. 2020).

Heavy rainfalls have the potential to provide additional breeding sites for vectors, e.g., mosquitos that ultimately provide resting sites and shelter for vectors (Githeko et al. 2000). Mosquitos have the potential to carry various pathogens like bacteria, viruses and protozoa. Thus, it is necessary to understand the expected outcomes of mosquito-borne diseases related to climate change. Similar to other disease patterns, favorable temperature conditions have the ability to influence mosquito activity, digestion, reproduction and blood meal frequencies (Martin et al. 2008). Therefore, climate change can modify the transmission and vector capacity of dynamics involved in mosquito-borne diseases (Reiter 2008).

Biting midges in the family Ceratopogonidae cause the transmission of diseases to livestock and wild animals by seeking a blood meal (Marquardt and Kondratieff 2005). Black flies in the family Simuliidae harbor devastating and important parasites such as filarial nematodes in birds, trypanosomes and Leucocytozoon (malarial parasites) (Marquardt and Kondratieff 2005; Reeves et al. 2007).

Various studies have reported that urban forests and city parks are causing the transmission of many ticks and tick-borne diseases (Hansford et al. 2017; Kowalec et al. 2017; Oechslin et al. 2017; Kubiak et al. 2019). Ticks feed on wild ungulates in fragmented habitats (Rizzoli et al. 2014; Selmi et al. 2018) and small vertebrates like lizards, birds, shrews and

rodents serve as the reservoir hosts of tick-borne pathogens (Amore et al. 2007). Furthermore, some urban forests have been invaded by chipmunks as alien species which can play a role in the transmission of many tick-borne pathogens (Marchant et al. 2017). Diversified genospecies can involve more vertebrate hosts such as lizards and birds to transmit the tick-borne diseases (Mannelli et al. 2012). The composition and moisture condition of soil can affect the distribution, survival and development stages of ticks (Hugh-Jones and Blackburn 2009).

Woodland encroachment and urban forests can have other consequences and can lead to increased transmission of tick-borne diseases to livestock and humans (Gilbert 2016). For example, *Ixodes* (*I.*) *ricinus* feeds on a wide variety of vertebrates, laying eggs on the ground and spending maximum life cycle stages off the host (Needham and Teel 1991). Various studies have indicated a positive association between the questing of nymphs and adults of I. ricinus with that of tree cover in France (Halos et al. 2010), Scotland (Gilbert 2013, 2016) and southern Norway (Vanwambeke et al. 2016). This is one of the most important tick species in terms of transmission of tick-borne diseases to livestock (*Babesia*, *Anaplasma* and rickettsia). Tick-borne fever, caused by *Anaplasma phagocytophilum* is the most important tick-borne disease in Europe and *I. ricinus* is the main vector behind this disease (Stuen 2007). Tick-borne fever is a major threat to sheep farming because more than 30% of mortality has been observed due to this disease in Europe (Stuen and Kjølleberg 2000). *Ixodes ricinus* is increasing its capability to bite companion animals and geographical distribution in many green areas of the cities (Rizzoli et al. 2014).

On a global scale, hares and rabbits in the Order Lagomorpha, host various tick species, such as *Hyalomma lusitanicum* and *I. dentatus* (Estrada-Peña et al. 2012; Hamer et al. 2012; González et al. 2016) by supporting their immature life cycle stages (González et al. 2016). As rabbits are very common and widespread in man-modified landscapes, where ticks are already causing problems (Rappo et al. 2013; Van Nunen 2015). It is suspected that *Perameles nasuta*, commonly called as long-nosed bandicoots, are the main hosts of Australia paralysis tick (*I. holocyclus*) (Lydecker et al. 2015). *Ixodes holocyclus* has a wide variety of hosts to feed on and then bites the companion animals and ultimately causes paralysis (Barker and Walker 2014; Van Nunen 2015).

Effects of Parasitic Diseases on Animal Health

Climate change has direct and indirect effects on animal health status and the primary reason is the change in the environment including magnitude and frequency of extreme events, precipitation, relative humidity and temperature (Forastiere 2010). Detrimental changes have been caused to the environment due to global climate change and alterations in land use. These effects have disrupted the natural ecosystem and they can result in an increased burden of parasitic diseases (Patz et al. 2000). Different processes are involved in this change (Patz and Confalonieri, 2005; Confalonieri and Aparicio 2011) and one of the most important is the increased population of humans in the forest areas. This has resulted into the transmission of naturally occurring diseases of wildlife to the immunosuppressant humans and domestic animals (Mandal 2011). Likewise, the same problems can be associated with the urban forests for the population of domestic and pet animals living in close proximity. An increase in the level of humidity provides favorable conditions for the survival of parasite cysts/oocysts, larvae and eggs (Semenza et al. 2012; Knapp-Lawitzke et al. 2016; Mignatti et al. 2016). Similarly, the intensity of rainfall favors the spread of cysts, oocysts and eggs through contaminated water (Jiménez et al. 2010), and urban forests are being managed for the same purpose. Thus, there is a dire need to keep all these factors in mind while planning an urban forest.

Changes in Animal Behavior and Population Growth

Some species of birds and mammals have a greater trophic spectrum with versatile behavior and they prefer to colonize in the urban and peri-urban areas so that they can find more trophic resources in these areas as compared to those available in the wild environment (Pozio and Murrell 2006). Several carnivores such as raccoon dog, raccoon, stone marten, badger, jackal, coyote and the red fox have established themselves in various cities of the globe by feeding on anthropogenic food sources and getting more opportunities in urban areas than in their natural environment (Parsons et al. 2018). In the same manner, urban forests can provide habitats to these carnivores and ultimately, they can cause serious parasitic and other diseases to livestock and pet animals. The population of wild carnivores has an increasing trend in the peri-urban and urban environment due to a lack of predators and hunting pressure and an abundance of available food resources (Bateman and Fleming 2012). It has been observed that recent wet, warm springs and winters have increased the population of rodents. Additionally, heavy rainfalls and favorable conditions have provided them the opportunity to promote their breeding and expand the population. These rodents can find shelter in urban forests and transmit various diseases to the surrounding populations of domestic animals and humans.

Migration of Species from One Continent to Another

In the previous century, 44 alien species of mammals entered Europe including many carnivores such as *Canis aureus* (the jackal), *Nyctereutes procyonoides* (the raccoon dog), *Procyon lotor* (the raccoon) and *Neovison vison* (the American mink). All of these species are considered to be good hosts of *Toxoplasma gondii* and *Trichinella* spp. (Széll et al. 2013; Pozio 2015; Kärssin et al. 2017; Cybulska et al. 2018; Shamsian et al. 2018). The entrance of these alien species occurred during the Neolithic age, however, a marked invasion was observed during the 20th century (Genovesi et al. 2012). *Sus scrofa* (central European wild boar) breed was introduced to southern Europe. As it has a larger size and greater reproductive rate, ultimately provided favorable conditions for the transmission of *Toxoplasma gondii* and *Trichinella* spp. along with the damage to the environment (Massei et al. 2015).

This is a need of time to think about the invasion of alien species from one continent to another but also those species which have found new areas in the same continent (Massei et al. 2015). Therefore, surveillance studies should be conducted about the presence of native and alien species in an area, and the same should be applied to urban forests.

Zoonotic Diseases

Zoonosis is another problem that results from the increasing interaction of humans and animals (Magouras et al. 2020) and it has been recently estimated that almost 60% of the emerging infectious diseases are of zoonotic nature (Taylor et al. 2001). Most of the emerging infectious diseases have wildlife origin and these diseases are emerging due to dynamic interactions between livestock populations, humans, wildlife and swiftly changing environments (Heymann et al. 2015). Geoclimatic variations and global warming have a huge influence on the epidemiology of zoonotic diseases by altering the dynamics of pathogens, vectors, hosts and their interactions (Anyamba et al. 2009, 2019). Native vegetation has been converted into anthropogenic habitats and this is one of the main drivers for the emergence of rodent-borne zoonotic diseases. This has enhanced the transmission of cross-species pathogens due to the increased interactions among domestic animals, wildlife, synanthropes and humans (Saker et al. 2004).

Concluding Remarks

Climate change and weather can affect the biology and distribution of insect-borne diseases. For instance, changes in the humidity level in temperate climates, precipitation patterns, global winds and temperature changes have a positive correlation with insect reproduction and population density. Thus, there are chances of the spread of tropical diseases in new areas other than their natural habitats. The world is witnessing a sharp reduction of skilled manpower involved in the research of ecosystem alterations due to increased interest in industrialization. This will further add up in the problem of changing environment, which needs to be addressed with dedicated funding and resources in the research institutes and at the university level.

Many research studies have indicated that change in the environment will have an impact on animal health and welfare. Thus there is a need for further epidemiological studies to understand the factors involved in the transmission of diseases from urban forests. The data regarding animal disease surveillance need to be prepared according to the changing climatic conditions with potential tools and techniques. Geospatial technology should be used to predict the connection of biological changes in the environment from outbreaks and abundance of insects, movement and quality of habitat.

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CHAPTER 02

PREVALENCE AND FUNDAMENTAL RESEARCH OF PREVENTION AND TREATMENT OF TOXOPLASMOSIS

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INTRODUCTION

Toxoplasma gondii is an intracellular parasite that causes toxoplasmosis, a zoonotic infection. Toxoplasmosis is a highly contagious zoonotic parasite disease caused by *Toxoplasma gondii* that affects people, livestock, poultry, and other animals (Dubey 2010). Infection with *T. gondii* varies by geography, season, and animal. Toxoplasmosis in humans is usually recessive, and it can cause a variety of congenital and acquired illnesses. Animals infected with *T. gondii* show changes in their development and reproduction, which can result in miscarriage, stillbirth and malformed fetuses in female animals, obstructing the growth of animal husbandry and causing economic losses (Elsheikha et al. 2020). More importantly, *T. gondii*-infected animal foods pose a serious risk to human health and life. Therefore, understanding the epidemiology of toxoplasmosis, particularly the epidemiological characteristics of toxoplasmosis in recent years, is critical for toxoplasmosis prevention and control. The epidemiological characteristics, diagnosis, treatment, and vaccine of toxoplasmosis are briefly reviewed in this paper with statistical analysis of the epidemiological survey of toxoplasmosis in humans and important animals.

Source of Infection

Pathogen

T. gondii was found in 1908, but its life history was only studied by international researchers in 1969, indicating that it has a more intricate life history. To complete its life cycle, *T. gondii* requires two hosts, namely intermediate host and terminal host. It has two modes of reproduction, asexual reproduction and sexual reproduction. Sexual reproduction occurs only in the small intestinal epithelial cells of the terminal host. Oocysts are the final product of sexual reproduction that can only be found in infected feline intestines. Tachyzoites and bradyzoites are the two basic stages of asexual reproduction. The terminal host ingests food or water contaminated with tachyzoites, cysts and oocysts. The capsule membrane dissolves after the terminal host digests it, and the tachyzoites, bradyzoites in cysts and sporozoites in oocysts penetrate the terminal host's small intestinal epithelial cells. The merozoites are first propagated to form sexual gametes and then the sexual gametes are propagated to form large and tiny gametes. The large and small gametophytes undergo sexual reproduction and combine to form oocysts, which overflow in the terminal host's long epithelial cells and are excreted with the terminal host's feces. The oocysts mature and become infectious after a period of external nutrition. The mature oocysts are ingested by the intermediate host, and the sporozoites in the oocysts undergo internal budding in the intermediate host's nucleated cells, eventually becoming tachyzoites. In host cells, tachyzoites represent the rapidly dividing stage and have the ability to spread to multiple and distant tissues within the host's body, triggering strong immunological responses. Then tachyzoites form pseudocysts in diverse tissues, which can last for a long time in intermediate hosts. Some tachyzoites can change into slow-reproducing bradyzoites despite chemotherapeutic medicines and host immunological protection. Unlike tachyzoites, bradyzoites split slowly and remain dormant inside a cyst that is mostly found in the brain and muscle, possibly due to the sluggish clearance of parasites caused by lower cellular renewal in these tissues compared with other organs (Elsheikha et al. 2020). When the immune system of the intermediate host is normal, no toxoplasmosis develops, resulting in a dormant infection (Dubey 2010).

Host

The main source of infection is toxoplasmosis-infected animals. *T. gondii* has a wide range of hosts, including practically all warm-blooded mammals and marine organisms. According to pathogenetic studies, *T. gondii* was found in 141 species of mammals (Dubey 2010). The epidemiological survey of the major hosts (cats, pigs, and humans) is examined in this chapter.

Cat

Toxoplasma cysts can be excreted by practically any type of cat. Cats can shed their oocysts during the three infection stages of *T. gondii*, namely tachyzoite, bradyzoite and oocyst stage (Dubey 2010). As a terminal host of *T. gondii*, cats infected with *T. gondii* excrete the oocysts from the feces, and the oocysts are highly infective after sporulation. Domestic cats (mostly stray cats) emit oocysts, which are one of the main causes of *T. gondii* infection in people and animals (Rahimi et al. 2015). In cats, *T. gondii* infection usually manifests as subclinical symptoms. Feline toxoplasmosis has a wide geographic distribution, with a global infection rate ranging from 30 to 40%

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(Dubey 2010), and infection rates in feral cats are often higher than those in domestic cats. The infection rates in some parts of Africa and Europe are higher than the world average infection level, such as a 97.4% positive serological test for *T. gondii* in wild cats in Egypt (Dubey 2010), 62.3% in Albanian cats (Spada et al. 2012), 70.9% in Algeria cats (Ouchetati et al. 2021), 40.3%-76% in Turkish cats (Dubey 2010; Can et al. 2014), 48.4% in Finnish cats (Jokelainen et al. 2012), 42.4% in Switzerland (Schreiber et al. 2021), 51.6% in Latvia (Deksne et al. 2013), 58.95% in France (Afonso et al. 2013), 60.8% in Estonia (Must et al. 2015), 32.3% in Cyprus (Attipa et al. 2021) and 24.2 to 60% in Spain (Montoya et al. 2018; Candela et al. 2022). The prevalence rates of *T. gondii* infection in cats in most of Asia are generally lower than the world average, such as 19.6% in Kuwait (Abdou et al. 2013), 30.4% in Iraq (Switzer et al. 2013), 24.5% in China (Ding et al. 2017; Li et al. 2022), 2.2 to 47.2% in Korea (Hong et al. 2013; Jung et al. 2017), 4.8 to 11% in Bangkok (Jittapalapong et al. 2010; Sukhumavasi et al. 2012), 40% in Iran (Sharif et al. 2009), 14.5% in Malaysia (Wana et al. 2020), 9 to 26.7% in Japan (Matsuu et al. 2017; Kyan et al. 2021) and 18.7% in Thailand (Huertas-López et al. 2021). For countries in Americas, the prevalence rates were 8 to 84.4% in Brazil (Cavalcante et al. 2006; Bastos et al. 2014; Magalhães et al. 2017; Lima et al. 2018), and 6 to 66.7% in the United States (Lilly et al. 2013; Ballash et al. 2015; Scorza and Lappin 2017). As the soul mate of humans, cats are inextricably linked to people's daily lives. *T. gondii*-infected cats pose a serious threat to human health. The number of pet cats is steadily increasing as people's living standards and spiritual levels improve. Therefore, controlling toxoplasmosis in cats is critical to preventing the disease.

Pig

T. gondii infection in livestock and poultry results in financial losses for the farming business and poses a potential risk for human infection. Pigs are significant carriers of *T. gondii* and are the main infection source of human infection. *T. gondii* infection in pigs usually manifests as subclinical symptoms. There were considerable geographical differences in the prevalence of porcine toxoplasmosis, with a global average infection rate of 30 to 40% (García-Bocanegra et al. 2010). Some parts of Africa and Europe had higher infection rates than the global average, such as 16.6 to 24.52% of domestic pigs seropositive for *T. gondii* in Spain (García-Bocanegra et al. 2010; García-Bocanegra et al. 2010; Herrero et al. 2016; Castillo-Cuenca et al. 2020), 52.8% in Austria (Dubey et al. 2020), 5.8 to 8.9% in Iberian (Pablos-Tanarro et al. 2018), 12.5% in Pernambuco (Samico Fernandes et al. 2012), 24.4% in Brandenburg, Germany (Bier et al. 2020). *T. gondii* infection in pigs was more common in China, with an average infection rate of about 40% in pigs nationwide and 20 to 45% in most provinces; The rates were 19.1 to 50.4% in Jilin Province, northeast China (Zhang et al. 2013; Xu et al. 2015), 9.94% in Henan province (Su et al. 2020), 70.0% in Guizhou province (Li et al. 2015), 53.4% in Zhejiang province (Yu et al. 2011), 13.8% in Shanghai (Zhang et al. 2020), and 27.0% in southern China (Zhou et al. 2010). Pigs kept for fresh pork consumption (feeder pigs, market pigs) in the United States are raised indoors in well-managed facilities for the most part of the life to avoid contact with rodents and cats. *T. gondii* prevalence has decreased dramatically in these well-managed facilities during the last decade. Seroprevalence was 23% in market pigs and 42% in breeder pigs (sows) in a statistically valid population-based nationwide survey conducted in America in 1983-1984. When pigs from these same areas were tested in 1992, the prevalence had reduced to 20.8% in breeders and 3.1% in finishers (Dubey 2010). Pork, as an important meat ingredient, is closely associated with the human existence, and pork with *T. gondii* poses a serious health risk to humans.

Human

T. gondii infection is widespread among humans and its prevalence differs among various regions and countries. Some investigations have pointed out that the prevalence of human infection varied from 4% in Korea to 92% in Brazil. Some of the variations in the report could be explained by sample size, age, and serological methods (Dubey 2010). *T. gondii* infection is mostly asymptomatic in healthy people, but it can be fatal in immunocompromised patients such as patients with malignant tumors, AIDS and organ transplant (Dubey et al. 2008). *T. gondii* infection in pregnant women and pregnant female animals (such as goats, sheep) can result in birth abnormalities, stillbirth or miscarriage. It is estimated that about one-third of the world's population is infected with *T. gondii.* The positive rate of *T. gondii* antibodies in European and American is high, about 25 to 50% (Petersen et al. 2007; Villena et al. 2007). *T. gondii* infection rate was 29% in Mexico (Ramirez et al. 2010), 33% in Auckland (Morris et al. 2004) and 50 - 80% in Brazil (Dubey et al. 2012). The positive rate of *T. gondii* antibodies in African was also relatively high. The infection rate was 36.7 - 62.1% in Morocco (Tlamcani et al. 2017), 39.8 - 53.2% in Algeria (Schneider et al. 1977; Messerer et al. 2014; Berredjem et al. 2017), 39.3 - 47.7% in Tunisia (Ben Abdallah et al. 2008; Sellami et al. 2010; Fakhfakh et al. 2013; Lachkhem et al. 2020), 38.5 - 47.7% in Libya (Kassem and Morsy 1991; Gamal and Jaroud 2015; Gashout et al. 2016) and 35.7 - 59.6% in Egypt (Youssef 1993; Ibrahim et al. 1997; Elsheikha et al. 2009; Abou Elez et al. 2017). The positive rate of *T. gondii* antibodies in Asian was also relatively high. The rate was 55-67% in Beirut (Bouhamdan et al. 2010), 37.9 - 43.9% in Turkey (Aynioglu et al. 2015; Alver et al. 2021), 43.9% in Turkey (Aynioglu et al. 2015), 27.9% in Palestine (Nijem and Al-Amleh 2009), 22% in Thailand (Andiappan et al. 2014) and 19.3 - 37.8% in Iran. *T. gondii* infection in Chinese people is low when compared with people in other countries. According to recent research, the overall positive rate of *T. gondii* antibodies in the Chinese population is 8.20 to 8.60% in pregnant women, but the antibody positive rate in cancer patients is 16.8%, which is significantly higher than the rate in the general population (Li et al. 2017; Dong et al. 2018). This fact has been found especially in certain types of cancer patients (Jiang et al. 2015; Cong et al. 2015; Zhou et al. 2018). The reasons for the high *T. gondii* antibody positive rate in tumor patients need to be further studied.

Way of Transmission

In humans, Toxoplasmosis is divided into two types, congenital infection and acquired infection. And the acquired toxoplasmosis is mainly through the ingestion of *T. gondii* cysts or oocysts.

Congenital Infection

In humans, toxoplasmosis is acknowledged as an essential public health problem. Congenital infection is that *T. gondii*

infects pregnant women or pregnant animals during pregnancy, and vertically transmitted to the fetus in the uterus through the placenta, affecting fetal development and resulting in abnormalities or stillbirths, as well as premature birth and miscarriage. Several studies have found that as the gestational age at the time of maternal infection rises, the risk of materno-fetal transfer also increases. However, the clinical severity of congenital toxoplasmosis is less noticeable when the infection occurs late in pregnancy (Cortina-Borja et al. 2010; Hotop et al. 2012; Wallon et al. 2013; Prusa et al. 2015). After the fetus is infected with *T. gondii*, it shows congenital defects. Therefore, women of reproductive age and first trimester should be routinely checked for serum *T. gondii* antibodies, which will effectively reduce the occurrence of congenital malformations and protect the health of newborns.

Acquired Infections

Ingestion of *T. gondii* cysts Infection

Uncooked animal food products such as meat, eggs, milk could contain the trophozoites or cysts of *T. gondii* not completely killed and people may be infected after ingestion. In addition, in the process of meat production, if the temperature is too low to kill the worms, cysts may still exist in the meat, or the raw meat may contaminate the relevant tableware and be ingested into the body, which may also cause infection. This is mainly infection in the form of cysts in the meat. The bradyzoites from the tissue cysts or sporozoites from the oocyst penetrate the intestinal epithelial cells and propagate in the intestine. *T. gondii* may spread locally to mesenteric lymph nodes and to distant organs by invasion of lymphatics and blood. A host may die because of necrosis of the intestine and mesenteric lymph nodes before other organs are severely damaged. Some tissue cysts survive for several days after the death of an infected animal, even though its tissues have begun decomposing (Dubey 2010). It is obvious to find that the transmission of this cyst through meat has a great impact on humans infected with *T. gondii*.

Ingestion of *T. gondii* Oocyst Infection

Cats and their felines are the ultimate hosts of *T. gondii*, with a large number of *T. gondii* oocysts in their feces. People can be infected by touching or ingesting these developed oocysts shed by felines exposed to *T. gondii*. Mature oocysts pollute people's drinking water or food, which is a more common form of transmission.

Genotype, Regionality and Seasonality

T. gondii pathogenicity is tightly linked to the strain's genotype and virulence. *T. gondii* has a genetically and geographically diversified population structure. Types I, II, III, and atypical genotypes are included in the standard classification of *T. gondii* genes. BrI, BrII, BrIII, African 1, China 1, and China 12 are all atypical genotypes (Howe and Sibley 1995). The use of PCR restriction fragment length polymorphism analysis (PCR-RFLP) to detect the genotype of *T. gondii* is now generally acknowledged (Yu et al. 2018). Type I strains are particularly pathogenic to outbred mice and immunosuppressed individuals, whereas types II and III strains are less pathogenic (Howe and Sibley 1995). Type II strains are the most common cause of human *T. gondii* infection in Europe, hence most strains isolated from congenital *T. gondii* pathological tissues are likewise type II (Ajzenberg et al. 2002). Followed by type I, type III strains are mainly found in animals (Howe and Sibley 1995). In South America, however, the majority of congenital infections are caused by atypical strains with high virulence (Pardini et al. 2019). The genotypes revealed a high level of genetic variability, and the gene sequences of these strains are distinct, despite the fact that they were made up of diverse lines and showed significant differences between populations. The majority of strains that cause severe toxoplasmosis in immunocompromised people are of unusual South American genotypes. *T. gondii* usually spreads through asexual reproduction in North America and Europe, and by sexual union in South America, according to these distinct gene sequences (Rajendran et al. 2012). The ToxoDB#9 (Chinese 1) genotype is the most prevalent one identified in Asia. In Asia, the clonal types I, II, II variant, and III were also common (Chaichan et al. 2017).

T. gondii infection is distributed worldwide. It is estimated that approximately one-third of the global population is seropositive for *T. gondii*, but infection rates differ greatly by region. Almost all Chinese provinces, municipalities, autonomous areas, and municipalities have proven the existence of this disease, which has a very variable infection rate. The seroprevalence of *T. gondii* was higher among ethnic minority blood donors than in other ethnic groups (Li et al. 2019). In contrast, Western populations have a greater rate of *T. gondii* antibody positivity (Dubey et al. 2012). All of this is attributable to the consumption of raw and semi-raw meat. Zones with severe *T. gondii* infection are mostly found in China's coastal subtropical areas. Because the coastal provinces of China have a greater seropositivity rate than the inland provinces of China, it can be concluded that coastal provinces have a higher *T. gondii* infection rate than inland provinces in China. This could be due to increasing maritime transportation in coastal provinces and increased interaction with foreign pathogens, resulting in a higher risk of infection (Pan 2018), whereas *T. gondii* infection rates are generally low in cold and dry places. Pathogens cannot survive or spread in environments that are relatively cold and dry.

The spread of *T. gondii* has a seasonality. For example, in pigs, the incidence of pig *T. gondii* is highest in the summer and autumn and the lowest in the winter and spring (Zhang et al. 2020). The higher the ambient temperature in the enclosure, the more probable the disease will spread, which may be more closely linked to the growth of insects (mosquitoes, ticks, etc.). Therefore, more attention should be paid to the control of *T. gondii* in summer and autumn.

Diagnosis

T. gondii can be diagnosed in two ways: pathogenic diagnosis and serological diagnosis. Etiological examinations, such as direct microscopic inspection, trophozoite isolation, and cyst investigation, are difficult to diagnose. These routine etiological examination procedures, on the other hand, are usually used in animal infection diagnosis or insect body isolation, as well as human pathological tissue investigation in special cases. Pathogenic diagnosis can be detected histologically on-site, and the detection time is short. However, because it is based on human factors observations, and the testing personnel's judgment standards differ, there is a subjective awareness

effect, and the likelihood of erroneous detection or missed detection is considerably to be increased. Although the accuracy of the cell infection test is higher than that of the animal infection test, it is insufficient and takes too long to detect. Overall, the developing approaches will gradually replace the inadequacies of pathogenic detection of pathogens (Pan 2018). *T. gondii* nucleic acid, serum circulating antigen, and specific antibody detection are now more widely employed in clinic. Conventional PCR, nest PCR, RT-PCR and other nucleic acid detection technologies are available. In theory, positive nucleic acid detection is only identified when *T. gondii* is present in parasitemia, diseased tissue, or body fluid and it is not suited for the diagnosis of hidden *T. gondii* infection in the human body. Immunological diagnosis technology has benefited from the advances in antibody cloning technology. Antibody detection for *T. gondii* can alleviate this difficulty, thus it becomes more commonly employed, mostly by enzyme-linked immunosorbent assay (ELISA) and agglutination tests. Although ELISA has a higher sensitivity than agglutination tests, such as modified agglutination test (MAT), indirect hemagglutination assay (IHA), and latex agglutination test (LAT), agglutination tests are superior for field epidemiological investigations since they do not require the use of a second antibody in the detection process.

Treatment

For toxoplasmosis, there are currently no preventive and specific therapeutic drugs. At present, pyrimethamine combined with sulfadiazine (PS) and trimethoprim combined with sulfamethoxazole (TMP-SMX) and spiramycin (SPI) are the first choices for the clinical treatment of toxoplasmosis, and the second-line therapeutic drugs are atovaquone and clindamycin, etc. (Deng et al. 2019). Although these regimens are effective against active infections, they are not conclusive cures. Western medicine treatment of toxoplasmosis has shortcomings. For example, it is easy to produce drug resistance and noticeable side effects for Western medicine treatment, while traditional Chinese medicine has the advantages of having a wide variety of selections and minimal side effects, which makes the treatment of toxoplasmosis with traditional Chinese medicine to be a well-deserved research topic (Chen et al. 2009; Zhang et al. 2018).

Traditional Chinese medicines have direct or indirect inhibitory effects on the proliferation of *T. gondii*, such as artemisinin and allicin. For example, Artemisinin, an extract of the plant *Artemisia carvifolia*, is an antimalarial drug with in vivo and in vitro activity against *T. gondii*. At a concentration of 0.4 g/mL, it can eliminate *T. gondii* in Plaques formed in fibroblasts with IC50 values in the very low micromolar range (Guo et al. 2018). De Oliveira et al. (2009) demonstrated that artemisinin treatment of mice infected with ME49 strain for 30 days resulted in a 100% survival rate, but the protection rate of mice infected with RH strain was only 20 to 50%. Researchers synthesized a series of unsaturated artemisinin-thiazole derivatives based on the principle of pharmacophore hybridization, which can effectively inhibit the proliferation of tachyzoites, thereby playing a role in killing *T. gondii* (Hencken et al. 2010). Further studies by Schultz et al. (2014) showed that the 10-position of artemisinin is connected to the thiazole ring linker, which can improve the activity of artemisinin derivatives against extracellular and intracellular *T. gondii*. In addition, it is reported that using dihydroartemisinin piperaquine tablets and artesunate tablets to treat toxoplasmosis encephalopathy can enhance the therapeutic effect, and the absorption of intracranial lesions is obviously improved, which is better than that using sulfamethoxazole and azithromycin alone (Chen and Deng 2016).

Currently, although there are no ideal drugs available, the development of promising drug candidates is still ongoing. Many preclinical in vivo and in vitro studies have shown that many compounds have therapeutic effects on both acute and chronic toxoplasmosis, and some of them show significantly higher anti-toxoplasma activity than current clinical anti-toxoplasma drugs (Qiu et al. 2020).

Vaccine

Almost all individuals are at a risk of being infected with toxoplasmosis sometime in their lifetime, so a vaccine to prevent human toxoplasmosis would benefit all people. A vaccine targeting *T. gondii* in livestock is needed not only to prevent the formation of cysts, minimizing transmission of the parasite to humans through eating raw meat, but also reduce the economic loss caused by animal abortion. Domestic cat vaccination can minimize oocyst excretion and increase livestock production, and in addition it can reduce the quantity of cysts in meat and lessen the risk of human infection. At present, several toxoplasmosis vaccines have been developed, such as live-attenuated vaccines, DNA vaccines, nanoparticle-based, exosome-based, and carbohydrate-based vaccines (Elsheikha et al. 2020). Unfortunately, the "Toxovax" vaccine is currently the only commercial veterinary live-attenuated *Toxoplasma* vaccine on the market, which is derived from the live-attenuated tachyzoite S48 strain and can be used to prevent infection in sheep and goats (Buxton and Innes 1995; Zhang et al. 2013; Katzer et al. 2014; Wang et al. 2019). The "Toxovax" vaccine strain was originally isolated from sheep, and after long-term proliferation in mice, its tachyzoites lost their pathogenicity to sheep and their ability to form oocysts in cats' intestine. The worm burden in the body was obviously reduced after immunizing sheep with the vaccine, and muscle and central nervous system lesions were alleviated, but the formation of tissue was not completely prevented. As a non-persistent infection strain, it has a good safety record, but there is still the possibility of reversion, as well as re-infection and transmission to immunocompromised animals and people. In 2015, researchers developed a non-reverting and non-replicating uracil auxotrophic type II live attenuated vaccine, which is a completely attenuated PruΔOMPDC irreversible strain obtained by knocking out the OMPDC gene in *T. gondii* type II Pru strains. The ability to replicate, virulence, and the ability to form cysts and chronic infection were all lost when the OMPDC gene was knocked out. Therefore, the vaccine is non-virulent and does not form cysts in the body after inoculating mice, so it will not cause chronic infection, and can induce mice to produce $CD8^+$-mediated complete protection against acute infection of type I virulent or type II attenuated strains. It also prevents cyst formation in type II strains. This research is another promising genetically modified live insect vaccine after "Toxovax" (Fox and Bzik 2015). In addition, the research on recombinant or natural molecular vaccines almost includes all the protein antigens of *T. gondii* life cycle stages except cysts, but ideal candidate molecular vaccines have not been screened. With the widespread application of CRISPR/Cas9 gene editing technology in the field of *T. gondii*,

more and more gene deletion strains have been constructed. For example, RHΔGRA17, PRUΔCDPK2, RHΔTKL1, RHΔNPT1, ME49ΔLHD, RHΔMORN1, and ME49ΔADSL may become candidate strains of live attenuated vaccines for *T. gondii* (Zhang et al. 2013). Although significant progress has been made in vaccine discovery, including many promising proof-of-concept vaccination trials in mice, none of the tested vaccines has been applied to human clinical trials (Elsheikha et al. 2020). In addition, *T. gondii* has a complex life cycle with several antigenically distinct developmental stages that elicit different immune responses. Thus, developing a vaccine targeting several developmental stages is not straightforward.

Summary

Toxoplasmosis, an important food-borne parasitic disease, will face severe challenges as society and economy develop, dietary patterns diversify, and pet cats are raised. International interconnection and frequent population movements increase the risk of *T. gondii* infection in humans and animals around the world. To effectively reduce the infection rate of human and animal food-borne *T. gondii*, it is necessary to strictly control the centralized and scientific breeding of domestic animals, strengthen pet management, strengthen food hygiene inspection and pre-market harmless processing, and extensively carry out scientific education of prevention. Toxoplasmosis prevention, control, and treatment will greatly benefit from the use of novel theories, technologies and techniques to conduct in-depth biology and clinical research on *T. gondii*, which is found in humans and animals.

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CHAPTER 03

DEVELOPMENT AND ADVANCEMENT IN VACCINES AGAINST *HAEMONCHUS CONTORTUS*

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INTRODUCTION

Haemonchosis is the infection caused by *Haemonchus contortus* (barber's pole worm) which is one of the most pathogenic and socio-economically important parasites affecting cattle, goats and sheep of tropical and subtropical areas of the world (O'Connor et al. 2006). Haemonchosis is clinically characterized by decreased wool production, anasarca and chronic wasting resulting in poor carcass quality, anemia, hypoproteinemia, submandibular edema and death in severe cases. Small ruminants like sheep and goat share a major contribution to the agricultural economy of world in terms of their wool, skin, milk and meat production (Roos 2009; Roeber et al. 2013; Emery et al. 2016). Estimates of annual losses caused by haemonchosis to livestock account for 30 to 300 million dollars all over the world (Roeber et al. 2013; Emery et al. 2016). Primarily these parasitic infections are controlled by using anthelmintics but non judicious use of anthelmintics have led to the development of resistance against these drugs (Saddiqi et al. 2011). Resistance against different broad spectrum anthelmintics like morantel, levamisole, imidothiazoles-tetrahydropyrines, benzimidazoles, pyrantel, closantel, ivermectins, and macrocylic lactones has been reported across different parts of the world (Kaplan 2004; Kaplan and Vidyshankar 2012). So, the development of anti-parasitic vaccines and immumological approaches to control these infections is need of the hour. For the development of cost-effective methods to control *H. controtus* infections, we need a comprehensive knowledge of biology of *H. controtus* and immune responses involved in host-parasite interaction (Nisbet et al. 2016).

Life cycle of *H. controtus* starts with the sexual reproduction in the abomasum of the infected host resulting in the production of 5000-15000 eggs by a single female every day. Eggs are passed into feces and hatch to first larval stage (L1) and then moult to second larval stage (L2) within 4-6 days and start feeding on the bacteria present in the dung. When conditions are favorable like temperature ranging from 24 to 29 °C, L2 moults to L3 but does not shed its cuticle. L3 larvae are capable of moving up to the blades of grasses with in moisture drops. During grazing these are taken up by the host and reach abomasum where shedding of cuticle takes place and they burrow themselves into the abomasum. Within 48 hrs after burrowing they ex-sheath into L4 stage and ultimately into early (L5) and then ultimately into late adult worms. These adult worms start feeding blood. Infective L3 stage or early L4 stage instead of directly proceeding to next stage become dormant in the gastric glands of abomasum and are metabolically inactive. This phenomenon is called as developmental arrest or hypobiosis (Michel 1974). During unfavorable environmental conditions for the parasitic development proportion of hypobiotic worms is greater as the eggs shed in the feces have lesser chances of survival and development into the next stage (Waller at al. 2004). This mechanism depends on the environmental factor as well as immune responses of the host and genes involved in this phenomenon can be used as target antigen for developing new drugs and vaccines for control of *H. controtus* infections.

Importance

In last two decades various antigens have been identified from *H. controtus* and protective efficacy of these antigens as recombinant subunit and vector vaccines have also been evaluated (Knox et al. 2003; Tak et al. 2015; Wang et al. 2017). Potential efficacy of the use of gut proteases of *H. controtus* including an aminopeptidase H11 (Smith et al. 1997), cysteine protease with fibrinogenolytic properties (Boisvenue et al. 1992), H-gal-GP (gut membrane glycoprotein complex) (smith et al. 1994) which is a gut protein complex containing metalloendopeptidases (Redmond et al. 1997) and aspartyl protease activities as vaccine components have been evaluated previously. Among these, an integral membrane protein H11 having molecular weight of 10 kDa was found a potential candidate antigen with resulted in >90% reduction in fecal egg count and >75% reduction in abomasal worm load. A vaccine named Barbervax® has been recently licensed to be marketed in Australia which contain two native integral gut membrane protein H11 and H-gal-GP from *H. controtus* (Nisbet et al. 2016). During evaluation of safety and serological outcome of this vaccine significant antibody titers have been detected in the vaccinated animals (Vanhoy et al. 2018).

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Efficacy evaluation of Barbervax® was carried out in two groups of periparturient ewes with two different nutritional supplements and results showed 80% reduction in fecal egg count and higher antibody titers of vaccinated ewes which suggested that *H. controtus* can be controlled by combined protective effect of vaccine and improved nutrition (Bassetto et al. 2018). But requirement of repeated vaccine doses to induce high level of antigen specific circulating antibodies was major drawback of this vaccine which has resulted in failure of application of vaccine at large scale and limited its access to international market. Recently use of soluble and recombinant protein vaccines to control *H. controtus* has unlocked a new insight into the field of host-parasite interaction (Wang et al. 2017). In current scenario vaccines can be suitable option to control parasitic infections but immunoregulatory properties and antigenic variation of parasites are major hurdles in the process of development of effective vaccines (Hewitson and Maizels 2014). Therefore, development of safe and effective vaccine for controlling *H. controtus* requires identification of molecular targets and utilization of advanced molecular techniques for structural as well as functional studies on potential candidate targets for vaccine development.

Immunity

Host's immune response to parasitic infection is complex and categorized into two stages; one being against the infective larval stage and other one against adult helminths. Immune reaction against helminths is mainly governed by level of eosinophilia and high levels of serum and mucosal immunoglobulins (IgA, IgE, IgG) which are associated with T-helper type 2 mediated response (Balic et al. 2006; Lacroux et al. 2006; Shakya et al. 2011). Resistance of host against *H. controtus* infection depends on many factors like specie, age, breed, nutrition and previous exposure to parasitic infection. Younger lambs are at a high risk of developing infection of *H. controtus* due to their poor immunity. Several events occur in the immunized host when it is exposed to parasite. These events include humoral and cell mediated responses characterized by recognition of antigens either somatic or secretory/ excretory by dendritic cells and presentation to T-cells as antigen presenting cells (APCs) (Meeusen et al. 2005). As a result, allergic inflammatory response is triggered which include mucosal eosinophils and mast cell, these cell release inflammatory mediators which check parasitic infection by decreasing egg production, preventing the establishment of larval and adult stage as well as paralyzing the worms (Jones et al. 1994; Emery 1996).

General immune response to helminths' infection consists of T-helper type 2 cells as a part of humoral immune response involved on the expression of cytokines at infection site and these cytokines are responsible for stimulation and activation of CD4+ T cells (Meeusen et al. 2005; Anthony et al. 2006). Helminths' infections are reported to induce strong Th-2 cell response characterized by production of high amount of interleukins (IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and IL-31) (Jackson et al. 2009; Maizels and Vazdanbasksh 2003; Wang et al. 2008) as well as high level of immunoglobulins (IgG1, IgG4, IgE) and stable mast cell and eosinophil responses. IL-4 and IL-13 were main regulators of humoral immunity stimulating B cells to produce IgE and regulating the production of major histocompatibility complex class II (Anthony et al. 2007). Generally, immunity against *H. controtus* is governed by eosinophils, mast cells, antibodies and inhibitory molecules (Bricarello et al. 2004; Balic et al. 2006). Development of various strategies to modulate or escape the immune response by helminths have also been reported such as infection of Ancylostoma caninum lead to upregulation of regulatory T cells which ultimately resulted in the suppression of Th-1 andTh-2 mediated responses (Ferreira et al. 2013). Similarly, study reported in-vitro suppression of maturation of dendritic cells by excretory/secretory products of Trichinella spiralis and both R- and S-from lipopolysaccharide induced upregulation of T regulator cells (Aranzamendi et al. 2012).

Exploitation of Genomic and Proteomic Profile of *H. controtus*

Exploitation of genomics and proteomics of *H. controtus* has resulted in identification of several novel vaccine components. According to genome-wide transcriptomic data of all stages of *H. controtus* , 23610 protein coding genes are responsible for reproduction, development, host-parasite relationship, immunity and disease (Schwarz et al. 2013). Proteomic analysis done by liquid chromatography tandem mass spectrometry (LC-MS/MS) and matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) has identified 107 identities from 102 spots (Yatsuda et al. 2003) which include zinc metalloproteases, aspartic and serine proteases, aminopeptidases (Karanu et al. 1993; Rhoads and Fettterer 1995; Shompole and Jasmer 2001) and excretory/secretory products of *H. controtus* (HcESPs) Hc-15, Hc-24, Hc-40 and apical gut proteins (Yatsuda et al. 2003; Schallig et al. 1997; Dicker et al. 2014). LC-MS/MS analysis at 3 days post infection also identified approximately 4400 unique proteins (Nagaraj et al. 2012). Twenty-two hundred (2200) protein identities were recognized during analysis of proteins differentially expressed between L3 and xL3 stages of *H. contortus* by 2D-DIGE (two-dimensional differential gel electrophoresis) and 124 of them expressed between L3 and xL3 (Wang et al. 2016). Previously 407 interacting proteins were identified with ranged from 13 kDa to 180 kDa by in-vivo analysis of HcESPs, 47 out of them were shared in among all developmental stages from L3 to adult (Gadahi et al. 2016). Analysis of whole protein extract from male and female *H. controtus* revealed 129 male specific, 123 female specific and 23 immunogenic proteins (Yan et al. 2010). Moreover, proteomic study of 2487 differential proteins from eggs, L3, L4 and adult male and female of *H. controtus* showed substantial protein profile transition among different stages (Wang et al. 2019). Purpose of reviewing genomic exploitation of *H. controtus* is to provide a major resource for scientific community extensive molecular, genomic, epidemiological studies and development of new and effective drugs or vaccines for the control of *H. contortus*.

History of Vaccine

In past various efforts have been made to develop an effective vaccine to control *H. controtus* . Several methods have been used for the development of vaccine which include DNA vaccines, recombinant subunit vaccines and protein vaccines which are under consideration and are in process for checking their safety and efficacy. Commercially development of vaccines against helminths started with the development of commercial vaccine against nematodes named Dictol in early

1950s. But soon research focus shifted towards the identification and development of ESPs and H-gal-GP based vaccines after the failure of previous vaccine against infective stage (Smith and Zarlenga 2006; Knox et al. 2003).

Types of Vaccines

Gut derived/ Hidden Antigens based Vaccines

Use of glycoprotein complex derived from gut of *H. contortus* as vaccine component resulted in >90% decrease in fecal egg count and approximately 70% decrease in fecal worm output in vaccinated animals (Smith and Smith 1996). This presents H-gal-GP as a strong immunogen and this a candidate for vaccine development. Glycan part of H-gal-GP was considered main immunogen (Knox et al. 2003). Other studies also support the immunogenic role of galectins as they decreased fecal egg count by 48% (Yanming et al. 2007), thus highlights the potential of use of galectin as antigens in vaccine against *H. contortus*. Another option is integral membrane glycoprotein complex H11 obtained from gut of blood feeding *H. contortus*. Five isoforms of H11 have been documented being H11-1, H11-2, H11-3, H11-4, and H11-5 (Roberts et al. 2013) but H11-1 is most immunogenic and its vaccination trails have reported 91% decrease in fecal egg count as well as 82% and 72% decrease in adult male and female worm burden respectively (Nisbet et al. 2016). Moreover, vaccination containing both H-gal-GP and H11 antigen have also be successful in reducing losses due to haemonchosis (Smith et al. 1999). But these combination vaccines have some demerits which include repeated dose requirement for developing good and long-lasting immunity (Ehsan et al. 2020). This is a major reason behind the failure application of these vaccines on large scale.

DNA based Vaccine

This method uses genetic engineering to produce a genetically engineered antigen and immune cells are directly exposed to antigen to produce diverse immune responses. DNA vaccines are considered superior that the conventional vaccine because of their ability to produce diverse immune responses. Development of an effective vaccine demands high level of specific antibodies against *H. contortus* in vaccinated animals. Study conducted by Zhao et al. (2012) showed that DNA fragments of caprine IL-2 and H11-1 served as good immunogens as they resulted in 57% decrease in fecal egg count and 47% decrease in abomasal worm burden in the vaccinated goats. Similarly in another study goat vaccinated with glutathione peroxidase and HC29 encoding gene resulted in 36% decrease in fecal egg count and abomasal worm burden with the production of appreciable level of HC29 specific antibodies IgG and IgA as well as intensification of CD4+ T cells (Sun et al. 2011). Moreover, extending studies over DNA vaccine also covered two more antigens from *H. contortus* named GAPDH and Dim-1 conjugate with pVAX1 recombinant plasmids which were administered to 10 months old goat. Vaccination with GAPDH resulted in 35% decrease in fecal egg count and 38% decreases in abomasal worm burden along with increased antigen specific antibodies as well as intensified CD4+ T cell population (Han et al. 2012) while vaccination with Dim-1 resulted in 46% decrease in fecal egg count and 51% decrease in abomasal worm burden (Yan et al. 2013).

Protein based Vaccines

Over last two decades several studies have been conducted to identify various antigen from *H. contortus* for efficient vaccine development which can provide high level of specific and long-lasting immunity. During blood feeding stages various proteins called as ESPs are released into the environment by *H. controtus* (Rathore et al. 2006; Marcilla et al. 2012). Survival of helminths within their hosts depends on their ability to modify host's immune responses (Maizels and Yazdanbaksh 2003). Ability of parasite to modulate its host's immune responses improves its survival within host. Nematodes contain two type of antigens one being soluble (ES products) while other one being somatic antigens that are present either on the surface or present within the parasite. ES antigen that induces immune response in host are known as natural antigens while those that do not do so are called as hidden antigens (Munn 1997). An antigen that is a good candidate for vaccine must be presented to antibodies and cells involved in regulating the immunity of the host. HcESPs contain antigen candidates for the development of vaccine that can provide up to 90% protection in sheep against *H. contortus* infections (Yatsuda et al. 2003). Previously study conducted by Schallig and Leeuwen in 1997 reported 99.9% decrease in fecal egg count and 97.6% decrease in abomasal worm burden of animals vaccinated using two adult somatic extracts enriched in ES-15kDa and ES24-kDa. Similarly, a reduction of >90 % in fecal egg count and 72 to 80% decrease in abomasal worm burden has been reported in animals vaccinated using gut membrane antigens of *H. controtus* (Jasmer and McGuire 1991; Andrews et al. 1997). All of these studies hint the potential of using ES antigen in the development of protective vaccine.

Binding Proteins as Vaccine Agents

When a parasite infects an individual, it releases large number of molecules into the host which are responsible for immune reactions within the host (Cox et al. 1990). Excretory/secretory (ES) products contain many proteins which act as immunogens and can be used as antigens in vaccine.

Challenges to the Development of Effective Vaccine

Various vaccine trails have been conducted in which vaccine against *H. contortus* produced by several strategies have been test for their safety and efficacy. Although advancement in the development of vaccine against *H. controtus* is obvious but at the same time none of the vaccine is completely capable of to eliminate haemonchosis completely or they nullify its transmission. These vaccines are only capable of reducing fecal egg count and abomasal worm burdens partially to varying extent. So, still a gap is present that can be filled and further and more comprehensive studies are needed for the development of an effective vaccine against *H. controtus* .

Diversity of *H. controtus*

Various studies have been conducted in past regarding the genetic diversity of *H. controtus* but a more comprehensive overview of genetic diversity is needed for the development of effective vaccines or drugs and knowledge about

epidemiology, molecular genetics and drug resistance of *H. controtus* . Study conducted by Charlesworth in 2009 on the population genetics of *H. controtus* revealed that genetic diversity is dependent on many factors that include population size, gene flow, geographical restrictions and life history. Therefore, extensive genetic diversity among a population is suggestive of a larger population size or increased rate of mutation in *H. controtus* (Gilleard and Redman 2016). Similarly, a high degree of genetic variation has also been reported among laboratory strains of *H. contortus* from different countries (Redman et al. 2008). On the other hand, various genetically different isolates have also been detected in sheep and goats from same geographical locality. Hunt et al. in 2008 infected ten sheep with five different laboratory isolates of *H. controtus* from different regions of Australia and reported increased fecal egg count and abomasal adult worm burden resulted from difference in establishment rate. Difference in the pathogenicity among genetically different isolates of *H. controtus* collected from different geographical areas of United States has also been reported (Gilleard and Redman 2016). Poeschel and Todd (1972) performed an experimental study to check pathogenicity of 18 isolates of *H. controtus* and reported that three of these isolates were more less while two were more pathogenic than the control. Furthermore, reporting of the fact that the antigens associated the development of protective immunity are also not conserved in different species and different isolates of a specie (Maizels and Kurniawan 2002) has further added to the challenges faced in development of vaccine against *H. controtus* . Keeping in view the facts about genetic diversity and difference in immune response to different parasitic stage has highlighted the need of development of a vaccine that can be effective to be used in young animals against L3 stage of *H. controtus* in pre-seasonal as well as eliminates the necessity of administration of booster shot in post-seasonal period. This task can probably be performed by developing a vaccine that contain the antigens from different stages of *H. controtus* such as it L3 surface antigen and H-gal-GP from L4 and abomasal parasites can be used in combination (Nisbet et al. 2016). Although it is a difficult task to be performed because it requires the collection of different parasitic stages from naturally infected individual and complete of their antigenic profile which needs financial resources (Willadsen 2008). But development of such an effective vaccine will revolutionize the control of *H. controtus* as well as will open a way for the development of vaccines against other parasitic diseases as well.

Genetic Diversity of Host

Genetic diversity of host basically affects the immune response to parasitic antigens. Effect of host genetics on the development of innate as well as acquired immunity against nematode infection in a herd has been studied previously (Smith and Zarlenga 2006). Generally, it was considered that vaccination can ensure 100 percent immunity in individual infected naturally in past in comparison to those involved in host parasite relationship for longer periods (Lightowlers et al. 2003). Studies reported variation of fecal egg count of grazing animals with the genetic diversity of host, furthermore demonstrated that a major cause of parasitic transmission was a small proportion of highly vulnerable animals within the population (Barnes et al. 1995). Similarly, vaccination of that small proportion of population that is highly vulnerable can be used to decrease pathogenicity and transmission of parasites in a herd (Smith and Zarlenga 2006). Parasitic infections can be more challenging in genetically less diverse populations. There has been an increase in the evidences supporting the hypothesis which suggests that genetic diversity host is also reduced as result of environmental changes, pollution, global warming and decrease in the geographical range of host specie (Ekroth et al. 2019). At the same time increase in the parasite dominancy has resulted in increased chances of co-infection of different parasites among genetically homogenous populations (Whiteman et al. 2006). Pathogenicity in a population might also depend on timing and intensity of parasitic infection (Ekroth et al. 2019). A potential association also exists between lower genetic diversity of host and new emerging infections. So, conserving genetic diversity of host and vaccination of most vulnerable individuals within populations might be a suitable strategy to control emerging parasitic diseases.

Complexity Due the Different Developmental Stages of Parasite

Like many other parasites life cycle of *H. contortus* also consists of various developmental stages that differ in their antigenic profile (Gadahi et al. 2016). Similarly, immune responses of host against each stage also differ greatly which makes vaccine development against *H. controtus* more challenging. Vaccine containing antigen from one stage fails to protect against infection by other stages of parasite. Combination of genetic diversity and multistage complexity of parasite raises questions on the idea that a vaccine containing antigen from a single stage of parasite can provide long lasting immunity. So, a vaccine that contain suitable antigens from all developmental stages of *H. controtus* can be suitable options for providing long lasting immunity against all developmental stages of the parasite. Moreover, another challenge in vaccine development is the ability of parasite to modulate immune responses of the host in such a way that causes a delay or inability of host to resist parasitic infection.

Composition of Effective Vaccine

In the process of vaccine development against *H. controtus* one thing that needs to be emphasized is the quality and durability of immunity produced by vaccine. In this context adjuvants are considered an important component of vaccine in regard of production of vaccine that has better stability, safety, lower requirement in terms of volume and frequency of vaccination as well as capable of producing high and rapid immunity by increased differentiation of B lymphocytes (Chauhan et al. 2017; Reed et al. 2013). Selection of adjuvant from those available nowadays depends on immunogenicity of antigen adjuvant complex as well as lesser side effects associated with the vaccine administration. Various adjuvants have been used and their outcomes have been recorded in host models (Stutzer et al. 2018). But these has some disadvantages such as use of saponins results in tissue damage at the site of administration that leads to induction of improper immune response (Chauhan et al. 2017). Recently a new technology named as microencapsulation of antigens has been developed as an alternative to conventional adjuvants used in vaccines that has proved to be more reliable, effective

and promising vaccine delivery system (Himly et al. 2017). When considering adjuvants as most effective vaccine delivery system studies have shown that vaccination of lambs containing native or recombinant type antigens of *H. contortus* named as Hc23 and rHc23 respectively and Al(OH)3 as adjuvant resulted a decrease of 70 to 80 %in fecal egg count and abomasal worm burden of vaccinated animals (Fawzi et al. 2014; Fawzi et al. 2015). Another study conducted on the efficacy of vaccine containing rHc23 antigen and Al(OH)3 as adjuvant as showed similar results (González-Sánchez et al. 2018).

Future Perspectives

Extensive studies have been conducted all over the world about the biology of *H. contortus* which include molecular genetics for the search of antigen candidates from various its developmental stages that can be used for the development of an effective and protective vaccine against haemonchosis as well as various efficacy trials have also been conducted to test efficacy and safety of these vaccines. In spite all of these efforts lack of an effective vaccine against all stage of *H. controtus* still persists. Researchers have explored most of the complexities associated with host-parasitic relationship, life cycle, development of resistance and antigenic diversity of *H. controtus* . Similarly, valuable and more specific as well as sensitive diagnostic techniques have been developed which would help minimizing losses caused by haemonchosis through an early diagnosis and treatment. Furthermore, studies on the molecular genetics, proteomics as well as transcriptomics have enabled us to use most advanced gene editing technologies like CRISPR-Cas technology for the development of an effective vaccine development tool. By the use of these modern technologies, we are determined to develop and effective and safe vaccine against *H. controtus* in near future.

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CHAPTER 04

DIAGNOSIS, TREATMENT AND CONTROL OF CANINE TICK DISEASE

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INTRODUCTION

With the change in China's social and demographic structure, the number of empty nest groups is increasing, and pets have gradually become a new emotional sustenance. The number of pet families has increased dramatically, especially the number of pet dogs. Dogs have become one of the most exposed and closely related animals with humans. Dog diseases, especially those shared by humans and dogs, have also drawn attention in public health and become a key epidemic prevention topic of public health (Harrus et al. 2007; Dobler 2010; Dantas-Torres 2010). Ticks and tick-borne diseases seriously endanger the health of dogs. People have paid more and more attention to this disease in recent years. Canine tick disease is a common ectoparasitic disease in dogs. It is an insect-borne infectious disease caused by ticks in dog skin sucking blood. Cases of canine tick infection have been reported all over the country, but the infected tick species are different. The distribution of tick species is related to different climates, soils, vegetation and hosts. Ticks are mainly parasitic on the surface of the dog and the inside of its limbs. Feeding by sucking the blood of the dog will not only cause redness, swelling and itching of the skin of the parasitic part and cause anemia symptoms of the dog, but also secrete the toxin into the blood circulation of the dog when sucking blood, affect the release of acetylcholine, and lead to motor nerve dysfunction, muscle paralysis, and even death. In addition, ticks carry a variety of pathogenic microorganisms, such as bacteria, viruses, rickettsia and protozoa, which can spread a variety of animal diseases, including some zoonotic diseases. Therefore, how to make a rapid and accurate diagnosis of canine tick disease, how to effectively prevent tick infection and avoid the harm of ticks, which are particularly important to protect dogs and people's health.

Pathogen and its Biological Characteristics

Species Classification of Ticks

Tick is also known as wall lice, flat lice commonly known as dog turtle, grass, cattle tick, grass tick, dog bean and cattle turtle. The most harmful species are Ixodes, Argasidae and Nuttalliellidae, but they belong to Arachnida, Acarida, Acari suborder, and Acaricoidea. More than 800 species of ticks have been found all over the world, including more than 700 species of Ixodidae, 150 species of soft ticks and 1 species of nanotick (only in southern Africa). There are 117 known species of ticks in China, among which hard ticks are also the most widely distributed. About 100 species have been found. Soft ticks are less distributed in China, with only slightly more than 10 species. At present, the species of ticks parasitic on the body surface of dogs in China, mainly include Haemaphysalis, sickle-shaped Haemaphysalis, *Haemaphysalis longicornis*, *Haemaphysalis campanulatus*, grassland leather tick, and bovine tick, all of which belong to the family Ixodes (Zhang et al. 2013; Zhang et al. 2017).

Morphological Characteristics of Ticks

The tick's body is oval. The ventral back is flat when not sucking blood. The back is slightly raised, and the adult body is 2 to 10mm long. The tick swells after full blood, such as red bean or castor seed, which can be up to 30mm. Epidermis leathery, abaxially, or with a crustaceous shield plate. The insect body is divided into two parts: false head and body. The false head is located in the front of the body, visible from the back of the body. The false head of soft tick is located at the front of the body and invisible from the back.

The false head of Ixodes is composed of jaw base, claw limb, suboral plate and whisker limb. The jaw base is connected with the front end of the body. It is a well-defined ossification area, which is hexagonal, rectangular or square. There is a pair of pore areas on the back of the jaw base of female ticks, which can sense and secrete body fluid to help lay eggs. One pair of claw limbs, extending from the center of the back of the jaw base, are important stabbing and cutting devices. One suboral plate is located on the ventral surface of the claw limb and forms an oral cavity when it is closed with the claw limb. There are inverted teeth on the ventral surface of the suboral plate, which are attached organs fixed in the host skin during blood suction. The two sides of the claw limb are whisker limbs, which are composed of four segments. The fourth segment is short and embedded in the small depression on the ventral surface at the end of the third segment. The body is bag shaped, mostly brown, symmetrical on both sides. The shield plate of male ticks covers almost the whole back, while the shield plate of female ticks only accounts for a part of the front of the back. Some ticks form different flowers on the trailing edge of the shield plate, which is called festoon. There are 4 pairs of feet on the ventral surface, and each foot has 6 sections, namely the basal section, rotator section, femoral section, tibial section, posterior tarsal section, and tarsal section. There is usually a

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gap on the base node. There is Haller's organ at the proximal end of the dorsal edge of the tarsal joint of foot I, which has an olfactory function. At the end, there are a pair of claws and a pad shaped claw process. The genital pore is located in the front half of the ventral surface, often in the horizontal line of the second and third pair of foot basal ganglia. The anus is located at the back of the body and often has the anal groove. A pair of valves are located at the rear and outer side of foot Ⅳ base joint, and the valve plate is wide. The male tick has a chitin plate on the ventral surface, and its number varies according to the genus and species of the tick.

The jaw body of soft tick is on the ventral side of the body and cannot be seen from the back. There is no hole area on the back of the jaw base. There is no shield plate on the back of the body, and the body surface is mostly granular verruca, or wrinkled and discoid depression. The valve plate is small and is located above the front of base joint IV. The genital foramen is located in the front of the ventral surface, and the sexual characteristics are not significant. The anus is located in the middle or later on the body, and some soft ticks still have anterior anal sulcus, posterior middle anal sulcus and posterior transverse anal sulcus, which are located in the front and back of the anus respectively. There is no spur at each basal joint. Although the tarsal joint has claws, it has no claw pad. There is an opening of basal gland between the foot basal ganglia I - II of adults and nymphs. The secretion of basal gland fluid can regulate water, electrolyte and hemolymph. When sucking blood, pathogens also pollute the host wound with the secretion of basal gland fluid, such as some species of blunt edged ticks.

Development of Ticks

From the development process from egg to adult, tick is an incomplete metamorphosis insect, including egg, larva, nymph and adult. After sucking blood, the mature ticks mate and land, crawl in the soil, grass roots, tree roots, corners of livestock houses, etc., and lay their eggs in the cracks on the surface. One to two weeks after laying eggs, the female ticks die, and the male ticks can mate several times in their life. Ticks lay eggs only once in their life and can produce thousands or even more than 10000 eggs. The eggs are small, spherical or oval, about 0.5-1mm in size, light yellow to brown in color, and often piled into clusters (Sun 2011). Under suitable conditions, eggs can hatch larvae within 2-4 weeks. The larvae look like nymphs, but they are small and have three pairs. The larvae fall to the ground after sucking blood for 2-7 days, molt and become nymphs, and then wait for the opportunity to invade various animals. Hard tick nymphs only have one stage, and soft tick nymphs go through 1-6 stages. Nymphs have 4 pairs of feet and no reproductive pores. Nymphs fall to the ground after 3-9 days of full blood, and can degenerate into adults after dormant for several dozens of days. The life cycle of ticks ranged from 2 months to 3 years. Most soft ticks take six months to two years. The life span of Ixodes ranged from one month to tens of months. The adults of soft ticks can generally live for 5, 6 years to decades due to multiple blood sucking and oviposition.

Living Habits of Ticks

Ticks are sensitive to sunlight, temperature and humidity, dryness and rainfall. Ticks generally begin to appear at 1℃, although they can survive at 6-11℃, their development is stagnant, and they begin to be active at 10-15℃, so the peak season of tick activity is in spring, summer and autumn, which is cold resistant. Ticks are more active in the early morning and evening. At this time, they often climb to the tip of grass leaves to wait and find the host. When other animals or people pass and stay, they climb to animals and people to sting and suck blood. At noon, when the temperature is high and the light is strong, they climb under the dead branches and leaves and remain dormant. The litter and grass vegetation in the forest area can form a micro environment suitable for tick activities. Therefore, the occurrence of tick disease has obvious seasonal and regional characteristics, and has strong regular adaptability to the periodic changes of the environment.

Most Ixodes live in forests, shrubs, open pastures, grasslands and mountain soils. For example, *Haemaphysalis sanguinalis*, *Haemaphysalis longicornis* and *Haemaphysalis biconicus* mainly live in agricultural areas and fields, and the activite season is from April to September. Gamasid ticks mainly live in grassland. Overwintering adults begin to appear in late February or early March of early spring, peak period in April and gradually decrease in May. The tick mainly lives in agricultural areas, and its active season in North China is from April to November. Soft ticks mostly inhabit in livestock pens, wildlife caves, bird nests and gaps in houses. Although the activity range of ticks is small, usually tens of meters, the activities of the host, especially the seasonal migration of migratory birds, play an important role in the spread of ticks.

Host Characteristics of Ticks

The hosts of ticks are widely distributed, including reptiles, birds and mammals, with a total of more than 130 species of 3 classes, 20 orders and some species will also invade humans. Ticks find their host in a very special way, mainly relying on their keen sense of smell, especially sensitive to animal sweat and CO2. When they are 15m away from the host, they can sense it and change from passive waiting to active attack. Once they contact the host, they climb up and turn on the parasitic mode. The larvae, nymphs and male and female adult ticks suck blood. After being full, they leave the host and return to the environment. But only adults can parasitize on animals, and other stages live in the environment. Most Ixodes invade the host during the day, and the blood sucking time is long, which generally takes several days. Soft ticks mostly invade the host at night, and the blood sucking time is short, usually from a few minutes to an hour. Ticks absorb a large amount of blood, which can swell several times to dozens of times after full blood in each development stage, and female Ixodes can even reach more than 100 times (Wang et al. 2013). Ticks are often selective in the host parasites, usually preferred areas that are sparse and not easy to disturb, such as auricle, periocular, neck, armpit, inner thigh, pudenda, femoral sulcus, perianal and caudal root.

Ticks change their hosts in their life cycle. It can be divided into four types according to the number of host changes:

(1) Single host tick: each stage of development is on a host, and the female lays eggs when she is full of blood. An example is *Boophilus microplus*.

(2) Two host ticks: larvae develop into nymphs on one host, while adults parasitize on the other host. An example is residual glass eye tick (*Hyaloma detritum*).

(3) Three host ticks: larvae, nymphs and adults parasitize on three host bodies respectively. Examples include whole ditch Ixodes and grassland leather tick. More than 90% of hard ticks are three host ticks, and most of the important vectors of tick-borne diseases are three host ticks.
(4) Multi host ticks: larvae, nymphs and adults of various instars and female ticks need to look for the host to parasitize and suck blood before laying eggs, and leave after being full of blood every time. About the size of mung bean before blood sucking. After sucking full blood, it can reach the size of a thumb. Usually, soft ticks are multi host ticks.

Hazards of Ticks

Tick-borne infectious diseases are an important part of insect-borne infectious diseases. They are a kind of diseases transmitted by ticks. Most of them are natural focal diseases, which have the characteristics of wide distribution, great harm and being easy to cause outbreaks. Countries all over the world attach great importance to the research and control of tick-borne diseases. China has a vast territory and complex geographical climate, which is suitable for the natural reproduction of many vector ticks. Tick borne infectious diseases are very popular in China and seriously affect the health of the people. Therefore, the prevention and control of tick-borne infectious diseases are becoming more and more important in the work of health and epidemic prevention.

Direct Harm

Both hard ticks and soft ticks fed by blood sucking and obtain nourishment through parasitism. They are usually painless when biting and sucking blood. However, in order to protect themselves, ticks will completely embed their mouthparts into the host skin, which will make animals itchy and fidgety. They often rub, scratch or bite the skin, resulting in congestion, bleeding, edema, horny hyperplasia and acute inflammatory reaction at the parasitic site, or secondary wound maggot disease. When the number of parasites reaches a certain amount, it can cause anemia, emaciation and dysplasia of diseased animals (Fan et al. 2016). If a large number of parasites are on the hind limbs of animals, it can cause hind limb paralysis; if parasitic between the toes, it can cause claudication. For working dogs such as police dogs, tick parasitism will distract their attention during training and work, resulting in reduced training and operational ability (Liu et al. 2013).
In the process of biting and sucking blood, the neurotoxin secreted by some hard ticks will enter the host with saliva, which can inhibit the release of muscle neurocholine, lead to the conduction disorder of the motor fibers in the host, cause ascending muscle paralysis, and lead to respiratory failure and death, which is called tick paralysis (Shao et al. 2017).

Disseminate Infection

Ticks can have a variety of pathogenic microorganisms in their bodies. They are carriers and communicators of many animal pathogens. They are the most important vector of human and animal diseases except mosquitoes. They can spread a variety of diseases. It is known that ticks can carry 83 kinds of viruses, 14 kinds of bacteria, 17 kinds of regressive fever spirochetes and 32 kinds of protozoa, most of which are important natural foci diseases and zoonotic diseases. So far, 18 kinds of tick borne infectious diseases have been found at home and abroad, namely Scottish encephalitis, Powassan encephalitis, kesanu forest disease, jaw musk hemorrhagic fever, Rocky Mountain spotted fever, button fever, Queensland tick borne typhus, paroxysmal rickettsiasis, human Babesia Forest encephalitis, Crimean Congo hemorrhagic fever, tick borne spotted fever in North Asia, Q fever, tulafellosis, Lyme disease, tick borne relapsing fever, human Ehrlichia disease and tick paralysis. The last 10 diseases are also distributed in China, which can directly or indirectly cause human and animal death (Dong et al. 2011). The biggest and most common hazard to dogs is babesiosis (Wang et al. 2013). In the process of biting and sucking blood from dogs, ticks inhale the red blood cells parasitized with Babesia into the body, and Babesia can be transmitted through eggs or metamorphosis. With the process of biting and sucking blood again, they transmit the new insect with the infectious ability to healthy dogs. Babesia parasitized in the red blood cells of dogs, causing the destruction of red blood cells, resulting in the symptoms of anemia in dogs. In addition to the symptoms of anemia, the affected dogs also showed symptoms such as elevated body temperature, depression, loss of appetite, hematuria and brown urine. With the development of the disease course, the affected dogs also showed hemolytic jaundice and progressive weight loss. If they were not treated in time, they would eventually lead to the death of the affected dogs (Bao et al. 2013).

Causes of Canine Tick Disease

Negligence of Daily Protection

Because ticks mainly live in grassland and woods, people and dogs are easy to come into contact with ticks during outdoor activities. If the dog is not checked in time after going out and returning, it is easy to carry ticks and cause infection. In addition, the open kennel allows dogs to go in and out freely and lie down everywhere, which increases the chance of infection with ticks.

Poor Living Environment of Dogs

The kennel is old, the walls are covered with caves, the internal and external environment is rarely clean, feces and grass accumulate, and the dog excreta and open channels are not often cleaned, which provide conditions for the breeding and reproduction of ticks.

Improper Management of Kennel

The diet is insufficient or of poor quality, and the dogs are malnourished and emaciated. Without regular insect repellent immunity, the resistance of dogs decreased significantly. There is no seasonal environmental disinfection and pest control in the places where dogs often move, which increases the possibility of tick breeding. These loopholes in feeding management, once individual dogs are occasionally infected with ticks, will cause the outbreak and spread trend of tick disease in the whole kennel.

Symptoms and Diagnosis

Clinical Symptoms

When dogs are parasitized by a small number of ticks, most dogs do not show clinical symptoms, but with an increase in

the number, they often have local irritation, causing pain and itching in the parasitic parts, constantly shaking their heads and scratching their ears, and often trying to get rid of pests by friction, scratching and licking. However, this practice often leads to local bleeding, inflammation, swelling, erosion, ulcer or suppuration. Each female tick sucks an average of 0.14ml of blood each time. Therefore, when a large number of ticks parasitize, it can cause anorexia, anemia, emaciation and dysplasia, and the resistance decreases significantly. If the insect body is parasitic around the orbit, it will make the dog's eyelids red and swollen and the conjunctiva red. Parasitic on the auricle and external auditory canal. The dog's ears cannot stand up and are often scratched with claws. Parasitic on the hind limbs, which can cause hind limb paralysis (neurotoxin effect). Parasitism between toes (even if there is only one) can cause claudication. Even after catching ticks, claudication will last for 1-3 days (Lin 2015).

In addition, ticks are also an important vector of other blood parasitic diseases, which can indirectly cause dogs to suffer from other diseases, such as eperythrozoonosis, babesiosis and other blood protozoan diseases. Sick dogs will have anemia, high fever, jaundice, dyspnea and other symptoms, and severe cases will die of exhaustion (Li et al. 2016).

Diagnosis

Clinical Examination of Dogs

A spherical protrusion is found attached to the skin in the mouth, eyelids, head, ears, inner sides of front and rear limbs and toes of the sick dog. A closer look will show that some insects drill into the skin, some oval insects stay outside the skin, and the whole body is brown. The size of insects varies, as shown in Fig. 1 and 2. The smallest insects are the size of millet, and the larger ones are the size of soybeans and even fingernails. At the same time, the affected dogs show pain, itching and agitation. They often rub the wall and the ground with their body, or scratch and bite a certain part of the body. The affected part has skin edema, inflammation, local bleeding or bleeding points and some have partial skin ulcers. Affected dogs have decreased appetite, rough coat, depressed spirit, malnutrition, emaciation and lack of activity. Individual dogs may also have a high fever.

Laboratory Inspection

Ticks were collected from the sick dog and soaked in 70% alcohol and brought back to the laboratory for treatment. The species were identified according to the morphological characteristics, as shown in Fig. 3 and Fig. 4. Under the microscope, the shape of the whole tick is long oval, with false head and body. There is a hard shield plate on the back of the insect body. The false head is in front of the body. The base of the false head is short and looks like a hexagon; There is a germinal hole in the middle of the front of the abdomen, and its anus is located in the middle of the rear, showing a hemispherical shape of the longitudinal fissure. The insect body has a pair of valve plates, on the posterior side of the fourth pair of foot basal ganglia, a pair of claws at the end of the foot, and a hastellar apparatus at the dorsal edge of the end of the first pair of foot tarsal ganglia (Zou et al. 2016). The diagnosis can be made through the comprehensive diagnosis of typical clinical symptoms and laboratory test results.

Auxiliary Diagnosis of Complications

Ticks are the transmission vector of babesiosis in dogs. Dogs infected with ticks are prone to secondary babesiosis. Therefore, collect the blood of sick dogs for blood smears, and check whether there is babesiosis infection in red blood cells with a microscope. It can also be diagnosed with babesiosis rapid detection test paper or PCR detection.

Treatment

Manual Removal

Once a tick is found attached to the dog's body surface, it shall be taken out with tweezers. It is forbidden to pull it out by hand, so as to prevent secondary skin damage caused by tearing tissue or broken mouthparts. Put a drop of iodine tincture, alcohol or vaseline oil on it, or scald it gently with cigarette butts and incense sticks. After the tick suffocates, it will naturally fall from the skin. Do not pull it (Zhang 2016), also anesthetize the ticks with a high concentration of alcohol to relax or kill the tick head. When pulling out the tick, clamp the head of the tick with tweezers close to the front end (including mouthparts) as far as possible, keep the hand stable, and pull out the body of the tick completely in a vertical state. Pay attention not to exert too much force, but the speed should be fast, so as to prevent the tick mouthparts from falling into the body and causing local inflammation. The captured ticks should be killed immediately and can be collected in closed containers and burned to death (Wang et al. 2016). After tick removal, the bitten wound should be disinfected with iodine tincture or alcohol immediately, and the physical condition should be observed at any time (Zhang et al. 2010).

Chemicals Applications

Spraying, bathing or washing the body surface of animals with liquid medicine can kill ticks on moving objects. Pyrethroids and formamidine with low toxicity and safety can be selected. For example, the effect of medicine bath with 1% trichlorfon, 0.5% malathion and 7.5% phoxim is very good (Zhang et al. 2013). It was reported that 0.04-0.08% cypermethrin bath has an obvious insecticidal effect, and there is no obvious adverse reaction in dogs. It can also be used as environmental insecticidal (Wen 2013). Chulanling medicine bath combined with subcutaneous injection of ivermectin has a good feedback effect (Li et al. 2015). 0.5% deltamethrin emulsion, or use 0.04-0.08% metoclopramide (saffron) and other solutions to bath for 15 minutes, and the insects can fall off by themselves (Yang et al. 2009); Dichlorvos with high toxicity and high efficiency can also be selected, and the tick killing effect is also very good, but we must pay attention to safety to prevent dogs from poisoning and death.

Environment Control

At the same time of sterilizing dogs, it is necessary to spray 0.003% deltamethrin emulsifiable concentrate (also known as betamethasone and diphtheria) or 0.75% DDT on kennels, dog training grounds, lawn activity sites and other places to jointly kill ticks. Single site and environmental tick control can be incomplete and will have potential hazards (Yang 2015).

Fig. 1: Ticks on the dog's head

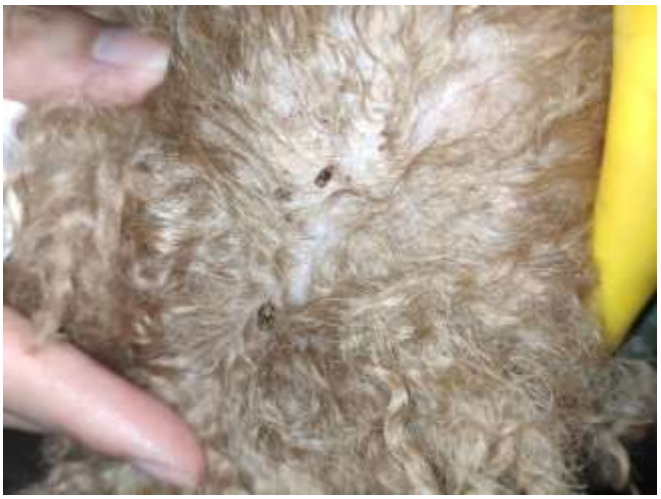

Fig. 2: Ticks on the back of the dog

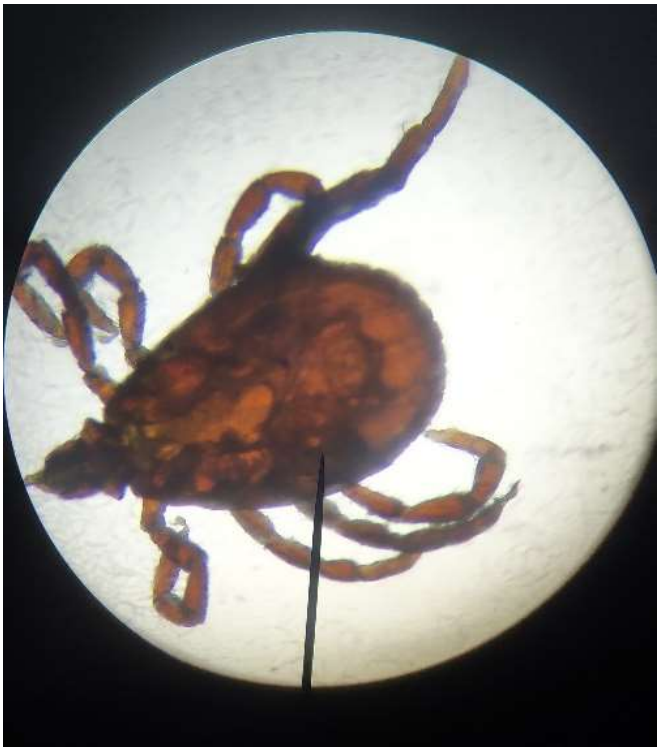

Fig. 3: Adult canine tick

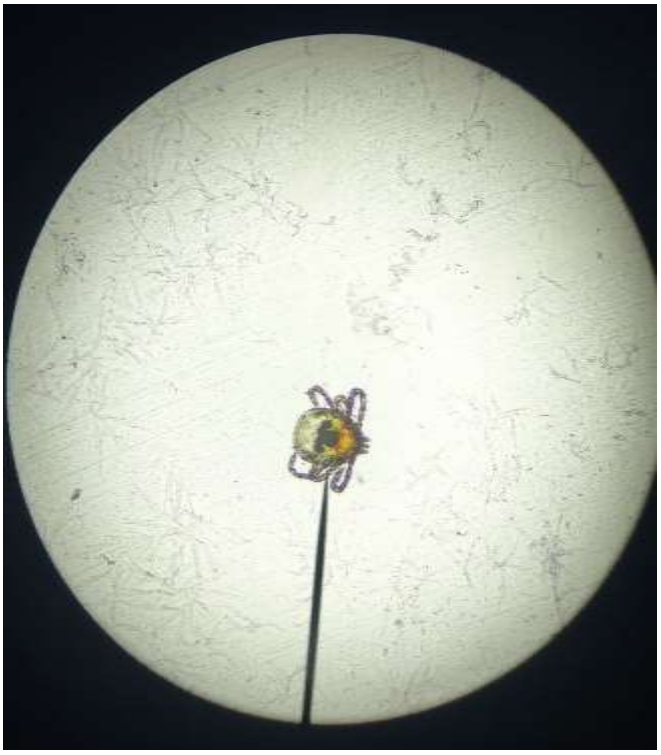

Fig. 4: Nmph of canine tick

Symptomatic Treatment

For local bleeding, edema, inflammation and other symptoms, "Wujigao (Compound beclomethasone camphor cream)", "Piyanping (Compound Dexamethasone Acetate Cream)", "Ailuosong (Mometasone Furoate Cream)" and other creams may be used for external use. In case of suppuration and other infection symptoms, antibiotic ointment such as ciprofloxacin and mupirocin can be used together. If there are neurological symptoms such as hind limb paralysis and claudication, you can take "cyproheptadine" and "vitamin C" orally and inject "vitamins B1 and B12" and other drugs, but most affected dogs will gradually reduce their symptoms with the removal of ticks (Chen et al. 2008; Chen et al. 2016). It is reported that veterinary acupuncture combined with drugs can also effectively to cure tick induced paralysis in dogs (Zhang 2010). If the symptoms of tick infection are serious, supplement body fluids (sugars and vitamins, etc.) to expand blood volume, and select anti-anemia drugs such as iron preparation to prevent serious dehydration and failure and maintain normal metabolism (Bao et al. 2010). Apply broad-spectrum antibiotics (such as ceftiofur) or antiviral drugs to prevent secondary or concurrent infection, and timely give Sodium bicarbonate to correct metabolic acidosis. At the same time, strengthen nutrition, feed easily digestible food to affected dogs, and do a good job in post-treatment care such as cold prevention and warmth preservation, so as to enhance the physique of sick dogs and recover as soon as possible.

Prevention and Control Measures

Prevention and Control Measures for Family Pet Dogs

Regular insect repellent: Parasitic infection is one of the important factors causing pet dog diseases. Many parasites can also infect humans and pose a threat to human health. Therefore,

pet owners should do a good job of regular insect repelling and clean up the parasites in and out of the pet dog. In addition, regular cleaning of the bad environment in pet living and activity areas, timely cleaning of excreta and keeping the feeding environment clean can effectively reduce the chance of pet dogs infected with parasites (Bai 2021). For example, Quanchongjing (Ivermectin) spray or Freon drops can be used to spray or drip on the ventral side of the dog's neck (Wang et al. 2014).

Fixed Pet Dog Rest Place

Since the date of purchase, pet dogs have established a close relationship with humans and have been favored by many pet owners. Many pet owners are used to eating and sleeping with their pets. However, such living habits are not healthy. Some pathogens (such as parasites) will sneak into people in the close contact between dogs and people, resulting in indirect infection of people (Yang et al. 2018). Therefore, in order to protect the health of pet dogs and people, it is necessary to set up a fixed rest place for pet dogs. On the one hand, an independent space can allow dogs to spend time alone and avoid behavioral problems such as separation anxiety due to excessive dependence on people. On the other hand, the fixed rest place is easier to clean, reduce the breeding of pathogens and ensure the sanitary quality.

Strengthen Daily Health Care

The health of animals mainly depends on the quality of feeding and management. Therefore, pet owners should strengthen the feeding and management of pet dogs and strengthen the daily health care of pet dogs in order to improve the body's resistance, including the following aspects: (1) reasonably match the diet and provide clean water to make the pet dogs grow healthily; (2) Regular outdoor exercise, control the amount of daily exercise and maintain the health of pet dogs; (3) Protect yourself before going out (e.g. wear an insect collar), do not go to the overgrown land when walking, and check and clean your body surface when returning (Chen 2017); (4) Wash and protect the whole body regularly to ensure that the dog is clean and refreshing; (5) Pay attention to the status of pet dogs, find abnormalities in time and deal with them as soon as possible.

Prevention and Control Measures for Kennels and Training Institutions

Environmental Protection

Regularly trim the grass in the scattered training site, and try to eradicate shrubs and weeds within the scope of dog activities. Clean up all supplies, utensils and insects in the sick kennel and burn them to completely eliminate pathogens and prevent secondary infection. The inside of the kennel can be fumigated with dichlorvos, and then it needs to be fully ventilated and smoke exhaust (Li 2010). Use soil mixed with 0.005% deltamethrin emulsion to block all gaps and cracks in the kennel, and then paint with lime milk to minimize the hiding place of ticks in the kennel.

Chemical Protection

Spray trichlorfon, malathion, phoxim and other anti-tick drugs on the inside and outside of the kennel, up and down, and the surrounding environment (including drainage ditch, dog cage, etc.). Pay attention to thoroughly remove the dirt on the ground and wall before spraying drugs in the kennel. Ticks in the gaps of walls and railings shall be sprayed with flame blowtorch after spraying drugs. In addition, some ticks have strong tolerance, so they can seize the favorable opportunity that ticks are in dormancy in winter and increase the work of tick killing and disinfection in winter, which can effectively prevent the outbreak of ticks in the coming year (Bao et al. 2013).

Drug Protection

Regular external insect repellent, especially for those dogs who like to go in the grass and shrubs, regular body surface insect repellent every month. "Compound non prednisolone drops","Fulaien", "baichongshuang" and "Miechongning drops" can be used to spray or drop on the neck and abdomen of the dog (Dong et al. 2022), which can effectively prevent tick bites, but do not let the animals lick them. Therefore, in the season of vigorous tick activity, drug tick control work should be more regular, comprehensive and meticulous (Yu et al. 2004).

Biological Protection

Because ticks mainly inhabit in grassland and forest, biological pesticides are used to spray the ground where ticks mainly occur, which has a long duration and is harmless to humans and livestock. It can be physically prevented and treated with "Algaecidal" (0.12% propylene glycol alginate). The drug is colorless and tasteless and harmless to human elements (Wang et al. 2013).

Strengthen the Management of Dog Breeding

Provide dogs with qualified feed and keep drinking water clean to improve their resistance and reduce disease (Shi et al. 2002). Optimize the kennel design, try to achieve one kennel for each dog as much as possible, and reduce the feeding density to reduce the opportunity of tick transmission (Liu et al. 2013). Conduct environmental sanitation and disinfection. Conduct a good job in the daily grooming of dogs, and maintain the health of dogs. In particular, during the grooming process, careful observation should be made to facilitate the timely detection of insect infection (Chen 2018). Ensure a certain outdoor exercise time, control the appropriate amount of exercise and enhance the dog's physique. Strictly control the entry and exit of dogs, strictly quarantine and conduct epidemic prevention, and carry out dog body inspection (brushing and swabbing of fur) and tick killing for dogs introduced or sold to prevent bringing in or bringing out ticks. Regular environmental tick control shall be included in the daily management work to achieve drug prevention in advance, especially the outbreak period of ticks is from June to October every year. During this period, the number of insecticides should be strengthened, and training in the main habitats of ticks such as grassland and forest shall be avoided as far as possible (He et al. 2005). In addition, often monitor the health status of dogs, and achieve early detection, early diagnosis and early treatment for dogs infected with tick disease. Once this tick disease occurs, appropriate drugs should be selected for treatment, and attention should be paid to the adjuvant treatment of nutritional supporting therapy.

Summary

With the rapid development of the pet industry, more and more people keep pet dogs, and pet dogs often go to the lawn and other outdoor environments, which are more vulnerable to ticks. Ticks have superior adaptability to the natural environment and have the ability to preserve pathogenic microorganisms and spread diseases. Therefore, the diagnosis, treatment and prevention of tick disease are related to the health of people and pets. In this chapter, the biological characteristics of ticks are discussed in detail, the common causes, clinical symptoms and diagnostic methods of dog infection with ticks are analyzed, and the specific prevention and control measures applicable to family pet dogs, kennels and training institutions are put forward, which is of great significance to the identification, diagnosis, treatment and prevention of canine tick disease, and provides reference for the prevention of human and canine co diseases.

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CHAPTER 05

IMMUNE EVASION MECHANISMS OF PARASITES WITH SPECIAL FOCUS ON *FASCIOLA HEPATICA*

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INTRODUCTION

Parasites are the living organisms (eukaryotes) that depend upon their host (definitive or intermediate) for their survival (Chulanetra and Chaicumpa 2021). They may be unicellular or multicellular organisms such as protozoa and metazoan. Parasites that are responsible for manifestation of disease in humans and animals can be classified in three main categories such as helminths, protozoans and arthropods (ectoparasites). Helminths and arthropods belong to kingdom Animalia whereas protozoans are classified into kingdom Protista (Verma 2021). Helminths have been further categorized into two phyla. Nematodes belong to the phylum Nematoda while flukes and cestodes are classified into phylum Platyhelminthes. Parasites have remarkable diversity in their life cycle and host (Cribb et al. 2003).

Although there is diverse taxonomy of the parasites, they share same mechanism to evade, overcome, and decrease the immune response of host to maintain their life cycle and parasitism due to which they are considered as the most successful organisms on earth (Chulanetra and Chaicumpa 2021). This chapter describes the most significant tactics employed by selected protozoa and helminths with special emphasis on *Fasciola* species to avoid, resist, withstand, inhibit, and alter the host immunity which is mounted against them.

Circumventing the Physical/physiological Barriers of the Host

In most cases, healthy skin is a powerful barrier that serves as the first check point against pathogens attempting to enter in the host. However, several helminthic parasites may infect humans by penetrating straight through the epidermis. Hookworm larvae (*Necator americanus*) and *Strongyloides stercoralis* (threadworm) a filariform larva can enter cutaneous surfaces of humans walking barefooted on contaminated ground. At helminth-penetrating location, the larvae generate a focused irritating region (ground itch), as well as rashes and papules. Humans that swallow contaminated substances can be infected by *Ancylostoma duodenale* larvae. Hookworm larvae that penetrate the skin can result into cutaneous larva migrans, which looks like a snake's track (Albanese et al. 2001). Urocanic acid is a metabolite of histidine which attracts *Strongyloides* spp. and is high in human and animal host skin and skin secretions (Safer et al. 2007).

The larvae of the mammalian *Schistosoma* spp. enter the skin of humans after their escape from snail into water. These infective larvae grow into adult parasites inside the mammalian host, causing various kinds of schistosomiasis based on the species of invading flukes. Urogenital schistosomiasis is caused by *Schistosoma haematobium*, which can lead to development of cancer of bladder (Ajibola et al. 2019) whereas *S. mekongi*, *S. mansoni* and *S. japonicum*, lead to intestinal and hepatic schistosomiasis (Elbaz and Esmat 2013). Schistosomes of aviary birds and animals are capable to enter in skin of human beings, but unable to mature further. Instead, they are limited to the site of penetration and result in cercarial dermatitis (Kolarova 2007; Horák et al. 2015). While some parasites, like trypanosomatids (*Leishmania* spp., *Trypanosoma* spp.), *Babesia, Plasmodium* spp., and filarial worms (*Oncocerca volvulus, Brugia malayi, Wuchereria bancroft, B. timori, Loa loa*) need vectors (ticks, hematophagous flies, mosquitoes and bugs) to transmit their invasive stages into their hosts.

Plasmodium-positive *Anopheles* bite can deliver up to 200 sporozoites into skin of human (Gomes et al. 2016). In addition to sprozoites, biting mosquitos also inject a variety of salivary constituents into the skin, including antihistamines, immunomodulators, anticoagulants, vasodilators, and platelet agglutination inhibitors, all of which aid in survival of sporozoites (Zheng et al. 2014). Even though many *Plasmodium* sporozoites are eliminated by host's innate defence components at the inoculation site, others manage to evade the immune system by diverse mechanical tactics, such as fast intercellular gliding movements (Vanderberg 1974). Invasion movement to invade hepatocytes, where the erythrocyte-infecting form, merozoites, is produced (Yuda and Ishino 2004; Tavares et al. 2013; Risco-Castillo et al. 2015; Gomes et al. 2016). *Plasmodium* sporozoite and its secretory organelles have a protein called thrombospondin related anonymous protein (TRAP) that enables sporazoite to interact with molecules of host skin surface and provide it with gliding movement to leave the skin via blood capillaries

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(Müller et al. 1993; Gomes et al. 2016). The sporozoites enter liver from bloodstream by penetrating the sinusoidal cell layer of liver and infecting the hepatocytes. The sporozoites employ perforin-like protein (PLP1) to avoid breakdown by lysosomes of hosts during movement across the cell (Patarroyo et al. 2011). The surface coat of sporozoites, circumsporozoite protein (CSP), attaches to the highly sulfated glycosaminoglycan chains which are generated by stellate cells and hepatocytes (Menard et al. 2013). The sporozoites live within the parasitophorous vacuoles, where they produce a large number of blood-stage merozoites.

The causative agents of sleeping sickness (*Trypanosoma brucei*) use the tsetse fly (obligate hematophagous) as a vector to transfer their infectious trypomastigote stage into human skin as fly bites for taking blood meal. Saliva of Tsetse fly comprises of anti-hemostatic chemicals for successful blood feeding. In addition to this, saliva also contains other multifarious constituents that aid in establishing a proper infection by transforming the human skin microenvironment into a trypanosome-friendly one. Thromboregulatory chemicals 5′-nucleotidase-related apyrase and nucleotide deaminase found in tsetse fly mouth limit the platelet aggregation and blood coagulation at the puncture site (Caljon et al. 2010). Antigen 5 is another allergen found in fly saliva that can induce hypersensitivity (type-1) (Caljon et al. 2009). Another constituent, Gloss 2, of tsetse fly saliva suppresses immune response of host by reducing the release of TNF-alpha, IL-6, IFN gamma and IL-10 from the host. Trypanosome transmission and blood feeding by the fly is facilitated by these mechanisms (Bai et al. 2015). Growth and settlement of trypanosomes in the host is also facilitated by the kinesin heavy chain 1 and arginase 1 proteins in case of *T. brucei* infection via suppressing the inflammatory response by host immune system (De Muylder et al. 2013). Trypanosome lytic factors (TLF1 and TLF2) are poisonous to trypanosomes. TLFs are usually found in human blood (Thomson et al. 2009). *Trypanosoma brucei*, on the other hand, has acquired ability to resist TLFs by releasing serum resistant associated protein, which inhibits TLF function (Thomson et al. 2009). As a result, they can progress to create sleeping sickness, which is characterized by neuropsychiatric symptoms such as sleep disturbance, disorientation, exhaustion, and seizures. If not addressed, the sickness might be deadly. Triatoma bugs are carriers of *Trypanosoma cruzi* which causes Chagas disease. Triatomine bugs like to attack peoples face. When bugs defecate, trypanosomes present in feces enter in the bite wound (Montes et al. 2006).

Parasitic worms that cause infection in human hosts through the mouth must surmount acidic environment of host's stomach. Ingested cysts of *Entamoeba histolytica* and *Giardia spp.* can resist the low gastric pH, allowing the trophozoites to germinate and develop in the host. *Giardia lamblia* infests the small intestine, feeds on nutrients in the digestive fluids, producing giardiasis in humans (Schofield et al. 1992; Hemphill et al. 2019). Oocytes of *Cryptosporidium parvum* also cause diarrhea in immunocompromised individuals (Striepen 2013). Trophozoites of *E. histolytica* break MUC2 mucin using glycosidases and cysteine proteases to penetrate the mucus layer and populate in the colon by binding with strong affinity to mucosa with their surface lectin, producing amoebiasis (Moncada et al. 2003; Lidell et al. 2006; Nakada-Tsukui and Nozaki 2016).

Echinococcus granulosus uses components of bile acid to induce the development of eggs into oncospheres, which then travel to the hepatic system through portal and lymphatic channel, where they generally grow into *Echinococcus* cysts. Onchospheres of *E. granulosus* can occasionally enter the pulmonary tissue, bones, brain, or any other organ forming hydatid cysts (Wen et al. 2019). Many parasites elude host immunity by staying at anatomical regions that are free of the host immune factors, such as hollow organs or inside the cells of host. To avoid the complement system and antibodies of host, red blood cells (RBCs) infected with merozoites of the *Plasmodium* spp. create rosettes with noninfected counterpart (Moll et al. 2015). Merozoites are insensitive to major histocompatibility complex and lymphocytes mediated killing because human red blood cells lack MHC molecules (Bowen and walker 2005). According to one study, a splenectomized squirrel monkey exhibited less *Plasmodium*-infected erythrocyte sequestration than an untreated animal.

Appropriation in Host

Several parasites elude host immunity by staying at immunological favored areas/regions that are free of the host's defense mechanism, such as within the host cells or body cavities. Blood cells lack major histocompatibility complex due to which parasites are able to survive within blood cells (Bowen and Walker 2005). Most of the life forms of blood parasites are not available in blood circulation after maturity (trophozoites and schizonts). This is made possible through a process known as sequestration which is also an immune evasion mechanism of the parasites (Miller 1969). *Babesia* spp. infect erythrocytes and generate molecules on their surfaces, causing infected red blood cells to attach to the vascular wall (Allred and Khedery 2004). In this way, they escape the elimination of themselves by the spleen.

Trichinella species, including *T. spiralis, T. britovi, T. nativa,* and *T. nelsoni* develop L3 larvae in muscle cells of the host that give rise to nurse cells which shield the parasites from host immunological identification while simultaneously supplying them with nutrients obtained from the host (Wu et al. 2008). Several parasites, such as *Taenia* spp., *Ascaris* spp., and *Opisthorchis viverrini,* reside in the host's body cavities, such as the gut lumen. They cannot be accessed by the immunoglobulins at these places, and the secretory IgA produced in the mucosal cells cannot activate the complement system. In addition, the epithelium of intestine produces many factors that have the potential to neutralize complement protein molecules (Sun et al. 1999; Andoh et al. 2001).

Antigenic Disguise

Several parasites have the host components in their coat such as carbohydrate conjugates and proteins to avoid being detected as foreign particles by the host immune cells. To escape the recognition by host immune system, adult flukes acquire antigenic proteins of the host such as erythrocyte associated antigens, integrins, complement proteins, collagen, monoclonal antibodies, CD44, and MHC (class-1) (Goldring et al. 1976; Snary et al. 1980; Braschi et al. 2006). *Schistosoma* spp. generate paramyosin muscular protein that interacts to host Fc segments of antibodies and complement 1 (C1) and complement 9 (C2) for antigenic disguise and action against complement (Laclette et al. 1992).

Onchocerca volvulus microfilariae (causative agent of river blindness) cover themselves with factor H which helps them to mask surface antigens (Meri et al. 2002). The host cells produce the outermost layer that encases the *Echinococcus* hydatid cyst developed in host organs. In this way, the pericyst is vital not only in parasite growth and sustainability, but also in escape from immune response by the antigenic masking process (Golzari and Sokouti 2014).

Different Developmental Forms of Parasites

Almost all parasites have a complicated lifecycle that includes many growth phases or variations that exhibit various surface antigens, driving the host to generate diverse/specific immune responses. In most cases, the immunity to one epitope of antigen is useless against the other epitopes.

Adult male and female parasites have different secretory and excretory products and protein profile as indicated by a proteomic study (Moreno and Geary 2008). Out of 228 proteins in microfilarial worm in a study, only 32 proteins are shared by male and female parasites (Moreno and Geary 2008). Different proteins in larval and adult parasite in both male and female indicate their different mechanisms for survival within the host (Reamtong et al. 2019). This antigenic variety renders them excellent escape from host's immune system. *Schistosoma* species have complex life cycle and includes various stages such as miracidium, sporocyst, cercariae, schistosomulae and adult (Khurana et al. 2005). After infection in the host animal, *Schistosoma* spp. express different proteins that have different biochemical composition (Gryseels et al. 2006; Colley et al. 2014; Smit et al. 2015).

Common Antigens of Host and Parasite

Many parasites have the ability to produce antigens that have molecular similarity with mammalian host components. By doing this, they are recognized as self and secure themselves from the host's immune response. Human *Schistosoma* spp. possess CRIT gene that has 98% similar nucleotide sequence with mammalian analogue (Inal 1999). Eggs and cercariae of *Schistosoma* are rich in CRIT (Deng et al. 2003). *Plasmodium* sporozoite protein has composition similar with host thrombospondin (Robson et al. 1988).

Resisting Killing by the Host

To finish the life cycle, parasites can avoid immune system by competing with phagocytic activity and avoiding the very deadly oxidative radicals and digestive enzymes of host cells in their surroundings.

Hemozoin, a substance produced by some blood parasites, interfere the phagocytic activity of the macrophages (Belachew 2018). *Leishmania* spp. produce nuclease that causes the digestion of neutrophil (Guimarães-Costa et al. 2014). Sand flies possess endonuclease in their saliva that also increase the chances of survival of *Leishmania* parasite (Chagas et al. 2014). *L. donovani* outer membrane is composed of lipophosphoglycan which prevent the phagosome maturation and neutrophil mediated damage. Promastigote is a life cycle stage of the *Leishmania* that survives within macrophages by this mechanism (Holm et al. 2001). *T. gondi* employs a number of mechanisms to evade of killing by the host cells and can enter host tissue directly via actin-based movement known as gliding motility (King 1998).

Avoiding Complement Mediated Killing

To avoid complement-mediated elimination, parasites have developed a number of ways. One of the mechanisms is the production of parasite proteins conjugating to complement components and impede the actions of the complement proteins. *Trypanosoma cruzi*, and the worms such as *Brugia malayi* and *Tichinella spiralis* avoid detection by the mammalian complement system by generating vertebrate calreticulin homologs which indirectly causes the suppression of complement classical pathway (Ferreira et al. 2004; Valck et al. 2010; Zhao et al. 2017). In human serum, *T. cruzi* calreticulin interacts with ficolins and mannose-binding lectin (MBL) and bring about inhibition of the lectin route for activation of complement (Sosoniuk et al. 2014).

T. cruzi also produces regulatory proteins for complement pathway and decay accelerating factors to suppress the complement activation (Norris and Schrimpf 1994; Shao et al. 2019). *T. cruzi* also enhances the survival by producing microvesicles that interact with complement 3 (C3) convertase enzyme (Cestari et al. 2012; Wyllie and Ramirez 2017; Shao et al. 2019).

Taenia solium and *Schistosoma* spp. are equipped with the mechanisms for complement inactivation. They perform this activity by producing paramyosin (Parizade et al. 1994) which bind with C3 and C8 causing the inhibition of membrane attack complex (MAC) formation.

Parasite's immune evasion strategies that have been included in this manuscript are summarized in Table 1.

Review of *Fasciola* Immune Evasion Mechanisms

Fascioliasis is one of the helminths borne zoonotic diseases of the livestock caused by *F. hepatica* and *F. gigantica* (Mas-Coma et al. 2005). The disease leads to high mortality and morbidity causing a huge impact on livestock business and is of great veterinary concern. (Mas-Coma et al 2019). *Fasciola* infection has recently been added to the World Health Organization's list of neglected illnesses, with clinical cases found in the America, Asia, Africa, Europe, and Oceania as well as other temperate countries (Mas-Coma et al. 2014; Mehmood et al. 2017; Mas-Coma et al. 2019). This is a major foodborne disease that is currently thought to impact approximately two million people in over 70 countries, with developing countries more severely affected (Mehmood et al. 2017; Mas-Coma et al. 2018).

The life cycle of *Fasciola* species is completed in two hosts. Sheep and cattle are their definite host while snails serve as the intermediate host (Bethony et al. 2006; Jourdan et al. 2018). It spread by the ingestion of encysted metacercariae. In the small intestine, metacercariae excyst, and change into new form called excysted juveniles (Moazeni and Ahmadi 2016). Furthermore, this infective stage of the parasite moves towards the liver through the intestinal wall of the animal. (Mas-Coma et al. 2014; Cwiklinski et al. 2016). The parasite causes many destructive changes in the host body leading to inflammation and finally reaches the bile ducts of host liver where it attains adult size having the ability to lay eggs. Different strategies are adopted by the parasites to hide/evade from immune response as shown in Figure 1.

Table 1: Summary of immune evasion tactics of the parasites

Evasion Strategy	Evasion Mean	Parasite	Factor/ Mechanism Involved	References
Overcoming host's physical and physiological barrier	Skin penetration	Hookworms	Larvae enter in skin through minute break in skin	Albanese et al. 2001
		Strongyloides stercoralis	Urocanic acid, a histidine metabolite that attracts	Safer et al. 2007
	Vector and Vector's salivary factors	*Plasmodium* spp.	Biting mosquitos inject antihistamines, immunomodulators, anticoagulants, vasodilators, and platelet agglutination inhibitors	Zheng et al. 2014
		T. brucei	Tsetse fly release thromboregulatory compounds	Caljon et al. 2010
	Mechanical damage by the vector	*T. cruzi*	Biting wound caused by triatomine bug	Montes et al. 2006
	Resistance to serum toxic molecules	*T. brucei*	Serum resistance associated proteins	Thomson et al. 2009
	Tolerate gastric acidity	*Giardia lamblia, Entamoeba histolytica, Cryptosporidium parvum*	Cysts; modification of microenvironmental condition in intestinal mucosa	Schofield et al. 1992; Striepen, 2013; Hemphill et al. 2019
	Breach intestinal mucosa	*Entamoeba histolytica*	glycosidases and cysteine proteases	Moncada et al. 2003; Lidell et al. 2006; Nakada-Tsukui and Nozaki 2016
	Digesting extracellular matrix	*Entamoeba histolytica*	Many kinds of proteases	Nakada-Tsukui and Nozaki 2016
	Exploit Bile acids and salts	*Echinococcus granulosus*	Development of its ingested eggs into oncospheres in the small intestine, which then travel to the hepatic system through portal and lymphatic channel, where they generally grow into Echinococcus cysts. Onchospheres of *E. granulosus* can occasionally enter the pulmonary tissue, bones, brain, or any other organ forming hydatid cysts	Wen et al. 2019
Sequestration in host's immunological privileged sites	Reside in blood cells	*Babesia* spp.	GPI anchor surface protein. Microneme proteins	Allred and Khedery, 2004
	Sequestration	*Babesia* spp.	Parasite induced red blood cells membrane proteins	Allred and Khedery, 2004
	Nurse cells	*Trichinella* spp.	Parasite induced host process that involve muscle cell response (de-differentiation and arrest)	Wu et al. 2008
	Reside in hollow organs	*Taenia* spp., *Ascaris* spp., and *Opisthorchis viverrine*	Avoid effective serum IgG and IgM Anti-complementary activity	Sun et al. 1999; Andoh et al. 2001
Antigenic disguise	Masking host/ host derived molecules	*Schistosoma* spp.	Erythrocyte associated antigens, integrins, complement proteins, collagen, monoclonal antibodies, CD44, and MHC (class-1)	Goldring et al. 1976; Snary et al. 1980; Braschi et al. 2006
		Schistosoma spp.	Paramyosin, Fc fragment of immunoglobulin and complement C1 and C9 protein	Laclette et al. 1992
		Onchocerca volvulus	Factor H	Meri et al. 2002
		E. granulosus	Host cells form outermost layer	Golzari and Sokouti, 2014
Parasites exist in different developmental forms	Different morphological forms	*Schistosoma* spp., *Trypanosoma* spp. and many others	Parasites exist in various forms and shapes. They express different genes during their life which in turn changes surface antigenic proteins	Gryseels et al. 2006; Moreno and Geary, 2008; Colley et al. 2014; Smit et al. 2015; Reamtong et al. 2019
Sharing of antigen between host and parasite	Complement resistance	*Schistosoma* spp.	Complement C2 receptor inhibitory trispannin (CRIT)	Inal, 1999; Deng et al. 2003
Resist killing by the host	Interrupt phagocytic activity, resistance to toxic chemical synthesis	*Plasmodium*	Hemozoin	Belachew, 2018
	Neutrophil resistance	*Leishmania* spp.	Nuclease/ nucleotidase	Chagas et al. 2014
Prevention of complement mediated killing	Interfere classical pathway	*Trypanosoma* spp.	Calreticulin homologs	Ferreira et al. 2004; Valck et al. 2010
		Trypanosoma spp.	microvesicles interact with complement 3 (C3) convertase	Cestari et al. 2012; Wyllie and Ramirez, 2017; Shao et al. 2019
	Inhibition of membrane attack complex (MAC) formation	*Taenia solium* and *Schistosoma* species	Production of paramyosin	Parizade et al. 1994

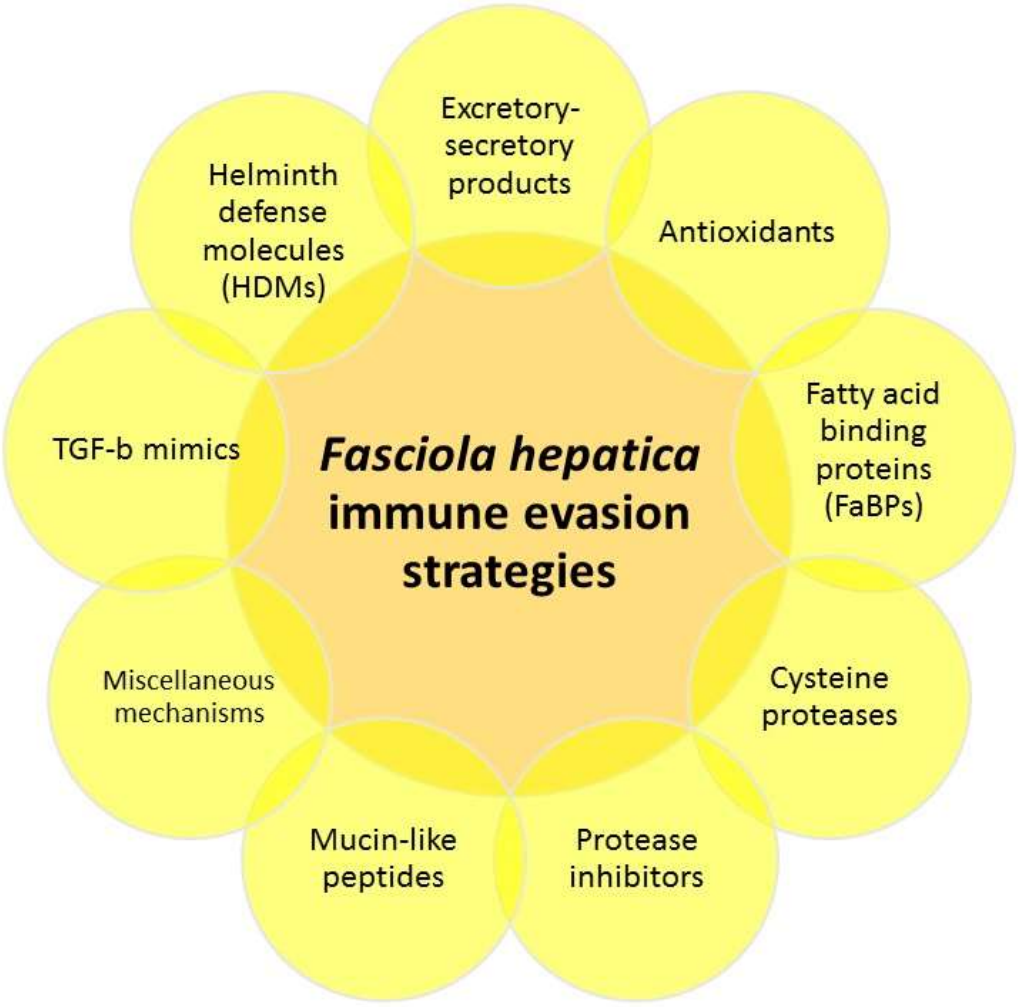

Fig. 1: *F. hepatica* immune evasion ways/ mechanisms.

Fatty Acid Binding Proteins (FaBPs)

Fasciola hepatica has FaBPs that arbitrate the lipid response in cells and is linked with metabolism and inflammation (Furuhashi and Hotamisligil 2008). Till now, there are four fatty acid binding proteins found in the excretory products of *F. hepatica* which have two roles *viz*. antioxidant and nutritive role for the *F. hepatica* (Robinson et al. 2009). Moreover, experimentally induced infection of *F. hepatica* causes decline of the immune system of host especially circulating cytokines (Ramos-Benítez et al. 2017).

Mucin-like Peptides

Some mucin-like peptides formed in the host's body act like excretory products and save the *F. hepatica* from the host immune system. These proteins are functionally and chemically similar to mucin (Cancela et al. 2010; Cancela et al. 2015). Experimental studies reveal that synthetic mucin quantity increases in the peritoneal cells of infected mice with *Fasciola hepatica* (Noya et al. 2017).

Excretory-secretory Products

F. hepatica releases various immunomodulatory molecules in the host body which are known as excretory-secretory (ES) products that alter the immune system of the host. These excretory-secretory products play an important role in the survival of the parasite in the host body. FhTLM, FhKTM, FaBP, TPx, Prx, and FhGST are the important excretory-secretory products of *F. hepatica* released in the host body (Jefferies et al. 2001).

TGF-β mimics

Bioinformatics approaches have shown that there are three transforming growth factor β (TGF-β). During parasitic development, TGF like molecules have a very important role, for example, recombinant *F. hepatica* TGF like molecules assist newly excysted juveniles (NEJ) sustainability and development by decreasing the nitric oxide (NO) production by the microphages (Sulaiman et al. 2016).

Antioxidants

F. hepatica has thioredoxin peroxidase/peroxiredoxin (TPx/Prx) antioxidant which detoxifies the metabolites of the immune system of the host increasing the survival chances of the parasite in the host body (McGonigle et al. 1997; McGonigle et al. 1998). *F. hepatica* produces Glutathione S-transferases (GSTs) which comprise about a total of four percent of their protein part and act as ES product protecting the parasite from free radicals (Chemale et al. 2006; LaCourse et al. 2012). *Fasciola hepatica* uses an important

antigen namely nFhGST which stops the Th1 responses as well as suppresses NF-kB pathway through JAK/STAT (Aguayo et al. 2019).

Cysteine Proteases

Cysteine proteases are the major part, about 80%, of ES product of *F. hepatica.* It plays an important role in the infestation of the *F. hepatica* (Robinson et al. 2008). A total of 5 classes of Fasciola cathepsin have been identified in which 2 are associated with the juvenile infective stage of the animal while three are specific to adult stage infection. FhCL1, FhCL2, and FhCL5 are found in adults while FhCL3 and FhCL4 in juveniles. FhCL3 secretion increased in the initial stage of immature juvenile infection which help the parasite in blocking the host immune system especially eosinophil (Carmona et al. 1993).

Protease Inhibitors

Kunitz serine protease has been recognized in *F. hepatica* (Bozas et al. 1995). Moreover, *F. hepatica* Kunitz type molecule (FhKTM) has exception against cysteine proteases (Smith et al. 2016). FhKTM has a specific role to deceive the immune system by impairing Th1 and Th17 responses by inducing a regulatory change in IL-27.

Helminth Defense Molecules (HDMs)

Helminths including *Schistosoma mansoni*, *Paragonimus westermani*, and *Schistosoma japonicum* have helminth defense molecules as secretory products which help the parasite to destruct the immune system of the parasites. It seems that the parasite secretes 8kDa protein during the whole life of infestation which acts as HDMs. Furthermore, HDMs divide into three groups: *Fasciola*/Asian fluke HDMs, Schistosome HDMs, and Sm16-like molecules. These groups have a unique structure, they have N-terminal peptide and helical structure which also contain hydrophobic C-terminal sequence (Donnelly et al. 2005). HDMs have a special feature that during parasitic infection. There is always secondary bacterial infections occurring at the parasitic site, but the host immune system does not trigger although there is tissue damage. It seems that the host immune system is suppressed (Onguru et al. 2011).

Miscellaneous Mechanisms

F. hepatica usually causes chronic infection in the animal due to the T helper cell 2 (Th2)/ regulatory response in the animal which helps the parasite and host tissue for support and integrity (McNeilly and Nisbet 2014; Dowd et al. 2017). In the initial phases of infection, immune response by T helper cell 2 and T helper cell 1 (Th1/Th2) gets activated along with cytokines including TGF-beta, IFNγ, IL-10, and 1L-4 activation. As *Fasciola* infection increases, the Th1 is suppressed and Th2 is amplified (O'Neill et al. 2000). Cytokines, IL-10 and IFNγ, are increasingly influenced by the mixed response at the initial stage of infection (Clery and Mulcahy, 1998). Sometimes in acute and chronic infections, TGF-β modulates IL-4 while IL-10 causes modulations in IFNγ (Donnelly et al. 2005; Flynn and Mulcahy, 2008). Another strategy adopted by *F. hepatica* to evade immune response of the host is the induction of the apoptosis of eosinophils (Escamilla et al. 2016).

Conclusion

In this manuscript, we have discussed various escaping mechanisms of parasites from host's immune response with special emphasis on *Fasciola hepatica* at molecular level. It has been revealed that parasites are endowed with diverse tactics/ mechanisms/ ways to overcome the cell mediated and humoral immune response. Moreover, these molecular entities/ factors and mechanisms can be targeted by designing novel vaccines and drugs to prevent and control disease against the respective parasite.

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CHAPTER 06

SELECTION OF PARASITES RESISTANT BREEDS OF SMALL RUMINANTS: A PERSPECTIVE

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INTRODUCTION

Gastrointestinal Parasitism and Associated Economic Losses

Parasites particularly those of gastrointestinal nematodes (GINs) have been recognized among major constraints for small ruminant population in terms of high morbidity and production losses round the globe (Aghazadeh et al. 2020). They directly affect the small ruminant production through reduced food consumption, delayed growth, low fertility, decreased milk production, skeletal growth, weight loss and mortality. Furthermore, GINs worsen the conditions where poor hygienic and managemental practice are there (Zanzani et al. 2014). Goats are more vulnerable to nematodes infection than other companion animals. Globally, occurrence of GINs in goats differs as it depends upon vegetation, geography, nutrition, season, climate, population of host and managemental exercises (Stadalienė et al. 2015). Moreover, influences of GINs are believed to become acute because of global warming (Morgan and Dijk 2012).

Small ruminants are exposed to different species of GINs during grazing. *Haemonchus* (*H.*) *contortus, Trichostrogylus* (*T.*) *axei, Teladorsagia (Te.) circumcincta, Chabertia ovina, Oesophgostomum columbianum, Strongyloides papillosus* and *Marsahlagia marshali* are the principal nematode species in this regard (Zajac and Garza 2020). Globally, GINs are commonly found in hot, humid, temperate, tropical and subtropical areas (Morgan and Dijk 2012). GINs have different predilection sites in their hosts e.g. *Heamonchus* and *Ostertagia* inhabit abomasa while *T. colubriformis* lodges in intestine. Various parasitic stages of GINs nourish or feed on blood and cellular secretions of host leading to direct and indirect losses (Malathi et al. 2021).

GINs affect the health and production of goats as well as cause massive financial and economic losses. Among direct losses, anemia, reduction in live weight, morbidity, production losses and mortality are of serious concern (Hoste et al. 2005). GINs negatively affect the health of animal and cause clinical and subclinical diseases, which ultimately lead to decrease in production and financial losses. In many countries, estimated financial loss due to parasitism includes millions of dollars annually. For example, the estimated loss of Australian sheep production system is one billion dollars per year (McLeod 2004). The financial effects of parasitism are also becoming prominent in such areas where their occurrence is not significant i.e. Netherlands, France, Denmark and Sweden (Waller et al. 2006). In UK, losses associated with haemonchosis and other GINs infections are £84 million (Nieuwhofa and Bishop 2005). In Pakistan, losses of approximately 8800 million rupees are attributed to GINs infection particularly haemonchosis (Qamar et al. 2011). According to some reports, approximately 26% of the goats have been found to expire due to haemonchosis and other GIN infections (McLeod 2004). On the other hand, cost of repeated anthelmintic treatments and poor management leads to parasite related indirect losses in small ruminants.

Strategies to Control Gastrointestinal Parasites of Small Ruminants

There are employed various methods and strategies to control GINs infecting the small ruminants. Some of these are very good to implement while some have limitations. The use of chemical compounds such as anthelmintics drugs are considered as first line of treatment for GINs of small ruminants (Nixon et al. 2020). However, the irrational use of anthelmintics is the leading cause of development of anthelmintic resistance (AR) in small ruminants. Development of AR is directly associated with repeated treatment with same brand, low quality anthelmintic preparations, under dosing and treatment of parasites free animals. Furthermore, lack of knowledge about selection of anthelmintic compound, method of administration of drug and re-exposure to resistant GINs parasites are other contributing factors. *H. contortus*; the parasite of major concern is notorious to develop resistance against specific as well as broad-spectrum drugs (Waller et al. 2006; Claerebout et al. 2020). In this prospect, non-chemical-based strategies for the control of GINs have greater scope and application in sustainable parasite round the globe (Imran et al. 2018; Burke and Miller 2020**)**.

In this scenario, husbandry practices, targeted selective treatments, biological control (use of nematophagus fungi, earth worm and dung beetles), pasture and grazing management, routine parasite monitoring strategies (Parasitic

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abundance assays, FAMACHA Scores), addition of nutritional supplements in feed, vaccine development, exploring the genetically resistant animals and integrated approaches are there to control GINs in small ruminants (Burke and Miller 2020; Claerebout et al. 2020).

Selective breeding of resistant breeds is an alternate tool for the control of GINs parasites of small ruminants. Exploring inherent resistance of goats to parasites would reduce dependence on anthelmintic drugs. Thus, reduced use of chemical anthelmintics would be optimistic approach for elimination of problem of AR and issues related to public health and environmental concerns. Sufficient data are available to support idea of breed resistance to parasites which demonstrates that small ruminant breeds are resistant, tolerant or resilient and susceptible to GI parasites (Saddiqi et al. 2010; Bishop 2012; Imran et al. 2018).

Breed Resistance to Gastrointestinal Parasitism

In the scenario of development of AR against GINs of small ruminants, selection of parasite resistant or resilient breed of sheep and goat is another auxiliary tool to control GIN parasites (Saddiqi et al. 2012; Zvinorova et al. 2016; Imran et al. 2018). Resistance from parasitological point view is known as host ability to limit faecal egg count during GIN infection. The rate of replication of a parasite inside its host is directly linked with level of resistance of the host (Doeschl-Wilson et al. 2012). It is a proven fact that selective breeding of animals against various diseases is possible. The term "tolerance" refers to ability of a host to perform or remain productive during course of parasitic infection (Bishop 2012). Breeding of such tolerant animals decreases their susceptibility towards parasites vigour without disturbing the invading parasite. The estimation of host tolerance is greatly depending upon the parasitic burden which make it difficult to measure in resistant animals (Bishop and Woolliams 2014). The term "resilience" is mentioned in various studies with different definitions. Doeschl-Wilson et al. (2012) defines it as ability of a host to maintain its performance during infection irrespective of parasite burden which may be confusing while measuring tolerance of host. Another confounding explanation resilience is the ability of a host to tolerate the parasitic burden without appearance of any clinical signs (Gunia et al. 2013). A comparatively more specific definition of this term is adaptation of an animal to diverse environments in its surrounding (Bishop and Morris 2007). Various parameters may be used to measure the resistance and resilience of an animal. Faecal egg counts and worm burden values in a host define its resistance status while PCV and live body weight are considered as parameters of resilience (Chiejina et al. 2010). Resistance to GINs can be calculated by checking of FEC directly or through determining the level of antibody produced or counting the blood and tissue cells in natural or artificially infected host population. Globally different breeds of sheep and goat from varying geography are screened for their inherent resistance to GI parasites listed as Table 1 and 2, respectively.

Parameters Associated with Breed Resistance

Faecal Egg Count

Faecal egg count (FEC) is an extensively used tool for evaluation of breed resistance towards GINs (Zvinorova et al. 2016; Imran et al. 2018). This is a heritable trait in sheep and goat (McBean et al. 2016). Its heritable ranges vary in goat (0.04-0.37) and sheep (0.2-0.4). Reports are available on heritability estimates of FEC in multiple goat breeds round the world. In Sannen goats of New Zealand, the estimated value of heritability was 0.05 in one-year goats while in case of Fijian goats, heritability values were 0.04-0.08 in Fiji (Bisset et al. 1992). Mandonnet et al. (2001) used different age groups (4, 8 and 10 month) of Serole goats to estimate heritability for FEC and values were 0.14, 0.17 and 0.33, respectively. These studies support the facts that heritability estimates with respect to FEC are associated with genetics of host and they tend to increase with age (Bishop et al. 1996). Faecal egg counts are reliable, practical and valuable criteria to measure the host resistance against GINs across the globe. This parameter is highly correlated with adult worm counts in lambs (Good et al. 2006; Shamim et al. 2016). While with respect to live weight of infected goats, FEC is negatively correlated in lambs of Scottish Blackface sheep (Bishop et al. 1996). A much significant correlation between growth rate and FEC in *Te. Circumcincta* infected small ruminants has been reported by Eady et al. (1998).

Several factors could be associated with variation in FEC such as inhibited growth of L_3, low egg production by adult parasites and inappropriate males and female ratio in abomasa (Bricarello et al. 2004; Prince et al. 2010). Worm fecundity and numbers directly affect the FEC values (Sonstegard and Gasbarre 2001). However, some other limitations are also associated with FEC values to determine the genetic resistance to GINs. Goats are more susceptible to GINs parasites as compared to sheep and heritability estimates for FEC are also lower in sheep (Baker et al. 2001).

The shedding of nematode eggs in faeces is a clear indication of an active infection in any host. The principle of FEC depends upon the number of eggs/burden and fecundity of parasite (Saddiqi et al. 2010). Variations in FEC have been reported in different species of sheep and goats which were exposed to natural and artificial infection with GINs (Gruner et al. 2003; Gonzalez et al. 2008). Significant differences in FEC values were also reported in Red Maasai and ¾ Red Maasai goats as compared to Dorper and ¾ Dorper goats, respectively (Mugambi et al. 2005).

Packed Cell Volume

The estimation of anaemia level in any individual is carried out by monitoring the Pack cell volume (PCV). The effects of blood feeding parasites are mainly estimated by determining the PCV (%) levels (Shamim et al. 2016; Imran et al. 2018). It is a phenotypic marker associated with genetically derived production parameters like wool growth and weight gain. There may be a negative correlation among PCV and FEC in parasite infected animals (Rout et al. 2011). In various parts of world where *H. contortus* is an endemic parasite, PCV is a low to somewhat moderately heritable trait in goats.

PCV heritability estimates range from 0.11- 0.33 and are similar in sheep (Baker et al. 2001). PCV heritability estimates in creole goats from India have been reported as 0.14, 0.33 and 0.10 (Mandonnet et al. 2001), and in Small East African goats from Kenya as 0.25 and 0.11 (Baker et al. 2001). Packed cell volume is considered as reliable marker for determination of host resilience in experimental as well as natural infection with various GINs (Chiejina et al. 2010; Imran et al. 2018). Both PCV

Table 1: Global distribution of some parasite resistant sheep breeds towards natural and artificial infection of gastrointestinal parasites

Sr. No.	Name of Sheep Breed	Country	References
1	Garole and Sahabadi sheep	India	Lalramhluna et al. 2020
2	Merino sheep	Australia	Brown and Fogarty 2016
3	Arsi sheep	Ethiopia	Abay et al. 2015
4	Local Awassi Sheep	Iran	Al-jebory et al. 2012
5	Gulf Coast Native sheep, Canaria Hair sheep, Florida Native sheep	USA	Gonzalez et al. 2008
6	Barbados Blackbelly lambs	Caribe	Terefe et al. 2007
7	Texel sheep	Ireland	Good et al. 2006
8	Scottish Blackface (responders)	U.K.	Davies et al. 2006
9	Red Maasai sheep	South Africa	Mugambi et al. 2005
10	Crioula Lanada sheep, Santa Ines sheep	Brazil	Bricarello et al. 2004
11	Black Belly, INRA 401 (resistant)	France	Gruner et al. 2003
12	Indigenous Sabi sheep	Zimbabwe	Matika et al. 2003
13	Rhön sheep	Germany	Gauly et al. 2002

Table 2: Global distribution of some parasite resistant goat breeds towards natural and artificial infection of gastrointestinal parasites

Sr. No.	Name of the Goat Breed	Country	References
1	Teddy, Beetal, Dera Din Pannah and Nachi goats	Pakistan	Imran et al. 2018; Shamim et al. 2016
2	Scottish Cashmere Tropical goats	UK	McBean et al. 2016
3	Black Bengal goats	Bangladesh, India	Dhara et al. 2015
4	Black Iraqi	Iraq	Al-jebory et al. 2012
5	Local Ardi and Imported Syrian	Saudi Arabia	Al-Seaf and Khaled 2012
6	Barbari, Local hill, Jamunapari and Barbari goats	India	Rout et al. 2011
7	West African Dwarf	Nigeria	Chiejina et al. 2010
8	Crossbred Cashmere	Scotland	Vagenas et al. 2002
9	Galla and Small East African	Africa	Baker et al. 2001
10	Savanna goats Creole goats	USA	Mandonnet, et al. 2001
11	Caninde, Bhuj and Anglo-Nubian	Brazil	Costa et al. 2000
12	Thai native goat	Thailand	Pralomkarn et al. 1997

and FEC are phenotypic markers associated with resistance to GIN parasitic infections and are helpful in identifying associated quantitative trait loci in resistant host (Davies et al. 2006). Imran et al (2018) determined that PCV and FEC virtuous indicators for estimation of naturally acquired resistance to GINs.

Total Serum Proteins and Albumin

Many of the parasitic infection are characterized by hypoproteinemia and hypoalbuminemia (Bordoloi et al. 2012). Therefore, total serum protein (TSP) and serum albumin (SA) can be used as indicator traits for assessing the resistance of breeds. Several studies have shown a substantial variation in levels of TSP and SA in parasite susceptible and resistant goats (Imran et al. 2018). Shamim et al. (2016) demonstrated this variation in different goat breeds which were exposed to natural and artificial infection of *H. contortus*.

Post Necropsy Worm Counts

It is generally accepted that parasite burden and health of infected host do not correlate always. In veterinary studies, post-mortem evaluations represent the actual picture of correlation between pathology and invading pathogen (Terefe et al. 2007). It always depends upon rate of establishment of infection and host response to pathogen. According to some studies, it is positively corelated but some claim that these are not associated with each other (Gauly et al. 2002; Dominik 2005, Idris et al. 2012).

Live Weight

Live weight is also a useful tool to determine the inherent resilience or resistance of small ruminants towards natural or artificial infection with GINs (Mugambi et al . 2005). The performance of such sheep and goats may be evaluated in terms of reduction or gain in live weight of animal. Live weight is moderately heritable trait which plays key role in determining the maternal effects of study animals. The live body weight is an important tool for numbers of reasons including selection, breeding, feeding and health care of animals (Moaeen-ud-Din et al. 2006).

Immunoglobulins

The immune system of goats produces IgA, IgE, IgG and IgM immunoglobulins in response to infection with GINs (Imran et al. 2020). In the past periods, some researchers studied the possible role of these Igs in immunity against GINs both at systemic and local levels. Among the antibody isotypes, IgM does not have a significant role (Nehra et al. 2019). However, IgE plays an important role in immunity against GINs. Most studies investigating the IgE response to helminth infections have been human and rodent models based. Parasite specific IgE as well as elevated level of IgE are considered as an important feature of host response during an active GIN infection (Miller and Horohov 2006). In humans, high levels of IgE in the serum during helminth infections is supposed to be among some important factors associated with immunity to GIN infections (Fitzsimmons et al. 2014). However, the significance of IgE in animals during infection with GINs is not well studied.

The role of IgE may be studied through goat specific monoclonal antibodies exposed to challenged infection with *H. contortus* and *Te. Circumcincta.* Furthermore, an IgE specific ELISA was also developed to monitor the Igs levels during experimental infections with GINs (Huntley et al. 1992; Kooyman et al. 1997). During haemonchosis, the IgE levels tend to increase at 2nd to 4th week PI while IgE levels and post

necropsy worm counts are negatively correlated (Kooyman et al. (1997). Huntley et al. (1992) studied the post infection IgE response to artificial infection with *Te. circumcincta* larvae in sheep. In sheep, without previous exposure the levels of IgE in lymph and serum started increasing at eight to fourteen days post infection, but a more rapid response was noted in formerly infected sheep while low levels of IgE towards adult antigen were observed by Huntley et al. (1992). However, a more vibrant IgE response towards ES antigen of *H. contortus* was recorded by Kooyman et al. (1997). Shaw et al. (1998) used natural and artificial infection with *Te. circumcincta* to infect the sheep and recorded some systemic levels of IgE. They observed peak levels of aforementioned antibody at 20-27 days post infection. Increased levels of IgE, eosinophils and mastocytes are three symbols of immune response to GINS. High levels of IgE and elevated eosinophil counts are observed in infection with GINs. Moreover, Interleukin (IL)-4 and IL-5 production is associated with higher levels of IgE and eosinophils. This indicates that Th1 and Th2 cytokines both may play a role in infection resistance. In both humans and animals, helminth infections have proven to be a potent stimulus of IgE responses (Fitzsimmons et al. 2014; Motran et al. 2018).

Cytokines produced by the T helper cell subset designated Th-2 regulate these responses in humans and mice (Liao et al. 2011). Mouse strains that are resistant to *Trichuris (Tr.) muris* can mount a Th2 response and can eliminate worms before they mature and can reproduce. Susceptible mouse strains that are treated with IL-4 are able to cure chronic *Tr. muris* infections. The importance of the Th2 response, but not the Th1 response, against helminth infection was similarly established in IL-4 or IFN knock out mouse. The IL-4 induced Th2 immune response regulates the production of antibodies, converting to IgG and IgE antibodies as described by Finkelman et al. (1988). The IgE response is strongly associated with allergic diseases and helminth infections, but it has been difficult to find the exact role of IgE in immunity towards GINs infection.

Serum IgE levels may increase may folds during infection with GINs that is proportionally larger as compared to other Igs (Cruz-Tamayo et al. 2021). Interestingly, during rise of levels of IgE, a minor amount of IgE level is specific to that parasite. This high level of disproportionately distributed IgE might be used for to soak IgE receptors present on effector cells. This will prevent the activation of the effector response or mechanisms (Albuquerque et al. 2019).

The immune response of small ruminants to GINs is characterized by increased levels of eosinophils, mastocytes and elevated levels of IgA, IgE and Th-2 type cytokines. In wool sheep selected for increased parasite resistance, IgA and IgE production increases after GIN infection, indicating an association between the antibodies and resistance. Greater numbers of immune cells are associated with low FEC and worm burdens in resistant breeds of sheep. Parasite resistant small ruminants have stronger Th_2-type immune response as compared to susceptible ones. It is a well-known fact that no single mechanism is responsible for the induction of immunity against GINs (Gonzalez et al. 2008). Even, immune response is not similar in case of different GIN species.

Role of Circulating Cells and Cytokines at Tissue Levels

Various studies have been conducted to determine the response of cytokines towards *H. contortus* infection by evaluation of abomasal tissues of sheep. These studies showed cytokines IL-3 and IL-4 response in immunized sheep within three days post infection. In some studies, cytokines upregulation is observed in immunized sheep as compared to susceptible ones. Along with cytokines, some other molecules such as lectins and galectin have been believed to play their role in immune expulsion of GINs (Dunphy et al. 2002). Faecal egg count and adult worm count methods are used to check protection level against GI nematodes (Robinson et al. 2011). During GI nematodes infection, increase in type 2 responses and increase in production of cytokines; IL- 4 and IL-5 increase in mast cells, intra-epithelial mast cell numbers, globule leukocytes and increased tissue eosinophil level is observed (Lacroux et al. 2006). In parasite resistant animals, hypersensitivity reactions and rapid rejection rate for larvae of *H. contortus* is observed (Balic et al. 2002). Rapid exclusion of larvae is related with immune response, increased influx, accumulation of globule leukocytes and mucosal mast cells from abomasal tissues (Huntley et al. 1992).

Genetic Marker for Parasite Resistance

Resistance against parasites is a variable character. Phenotype of quantitative trait depends upon additive effect of genes. Parasite resistant phenotype is due to involvement of minor and major genes of different environmental factors to genetic variation (Beh and Maddox 1996). Quantitative trait loci (QTL) was used first time for identification of genetic markers for parasite resistance. Previous studies suggest that QTL for strongyles is present on 3rd and 20th chromosome, respectively (Coltman et al. 2001; Davies et al. 2006). These QTLs are affecting the FEC and result in reduction of FEC up to 98 % (Schwaiger et al. 1995) while in another study there is no significant effect on FEC reduction (Marshall et al. 2009). According to these, QTLs study genome regions involved in parasite resistance during immune response. QTLs for resistance to internal parasites of sheep and goats are also reported (Dominik 2005). Based on segregation analysis; resistance effects for *H. contortus* are reported from major gene but confirmation through genetic marker is still needed. Issues related with the use of QTLs are the basic hurdle in finding proper phenotype for resistance and complex biological mechanism. In livestock production, related traits are controlled by many genes from different QTLs. These are composed of many genes involved in host protection mechanisms (Charon 2004).

So, number of QTLs are required for immunological response of host as described by previous studies (Behnke et al. 2003; Menge et al. 2003). QTLs participate in resistance development against GI nematodes and are identified on more than 20 chromosomes (Dominik 2005; Bishop and Morris 2007). These QTLs are present on MHC region and interferon-gamma (Davies et al. 2006). Creole breed genome was scanned first time for GI nematodes resistance and identified 13 QTLs involved in resistance, immune criteria and resilience (de la Chevrotière et al. 2012). So, resistance against GI nematodes is controlled by many genes (Bishop 2012). Genomic application will help in better understanding of genetic mechanism involved in resistance process (Goddard and Hayes 2009). Parasite resistance and susceptibility are inter-related and depend upon the immune response magnitude towards GINs during secondary infection. Humoral immunity also plays important role towards resistance against nematodes.

Major Histocompatibility Complex

Major histocompatibility complex (MHC) is known as clusters of genes involved in immunological and non-immunological responses and present in all vertebrates except some fish species (Wieczorek et al. 2017). Genes belonging to class I play important role in susceptibility to infections and class II genes involved in resistance against disease and regulation of hypersensitivity reactions and genes belonging to class III which regulate innate and adaptive immune responses are present in MHC (Friedhoff et al. 1988; Sayers et al. 2005). MHC plays an important role in providing resistance against nematodes, autoimmune diseases and several other pathogens (Liu et al. 2006; Stear et al. 2007).

According to Stear et al. (2007), MHC genes are considered as best potential genes for disease resistance. Genes present in MHC are known as markers and help in disease resistance selection. Selection based on use of genetic markers is known as marker assisted selection (Dekkers 2004). Variations among MHC are responsible for resistance selection at individual and population level (Trowsdale 2011). Bovine, caprine and ovine association among nematode infection and MHC is observed in different animal species i.e. sheep, cattle, mice and pig (Stear et al. 1990). Cluster of genes present in MHC are divided into two types, MHC-I and MHC-II based on their surface proteins. Antigens belonging to MHC -II are reported more active in resistance production as compared to MHC-I (Sayers et al. 2005). For regulation of immediate hypersensitivity reactions in humans, genes present in class II are more important as compared to class I (Friedhoff et al. 1988). Class II genes are also active in murine models of nematode infection as compared to class I (Yasmeen et al. 2014). According to Schwaiger et al. (1995), DRB1 allele present on ovine MHC loci is important from resistance point of view against *Te. circumcincta* and affect FEC due to its involvement in antibody response and antigen presentation. Specificity of antibody against nematodes is determined through MHC. According to Stear et al. (1998), W9 antigen is related with susceptibility while CA45 is related with resistance. Major antigens related with susceptibility and resistance against *Trichostrongylus axei* and *H. placei* are W7, CA36, CA5, Eul2, W8 and W9 (Stear et al.1990). During genetic variation, some contribution is provided by BoLA. Antigens associated with decrease worm egg concentration are CA36 and W7. *H. placei* count is significantly affected by BoLA consisting of four major antigens (CA36, W8, CA5, W7) (Stear et al. 1990). Sheep leukocytes antigen are interrelated with resistance against *T. colubriformis*. Bovine leukocytes antigen is related with *T. axei* infections having W9 antigen (Stear et al. 1990; Yasmeen et al. 2014).

Histamine Concentration and Breed Resistance

The lethal effects of eosinophils against GINs are intensified by derivatives of mast cells primarily through histamine followed by T-lymphocytes and macrophages derived complement factors (Meeusen 1999). Histamine derived immune system immediately expels the parasites from host and a significantly higher concentration of histamine is observed in abomasal mucosa of resistant small ruminants. The immunological functions of certain immune cells like dendritic cells, B-cells, T-cells, epithelial cells, granulocytes and type 2 cytokines are greatly affected by concentration of histamine (Miyamoto et al. 2006). Higher concentration of mucosal histamine imposes

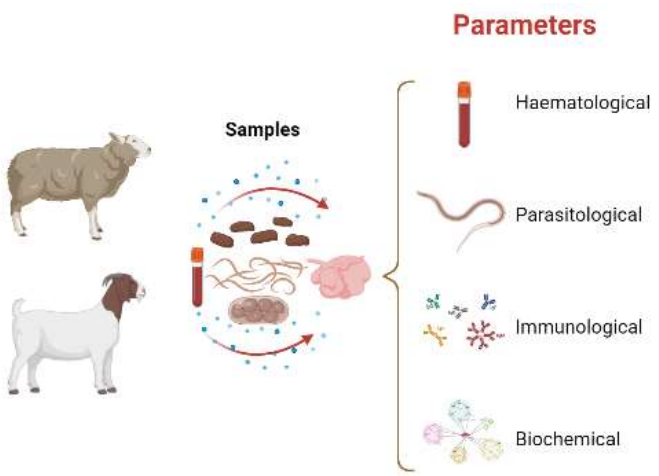

Fig. 1: An overview of parameters used for evaluation of parasite resistant breeds of small ruminants towards natural and artificial infection of gastrointestinal parasites.

detrimental effects on survival of parasitic worms in terms of loss of motility and fecundity, hence, facilitates the movement of antibodies through lumen of abomasa (Bryce et al. 2006). Histamine released by mast cells results in peristaltic movements and leads to mechanical expulsion of parasites. Gastric secretions like histamine results in decrease egg producing capacity of *H. contortus* worms.

Histamine released and caused vascular permeability in response to parasite's antigens. Histamine was secreted during whole infection period in artificially infected goat breeds. Minimum level in blood may be observed at day zero of infection while maximum concentration may be seen during 2nd to 6th weeks post infection in goats (Imran et al. 2020). According to Harrison et al. (2003), higher levels of histamine and Abs are observed in sheep infected *T. colubriformis*. A persistent concentration of histamine in plasma of infected goats is reported in various studies (Harrison et al. 2003; Imran et al. 2020). Histamine is involved in resistance development again parasites, minimizing immunopathological complications and modification of immune responses (Bourne et al. 1974). An overview of some parameters used for evaluation of parasite resistant breeds of small ruminants towards natural and artificial infection of gastrointestinal parasites in given in Fig. 1.

Concluding Remarks

Small ruminants are considered as integral part of various farming systems, particularly for non-agriculture community and resource poor farmers. Gastrointestinal parasitism particularly with those of nematodes remains a major constraint associated with the production of small ruminants under grazing/browsing conditions. Considering the devastating effects of GINs, control strategies that usually adapted are chemotherapy, vaccination, pasture exposure, ethnoveterinary practices, pasture and grazing management, but all these have their own limitations such as anthelmintic resistance, drug residues, cost of purchase, efficacy and environmental concerns. Genetic selection of lines or breeds of hosts (small ruminants) is a complementary tool used to control GI parasitism globally. Different breeds of small ruminants respond differently towards natural and challenge infection with *H. contortus*. This variation in response is the basis of selective breeding of resistant breeds of small

ruminants. Selected breeding of resistant breeds in the area will definitely enhance the economy of the herd owners in terms of negligible parasitic infections, cutting off treatment cost, low morbidity/mortality and high production. In conclusion, it can be stated that selective breeding of parasite resistant sheep and goat breeds should be carried out at rural as well as commercial level. Selectively bred animals will be more productive and disease resistant. This practice will not allow the establishment of disease and consequently may reduce the use of anthelmintics. This will ensure availability of chemical free meat and milk for human consumption. Ultimately, this effort will be a way forward to achieve the goal of secure food for human consumption.

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CHAPTER 07

ANAPLASMOSIS

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INTRODUCTION

Anaplasmosis is caused by obligatory intra-erythrocytic bacteria called Anaplasma (Order: Rickettsiales, Family: Anaplasmatacae, Genus: Anaplasma). The disease caused by Anaplasma is called anaplasmosis and is characterized by anemia, high fever, jaundice, abortion and death (Waruri et al. 2021). There are different species of genus Anaplasma (A.) cause disease in humans and animals including *A. marginale*, *A. phago¬cytophilum* and *A. ovis* (Fishbein et al. 1994; Dumler and Bakken 1998; Dumler and Broqui 2004; Zobba et al. 2020). Anaplasmosis is transmitted through infected tick-bite, biting flies and contaminated fomites. Bovine anaplasmosis is commonly known as gall sickness and is prevalent throughout the world (Rochlin and Toledo 2020).

Etiology

The word Anaplasma is a combination of Greek words ana means without and plasma means molded or produce (Dumler et al. 2004; Atif et al. 2021). On the basis of phylogenetic analysis, family Anaplasmataceae includes following genera Anaplasma, Aegyptianella, Candidatus, Neorickettsia, Ehrlichia and Wolbachieae (NCBI gene bank information). Genus Anaplasama comprises of various species including A. marginale, *A. centrale*, *A. ovis*, *A. bovis*, *A. phagocytophilum*, *A. platys* and *A. odocoilei* (Dumler et al. 2001). *A. marginale*, *A. centrale*, *A. bovis* and A. ovis are the species prevalent in ruminants (Table 1). *A. phagocytophilum* causes granulocytic anaplasmosis in both equines (EGA-Equine Granulocytic Anaplasmosis) and humans (HGA-Human Granulocytic Anaplasmosis).
Development of Anaplasma spp. occurs in the vertebrate host and arthropod vector. In case of vertebrate host, Anaplasma persists in their bodies for long duration and they act as reservoir host. When a vector feed on infected reservoir host, becomes infected. Anaplasma spp. grows in gut cells as well as in salivary glands of ticks and is transmitted through tick saliva during blood feeding (Villar et al. 2016).
Occurrence of species of genus Anaplasma is sporadic throughout the world but endemic in tropical and subtropical regions of world due to high tick burden in these areas and high risk of carrier animals (Smith et al. 1986; Wickwire et al. 1987; Allred et al. 1990; Rodriguez et al. 2000; de la Fuente et al. 2001; Palmer 2001; Maggi and Krämer 2019). *A. marginale* infected final hosts as well as vectors (ticks) acts as reservoirs of infection to other susceptible hosts. *A. marginale* only resides inside the cytoplasmic vacuoles of erythrocytes (Richey 1981; Raboloko et al. 2020). About four rickettsias are present in each erythrocyte while more than 70% of erythrocytes become infected with Anaplasma during the acute phase of the disease. Reticuloendothelial system removes infected erythrocytes which results in development of anemia and icterus (Richey 1981; Saetiew et al. 2020).

Transmission of Anaplasma Species

The mode of Anaplasma species transmission is based on the ecological conditions and availability of final host. Different geographical strains of Anaplasma are identified which have different morphological features, characteristics of antigen and their ability to disseminate by vectors (Cabezas-Cruz et al. 2013).
Various routes observed for the transmission of Anaplasma species includes vectors, fomites and transplacental transmission. Ticks play the role of biological vector whereas, flies are mechanical vectors. There are about twenty species of ticks; belonging to genus Rhipicephalus and Dermacentor, involved in the transmission of Anaplasma species (Aubry and Geale 2011). Unlike Genus Babesia, trans-ovarian transmission (transmission from female ticks to larvae) of Anaplasma species does not occur in ticks. So, persistent infection in host is needed for spread of Anaplasma species. Hematophagous flies including families Culicidae, Muscidae and Tabanidae are important in the transmission process of Anaplasma species. Fomites include surgical instrument, needles and piercing objects. Infected mothers may pass the infection to their claves, which end up in calf mortality (Kocan et al. 2010). It is noteworthy that there is still a conflict on transplacental transmission of Anaplasma species as some reports did not verify transplacental transmission (Aubry and Geale 2001). This conflict could be due to genetic variations among Anaplasma species and level of host immunity.

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Table 1: Molecular diagnosis of various species of genus *Anaplasma*

Species	Molecular test	Animal screened	Reference
A. phagocytophilum	Nested PCR	Goat	Tumwebaze et al. 2020
A. ovis	PCR, PCR-RFLP	Goat	Aguirre et al. 2006
A. marginale	PCR-RFLP	Cattle	Ayyez et al. 2019
Anaplasma spp.	PCR, ELISA, Nested PCR	Sheep	Fuente et al. 2001

Characterization of Genus Anaplasma

Below given is the detailed description of various species of genus Anaplasma:

A. marginale

A. marginale is an obligate species of Anaplasma which causes 'bovine anaplasmosis' or 'gall sickness'. A variety of wild and domestic ruminants are infected by A. marginale including Bos taurus, Bos indicus, Bubalus bubalis, Cervids and Giraffa etc. (Kocan 2003; Aubry and Geale 2011; Ayyez et al. 2019). Unlike transovarial transmission of Babesia, transstadial transmission occurs in case of *A. marginale* (Kocan et al. 1981; Stich et al. 1989; Kocan et al. 1992; Madison-Antenucci 2020). Transmission of *A. marginale* occurs through ticks, biting flies, fomites. Moreover, tans-placental transmission occurs during second and third trimester of pregnancy (Dikmans 1950; Zaugg 1985; Atif et al. 2021). More than 20 species of ticks are involved in transmission of *A. marginale* (Dikmans 1950; Kocan 2003; Da Silva et al. 2015). The mechanical routes of *A. marginale* transmission are significant in tick free areas (Dikmans 1950; Da Silva et al. 2013; Solomon and Tanga 2020). Clinical signs of gall sickness include high fever, emaciation, abortion, lethargy and death in few cases (Barbet et al. 1999; Kocan 2003; Sazmand et al. 2020). Incubation period of *A. marginale* is variable from days to months. During acute phase of infection, *A. marginale* infects up to 70% erythrocytes. During blood smear examination of infected animals, 2 to 4 intra-erythrocytic inclusion bodies are seen, and this could be major reason of high erythrocytic destruction (Barbet et al.1999; Kocan et al. 2010; Berthelsson et al. 2020).

A. centrale

A. central, is morphologically similar to *A. marginale* but it resides inside the erythrocytes but its inclusions are more central (Khodadadi et al. 2021). *A. centrale* was identified by Sir Arnold Theiler in 1911. Prevalence of A. centrale is high in tropical and subtropical areas (Rar and Golovljova 2011; Sarangi et al. 2021). It causes mild anemia in infected animals being less pathogenic by its nature and that is why it is preferred to be used as vaccine agent against anaplasmosis (Zaugg 1985; Kocan 2003; Rajput et al. 2005; Abdullah et al. 2021). *A. centrale* transmission occurs through ticks, biting flies and fomites. Only tick species involved in transmission of *A. centrale* is Rhipicephalus simus (Calleja-Bueno 2021) but with the help of molecular techniques, its presence in Haemaphysalis punctate and Amblyomma sp., has also been reported (Palomar et al. 2015; Teshale et al. 2015). *A. centrale* cause mild disease in cattle (Rar and Golovljova 2011). For control of bovine anaplasmosis live attenuated vaccine of *A. centrale* is being used in Australia, Africa, Latin America and Israel (Kocan et al. 2010; Waruri et al. 2021).

A. bovis

A. bovis was first time identified in cattle in 1936. Unlike *A. marinale* and *A. centrale*, *A. bovis* is also an obligate intra-monocytic species of Anaplasma in cattle (Donatien and Lestoquard 1936). Prevalence of *A. bovis* has been reported in United States, Europe, Asia and Africa (Uilenberg 1995; Goethert and Telford 2003; Kawahara et al. 2006; Santos and Carvalho 2006; Ceci et al. 2014; García-Pérez 2016; Consolaro et al. 2019). *A. bovis* can cause disease in cattle, buffalo, goat, dog, deer, Mongolian gazelle, raccoon, leopard cat, cotton-tail rabbit and eastern rock sengi but bovines (cattle and buffalo) are the usual host (Goethert and Telford 2003; Guo et al. 2020). Tick genera involved in its transmission are Amblyomma, Hyalomma, Rhipicephalus and Haemaphysalis (Uilenberg 1995; Dumler et al. 2001; Goethert and Telford 2003; Harrus and Waner 2011; Palomar et al. 2015). Clinical signs develop in infected cattle and buffalo include fever, emaciation, decrease in weight gain, icteric mucous membranes, inflamed pre-scapular lymph node and sometime death may occur in later stages of the disease (Donatien and Lestoquard 1936; Uilenberg 1995; Uilenberg 1997; Santos and Carvalho 2006; Arulkumar et al. 2016).

A. ovis

A. ovis is an obligate intra-erythrocytic species of Anaplasmain sheep. It was first identified by Bevan in 1912 (Bevan 1912; Dumler et al. 2001; Peng et al. 2021). *A. ovis* is prevalent in USA, Asia, Africa and Europe (Al-Hosary et al. 2021). *A. ovis* cause disease in small ruminants including wild ruminants (Bevan 1912; Kuttler 1984; Friedhoff 1997; de la Fuente et al. 2008; Li et al. 2015; Enkhtaivan et al. 2019). It also has zoonotic importance as it has been detected in humans by Chochlakis et al. (2010). Intra-erythrocytic inclusions observed during A. ovis, are observed at central position in 35–40% cases and at marginal positions of erythrocytes in 60 to 65% cases (Shompole et al. 1989; Primo et al. 2019). Clinical signs observed during the course of the disease are emaciation, anemia, fever, depression, rumen atony, decreased production, abortion and death of the animal (Yousif et al. 1983; Manickam 1987; Friedhoff 1997; Yasini et al. 2012; Tumwebaze et al. 2020). These signs of the disease depend on the breed, age and body condition score of the animal (Splitter et al. 1955; Shompole et al. 1989; Tumwebaze et al. 2020). Development of anemia due to A. ovis is the main cause of huge economic losses at livestock farms especially in developing countries, as sheep and goats are mainly reared in areas of tropics and subtropics (Jensen 1955; Zaugg 1985; Lacasta et al. 2021).

A. platys

A. platys, an also an obligate intracellular pathogen which resides in platelets, was observed initially during blood examination of dog in U.S.A. (Florida) in 1978. Later on, it was detected in almost all continents (Harvey et al. 1978; Suksawat

et al. 2001; Sanogo et al. 2003; Sparagano et al. 2003; Brown 2008; Aguirre et al. 2006; de la Fuente et al. 2006; Kawahara et al. 2006; Melo et al. 2016). A. platys mainly affect canines but it has been also reported in foxes, cats, camels, cattle, deer and humans (Harvey et al. 1978; Maggi et al. 2013; Qurollo et al. 2014; Cardoso et al. 2015; Dahmani et al. 2015; Li et al. 2015; André et al. 2020). In canines, A. platys causes the disease namely Canine Cyclic Thrombocytopenia (CCT). Disease is characterized by fever, severe emaciation, loss of appetite, lethargy, respiratory distress, increased mucus secre¬tion and ocular discharge, muzzle hyperkeratosis and splenomegaly (Sainz et al. 2015; Brandão 2019). Severity and type of clinical signs depend mainly on dog species. Transmission of A. platys occurs through tick species: Rhipicephalus sanguineus, Rhipicephalus turanicus, Dermacentor auratus, Haemaphysalis longicornis, Ixodes persulcatus, Haemaphysalis longicornis, chewing louse, Heterodoxus spiniger and blood transfusion (Harvey et al. 1978; Parola et al. 2003; Martin et al. 2005). Severe thrombocytopenia is observed during the course of disease in dogs which may reoccur after two weeks of incomplete recovery. Thrombocytopenia probably occurs due to direct damage of platelets by pathogen and immune mediated mechanism (immune cells) (Harvey et al. 1978; Piratae et al. 2019). Clinical signs of the disease are fever, anorexia, emaciation, pale mucus membranes, lethargy, pin point hemorrhages on oral mucosa and skin, lymph adenomegaly and epistaxis (Bradfield et al. 1996; Bouzouraa et al. 2016).

A. phagocytophilum

A. phagocytophilum, found in granulocytes of host, has great importance with respect to veterinary and public health (Dugat et al. 2015). A sheep was affected with a tick-borne disease of unknown etiological agent during 1932 in Scotland whose causative agent was described in 1940 as *A. phagocytophilum*. The host range of *A. phagocytophilum* includes human, carnivores, ruminants, reptiles, birds and rodents. Ehrlichia was known to cause disease in humans and horses and previously it was known as Ehrlichia equi (Stuen et al. 2002). During the course of the disease about 40% of the patients need hospitalization. Rate of the mortality of disease is 7-10 % in USA but in Europe there is no mortality (Fishbein et al. 1994; Dumler and Bakken 1998; Blanco and Oteo 2002; Dumler and Broqui 2004). Situation aggravates leading to death in case of prevailing health issues viz; intravascular coagulation, renal failure, cardiac enlargement, coma and seizures. The disease is more harmful in immune-compromised patients of old age and children (Fishbein et al. 1994; Dumler and Bakken 1998; Rocco et al. 2020). In ruminants, disease is characterized by rise in body temperature, in appetence, drop in milk and meat production and abortion. Death occurs in infected animals which remains unattended during the course of the disease (Stuen et al. 2002; Rodino et al. 2020). *A. phagocytophilum* is transmitted by ticks belonging to genera Ixodes, Dermacentor, Haemaphysalis and Amblyomma in USA, Europe and Asia (Holden 2003; Santos et al. 2004; Woldehiwet 2010; Clark 2012; Paulauskas et al. 2012; Tomanović et al. 2013; Rochlin and Toledo 2020). Transstidial transmission of *A. phagocytophilum* has also been reported (Baldridge et al. 2009; Dugat et al. 2015). *A. phagocytophilum* leads to granulocytic anaplasmosis in ruminants and clinical signs include fever, anorexia, abortion and decrease in milk production (Woldehiwet 2006; Stuen et al. 2002; Dugat et al. 2015). Furthermore, it leads to decrease in immune status of the animal which tends to increase in susceptibility of animal towards secondary or opportunistic bacterial infection (Woldehiwet 2006; Kahn et al. 2019).

Pathogenesis

Anaplasmosis is commonly categorized into four phases; incubation phase, developmental phase, convalescent and carrier phase. Incubation phase is the duration from inoculation of pathogen to a susceptible host until 1% of erythrocytes become infected (clinical signs develop). The duration of each phase of the disease is directly linked with the total number of pathogens entered into host animal. During this stage the PCV remains constant; erythrocytes are produced at the same rate as they are destroyed and no clinical signs are seen. The developmental stage is characterized by onset of clinical signs associated with mortality and sporadic abortions (Coetzee et al. 2010). Clinically recovered animals develop persistent infections and remain as carriers with undetectable parasitemia consequently act as reservoir of infectious agent (Kieser et al. 1990). The pathogen enters erythrocytes through endocytosis and exit by exocytosis and attack on other fresh erythrocytes. Infected erythrocytes are destroyed by reticulo-endothelial cells which leads to development of hemolytic anemia and icterus. Oxidative stress occurs due to imbalance in antioxidants and oxidant ratio. Due to increased production of reactive oxygen species (ROS), there is increase in oxidant level on cellular level. This increased level of oxidants leads to cellular damage and lipid peroxidation "LPO".

Economic Importance of Anaplasma

Anaplasma can cause huge economic losses in terms of decrease in productivity of animal, abortion, increase in treatment cost and mortality of infected animal. It can also cause death in new borne calves because of its trans-placental transmission. According to FAO report, the rate of mortality in case of anaplasmosis is 5 to 70%. In Norway, *A. marginale* leads to mortality of more than 0.3 million lambs each year (Stuen et al. 2018).

Diagnosis

Parasitological diagnosis provides direct evidence of Anaplasma infection. It includes microscopic examination of ethanol fixed Giemsa-stained thin blood smears. This is regarded as the best method of identifying *A. marginale* and to determine the parasitemias of the animals (Yoshihara et al. 2003; Siddiki et al. 2010). Inoculation of susceptible laboratory animals with blood of suspected animals to demonstrate presence of *A. marginale* is another technique (Siddiki et al. 2010). Nucleic acid detection methods for Anaplasma infection includes PCR, qPCR, RT PCR and LAMP. 16S ribosomal gene is the most targeted gene for the detection of anaplasma species (Atif et al. 2021). Whole blood preserved in EDTA coated tubes can be used for the diagnosis of anaplasma species (Hebels et al. 2014). Now a days rapid and sensitive real time assay is using more as compared to nested PCR (Atif et al. 2021).

Treatment

Doxycycline is the drug of choice for treatment of *A. platys* infection in canine (Sainz et al. 2015; Paterson et al. 2020). For

control of bovine anaplasmosis live attenuated vaccine of A. centrale is being used in Australia, Africa, Latin America and Israel (Kocan et al. 2010; Waruri et al. 2021).

Conclusion

Although Anaplasmosis has history of more than a century, still it is one of the most challenging parasites of human and animal health due to variety of species that are present in different hosts. Anaplasmosis is a bacterial disease that causes severe infection and leads to liver damage, severe anemia occurs due to lysis of erythrocytes which causes death of the animals. Disease is more prevalent in young animals. The only way to combat anaplasmosis is to adopt good precautionary measures.

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CHAPTER 08

LEISHMANIASIS

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INTRODUCTION

The advent of discernable abrasions alike to cutaneous leishmaniasis happened since the seventh century BCE on tablets from King Ashurbanipal (some of which might have started from significantly before texts from 1500 to 2500 BCE). In the 10th century CE, Persian physicians such as Avicenna described the balkh sore in detail (Cox 2002). Alexander Russell transcribed one of the most extensive clinical elucidations of the condition in 1756 after seeing a Turkish patient. Kala-azar (black fever) is the term used by specialists on the Indian subcontinent to describe it. Pre-Inca pottery portraying distorted faces and skin lesions from the first century CE in Ecuador and Peru shows indication of the cutaneous manifestation of the ailment. In the Americas, pre-Inca pottery portraying distorted faces and skin lesions from the first century CE in Ecuador and Peru marks confirmation of the cutaneous manifestation of the disease. Some Inca documents from the 15th and 16th centuries, as well as Spanish colonial texts, reference "valley sickness," "Andean sickness," or "white leprosy," all of which are liable to allude to the cutaneous variety (World Health 2010).

However, it is assumed that Surgeon David Douglas Cunningham (Major of the British Indian armed force) may have perceived it in 1885 but was unable to link it to the sickness (Cunningham 1885; Cox 2002). Peter Borovsky, (a Russian military specialist) positioned in Tashkent, investigated the cause of "oriental sore," which is also termed as sart sore and issued the very first precise explanation of the pathogen in 1898, appropriately describing the organism's relationship specifically towards host tissues and decorously stating it to the protozoa. However, because his findings were published in a low-circulation journal based in Russia, they were not widely recognized during his era (Hoare 1938). In 1901, in smears taken from the spleen of a patient who passed on from "dum-dum fever" (Dum Dum is a neighborhood near Calcutta), Leishman detected organisms and hypothesized that they were *trypanosomes*, which had been discovered for the first time in India (Leishman 1903). Shortly after that, Commander Charles Donovan (1863–1951) set up the discovery of *Leishman-donovan* in smears from the individuals in Madras (southern India) (Donovan 1903). Therefore, Ronald Ross was the one who suggested *Leishman-Donovan* bodies as intracellular phases of a novel parasite and accordingly termed as *Leishmania donovani* (Ross 1903). Charles Donovan first postulated the relationship with the ailment kala-azar, and Charles Bentley's detection of *L. donovani* in kala-azar patients proved it firmly (Gibson 1983). Ernest Struthers and Lionel Napier of the School of Tropical Medicine in Calcutta hypothesized sandfly transmission, which was later confirmed by his colleagues (Gewurtz 2017). Allied forces fighting in Sicily during WWII faced a large outbreak of the disease and Leonard Goodwin's findings revealed pentostam an effective treatment (Goodwin 2009).

Parasite Cell Morphology

Leishmania parasites have a variety of cell types (developmental forms) and cell morphologies that are specific to the host or vector. Some of these developmental forms are proliferative while others are quiescent and pre-adapted for transmission to the next host as found in other parasites like *Plasmodium* and *Trypanosomes* (Sinden et al. 1978; Shapiro et al. 1984; Bates 1994; Matthews 2011). *Leishmania* possess two different types of cellular shape and form including the promastigote morphology in the sand fly and the amastigote morphology in the mammalian host.

The fundamental cellular architecture is defined by cross-linked sub-pellicular corset microtubules and is however conserved between the two *leishmania* cell forms (Ogbadoyi et al. 2003). Cell division is dependent upon microtubules addition and extension into the prevailing assortment which is maintained all over the cell cycle. The nucleus along with the collection of single-copy organelles including the Golgi apparatus and the mitochondrion are found within the cell. At the anterior portion of the nucleus there is a mass of concatenated mitochondrial DNA called as kinetoplast that is directly related to the basal body from which the flagellum prolongs (Ogbadoyi et al. 2003; Wheeler et al. 2016). There is a vase like structure at the foundation of flagellum formed by an inward folding of cell membrane called as flagellar pocket which plays significant role in these parasites being the only site of various processes such as endocytosis and exocytosis and ultimately a critical contact between both parasite and its host environment (Lacomble et al. 2009).

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Leishmania cell is comprised of a series of few modular structures including the basal body–mitochondrial kinetoplast unit, flagellum, and Golgi–flagellar pocket neck (Ogbadoyi et al. 2003). These modular pieces are subsequently arranged in relation to one another resulting in the various cell morphologies observed (Hayes et al. 2014; Sunter and Gull 2016).

Epidemiology

Leishmaniasis is a tropical illness that affects the poorest people in more than 90 countries across Africa, Central and South America, Asia, and the Middle East. Ongoing approximations of prevalence of cutaneous leishmaniasis range from 700,000 to 1.2 million cases annually (Thakur et al. 2020), by roughly 95 percent of cases happening in the Central Asia, Mediterranean basin, Americas, and the Middle East (Mohammed and Al-khafaji 2021), despite the fact that it is likely underreported. Visceral leishmaniasis (VL) cases are estimated to be less than 100,000 annually, (down from 400,000 in previous estimates) (Servadio et al. 2020) over 95% cases conveyed to the WHO particularly from India, Somalia, Ethiopia, Kenya, Nepal, Brazil, China and Sudan (Pandian et al. 2021). An immunocompromised state poor hygiene, population morbidity, malnutrition and poverty are all risk factors for leishmaniasis (Alves et al. 2020).

The *leishmania* parasite has been identified in over 20 species and is spread by over 70 diverse varieties of phlebotomine sand flies subdivided into Lutzomyia in the New World in addition to Phlebotomus in the Old World (Rodriguez et al. 2018). Because of the enormous number of species, leishmaniasis has been alienated into double geographical areas: the New World and the Old World. Southern Europe, Africa, the Middle East, and Asia belong to Old World, which refers to the Eastern Hemisphere. While, the Western Hemisphere, notably United States, Central America, South America, and Mexico referred as the New World (Moreira et al. 2019).

Mostly, sandflies are nocturnal marking their presence all over the world (Pan American Health 2019). Additionally, subtropical species complete their life cycle during the summer (Pan American Health 2019). On the other hand, tropical species complete their life cycle all year long.

Although the bulk of cutaneous *leishmania* victims in the United States are allied to travel or colonization (Samarasinghe et al. 2018), the WHO designated the country as leishmaniasis prevalent in the year 2015. Furthermore, in a recent investigation, 69 new instances of leishmaniasis were reported in Texas. Among these, 41 (59%) were found to be the case of autochthonous CL without any set of experience of movement outside the United States (McIlwee et al. 2018). The rate of recurrence and topographic dissemination of leishmaniasis are projected to rise because of climate change (Zhao et al. 2021).

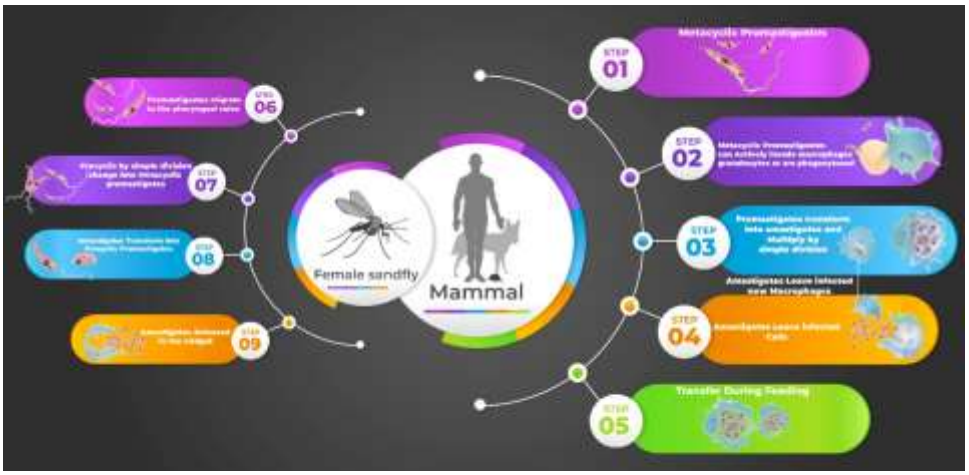
The genus *leishmania* contains 22 species, which are further classified into the subgenera *viannia* and *leishmania* based on their proliferate mode in the sandfly's digestive tract. Host variables, symptom features and geographical preferences vary per parasite species. In East Africa (Ethiopia, Sudan, Kenya and Somalia) and South Asia (Nepal, India-Bangladesh), *L. donovoni* appears as VL spares mature adults owing to developed immunity and commonly predominates among younger persons (Bern et al. 2006). On the other hand, *L. infantum* (also known as *L. chagasi* in the regions of Latin America) occurs predominantly in Pakistan, Brazil, Iran, Middle East and Mediterranean. Whereas *L. mexicana* (CL) was responsible to be the cause of all endemic cases of leishmaniasis in Texas (McIlwee et al. 2018).

Life Cycle

Leishmania, resembling many protozoan parasites, possess eugenic life cycle that comprises a mammalian host along with an insect vector. The leishmaniasis parasite is transferred to various animals or humans by the female phlebotomine sand fly, which is usually active at night ("from dusk till dawn"). The life cycle of *leishmania sp.* is divided into two phases including promastigote and amastigote (Pan American Health 2019; Gurel et al. 2020). The promastigote form contains a flagellum that allows it to move around in the intestines of the sand fly (Pan American Health 2019, Kipp et al. 2020). When the sand fly feeds on the host's blood (mammalian), promastigote form is introduced into the epidermis, this is immediately phagocytized by the host's mononuclear cells, changing it into the amastigote form, commonly recognized as the *leishman-donovan* body. (Fig. 1) (Pan American Health 2019; Kipp et al. 2020). After that, amastigotes proliferate inside the he host's reticulo-endothelial system and grow generating asymptomatic or symptomatic sickness depending on the circumstances underlying the host and parasite species (Pan American Health 2019). Amastigotes can spread through the bloodstream and lymphatic system, causing visceral and mucosal illness (Pan American Health 2019). It has been revealed that the LRV1(*Leishmania* RNA Virus) infects both *L. Viannia (V.) braziliensis* and *L. Viannia (V.) guyanensis* triggering a hyperimmune response via toll-like receptors, and mucosal obliteration and metastatic infection (Hartley et al. 2012). Although *L. major* (LRV2) has been found to have LRV separately of the *Viannia* subspecies, no link has been found between clinical phenotype and severity (Scheffter et al. 1995). *Leishmania sp.* transmission is primarily compelled by symptomatic infection followed by post-kala-azar dermal leishmaniasis (PKDL) (asymptomatic cases are assumed not to infect sandflies) (Mondal et al. 2019, Le et al. 2019). In some locations, humans are prerequisite for anthroponotic transmission in order to sustain the life cycle as is the case with *L. donovani* (which causes visceral leishmaniasis in India) and *L. tropica* (which causes cutaneous leishmaniasis in the New World) (Burza et al. 2019; Pan American Health 2019). Whereas Animals can continue to live their lives and may or may not show signs or symptoms of sickness. Susceptible hosts include edentates, monkeys, marsupials, rodents and dogs. Dogs have been considered as the most important *L. infantum* animal reservoir (Faye et al. 2010; Burza et al. 2019).

Pathogenesis

Leishmaniasis is traditionally assumed to be caused by a discrepancy of CD4+ helper cells between TH1 and TH2 (Scott and Novais 2016). Those with a main TH1 response possess exceptional parasite management and trivial intensities of parasitemia; nevertheless, due to hyperactive cellular immunity and cellular damage, they are predisposed to mucocutaneous illness (Scott and Novais 2016, Bennett et al. 2015). Antibody neutralization is ineffectual towards the intracellular parasite, hence those having a TH2 response holds greater parasite load (Scott and Novais 2016). Disseminated infection, leading to visceral ailment and DCL (disseminated



cutaneous leishmaniasis) in the New World, is more common in TH2 responders (Scott and Novais 2016). DCL manifests itself clinically as a slew of lesions all over the body.

Clinical Symptoms

Leishmaniasis can exhibit itself in a variety of ways, the three most common phenotypic groups are visceral leishmaniasis (VL), cutaneous (CL) and mucosal (ML). These categories can be further subdivided to include ML of the Americas, VL, CL of the New World, CL of the Old World, post-kala-azar CL, *Leishmania* recidivante, diffuse CL, and disseminated CL (Bennett et al. 2014) Infection might be asymptomatic or subclinical in some people, but it can also manifest as chronic, acute, or subacute disease in others.

CL lesions usually appear as a single non-suppurative papule on the spot at which vector, sand fly bite (often on unprotected regions including extremities and face), though numerous lesions can arise. CL can be included in New World forms (*L. viannia*, *L. amazonesis, L. venezuelensis* and *the L. mexicana*, subgenus, which comprises *L. V. guyanesis*, *panamensis* and *braziliensis*) as well as Old World forms (*L. tropica, L. major*, *L. aethiopica* and generally *L. donovani* and *L. infantum*) (Herwaldt et al. 1992; Bennett et al. 2014). The papules develop into painless ulcers with heaped-up edges over weeks to months, which might naturally heal over the period of months to years or leave blemishes and deformities (Berman and Neva 1981). Atypical cutaneous signs include disseminated, zosteriform, verrucous, psoriasiform, sporotrichoid, nodular, eczematous, and/or erysipeloid (Ortiz et al. 2016; Pan American Health 2019). Insignificant satellite abrasions external to the plaque/ulcer (nodular lymphangitis) are another unusual appearance. A naso-oropharyngeal exam should be performed proceeding all patients with CL as a proof of mucosal lesions (Aronson et al. 2017). Recidivins of Leishmaniasis (most typically associated with *L. tropica*) appear as satellite lesions encircling existing blemishes and are frequently misdiagnosed as cutaneous tuberculosis (Burza et al. 2019).

ML (alias espundia in Latin America) is the greatest damaging form of the ailment, causing facial abnormalities ages after the first cutaneous symptoms have fixed. The parasite propagates to the nasopharyngeal mucosa from amastigotes on the epidermis through lymphatic and hematogenous systems, which is frequently produced by the *L. Viannia* subgenus (Herwaldt et al. 1992). Mucosal lesions can co-occur with cutaneous symptoms, albeit this is less common. Patients frequently describe chronic nasal symptoms such as discharges, epistaxis, and pain, and physical examination commonly reveals ulceration, bleeding, and inflammation (Weller et al. 2005). Mucosal involvement of the mouth and nose occurs first, followed by laryngeal and oropharyngeal involvement later in the ailment. ML does not heal on its own, unlike cutaneous illness (Berman and Neva 1981). Damage of the nasal septum can take place after the cartilaginous septum classified anteriorly is affected. Other issues include the anterior nose collapsing and the nose and mouth being destroyed (Walton et al. 1973; Marsden 1986).

VL (alias kala-azar, which refers black fever in Hindi) is the most fatal form of leishmaniasis and can induce broad-spectrum infection distressing the hematogenous, spleen, lymphatic systems, liver, and *L. chagasi* (alike *L. infantum* but established in the New World). On the other hand, *L. donovani* and *L. infantum* (in the old World), are the species most commonly associated with visceral disease (Herwaldt et al. 1992). Other species linked to visceral sickness include *L. tropica* from Old World, which usually causes dermatologic indications the discovery related to this happened in the total of seven servicemen suffering from visceral symptoms in the 1990s during the Persian Gulf conflict. Cases of visceral illness have also been linked to *L. amazonensis* (Herwaldt et al. 1992). Fever, pancytopenia, hypergammaglobulinemia and hepatosplenomegaly are described as severe signs of the condition in a report from Sudanese patients (Seaman et al. 1996). Fatigue, stomach pain, and unintentional weight loss are common subjective symptoms documented in the past of individuals with VL. VL is an unscrupulous infection in HIV patients, accounting for 25–70% of HIV co-infections known in Europe (World Health 1994). Following the first VL syndrome, post-kala-azar dermal leishmaniasis causes macular hypopigmentation and cutaneous nodules and papules on the face (Mukhopadhyay et al. 2014).

Diagnosis

Although government and individual efforts can be beneficial in reducing disease risk even then persistent investment is required to lower infection threat for native individuals, therefore prompt identification is correspondingly critical in great-prevalent areas. In the case of VL, microscopy of surgery or aspirate models is the gold standard while in the case of CL, histology/direct microscopy is the gold standard (Srivastava et al. 2011, Vega et al. 2009). Other methods, majorly profligate diagnostic antibody/antigen testing, PCR and latex agglutination

are becoming more popular (Thakur et al. 2020, Sundar and Singh 2018). These possibilities are particularly appealing to rural medical centers looking for decentralized point-of-care options although antibody testing, specifically, has inadequate efficacy for recurrent infections (van Griensven and Diro 2019). Furthermore, PCR is becoming increasingly crucial for identifying specific species of infection to provide patients with personalized treatment (Moreira et al. 2018).

Therapeutic Agents against Leishmaniasis

Treatment for leishmaniasis has a number of difficulties after diagnosis. Unfortunately, the WHO-recommended pentavalent antimonial combination regimen, despite its low cost, can have serious impediments such as renal, heart and liver toxicity (van Griensven and Diro 2019, Aronson et al. 2016). Furthermore, treatment effectiveness contrasts through region on account of parasite resistance towards first line antimonial, stated to be as great as 40%–60% in India's Bihar and Algeria (World Health 2015). The most notable development from this standard in contemporary years has been intravenous injection of liposomal amphotericin B, which might abolish all systemic leishmaniasis with short intensities of toxicity — but at a significantly higher cost (Aronson et al. 2017; Shirzadi 2019; van Griensven and Diro 2019). Surgery (Azab et al. 1983), cryosurgery (Panagiotopoulos et al. 2005), heat therapy (Bumb and Satoskar 2011; David 2018), and a range of localized drug-delivery techniques for localized lesions (Handler et al. 2015) have all been investigated as additional treatment options. Finally, plastic surgery can be used to correct disfiguring scars that commonly persist after CL and MCL clearing (Frolich and Kaplan 1967).

Chemotherapeutics

Pentavalent antimony was once the first-line medicine for leishmaniasis treatment; however, it has been linked to cardiotoxicity (Sundar et al. 1998), cirrhosis, pancreatic toxicity (Gasser Jr et al. 1994) and the risk of resistance (Thakur et al. 1998). Amphotericin B (and lipid formulations) became second-line treatments as a result. Miltefosine has since been used in VL and CL; it has the benefits of being an oral medicine with good efficacy and a short course, but it also has the drawbacks of teratogenicity and drug resistance.
Amphotericin B, miltefosine, paromomycin, and petamidine are examples of existing medications that have been repurposed treating leishmaniasis. Antifungal azoles have also been explored for leishmaniasis, with itraconazole outperforming ketoconazole and fluconazole in suppressing the growth of most *leishmania* strains (Croft and Yardley 2002). Paromomycin was found to be effective in Indian patients with VL in a multicenter trial, but less so in a Sudanese group (Sundar and Singh 2018). Pentamidine is administered intramuscularly or intravenously, although there is no oral formulation. It has the advantage of being a quick treatment, although its efficacy varies depending on the *leishmania* species, and its use has been linked to dysglycemia and other adverse effects.

Combination Chemotherapy

Combination chemotherapy has been created to minimize drug resistance, shorten treatment duration, enhance compliance, and hence lower therapeutic costs. Liposomal amphotericin B plus miltefosine, liposomal amphotericin B plus paromomycin, miltefosine plus paromomycin, and sodium stibogluconate/ meglumine antimoniate plus paromomycin are among the different combinations (Sundar et al. 2008; World Health 2010; Omollo et al. 2011).

Local Therapeutic Remedies

To avoid toxicity from systemic drug use, local treatments for restricted CL have been developed. CL has attempted photodynamic treatment (PDT), cryotherapy and thermotherapy as explained below.

Photodynamic Therapy

Photodynamic therapy (PDT) comprises the application of aminolaevulinic acid (ALA) or methyl-aminolaevulinate to the skin, followed by laser or strong pulsed light irradiation. The principles underpinning the use of PDT for the treatment of CL have been studied in a few mechanistic studies (Akilov et al. 2007; Kosaka et al. 2007). ALA-PDT did not show any antileishmanial effects in mechanistic and in vitro investigations (Akilov et al. 2007). Topical ALA-PDT, on the other hand, caused substantial tissue damage and a considerable reduction in parasite load in vivo trials. Infected skin had a lower number of macrophages and a higher amount of interleukin-6. The destruction of host cells is the mechanism via which ALA-PDT for CL has antileishmanial effects. Topical ALA-PDT is not advised in clinical practice due to a lack of data.

Cryotherapy

At temperatures below freezing, the production of intracellular and extracellular ice crystals, as well as alterations in cell membrane, causes damage to infected cells and the killing of amastigotes. Cryonecrosis causes the release of antigenic chemicals, which trigger an immune response and allow other lesions to heal. Cryotherapy may be a viable treatment option for CL, especially given the numerous drawbacks of chemotherapy. It has demonstrated excellent results in individuals with skin lesions ranging in size from 10 to 30 mm, those with fewer lesions, and those who have had their lesions for less than three months (Salmanpour et al. 2006). Cryotherapy combined with intralesional sodium stibogluconate was shown to be quite effective, with CL lesions healing completely (El Darouti and Al Rubaie 1990). Another study found that combining itraconazole with cryotherapy improved CL lesions by 80.9%, and the risk of liver toxicity was minimized since the itraconazole dose could be reduced (Al-Mutairi et al. 2009).

Thermotherapy

Heat-sensitive *leishmania* species that cause cutaneous illness cannot survive in temperatures in more than 39°C (Berman and Neva 1981; Sacks et al. 1983). As a result, thermotherapy has been proposed as a treatment for cutaneous leishmaniasis lesions. In individuals with CL, RF (radiofrequency) therapy, a type of thermotherapy, has been tested. In a trial from Guatemala, patients with CL had a cure rate of 73%, which was similar to the rates acquired with a complete pentavalent antimonial medication (Navin et al. 1990). Heat penetrates

uniformly to a profundity of 4 mm in radiofrequency, warming and killing amastigote forms of *leishmania* in the upper dermis while causing little damage to the surrounding skin. RF thermotherapy had been proved to possess a bit lesser cure frequency than systemic pentavalent antimonial medicines in two randomized investigations, but it was more cost-effective and had fewer adverse effects (Vega et al. 2009; López et al. 2012). The cure rate of once-every-three-weeks thermotherapy was 73%, whereas once-weekly thermotherapy boosted the cure rate to 81 %(Sadeghian et al. 2007).

Immunotherapy

Immunotherapy is based on the manipulation of the immune response for preventative and/or therapeutic aims using organic molecules (Roatt et al. 2014). Immunotherapy, either directly or indirectly, enhances the natural host defense, restores compromised effector functions, or reduces the host's excessive response (Okwor and Uzonna 2009). Interferons (IFNs), vaccines, and protein immunomodulators, or a combination of these are used to treat leishmaniasis. It was shown that the immunomodulator protein aggregation magnesium–ammonium phospholinoleate-palmitoleate anhydride improved clinical symptoms in canine VL and greatly reduced parasite burden in the skin (Santiago et al. 2013). Immunotherapeutic and chemotherapeutic medicines are combined to have a synergistic effect in activating the immune system, as well as direct pharmacological action on the infectious agent (Roatt et al. 2014).

Interferons

IFNs are cytokines that are generated commercially using recombinant DNA technology. They have a variety of biological effects, including immunosuppressive effects. The cytokine IFN- may cause macrophages to destroy *leishmania* intracellularly (Murray et al. 1983). Sodium antimony gluconate is used to deliver IFN-1b protein. In patients with VLs who were resistant to monotherapy with pentavalent antimonial treatment, it was found to be well tolerated and efficacious (Badaro 1988; Sundar et al. 1994). In untreated instances of VL, the use of IFN- resulted in quicker parasitological control (Squires et al. 1993; Sundar and Murray 1995). as well as improved clinical efficacy of pentavalent antimonial therapy (Sundar and Murray 1995). Squires et al. 1993 found that a month of combined IFN- and pentavalent antimonial therapy resulted in a negative spleen culture more quickly in individuals with VL (Squires et al. 1993).

Phytochemical Agents

Plant-based conventional treatments have been employed in the treatment of infectious diseases since ancient times. Plant extracts and specific bioactive compounds isolated from plants are being used to treat leishmaniasis and other microbial infections as either direct medicinal sources or as herbal medications developed from them (Oryan 2015). NTDs, along with further ailments including protozoan (and their vectors), helminth (and their vectors), fungal, bacterial (and their vectors), ectoparasitic, and finally the viral infections have been treated with herbal medications derived from plants for millennia (and their vectors). Attributable to their innocuous, environmentally approachable, and lucrative features, medicinal plants become more favorable than other chemotherapies. Furthermore, natural chemicals derived from plants are regarded as a safe and effective treatment for leishmaniasis (Silveira et al. 2020). Leishmanicidal effects were seen in Ageratum conyzoides, Bidens pilosa, and Eugenia uniflora. When compared to other plant species, Bidens pilosa (root) exhibits the lowest IC50 value (1.5 g/ml) for antileishmanial characteristics (against *L. amazonensis*, promastigote) (Metwally et al. 2016; Rossi et al. 2017) Eugenia uniflorae essential oils have been shown to avert the progression of together the parasitic forms of *L. amazonensis*, the promastigote and amastigote, while Ageratum conyzoides has been used to treat infection caused by *L. donovani* (amastigote form) (Silveira et al. 2020). The major component of Melampodium divaricatum and Casearia sylvestris essential oils, E-caryophyllene, has been reported to have a promising antileishmanial (Coutinho De Oliveira et al. 2020) reaction against *L. amazonensis* (IC50 values of 10.7, 10.7, and 14.0 g/ml) (Moreira et al. 2019). Furthermore, the active components 1,8-cineole, -pinene, and p-cymene from Protium altsonii and P. hebetatum (Burseraceae) showed dose-dependent amastigote inhibition, with IC50 values of 48.4, 37, and 46 g/ml, respectively (Moreira et al. 2019). The butanol fraction of K. odoratissima displayed antileishmanial activities against *L. major* promastigote and amastigote with an IC50 value of 154.1 g/ml (Mirzaei et al. 2020).

Use of Vaccines

Whole-killed parasites (Coutinho De Oliveira et al. 2020, Nagill and Kaur 2011). fractionated *leishmania* antigen (Giraud et al. 2019; Coutinho et al. 2020) recombinant proteins (made by genetically engineered cells) (Kumar and Samant 2016; Duthie et al. 2018; Hobernik and Bros 2018) and live-attenuated pathogens (Karmakar et al. 2021; Soto et al. 2021) are among the vaccines that have been synthesized and are under clinical trials. Third-generation vaccinations are being researched.

Future Perspectives

In certain species of *leishmania*, endochin-like quinolones are competitive inhibitors of the cytochrome bc1 complex which might influence the mitochondrial electron transport chain (ETC). Hydroxynaphthoquinone–buparvaquone works by decreasing adenosine triphosphate (ATP) levels in amastigotes, causing ETC inhibition (Ortiz et al. 2016).

New bisphosphonium salts produced from benzophenone are leishmanicidal, causing the parasite to die by targeting complex II of the parasite's respiratory chain. Artemisinin inhibited the growth of *l*eishmania parasites by causing cell cycle arrest and apoptosis (Sen et al. 2007).

Tafenoquine affect *leishmania* promastigote's bioenergetic metabolism, resulting in a rapid decline in intracellular ATP levels, apoptosis, and so mitochondrial failure (Carvalho et al. 2010).

Ergosterol and 24-methyl sterol are the primary sterols required for trypanosomatid growth and viability; thus, the sterol and fatty acid metabolic pathway could be a potential therapeutic target in leishmaniasis. Simvastatin, atorvastatin, and resveratrol were reported to decrease the growth of *Leishmania donovani* promastigotes in experiments (Dinesh et al. 2014). Antileishmanial medicines could possibly target other enzymes involved in sterol production.

Recently, alkylphospholipid analogues have been shown to be potential target medicines. In lab tests, edelfosine destroyed both promastigotes and amastigotes of *leishmania* (Villa-

Pulgarín et al. 2017). Polyamines have an important role in cell survival, growth, and proliferation. Antileishmanial activity can be triggered by a variety of enzymes involved in polyamine and folate metabolism.

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CHAPTER 09

PARASITIC DISEASES OF FANCY BIRDS

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INTRODUCTION

Parasitism is a relationship between organisms of two various species in which one gets benefits at the expense of the other. The diversity of parasites has been identified and detected in different animal species including birds. Although, several parasite species have been isolated from birds; however, the presence of the parasites does not show a confirmed association with disease occurrence. There are around 18,000 species of birds found worldwide (Barrowclough et al. 2016). The organism's pathogenicity depends on (1) the infected species of the concerned host (2) the frequency of parasites presents either in or on the host (3) some internal factors by host response. Parasites can affect fancy birds like pigeons, parrots, peacocks, doves, ducks, love birds, and chickens adversely because some of these have zoonotic importance. This increased hazard of parasites exposure can lead to serious issues or even death in birds, especially after long captivity and stress and adaptation to new environments (Krone et al. 2002). External and internal parasites can affect the fancy birds and the main source of these parasites is contaminated food, water, and unhygienic conditions. Internal parasites can cause a loss in weight, inactivity, lethargy, retarded growth, and diarrhea. These internal parasites can be lethal for birds, especially at a young age. The entire life cycle of internal parasites denotes that there must be numerous treatments for complete eradication. The routine conventional parasitological techniques for preliminary diagnosis include morphological identification, comparison of parasites, and fecal smear observation (Kompalic-Cristo et al. 2005).
The diagnostic efficiency of these tests has not been up to the mark. Due to the low sensitivity and specificity of the routine tests, molecular diagnostic techniques are the best options to differentiate diverse parasites that are responsible for numerous parasitic infestations (Jardim et al. 2006). The traditional analysis is cost-effective, relatively cheap, and does not require special equipment and trained personnel. On contrary, the molecular techniques are slightly difficult and require a high cost, but the presence of an organism can be determined on nucleotide sequence-based (Shen et al. 2020).

Etiology and Epidemiology

Birds have crucial importance in the maintenance of the environment. In Pakistan, it is the hobby of people to rear fancy birds like pigeons, parrots, peacocks, doves, love birds, and chickens (Hussain 2016). The term "Domestic or Pet bird" primarily confers to the birds kept solely for fancy usage and mainly involves the sparrows, finches, and canaries (Tully et al. 2009). These birds can easily be kept in captivity and reared by giving special attention. The environment of Pakistan is also favorable for the production of these fancy birds (Hussain 2016). It is very feasible and economical to rear the fancy birds in Pakistan. Concerning the business point of view, these are very beneficial due to low investment and high profit. However, mismanagement and carelessness lead to a severe attack of diseases leading to death (Hussain 2016). Birds play an important role in the spread of zoonotic and other diseases such as Newcastle disease, Influenza Salmonellosis. Parrot fever or psittacosis is also an important zoonotic disease that is spread by parrots, pigeons, and other fancy birds. Some parasites are the primary parasites while others are opportunistic causing mild to severe damage to the birds. These parasites affect the visceral organs like kidneys, muscles, respiratory tract, gastrointestinal tract, including blood and skin. (Doneley 2009). Trichomonas is a widely distributed disease caused by *Trichomonas gallinae*; a single-celled protozoan parasite. It is of critical importance to control the parasites for the maintenance of health measurements. These parasites are prevalent in wild and caged birds (Taku Awa et al. 2014). One of the important primary protozoan infections in birds is cryptosporidiosis affecting mainly the respiratory and gastrointestinal systems. The morbidity rate is very high in this disease (Ryan 2010). Black flies and red mites are also important external parasites. Black flies are also responsible for the spread of the Leucocytozoon spp protozoan parasites (Lin et al. 2000).
Heterakidosis caused by *Heterakis gallinarum* and other Heterikis spp is also one of the important diseases of the birds (Amundson et al. 2016). In general, caged birds are more prone to infections as compared to wild birds due to their more confined and suffocated environment (Ombugadu et al. 2018). Close bound and unhygienic environmental conditions play an important role in the transmission of parasitic diseases. Some birds may have a subclinical infection of parasites which plays important role in the disease transmission (Doneley 2009). Molecular techniques have been performed to detect the presence and characterize the disease-causing parasites in birds. *Trichomonas gallinae* identification has been performed by Qiu et al. (2017) through PCR assay. Pair of primers was developed i.e.,

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TgF2/TgR2 based on the nuclear ribosomal DNA and characterized through phylogenetic analysis (Qiu et al. 2017).

Madani and Peighambari (2013) used nested polymerase chain reaction (PCR) to analyze the samples from fancy pigeons. Thirty-two samples were tested out of which 12.6% were found positive for *Chlamydia psittaci* by using CTL/CTU primers to amplify gene (ompA) DNA and using AluI restriction enzymes. Four restriction patterns were used to perform restricted fragment length polymorphism of the ompA gene. The analyses revealed the existence of four genotypes A, B, I, and J, and also discovered that the *C. abortus* and *C. psittaci* were closest to the new genotypes I and J.

The presence of Leucocytozoon spp in the blood of birds and black flies was determined by PCR. The sequencing revealed the presence of the two closely related haemosporidian parasites including Haemoproteus spp. and Plasmodium spp (Jones et al. 2015). In Avian populations, the protozoan parasites like Haemoproteus occur to a great extent and are normally found in the blood of hosts. These hosts are found everywhere on the earth. According to a recent study, by using a molecular technique like PCR the *Haemoproteus columbae* was found in Iranian pigeons with a prevalence rate of 23.18% out of (51/120) (Doosti et al. 2014). These parasites are transmitted by biting flies (Tanigawa et al. 2013). Eljadar et al. (2012) A investigation of pigeons revealed the prevalence of different endoparasites and ectoparasites in Tripoli region of Lybia. This study revealed that 55% and 76% were infected with *Trichomonas gallinae* and Haemoproteus spp. respectively. While in pigeons, the prevalence of the intestinal helminths was 56%. Prevalence of three species of class Nematoda, *Heterakis gallinarum*, *Ascaridia galli*, and Capillaria spp was found respectively at 18%, 22%, and 4%. The prevalence of three species of class Cestoda like *Raillietina tetragona*, *Raillietina echinobothrida* and *Raillietina cesticillus* was 2%, 32%, and 4% respectively. The fecal flotation method was used to detect prevalence in pet birds. Usually, 35.6% of the birds were found infected with parasites i.e., zoo birds and domestic birds with the ratio of 42.2% and 27% including Ascaridia, Strongyles, Strongyles-Capillarids, G. duodenalis Assemblage, Coccidia, Cryptosporidium, Porrocaecum-Capillarids, Porrocaecum, and Syngamus-Capillarid with the ratio of 6.8%, 5.5%, 8.9%, 5.3%, 4.1%, 4%, 2%, 2.7%, and 0.7% respectively. Zoo birds were most likely to harbor different parasitic diseases as compared to domestic birds. Whereas indicative birds are parasitized (Papini et al. 2012). Few zoonotic parasitic diseases like Giardia duodenalis and Cryptosporidium spp. were found in caged birds which have the public health significance, so the clinician should know about them (Quah et al. 2011).

Poultry was also infected with intestinal parasites with a prevalence of 62%, the birds were found infected with various species of parasites including Ascaridia galli, Heterakis gallina, coccidia, Capillaria annulata (Threadworm), Syngamus trachea, and Tapeworm. Among the helminths, the Ascaridia galli was the most dominant species found with a ratio of 17.2% (Heyradin et al. 2012). It was suggested that a sustainable control methodology after the determination of the high prevalence of diverse diseases and parasitism could be a major imperative to creation in the study area (Rufai and Jato 2017).

Parasites of the Circulatory System

Protozoal Parasites of the Circulatory System of Pet Birds

The canaries, raptors, and *Columbiformes psittacines* were high in Leucocytozoon spp, Plasmodium spp., Cacatua spp. Haemoproteus spp, and toxoplasma spp. (Rae 1995; Masello et al. 2006). Toxoplasmosis is also diagnosed in canaries (Dubey 2002).

Parasites of the Gastrointestinal System

Giardiasis of the Gastrointestinal System of Pet Birds

Giardiasis was commonly reported in many species of fancy birds but mostly it is present in cockatiels. Adult birds may be the carriers of giardiasis (Malik et al. 2021). Giardiasis is transmitted via direct contact or ingestion of infective cysts. Affected cockatiel develops minor clinical signs of feather pulling from the inner thigh and axillary regions, along with vocalism. (Krautwald-Junghanns et al. 2008). There is no evidence of a relationship between the appearance of clinical signs of giardiasis and the incidence of the disease. Cockatiels that are infected with it have capacious and "popcorn" appearance droppings.

Diagnosis

There are many diagnostic techniques to detect Giardia spp. from droppings of fancy birds. One of them is the zinc sulfate flotation test of bird's droppings to detect cysts of Giardia. A direct saline smear of fresh feces is also used to detect the motile trophozoite stage of Giardia (Hooshyar et al. 2019). ELISA and PCR tests are used for Giardia spp. antigen detection from feces (Cook 2008).

Treatment

Metronidazole with a dose rate of 25-50 mg/kg, per oral, after every 12-24 hrs for 5-7 days, and Carnidazole with a dose rate of 20-30 mg/kg/day orally, for 1-2 days is used for the treatment of giardiasis.

Trichomoniasis of Pet Birds

Trichomoniasis has been reported in many species of fancy birds, including Passeriformes Columbiformes, Galliformes, Psittaciformes, and Falconiformes (Nemeth et al. 2016). *Trichomonas gallinae* (those prey birds which are affected with trichomoniasis named as "frounce" and in Columbiformes called as "canker") is occasionally seen in pet birds, and more notably in budgerigars (Brandão and Beaufrère 2013). Clinical signs of Trichomoniasis may include dysphagia, anorexia, dyspnea, and weight loss. Postmortem findings in raptors and Columbiformes may include whitish-yellow and caseous lesions present on the mucosa of the crop, oropharynx, and esophagus (Bunbury et al. 2007). Budgerigars have increased salivation and regurgitation, without clinical evidence of oral lesion (Fennoscandia 2010). There are two routes of transmission, the first one is a direct transmission from parents to offspring during feeding and the second is the indirect transmission from ingestion of contaminated food and water.

Table 1: Prevalence review of various fancy birds parasites in different countries

Bird species	Parasitic specie	Gender	Percentage prevalence	Country	Reference
Gallus gallus Domesticus	*Argas persicus*	-	61.2	Iraq	Abdullah 2013
		-	7.46	Nigeria	Edosomwan and Lgetei 2018
	Ascaridia galli	-	1.15	Nigeria	Edosomwan and Lgetei 2018
		-	32.5	Khumasi Ghana	Asumang et al. 2019
		-	21.29	Pakistan	Fatima et al. 2015
		-	31	Iraq	Abdullah 2013
	Capilaria Spp.	-	14.5	Kumasi Ghana	Asumang et al. 2019
		-	1.72	Iraq	Abdullah 2013
	Cuclotogaster heterographus	-	4.48	Nigeria	Edosomwan and Lgetei 2018
		-	10.42	Iraq	Abdullah 2013
	Davainae proglottina	-	5.38	Nigeria	Edosomwan and Lgetei 2018
		-	2	Kumasi Ghana	Asumang et al. 2019
		-	3.45	Iraq	Abdullah 2013
	Eimeria species	-	1	Iran	Badparva et al. 2015
		-	50	Jorden	Alasadiy et al. 2020
	Goniocotes gallinae	-	54.17	Iraq	Abdullah 2013
	Goniodes gigas	-	17.91	Nigeria	Edosomwan and Lgetei 2018
			39.58	Iraq	Abdullah 2013
	Haemproteus spp.	-	13.2	Iraq	Alasadiy et al. 2020
		-	24.4	Pakistan	Alasadiy et al. 2020
	Liperus caponis	-	17.91	Nigeria	Edosomwan and Lgetei 2018
	Leucocytozoon spp.	-	0	Iraq	Alasadiy et al. 2020
		-	13	Pakistan	Alasadiy et al. 2020
	Menacanthus stramineus	-	72.92	Iraq	Abdullah 2013
	Menopon gallinae	-	22.39	Nigeria	Edosomwan and Lgetei 2018
		-	37.5	Iraq	Abdullah 2013
	Prosthogonimus species	-	1.5	Kumasi Ghana	Asumang et al. 2019
	Raillietina spp.	-	9.5	Kumasi Ghana	Asumang et al. 2019
		-	7.5	Iran	Badparva et al. 2015
		-	55.17	Iraq	Abdullah 2013
Peacock	*Argas persicus*	-	5.94	Pakistan	Khursheed et al. 2014
	Amyrsidea minuta	-	7.14	Algeria	Marniche et al. 2017
	Amidostomum spp.	-	20.8	Algeria	
	Amidostomum spp.	-	25	Algeria	
	Columbicola columbae	-	9.9	Pakistan	Khursheed et al. 2014
	Capilaria. spp.	-	25	Algeria	Marniche et al. 2017
	Cooperia spp.	-	4.2	Algeria	
	Cyathostoma branchalis	-	8.3	Algeria	
	Colpacephalum tausi	-	50	Algeria	
	Chilomastix spp.	-	16.7	Algeria	
	Echidnophaga gallinacean	-	6.93	Pakistan	Khursheed et al. 2014
	Eimeria spp.	-	58.3	Algeria	Marniche et al. 2017
	Giardia spp.	-	4.2	Algeria	
	Lipeureus caponis	-	7.14	Algeria	
	Menacanthus stramineus	-	10.89	Pakistan	Khursheed et al. 2014
	Menopon spp.	-	7.14	Algeria	Marniche et al. 2017
	Menacuntus spp.	-	28.57	Algeria	
	Strongyloides spp.	-	16.7	Algeria	
Columba livia	*Ascaridia columbae*	M	10.2	Nigeria	
		F	12.4	Nigeria	Adang 2012
		M	27.8	Pakistan	
		F	40	Pakistan	
		-	21.81	Iran	Dehghani-samani et al. 2020
	Ascaridia galli	-	22	Libya	Alkharigy et al. 2018
		-	3.3	Nigeria	Adang 2012
		M	3.1	Nigeria	
		F	3.5	Nigeria	
		-	7.27	Iran	Dehghani-samani et al. 2020
	Amoebotaenia cuneate	-	5	Nigeria	Edosomwan and Lgetei 2018
		M	0.8	Nigeria	Adang 2012
		F	0.9	Nigeria	
	Capillaria contorta	-	10	Nigeria	Edosomwan and Lgetei 2018
	Cryptosporidium meleagridis	-	2.7	Iran	Badparva et al. 2015
		-	3.63	Iran	Dehghani-samani et al. 2020
	Chelopistes meleagridis	-	5	Nigeria	Adang 2012
	Columbicola columbae	-	35	Nigeria	Edosomwan and Lgetei 2018
		-	82	Libya	Alkharigy et al. 2018

		-	63.8	Nigeria	Adang 2012
		M	66.9	Nigeria	
		F	60.2	Nigeria	
		-	86.66	Pakistan	Khan et al. 2018
		-	56.36	Iran	Dehghani-samani et al. 2020
	Dermanyssus gallinae	M	1.6	Nigeria	Adang 2012
		F	3.5	Nigeria	
	Eimeria labbeano	-	23.63	Iran	Dehghani-samani et al. 2020
	Goniodes dissimilis	-	20	Nigeria	Edosomwan and Lgetei 2018
		-	10.8	Nigeria	Adang 2012
		M	10.2	Nigeria	
		F	11.5	Nigeria	
	Goniocotes gallinae	-	18	Libya	Alkharigy et. al 2018
	Lipeurus caponis	-	25	Nigeria	Edosomwan and Lgetei 2018
		-	16.36	Iran	Dehghani-samani et al. 2020
	Menopon gallinae	-	15	Nigeria	Edosomwan and Lgetei 2018
		-	6.3	Nigeria	Adang 2012
		-	3	Libya	Alkharigy et al. 2018
		M	3.1	Nigeria	Adang 2012
		F	9.7	Nigeria	
		-	21.81	Iran	Dehghani-samani et al. 2020
	Pseudolynchia canariensis	-	1	Libya	Alkharigy et al. 2018
		-	37.1	Nigeria	Adang 2012
		M	38.6	Nigeria	
		F	35.5	Nigeria	
		-	36.36	Iran	Dehghani-samani et al. 2020
	Raillietina echinobothrida	-	85	Nigeria	Edosomwan and Lgetei 2018
		-	32	Libya	Alkharigy et. al 2018
		M	11	Nigeria	Adang 2012
		F	10.6	Nigeria	
		-	18.18	Iran	Dehghani-samani et al. 2020
	Trichomonas gallinae	-	56	Saudia Arabia	Albeshr and Alrefael 2019
		-	67.27	Iran	Dehghani-samani et al. 2020
		-	75.78	Turkey	Alkharigy et al. 2018
Duck	*Anaticola cassicornis*	M/F	100	Bangladesh	Begum et al. 2018
	Ascaridia galli	M	43.75		
		F	85.71		
	Colpacephalum turbinatum	M	25		
		F	50		
	Cotugnia digonopora	M	31.25		
		F	50		
	Echinoparyphium recurvatum	M	25		
		F	35.71		
	Echinoparyphium elegans	M	18.75		
		F	28.57		
	Echinostoma trivolvis	M	12.5		
		F	14.28		
	Echinostoma revolutum	M	18.75		
		F	21.43		
	Goniocotes hologaster	M	75		
		F	100		
	Goniocotes gigas	M	37.5		
		F	35.71		
	Holomenpon leucoxanthum	M	56.25		
		F	64.28		
	Hymenolepis lanceolate	M	43.75		
		F	78.57		
	Hymenolepis columbia	M	62.5		
		F	85.71		
	Lipeuris caponis	M/F	100		
	Menopon gallinae	M	93.75		
		F	100		
	Menacanthus stramineus	M	62.5		
		F	71.43		
	Psilochasmus longicirratus	M	18.75		
		F	35.71		
	Raillietina bonini	M	62.5		
		F	64.28		
	Raillietina cesticillus	M	56.25		
		F	57.14		

	Raillietina echinobothridia	M	50		
		F	50		
	Sobolevicanthus spp.	M	25		
		F	35.71		
Psittaci forms	*Ascaridia galli*	-	25	Bangladesh	Hasan et al. 2018

Raptors may get the infection by ingesting infected doves or pigeons. A sample of a warm saline amount of material from the oral cavity may reveal the flagellated organism during microscopic examination (Anderson et al. 2009). Treatment protocols against Trichomonas may include ronidazole with the dose rate of 6-10 mg/kg/day, through the oral route, for 7-14 days, carnidazole with a dose rate of 20-30 mg/kg, per os, for 1-2 days, or metronidazole with a dose rate of 25-50 mg/kg, orally for every 12-24 hours for 5 days (Cousquer and Parsons 2007).

Other Protozoal Diseases of Pet Birds

In other protozoan parasites, the coccidial oocyst is much more prevalent in Columbiforme or gallinaceous birds, although it is rarely present in passerine and psittacine birds.

Cryptosporidiosis

It also affects many species of fancy birds, but it is considered the second most important pathogen after the primary pathogens (Fayer 2010).

Plasmodium spp.

Canaries, gyrfalcons, and penguins are highly affected by plasmodium spp. Plasmodium is spread by a certain type of mosquito that causes malaria (Mirza 2014).

Toxoplasmosis

Is a highly fatal protozoal disease having a coccidian-like oocyst, it causes splenomegaly and hepatomegaly in canaries (Twentyman 2001).
In other protozoan parasites, the coccidial oocyst is much more prevalent in Columbiforme or gallinaceous birds, although it is rarely present in passerine and psittacine birds (Cole and Friend 1999).

Roundworms of Pet Birds

Nematodes of various species and genera infect pet birds and wild birds. That remains a big source of transmission of roundworms to housed and outdoor captive parrots (Huang et al. 2017). The main route of transmission is direct ingestion of embryonated ova of roundworms. Most commonly observed clinical signs are weakness, loss of condition, emaciation, anorexia, and ultimately death of pet birds. Intestinal obstruction has been observed in the case of heavy infestation. We can diagnose nematode infection through the fecal flotation examination technique. Ivermectin is a drug of choice against ectoparasites and endoparasites with a dose rate of 0.2 mg/kg, oral, subcutaneous, or intramuscular (IM) and repeated after every 10-14 days. Pyrantel pamoate and fenbendazole are respectively used with the dose rate of 4.5 mg/kg and 20-50 mg/kg per os, repeated after every 10-14 days (Basit et al. 2014). During warm climates in outdoor aviaries, deworming is recommended after every 6 months with one of these anthelmintics (Elsheikha and Patterson 2013).

Cestodes of Pet Birds

Cestodes are not commonly present in domestic bred birds (Smith 1996). Tapeworms mostly infect the cockatoos, finches, and African grey parrots. Earthworms, slugs, and various types of arachnids serve as an intermediate host during the transmission of tapeworm. Pet birds show some sort of clinical signs like thriftiness, with or without diarrhea and some are asymptomatic. The fecal flotation examination technique is used to detect eggs of tapeworm (Willis and Wilkie 1999).
Praziquantel is a drug of choice against tapeworm with a dose rate of 5-10 mg/kg, oral or IM route, once a week. It's hard to develop tapeworm infection in household birds without an intermediate host.

Parasites of the Integumentary System

Knemidocoptes pilae is also called *Cnemidocoptes pilae*, which causes a scaly face in budgerigars and psittacine species (Jackson et al. 2015). In budgerigars, white, porous, and proliferative hard covering involves the base of the upper beak, corner of the mouth, periorbital area, legs, and vent. In canaries and European goldfinches, crusts form on the surface of tassel foot, legs, and digits (Powers 2011). The burrowing and non-burrowing mites can be recovered from skin or facial scrapings of budgerigars, it's also a pathognomonic clinical sign of the disease. Skin scraping of legs is generally not recommended due to hemorrhages developed after scraping in passerines affected with Knemidocoptes (Mangus et al. 2021). Ivermectin and moxidectin are mostly used with a dose rate of 0.2 mg/kg, per oral, IM, or topically and it is repeated after every two weeks.

Feather Mites of Pet Birds

Dermanyssus gallinae is the red mite of poultry. It also affects Psittacine birds. Those birds which live in outdoor aviaries and nest boxes are affected by *Dermanyssus gallinae* (Dorrestein 2003) Due to heavy infestation of mites, birds pluck their feathers, and it is also observed by the owner. Many other factors also play important role in feather plucking which includes, including husbandry, behavioral and systemic factors which are linked with each other.
Fancy birds with a severe infestation of mites are treated with, 5% carbaryl powder, pyrethrin sprays, and ivermectin with a dose rate of 0.2 mg/kg, per oral or intramuscular which is repeated after every 2 weeks. 5% carbaryl powder mix with nest box substrate is used to treat nest boxes of birds. Cages and wooden nest boxes should be cleaned thoroughly with insecticide spray. All the old wooden nest boxes should be discarded.

Parasites of the Respiratory System

Air Sac Mites of the Respiratory System in Pet Birds

In birds, *Sternostoma tracheacolum* is a parasite that affects the lungs, trachea, and respiratory tract of birds (Dorrestein 2009). Its life cycle is not well understood yet. In lower infection, no systems are affected in birds. However, heavy infection is present when the intensity of infection increases. Signs of the disease include high voices from the lungs of infected birds, nose licking, and open mouth breathing. The saliva drools out from the mouth of the infected birds. When the stress on birds increases due to heavy flight or exercise, the condition of the bird loses. Due to this disease death rates increases. If the recovery after treatment occurs, it shows there is a response to treatment. Quickly treat the birds after signs appear. After every two weeks, Ivermectin should be administered with a dose rate of 0.2-0.4 mg/kg, oral or IM route. Moxidectin with the dose rate of 0.2 mg/kg, oral or topically, and repeated in 2 weeks may be administered.

Conclusion

A variety of parasites is present in birds throughout the globe. Some external and internal parasites cause heavy infection and infestation in birds which leads to severe economic losses leading to a decrease in international trade of fancy birds. Proper hygienic measures and effective treatment may decrease the disease tendency in birds.

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CHAPTER 10

FILARIASIS: NEW INSIGHTS INTO AN OLD DISEASE

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INTRODUCTION

Filariasis is a vector-borne parasitic disease of almost all kind of animals and humans which causes high economic losses in tropical and subtropical areas of the world. Causative agents of this disease are the filarial worms which belong to superfamily *Filarioidea*. Morphologically, these filarial worms are thin and long with no buccal capsule and pharynx. Esophagus has two portions; muscular on anterior side and glandular on posterior side. Males are usually smaller than females having unequal and dissimilar spicules. However, in females, vulva is located on the anterior side where fully developed larvae are born. The larvae of filarial worms are called microfilariae which usually live in the blood, lymphatic system and body cavities of the host.

Filarioidea include three families namely *Filariidae*, *Onchocercidae* and *Setariidae* (Soulsby 2005). Most of the members included in these families are host specific including animals and humans. For examples, *Wuchereria bancrofti* is only pathogenic to humans causing elephantiasis which leads to a permanent disability in humans. Similarly, species of genus *Setaria* are only pathogenic to animals. However, most of the parasites included in family *Filariidae*, and *Onchocercidae* are associated in causing disease in both animals and humans. Nevertheless, few species among these families are non-pathogenic to animals, reports are available on the occurrence of these parasites in animals. Such species use these animals as their reservoir host till they find their definitive host i.e., humans (Soulsby 2005; Allen et al. 2008). Most important genera of family *Filariidae* include *Wuchereria*, *Brugia*, *Mansonella*, *Dirofilaria*, *Parafilaria*, *Ornithofilaria* and *Bhalfilaria*. Members of genus *Wuchereria*, *Brugia*, and *Mansonella* are mostly zoonotic in nature, however, they can also be found in different animals such as dogs, cats, horse, donkey, camel and small ruminants. Species of these genera have been reported as the main causative agent of lymphatic filariasis in humans. For example, *Wuchereria* (*W.*) *bancrofti*, *Brugia* (*B.*) *malayi*, *Brugia timori*, *Mansonella* (*M.*) *ozardi*, *Mansonella perstans*, have been involved in obstruction of lymphatic system, swelling, enlargement of limbs, breast and testes in humans (Deshpande et al. 2020). Genus *Dirofilaria* (*D.*) is mostly host specific and is prevalent in dogs, cats, foxes and wolf. Different species of *Dirofilaria* have been reported and are mostly present in America. Furthermore, species of *Parafilaria*, *Ornithofilaria* and *Bhalfilaria* are non-pathogenic to humans and are mostly present in animals including cattle, buffalo, sheep, goat, camel, horse and other wild animals (Soulsby 2005).

Similarly, species of family *Onchocercidae* are mostly present in sub-cutaneous tissue of animals and a leading cause of river blindness in both animals and humans. Most important species of genus *Onchocerca* is the *Onchocera* (*O.*) *volvulus*, which is transmitted by black flies of genus *Simulium* and causes keratitis in animals, most importantly in cattle. However, onchocerciasis have also been reported in humans in Africa, Asia and United States (Deshpande et al. 2020).

Geographical Distribution

Filariasis most importantly elephantiasis is a worldwide problem which is the second most common cause of permanent disability. It is a vector-borne disease and mostly observed in tropical and subtropical areas of the world. Approximately, 120 million people in 73 countries including Asia, Africa, Western Pacific and some areas of America have been infected with lymphatic filariasis. *Wuchereria* (*W.*) *bancrofti* is responsible for 90% of infection in these areas. Less contributing parasite is *B. malayi* which is prevalent in China, Philippines, India, Indonesia and Malaysia. However, *B. timori* is the least contributing filaria which commonly occurs on Timor Island of Indonesia. Onchocerciasis is another form of filariasis which is commonly present in Saudi Arabia, Yemen and Latin America. However, it is highly prevalent in Africa and a major cause of river blindness (Riches et al. 2020).

Filarial worms are usually pathogenic in human population, but they may infect almost all animal species. Unfortunately, the scientists have mainly focused on estimating the prevalence of different filarial parasites in the humans and a very little attention was given to locate the possible reservoirs and carriers for these zoonotic filarial worms such as domestic animals. Nevertheless, many species of microfilariae have also been reported from dogs, horses, sheep, goats, cattle and buffalos (Azzay et al. 1998; Hassan et al. 2015; Radwan et al. 2016). Ekong et al. (2012) found from his experiment in Nigeria that more than 60% of filarial species were zoonotic in nature. *D. immitis* is most commonly present in dogs and is known as dog heart worm. From 424 clinically sick dogs presented at teaching veterinary hospital Guwahti, Assam revealed 5.42% cases of microfilariae infection (Bhattacharjee and Sarmah 2014). Furthermore, 37 million people around the globe are infected with Onchocerciasis (river blindness). In 2012, *Onchocerca* spp. was also reported from dogs. Similarly, another study revealed 12.8% prevalence of *Dirofilaria* in Baix

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Llobregat region, Spain (Hassan et al. 2015). Reviewing the literature, it was found that filariasis is being neglected in many regions of the world including Pakistan. In Pakistan, there is a scarcity of knowledge regarding the occurrence of different filarial worms and very few cases of elephantiasis have been reports in humans and animals as well.

First case of Onchocerciasis was reported in 1967 in wolf in Russia and the species identified was *Onchocera* (*O.*) *lupi*. After few years in 2011, ocular *O. lupi* was also isolated from human eye in Turkey. Interestingly, no case was reported in dogs at that time. During last decade, there has been observed an increasing trend of zoonotic cases of *O. lupi* in America, Middle East and Europe (Gracio et al. 2015). Likewise, *Seteria* spp. parasitize mostly the animals such as sheep, goat, horse, cattle and buffalo. These species occasionally cause fibrinous peritonitis in some accidental hosts. Similar case of *Seteria* infection was observed in Iran, where *Seteria equina* was isolated from left eye of fifteen years old girl. Similarly, four human cases of subconjunctival infection associated with *Seteria labiatopapillosa* were reported in Romania who were presented with swelling, photophobia and tearing signs (Panaitescu et al. 1999).

Transmission Dynamics

It is important to understand the transmission cycle of filarial worms between humans, their biological vectors, geographical distribution of different microfilariae and the competency of vector for filarial worms. Biological vector of this disease are black flies and five genera of the mosquitoes namely A*edes*, A*nopheles*, *Culex*, *Mansonia* and *Ochlerotatus*. Infective stage is called microfilariae which are consumed during a blood meal. Within the mosquitoes, they lose their outer cuticle and migrate to the thoracic muscles where they undergo larval development and L3 stage is formed. L3 migrates to the proboscis from where they can infect other vertebrate host during another blood meal. These L3 find their way in through a bite wound and after penetration L3 larvae molt into L4. These L4 migrate to their predilection sites such as lymphatic vessels and serous cavities and mature into adult worms (Chu et al. 2013). A complete layout of life cycle of filarial nematodes in the vector and host (animal and human) has been described in Figure 1.

Unlike malaria and arboviruses, the transmission of filariasis is inefficient and many bites by the infected mosquitoes are required to start a new infection. Many factors contribute to their inefficient transmission. For example, very limited number of microfilariae are ingested by the vector and cannot multiply within the vector. Secondly, during blood meal, they are deposited on the skin rather then injected by the mosquito saliva and they need to find their way into the bite wound. Additionally, only those mosquitoes will contribute to transmission that survive for more than ten days. So, the transmission of filariasis is less efficient as compared to other vector-borne parasites (Malhotra et al. 2006).

Transmission potential can be used as a tool to estimate the risk of lymphatic transmission. It can be calculated as the average number of microfilariae per infective mosquito and the average biting rate of vector in a specific time period. Transmission potential can be measured as monthly and annual. However, the annual measurement of transmission potential is more suitable because the monthly transmission potential may vary based on the season and biting density (Thiele et al. 2016). Unfortunately, the annual transmission potential of filariasis in relation to vector-parasite relationship and competency of different mosquitoes has not been estimated so far.

Microfilarial periodicity is the daily pattern of circulation in the peripheral blood which is different for different microfilariae. In nocturnal periodicity, density of microfilariae greatly increases during the night while inexistent during the daytime. However, the density of some microfilariae is very high during the day showing diurnal behavior. Sometimes, the microfilariae can be seen during the day as well as at night which are called aperiodic microfilariae. Furthermore, diurnal sub-periodic behavior has also been reported wherein, high density of microfilariae is present at night still the microfilariae can be seen during night (Ekong et al. 2012).

Microfilariae of *W. bancrofti* are said to have nocturnal behavior because they mostly present in peripheral blood of humans at night than day light. However, in case of *Loa loa*, the microfilariae exhibit diurnal behavior. It has been observed that periodicity of these microfilariae is a behavior of parasites rather than the host because the microfilariae are accumulated in the lungs at day light mediated by a fixation force while the switch mechanism allows the microfilariae to be released in the peripheral blood. Fixation force is dependent on the presence of oxygen tension, which means the parasites accumulate in the lungs at day when there is more oxygen available in the lungs (Allen et al. 2008).

Periodicity of microfilariae also depend on the biting behavior of the vector ensuring their transmission. Moreover, some vectors prefer to seek blood from their host inside the houses while some prefer to feed when the hosts are outside. For example, the *Anopheles* mosquitoes prefer to feed on host during the night ensuring the transmission of *W. bancrofti* in Africa, South America, Middle East and South Asia (Deshpande et al. 2020). So, an appropriate method should be implemented to prevent biting of the mosquitoes.

Pre-disposing Factors

Humans are the primary host for many filarial worms. However, animals can also be infected with these parasites and may act as reservoirs for zoonoses. Different factors may be associated with the occurrence of filarial worms or with the emergence and re-emergence of filariasis.

Age

Lymphatic filariasis can occur in almost all age groups. Children may acquire infection sporadically in the age of six months which remain asymptomatic reservoirs for 10-15 years. However, infection rate has been found to rise with age of 20-40 years. Due to poor diagnosis and false negative results, it appears only in small percentage of infected individuals. With the development of new diagnostic tools, it is revealed that the filariasis is a childhood-acquired disease and almost one third of the children are asymptomatically infected before the age of five years. In Kenya, Malhotra et al. (2006) screened neonates from 159 pregnant women having *W. bancrofti* infection. Some children were lacking filarial specific T-cell response at birth which indicated 13-fold increased risk of developing childhood infection.

Mostly, the infected people remain asymptomatic and may not develop clinical symptoms throughout their life. Yet, many people develop clinical symptoms in later stage of their life after

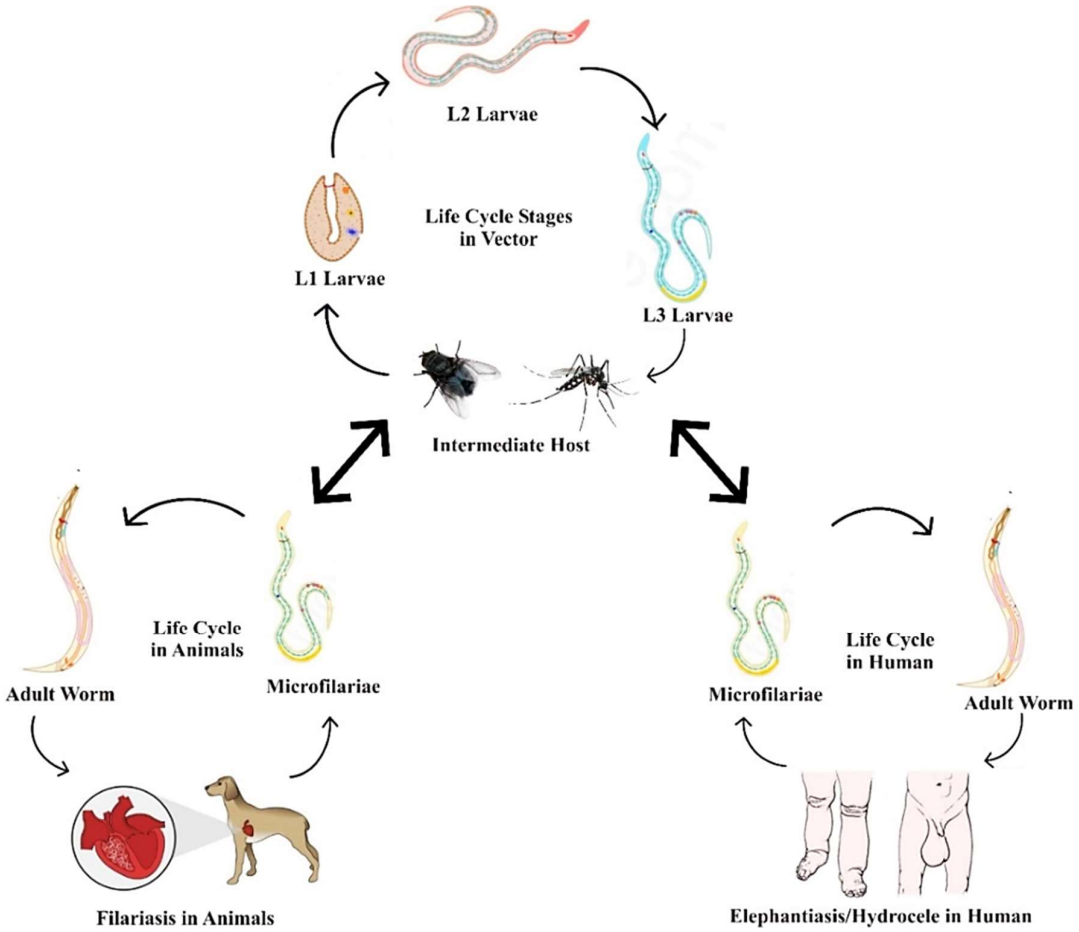

Figure 1: Transmission dynamics of filarial worms

the microfilariae will have damaged the lymphatic vessels with accumulation and obstruction. Initially, the condition is misdiagnosed with adenitis, but after puberty, the infected people begin to show characteristic clinical symptoms like elephantiasis and hydrocele. Additionally, filarial infection may lead to tropical pulmonary eosinophilia symptomized by cough, wheezing and shortness of breath. This condition is most common in Asia and is characterized by high level of IgE and anti-filarial antibodies (Solismaa et al. 2008).

Density of Infection

It is still unclear that how much or minimum level of microfilariae in an infected individual permit the infection of mosquitoes. Hawking (1962) reported that an individual with one microfilaria/mm^3 of blood was able to infect 2.6% of mosquitoes fed on him. Besides, Omori (1962) observed that the mosquitoes did not survive when they were fed on a carrier having ≥80 microfilariae/mm^3 of blood. The major reason behind the death was the maturity of a number of microfilariae in the mosquitoes.

Social Factors

Awareness, sleeping habits, urbanization, deforestation and industrialization are closely associated with filariasis. Furthermore, migration of people, vector population, import, export of goods are also some social factors associated with filariasis. Elephantiasis or lymphedema of legs cause permanent disability which hinders in the working capacity of the individual and cause suffering and economic loses (Malhotra et al. 2006).

Vector Population

Mosquitoes are the biological vectors for most of the filarial worms. Population density of mosquitoes is very high in tropical and subtropical areas of the world due to high temperature and humidity which are the favorable conditions for mosquito population. Furthermore, the host range for these vectors is very large including different animals which enables the filarial worms to use these animals as carriers (Thiele et al. 2016).

Host Immunity

Immunity of the host can limit the number of microfilariae to the threshold level. Mostly the filarial worms in subcutaneous tissues and skin become dead and remain encapsulated in the body. Furthermore, the immune system triggers the T-cell response that help to eliminate the microfilariae circulating in the blood (Malhotra et al. 2006).

Migration of People

Migrators pay major contribution in the emergence of a disease in disease free areas. Though, the migrated individuals from

endemic areas are asymptomatic, but they may be a carrier of filarial infection (Ekong et al. 2012).

Gender

Gender of the host is not apparently associated with the transmission of filarial infection as there is an equal chance of getting bite from the mosquitoes (Thiele et al. 2016).

Parafilariais/ Summer Bleeding

Parafilariais or summer bleeding is a skin disease of domestic and wild animals which is usually caused by filarial nematodes of genus *Parafilaria*. Two important species of *Parafilaria* are *Parafilaria* (*P.*) *bovicola* and *Parafilaria (P.) multipapillosa*. *P. bovicola* mostly infect cattle and buffalo and distributed worldwide. However, it is highly prevalent in Asia, Africa and some European countries such as Scandivania and Russia. Another species called *P. multipapillosa* is pathogenic to animals and most commonly parasitize horses, mules and donkeys. This species is abundantly prevalent in Eastern Europe (Caron et al. 2013). However, both species do not parasitize sheep, goat, dogs, cats and poultry. Prevalence of these parasites varies depending upon the region, vector population and season. It has been reported that half of the cattle population in South Africa is infected by *P. bovicola*. Similarly, about 15% prevalence of *Parafilaria bovicola* has been reported in Belgium (Losson and Saegerman 2009).

Morphologically, *Parafilaria* adult worms have whitish cylindrical body with 6cm length while females are longer than males. Like other round worms, *Parafilaria* have a tubular digestive and nervous system but do not have excretory and circulatory system. Adult microfilariae reside in the skin of their host where the female worm cause focal cutaneous hemorrhages from where the blood start oozing for few hours, followed by the clotting and drying on the skin (Soulsby 2005). These bleeding spots mostly occur on the forequarters of the animal and usually seen in the spring and early summer. Due to this reason, the condition is called as summer bleeding, which facilitates the female worms to perforate the skin nodules and lay eggs in the blood dripping from the wound. *Musca* flies ingest *Parafilaria* eggs while feeding on the bleeding spots. In the vector, they undergo development and L3 larvae are formed which are again transmitted to animal during next meal. Parafilariasis in animals damage the skin and underlying muscles, which leads to condemnation of carcasses (Hamel et al. 2022) and cause huge economic losses to the meat industry.

Onchocerciasis

Onchocerciasis is a parasitic disease of farm animals including cattle, buffalo and horses. However, some species of *Onchocerca* have also been reported in dogs and humans (Soulsby 2005). The species of *Onchocerca* are distributed worldwide, particularly, in tropical and sub-tropical areas of the world. Most common species of veterinary importance include *O. gibsoni, O. gutturosa, O. dukei, O. ochengi, O. armillata, O. reticulata, O. raillieti, O. cervicalis, O. lupi and O. volvulus*. Most of these species are host specific with specific geographical area, for examples, *O. dukei*, *O. ochengi* and *O. armillata* are parasites of cattle and mostly prevalent in Africa. Similarly, some other species like *O. gutturosa* and *O. gibsoni* are prevalent in Australia, Africa, Europe and America. Some of the parasites also infect horses all around the world such as *O. cervicalis*, *O. reticulata* and *O. raillieti*. In Europe and America, *O. lupi* has been occasionally found in dogs, however, rare cases of human infection have also been reported. Among all the species of *Onchocerca*, *O. volvulus* and *O. lupi* are zoonotic in nature and have been associated in causing river blindness in humans (Macfarlane et al. 2020).

Adult *Onchocerca* can be 50cm long having flexible but rough cuticle. The cuticle in *Onchocerca* is transversally striated forming structures like rings. Females are viviparous in this genus. In the life cycle of *O. gibsoni*, midges of genus *Culicoides* act as intermediate host whereas, in *O. gutturosa* and *O. dukei,* black flies of genus *Simulium* act as intermediate host to transmit these parasites into other animals. The incidence of onchocerciasis in cattle may be high, for example, 100% of cattle may be infected with *O. gibsoni, O. lienalis and O. gutturosa* in Australia and 50 to 90% cattle may be infected with *O. dukei.* Likewise, 22-61% of horses have been infected with *O. cervicalis* in USA (Soulsby 2005).

The adult worms of *O. gibsoni* reside in subcutaneous tissue by producing nodules (worm nest). These nodules are surrounded by fibrous tissue, which becomes thickened with the passage of time. Furthermore, the degeneration of tissue occurs, and calcification takes place forming a rigid nodular swelling under the skin. In such cases, the infected animals do not show any clinical signs, however, the carcasses of such animals are severely damaged and should not be recommended for sale (Lagatie et al. 2018). The accumulation of microfilariae in the subcutaneous tissues leads to popular or exudative dermatitis with alopecia and sever pruritis. Nevertheless, this condition may be due to allergic reactions to the bites of insect vectors. *O. volvulus* is commonly involved in ocular onchocerciasis which is most common in humans, cattle and horses. It causes river blindness in the infected patients and is characterized by conjunctivitis, keratitis, uveitis and kerato-conjunctivitis (Bennuru et al. 2020).

Adult worms of *Onchocerca* can most commonly be seen in the hip region of animals on post-mortem. However, the skin nodules containing adult worms can also be palpated. Furthermore, the microfilariae can be detected by skin snip method under microscope. Skin patch testing with diethylcarbamazine can also be effective in the identification (Alhassan et al. 2016). Some of the tests that can be used in identification of microfilariae are listed in Table 1.

Dirofilariasis/Dog Heartworm Disease

The causative agents of this disease are the filarial species of genus *Dirofilaria*. Several species of *Dirofilaria* have been reported, however, the most important parasite is the *Dirofilaria immitis*. The definitive host of this parasite is primarily dogs, cats, fox and wolf in which it resides in the right ventricle and pulmonary artery. Due to this reason, the *D. immitis* is known as dog heart worm. Males of this parasite are 12-16 cm long and females are 25-30 cm long. Microfilariae of this parasite can be always seen in the blood. However, the periodicity of their microfilariae is different in different regions of the world. For example, in USA, the concentration of microfilariae is maximum at 16.30 hours, while in France, maximum concentration can be observed at 20.00 hours (Soulsby 2005).

Transmission of *Dirofilaria* spp. is facilitated by the intermediate host i.e., a variety of mosquito species belonging to genus

Table 1: List of diagnostic tests for the detection of onchocerciasis infection

Test name	Principal	Target	References
Tissue biopsy	Microscopic examination of skin snip biopsy	Microfilaria	Alhassan et al. 2016; Unnasch et al. 2018
Mazzotti patch test	Microfilaria-induced inflammatory reaction by diethylcarbamazine	Microfilaria	Mazzotti 1951
Elisa	Screening of surface and secreted Ags by serum IgG and IgM	Adult *Onchocerca* AGs; Ov7, 10, 16, 20 and Ov 33	Lagatie et al. 2018
qPCR	Amplification of genomic DNA	Targeted gene	Zimmerman et al. 1994
LAMP	-	0-150, Cox1 and miRNA	Alhassan et al. 2016; Macfarlane et al. 2020
Biomarker	Lateral flow immunoassay	NATOG (N-acetyltyramine - O-glucuronide)	Denery et al. 2010
Lateral flow immunoassay and Mass spectrometry	Liquid chromatography tandem mass spectrometry method (LC-MS/MS)	Phospholipids	Bennuru et al. 2018; Bennuru et al. 2020

Aedes, *Anopheles* and *Culex*. Dogs become infected when infected mosquito takes a blood meal. During initial infection, the parasites can be found in the submuscular membrane and subcutaneous tissue. However, after three months of infection, these parasites migrate and reside in the heart and pulmonary artery. Further after two weeks, when the parasites mature into adults, microfilariae are shed in the blood where they can survive for two to three years. Most of the infected dogs do not show any clinical signs except for the presence of microfilariae in the peripheral blood. However, in heavy infections, these parasites cause mechanical interference in the functioning of the heart. Large number of *D. immitis* can accumulate in right atrium, right ventricle and pulmonary artery resulting in pulmonary hypertension which leads to congestive heart failure (Cuervo et al. 2013; Ciuca et al. 2018).

D. immitis is highly prevalent in United States, Canada, Mexico and Brazil. It has been reported that the prevalence of these parasites is much lower in colder climates as compared to warmer areas. For example, maximum prevalence of *D. immitis* in Canada has been reported to be 8.4% (Klotins et al. 2000). However, in United States, maximum prevalence of 40% has been reported (Lee et al. 2010). Similarly, the climate of Argentina is comparatively lower, and 74% prevalence has been observed in Argentina (Simon et al. 2012).

Other *Dirofilaria* species such as *D. repens, D. corynodes, D. conjuntivae, D. roemeri, D. tenuis* and *D. ursi* have been sporadically reported in different countries (Vezzani et al. 2017). *D. repens* reside in the subcutaneous tissue of the cats and dogs and has been reported in Italy, France, India, Sri-Lanka and South-east Asia. Unsheathed microfilariae of this parasite can be seen in blood and in lymphatic system. *D. repens* has also been found from the subcutaneous tissue of man. Similarly, *D. conjuctivae* has been found associated with hazelnut sized nodules on the head and upper eyelid of humans (Soulsby 2005).

Public Health Significance

According to the World Health Organization, almost 120 million people have been infected by these filarial worms. There are eight known reported species of filarial worms including *Wuchereria* (*W.*) *bancrofti*, *Mansonella* (*M.*) *ozzardi, Brugia* (*B.*) *timori, Loa loa, Mansonella* (*M.*) *Streptocerca*, *Onchocerca* (*O.*) *volvulus*, *Mansonella* (*M.*) *perstans*, *Dirofilaria* (*D.*) *immitis*, and *Brugia* (*B.*) *malayi* (Soulsby 2005).

Filariasis exists in three forms such as lymphatic filariasis, subcutaneous filariasis and serous cavity filariasis based on the predilection sites of the parasite. Lymphatic filariasis is also called elephantiasis because in this condition, filarial worms block the lymphatic system and prevents the circulation of lymph. It leads to the fluid accumulation in the body extremities most likely in the arms and legs resulting in the enlargement of arms and legs. Lymphatic filariasis can also affect external genitalia of males and females. In males, it causes enlargement of scrotum due to which penis retract under the skin and become thickened and painful. Furthermore, spermatic cord become thickened and the infected males experience pain and burning sensation. In females, a tumorous mass covered with thick and ulcerated skin may develop between the legs and may be accompanied by lymphadenopathy of the legs. Moreover, enlargement of breasts is also very common in infected females (Fassari et al. 2021).

Lymphatic filariasis is mostly caused by W. bancrofti, B. timori and B. malayi which are the most common pathogenic species of filarial worms. These parasites are associated with causing permanent disability in humans. Among 120 million infected people, W. bancrofti is responsible for 90% of infection, while B. malayi contributes only 10% (Ndeffo-Mbah et al. 2006). First case of elephantiasis (lymphatic filariasis) was reported in Korea in 1927 and known by several names like Soojongdari, Pijoeng, Pinaerim. Later, the infection goes on increasing and the infection rate goes to 12-26% by 1970. In 2012, another study was conducted in Korea to evaluate the main source of the disease in which *Anopheles* sp. and *Culex pipiens* were found the main vectors spreading these parasites in human population (Riches et al. 2020).

In asymptomatic microfilaremia, clinical diagnosis is very poor and there may be acute episodes of adeno-lymphangitis (Alonso 2009). However, in chronic cases, swelling of limbs (elephantiasis) and hydrocele are the most typical signs in humans. Despite of no systematic data available in India, there were 20.32 million people showing symptoms of chronic filariasis among which male population contributed 79% (Ndeffo-Mbah et al. 2006).

Onchocerciasis is another filarial disease which is also known as river blindness. Causative agent of this disease is *O. volvulus* which is transmitted by black fly of genus *Simulium*. Black flies breed near fast-flowing rivers and streams based on which it is called as river blindness. Onchocerciasis is the second leading cause of blindness throughout world and 0.5 million people were blind due to onchocerciasis. It mostly occurs in rural communities and cause permanent blindness. Microfilariae of *Onchocerca* can frequently be detected in skin snips of animals and humans but occasionally in blood streams with chronic infections. Global prevalence of onchocerciasis was estimated 20.9 million in 2017 and 205 million people living in endemic areas were at risk of onchocerciasis (Thiele et al. 2016).

Dirofilariasis is another zoonotic disease which is mostly present in dogs and cause heart disease, hence known as dog heart worm. Commonly known *Dirofilaria* species which are

zoonotic in nature include *D. immitis* and *D. repens*. In Thailand, several human cases of ocular filariasis were associated with *D. immitis* and *D. repens*. Furthermore, molecular analysis of ocular sample collected from a 67-year-old woman showed a new species of *Dirofilaria* named as *D. hongkongensis* (Dantas-Torres and Otranto 2020).

Genus *Mansonella* includes nine filarial species, among which, *M. ozzardi, M. perstans* and *M. streptocerca* are well known zoonotic parasites. These parasites are transmitted by female *Culicoide* and reside in the skin of their host. However, their microfilariae can be found circulating in the peripheral blood. Recently, another new species called *Mansonella* sp. DUEX has been discovered from febrile children in Gabon. Clinical manifestation of these *Mansonella* is poorly defined, however, some common signs like joint pain, itching, lymphadenopathy, swelling, eosinophilia and vague abdominal pains can be observed in infected patients (Alhassan et al. 2016).

Host immunity against Filariasis

Helminth infections usually occur due to weaker immune system of the host or immune-compromised individuals. The condition becomes severe when parasite evade the immune system, residing in the body for a longer period and causes ineradicable infection. Lymphatic filariasis is usually characterized by elephantiasis, lymphedema and hydrocele. These conditions usually develop by Th-1 immune response which is characterized by elevated IFN-gamma, TNF-alpha and Th-17 cells. These cells induce vascular endothelial growth factor which leads to dysfunction of lymphatic system and cause lymphedema (Spencer et al. 2001; Grencis 2015).

Helminths may differ from each other due to different types of glycoconjugates, but these molecules play a significant role in the development of Th-2 immune response. Likewise, T-cells are the main immune cells in filarial infections. Babu and Nutman (2014) observed that nude mice without T and B-cells were more susceptible to Brugian infection and concluded that T-cells are essential for complete eradication of lymphatic filariasis. Furthermore, macrophages attach to the microfilariae and secrete myeloperoxidase and nitric oxide, which ultimately kill the microfilariae (Kalyanasundaram et al. 2020).

During filarial infection, the parasites damage the host tissue against which cytokines (IL-25, IL-33 and thymic stromal lymphopoietin (TSLP)) are released. These cytokines activate innate immune cells, particularly, lymphoid cells to secrete IL-4, 5, 9, 10 and IL-13, which induce Th-2 immune response. Meanwhile, these innate immune cells also activate humoral immunity, which ultimately produce IgE, IgG4 in humans. However, IgG1 immunoglobulin has been observed in case of mice. Furthermore, localized eosinophilia and hyperplasia of goblet and mucosal mast-cells has been reported in filarial infections (Gurish et al. 2004; Bennuru et al. 2018).

As far the cell-mediated response is concerned, Th-1, Th-2 and Treg cells also produce IL-10 (immune-regulatory cytokines) in human filariasis. Interestingly, the production of IFN-gamma and NO has also been reported to play role in protection against filariasis in mice which indicated that both the Th-1 and Th-2 immune responses are responsible for protection of filarial infection. Moreover, the production of immunoglobulins such as IgG, IgE and IgM is involved in anti-filarial antibody response. IgG helps in clearance of filarial nematodes from infected patients by inducing antibody dependent cell mediated cytotoxicity (Chauhan et al. 2018).

In short, the Th-2 immune response is required against filariasis with minor contribution of Th-1 response and innate immune cells.

Prevention and Control

Almost 859 million people in 50 countries are at-risk of filariasis and need preventive chemotherapy. In response of filariasis, WHO has launched Global program to eliminate lymphatic filariasis (GPELF) in 2000 with two main objectives i.e., to stop the spread of disease with annual treatment of humans living in the endemic areas and to alleviate the suffering of filariasis. WHO started mass drug administration program (MDA) in endemic areas for population at risk. According to MDA regimens, albendazole (400mg) should be used twice a year in areas co-endemic with loiasis. However, in onchocerciasis endemic countries, ivermectin (200µg/Kg) must be added in combination of albendazole (400mg). Similarly, diethycarbamazine citrate (DEC: 6mg/Kg) with albendazole (400mg) are recommended for the areas free from onchocerciasis. Studies have shown that these combinations of drugs are very effective for almost all microfilariae of different filarial worms (Radwan et al. 2016).

In case of *Parafilaria* infection in animals, it is very difficult to treat and control the infection due to the long period before the manifestation of infection. The drugs are less effective during this period. However, treatment with macrolytic lactones (ivermectin @ 200mcg/Kg) or nitrooxynil (20mg/Kg) at three days of interval can control Parafilariasis. Preferably, the infected animals should be treated 70 days prior to slaughtering to reduce the skin lesions. Similarly, virbac that contains 1% ivermectin and nitroxinil, can also be used to treat *Parafilaria* infection. Secondly, vector population i.e., *Musca domestica* can also be controlled on the farm to prevent *Parafilaria* infection (Diakou and Prichard 2021).

Other preventive measures must include the cleaning of wounds and washing of infected limbs with lukewarm water to minimize the oedema and swelling. Good hygienic conditions, routine exercise and amputation of affected limbs should be implemented to minimize the suffering. Furthermore, another contributing measure in controlling the spread of infection is the control of vector mosquitoes and black flies. It provides the cheapest source of getting rid of filarial transmission and to stop the emergence and re-emergence of filarial infection all over the world. Insecticide-treated nets, indoor residual sprays can help humans in avoiding the mosquito bites (Ekong et al. 2012).

Nature Against Filarial Worms

Nature plays a significant role in the transmission of filarial worms throughout their transmission dynamics. It is already known that a considerable temperature and high humidity are essential for the growth and survival of insect vectors. Despite, such conditions are also contributing factors for the development of filarial worms in the vector. Bancroft (1899) reported that 16-17 days are required for the development of filarial worms in their vector. Furthermore, it has been reported experimentally that the development of filarial worms in mosquitoes require two weeks under 27°C and 90% humidity. Furthermore, the complete cycle takes 10-14 days under high temperature and moisture, but it takes 6 weeks if the temperature is very low (Chandra 2008).

Another natural factor of limiting the filarial transmission is the lack of synchronization between the period of vector population density and transmission season. It has been reported that the vector density e.g., mosquitoes is very high in rainy seasons as compared to dry season i.e., winter and summer due to flooded breeding sites (De and Chandra 1994; Chandra 2008). On the other hand, the best time for the transmission of filarial worms in vector population in endemic areas is summer season and hot months of rainy season. Experiments have revealed that a sharp reduction happens naturally between the number of microfilariae ingested and the number of microfilariae developed in the mosquito. It means that not all the ingested microfilariae develop in L3 stage in the mosquitoes, rather some microfilariae may be damaged or deformed by the buccopharyngeal armature of the mosquitoes. Additionally, the microfilariae can be deformed in the body of vector due to change in temperature and humidity (Hati et al. 1989; Chandra and Rudra 2006).

Vector mortality is another hindrance factor of nature in the filarial transmission. It has been observed that all the microfilariae- infected mosquitoes cannot survive the period which is required for the development of L3 larvae (infective microfilariae) (Yamamoto et al. 1985). Different scientists have recorded varying degree of daily mortality rate in mosquitoes i.e., 14-47% in different areas of the world (Dryer et al. 2005; Chandra et al. 2006). Moreover, many scientists have recorded significantly higher prevalence of infection rate as compared to infectivity rate in an endemic area. It also indicates the substantive vector mortality in a given period (Holmes 1986; Chandra et al. 1996).

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CHAPTER 11

COCCIDIOSIS IN RUMINANTS

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INTRODUCTION

The genus *Eimeria* (known as coccidia) contains a large number of species infecting vertebrate animals. To date is accepted that there is only a single host species for each species of *Eimeria* (Kreier and Baker 1987). *Eimeria* contains species that are important pathogens for domesticated animals (Kreier and Baker 1987). *Eimeria* species are usually parasites of the gastrointestinal tract, and most species of this parasitic genus are exclusively located in the intestine (Figure 1). They are not transmissible between different domestic ruminant species. *Eimeria* species conform a group of economically important parasites and a common cause of diarrhea in young ruminants. In this context, coccidia-related disease, also known as eimeriosis or coccidiosis (Keeton and Navarre 2017).
On the other hand, *Eimeria ovina, E. ahasta, E. ovinoidalis, E. faurei, E. crandalis,* and *E. intricati* are the most common species which infect sheep (Mohamaden et al. 2018). *Eimeria ninakohlyakimovae, E. hirci, E. aspheronica,* and *E. arloingi* are the most prevalent species in goats (Mohamaden et al. 2018); while *E. bovis*, *E. zuernii*, *E. canadensis*, *E. ellipsodalis*, *E. alabamensis*, *E. pellita*, *E. auburnensis* and *E. cylindrica* are the most common species in cattle (Heidari et al. 2014).

Etiological Agent

Eimeria spp. are protozoa that are part of the order Coccidia, which infect different vertebrate's species (Duszynski 2001; Trefancová et al. 2021). Currently, more than 1800 species of *Eimeria* are recognized; infection by these parasites is associated with the presence of enteritis, diarrhea, dehydration and weight loss in animals (Burrell et al. 2020).
Eimeria species belong to the phylum Apicomplexa, as do other protozoa of the order Coccidia (*Toxoplasma* spp.) that are also part of this phylum, possess a complex endomembrane system with specialized secretory organelles for invasion, residence and replication (Tenter et al. 2002; Tomavo et al. 2013; Marugan-Hernandez et al. 2021).
Eimeria spp. have an affinity for a specific host, even affecting the epithelial cells of a specific portion of the intestinal tract (Cowper et al. 2012). Unlike other species, humans are not affected by *Eimeria* spp. but by *Cyclospora* spp. a parasite similar in terms of pathogenicity and genetics (Liu et al. 2016). The production species in which *Eimeria* spp. infections have a significant impact are domestic fowl, swine and ruminants, although these parasites can infect other species such as fish and reptiles (Walker et al. 2013; Lucas et al. 2014). A wide variety of disease-causing *Eimeria* are recognized in ruminants.

Generalities

Eimeriosis or coccidiosis in small ruminants is considered one of the infectious-contagious diseases of worldwide distribution with great productive impact.
The presence of this disease is in a humid environment and another characteristic is overcrowding, although it is also observed in arid climates, under breeding systems where the population density is considered a predisposing factor. This parasitosis particularly affects young animals at the weaning stage, causing diarrhea, growth retardation and even death (Taubert et al. 2008). Currently, 12 different species of *Eimeria* with infective capacity are recognized in cattle, 11 in sheep and 9 in goats.
The disease is acquired when ruminants ingest the oocysts of the parasite through feed. The repeated exposure of the animal to *Eimeria* spp., and the constant replication of the parasite in the schizogony and gametogony stages in the intestine, cause damage and consequent clinical signs; the sporogony stage gives rise to the infective phase, the oocysts that are disseminated through the feces into the environment, contaminate the soil, pasture and feed, contributing to the transmission of the parasitosis in other animals (Burrell et al. 2020; Felici et al. 2021).

Biology

According to Tenter et al. (2002), coccidia have been found in almost all animals, including humans. During the 19th and early 20th centuries, classification of protozoa was based primarily on locomotion organelles, but with increasing knowledge about their morphology, biology, life cycle, and host specificity, a wide range of phenotypic characters were used to classify them into different taxonomic groups. In the 21st century, classification takes also into account phylogenetic studies and ultrastructural, biochemical, and molecular-biological data that have been generated from diverse studies of a wide range of protozoa.
Coccidia of the family Eimeriidae, such as *Eimeria* spp. and *Cystoisospora* spp. are parasites that fulfill their biological cycle in a single host; these parasites are known as monoxenes. Those belonging to the family Sarcocystidae, such as

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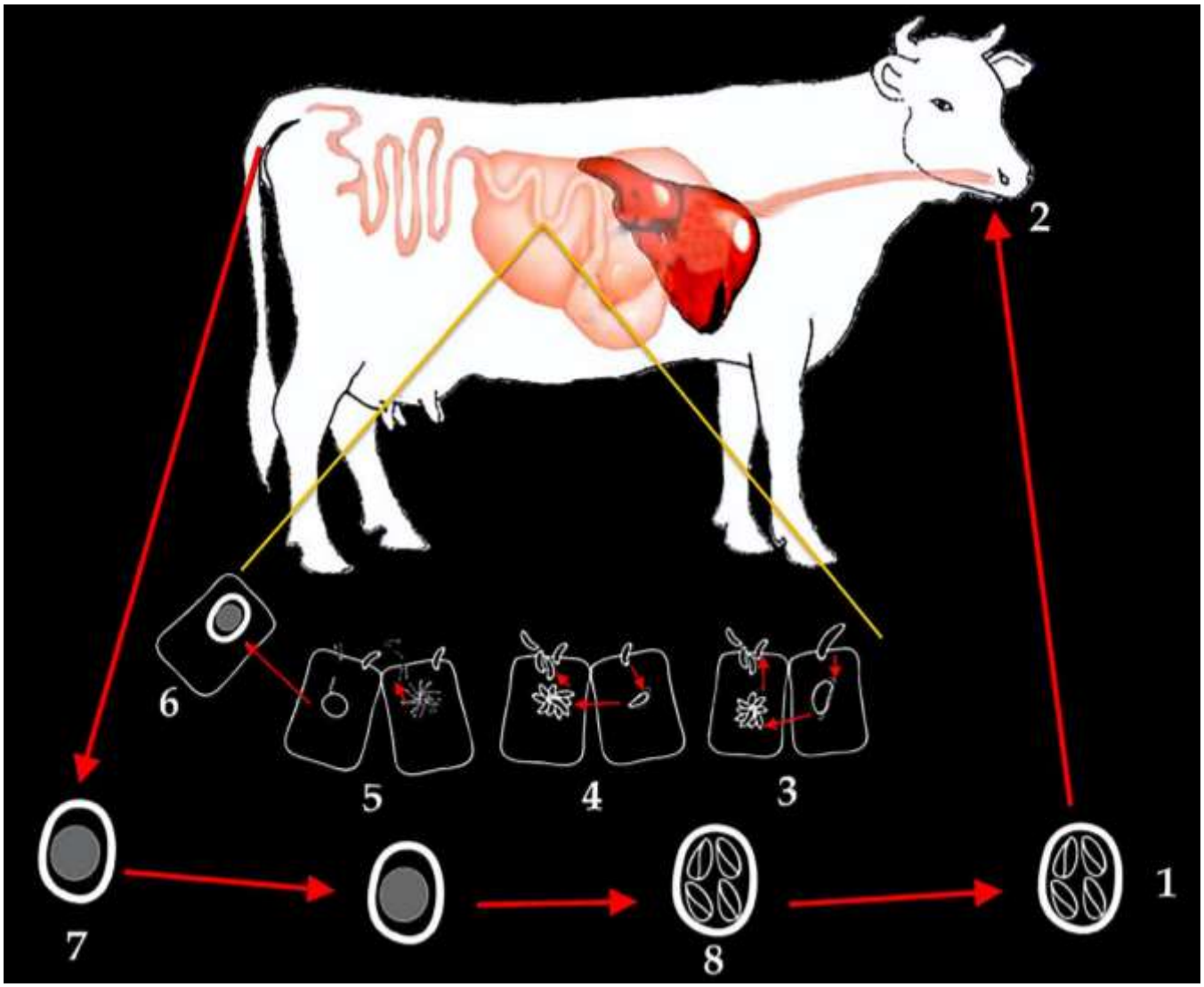

Fig. 1: General *Eimeria* life cycle. 1. Sporulated oocyst. 2. Host ingests sporulated oocyst. 3. Sporocyst containing sporozoites are released in the small intestine and penetrate intestinal cells and transform into merozoites. 4. Merozoites multiply asexually within cells causing it to burst. These merozoites will then infect new cells and can repeat this cycle several times. 5. Some merozoites will infect new cells and transform into either macrogametocytes or microgametocytes. Microgametocytes will emerge from the host cell and fertilize a macrogametocyte, which will develop into an oocyst. 6. Unsporulated oocyst. 7. Unsporulated oocyst is passed through feces into the environment. 8. Oocyst become sporulated in the environment (Figure made by Carlos Ramón Bautista-Garfias).

Toxoplasma spp. and *Neospora* spp. are heteroxen, that is, they initiate their life cycle in a variety of intermediate hosts and conclude it in the intestine of a definitive host (Wohlfert et al. 2017). Infections by *Eimeria* spp., in ruminants and the severity of its clinical signs depend on different factors such as the ingested parasite load, the *Eimeria* species involved, the concomitance with other infections, the age and the state of immunocompetence of the host (Bangoura et al. 2012; Das et al. 2015; Carrau et al. 2018), as well as other factors like herd husbandry conditions (intensive, semi-intensive or extensive system), zootechnical purpose (milk or meat production), conditions that may generate animal stress and other environmental particularities such as soil type and pH, vegetation present in the region, solar radiation, temperature, humidity, rainfall and altitude (Sun et al. 2018; Chaiyos et al. 2018).

The range of survival of *Eimeria* spp. in climatic conditions is very wide, although the disease is more frequent in ruminants raised on moderate humidity and heat (Keeton and Navarre 2018), with a higher incidence in intensive production systems. In a study conducted in Mexico, Rodríguez-Vivas et al. (2017) reported the economic losses due to coccidiosis in small ruminants for US $23.7 million only for Mexico. In a retrospective study by Alcalá et al. (2020), it was determined that this disease is widely distributed in Mexico, regardless of the geographical area and climatic conditions in which ruminants develop.

The most frequently reported species in cattle were *E. bovis*, *E. zuernii* and *E. alabamensis*; in sheep is *E. crandallis*; and in goats, *E. ninakohlyakimovae* and *E. christenseni*, all of these are pathogenic. This study also describes that the presence of these and other *Eimeria* species in cattle and sheep is influenced by the time of year, the size of the herd or flock, the production system and the age of the animals. The months with higher humidity, farms with higher numbers of animals and under semi-intensive and intensive production systems, as well as calves under one-year-old and lambs up to six months old, were frequently reported with infection by *Eimeria* spp.; it is worth mentioning that for the goat species, the most relevant factor for the presence of coccidiosis was the size of the herd.

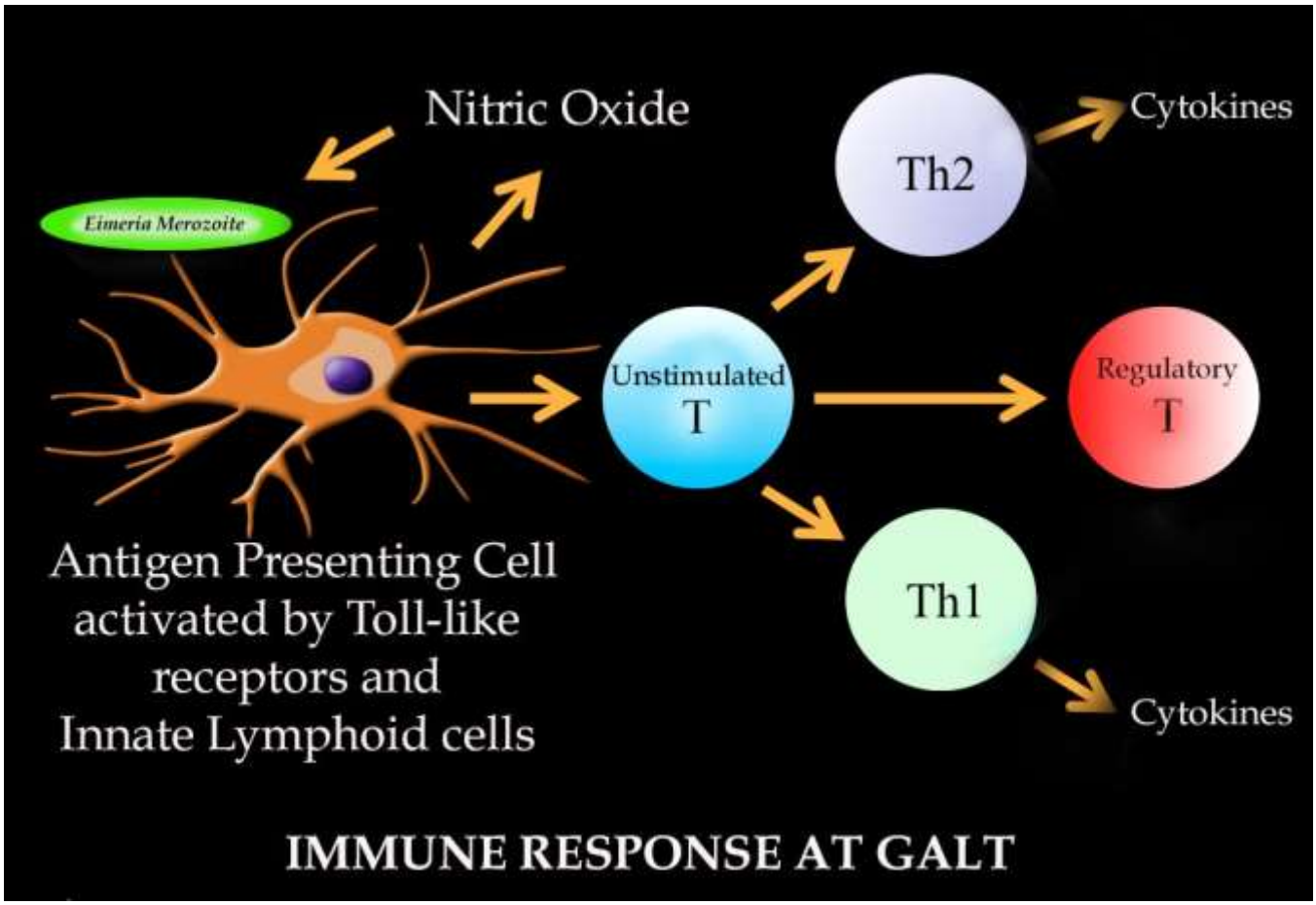

Fig. 2: The immune response at GALT (Gut Associated Lymphoid Tissue) against *Eimeria* spp. Th1: T helper 1; Th2: T helper 2. (Figure made by Carlos Ramón Bautista-Garfias based on Mirchandani et al. 2012; Min et al. 2013; Shivaramaiah et al. 2014; Iwasaki and Medzhitov 2015).

Life Cycle

The general life cycle of *Eimeria* has basically two stages: the exogenous phase (sporogony) and the endogenous phase (schizogony and gametogony) (Allen and Fetterer 2002). The initial infective unit of all *Eimeria* spp. is the sporozoite. The sporozoite of every apicomplexan parasite is characterized by a unique complex of structures specialized in the invasion of the host cells (Augustine 2001). The sporozoite is the beginning and the end of the life cycle of any coccidian (Bowman 2014). Sporozoites are the infective forms found in sporulated oocysts and are the result of protoplasm segmentation (Bowman 2014). Oocysts are ovoid and vary in size and shape according to the species.

The life cycle involves the following steps: The host ingests a sporulated oocyst (Figure 1: 1, 2). The life cycle process is described below: 1. Sporulated oocyst. 2. Host ingests sporulated oocyst. 3. Sporocyst containing sporozoites are released in the small intestine and penetrate intestinal cells and transform into merozoites. 4. Merozoites multiply asexually within cells causing it to burst. These merozoites will then infect new cells and can repeat this cycle several times. 5. Some merozoites will infect new cells and transform into either macrogametocytes or microgametocytes. Microgametocytes will emerge from the host cell and fertilize a macrogametocyte, which will develop into an oocyst. 6. Unsporulated oocyst. 7. Unsporulated oocyst is passed through feces into the environment. 8. Oocyst become sporulated in the environment.

Immunological Aspects

Both innate and adaptive immunity are part of the whole immune responses against pathogens (Takeda K and Akira S, 2005; Bautista 2016; Mirchandani et al. 2012). In the case of *Eimeria* infections, the Gut Associated Lymphoid Tissue (GALT) has an important role in the first line defense against this parasite genera (Shivaramaiah et al. 2014). A larger group of tissues called mucosal-associated lymphoid tissue (MALT) is responsible for conferring immunity across mucosal surfaces in different areas of the body; GALT is a component of MALT (Lillehoj and Trout 1996).

Early investigations in both mammalian and avian species have revealed that the cellular immune responses through T cells and their associated cytokines play an important role in anticoccidial immunity (Rose and Hesketh 1979; Rose at al. 1979; Wakelin et al. 1993). Acquired immunity to murine coccidiosis is attributed more to T cells than B cells (Rose et al. 1979). Several immune cell types including NK cells, dendritic cells, and macrophages are involved in innate immune responses to avian coccidiosis (Min et al. 2013). It is important to mention that a subset of T cells (innate lymphoid cells - ILCs) participates in the overall immune response against parasites (Koyasu and Moro 2012). The information about ILCs indicates that they are vital regulators for gastrointestinal mucosal homeostasis through interactions with other structural and stromal cells in gut epithelial barriers (Fan et al. 2019). It has been indicated the innate immune response controls adaptive immune response against different pathogens (Iwasaki and

Medzhitov 2015). The general immune response against *Eimeria* spp. is shown in Figure 2. It is important to point out that the stimulation of the innate immune system by *Lactobacillus casei* in broiler chickens confers a protective immune response against a challenge with pathogenic *Eimeria* species (Bautista-Garfias et al. 2003), this finding represents a sustainable control measure of coccidiosis in ruminants that must be evaluated in the future.

Epidemiology

Coccidiosis is a globally distributed disease, but it is a very important animal health problem in developing countries (Squire et al. 2019), reported to be distributed in several countries in all continents (Macrelli et al. 2019; Alcala-Canto et al. 2020; Jansen et al. 2020; Olmos et al. 2020; Silva et al. 2020; Trejo-Huitrón et al. 2020; Gondipon and Malaka 2021; Thanasuwan et al. 2021; Mohammed et al. 2021; Yan et al. 2021). Pathogens that cause coccidiosis in ruminants are found in temperate, subtropical and tropical climates. (Alcala-Canto et al. 2020).

Although prevalence is usually 60% or higher among herds and flocks (Macrelli et al. 2019; Squire et al. 2019), coccidiosis is a disease that affects mainly young animals: calves between six and 1 year of age are most susceptible, as well as lambs from one to six months of age (Sudhakara-Reddy et al. 2015; Keeton and Navarre 2018; Silva et al. 2020). The chronic or subclinical presentation of the disease is most often seen in growing animals and occurs mainly in the wet seasons (Constable et al. 2016).

This disease is relevant to ruminant production; the economic impact was valued in 2012 as a 6-9% reduction in gross margin (Lassen and Østergaard 2012). The economic losses are a combination of costs, including prevention and treatment, and morbidity and mortality (Silva et al. 2017; Squire et al. 2019), resulting in adverse effects on feed intake, growth rate, fertility, wool growth and milk yield (Squire et al. 2019; Silva et al. 2020).

Transmission

Ruminants become infested with the pathogens when they ingest the oocysts with contaminated feed or water (Constable et al. 2016; Alcala-Canto et al. 2020). The main source of sporulated oocysts is contamination of the environment in which ruminants are found, since oocysts can survive for weeks or months in favorable conditions of heat and humidity (Silva et al. 2017; Keeton and Navarre 2018; Macrelli et al. 2019; Trejo-Huitrón et al. 2020; Gondipon and Malaka 2021). A large number of animals in a confined environment causes the environment to have a higher concentration of feces, which increases environmental contamination, although pathogens are also prolific in paddocks and feedlots, especially if water accumulates in nearby areas (Keeton and Navarre 2018; Macrelli et al. 2019). Healthy animals one-year-old or older also act as a reservoir for lambs or calves (Keeton and Navarre 2018; Bangoura and Bardsley 2020; Silva et al. 2020; Mohammed et al. 2021).

Factors related to the pathogen species involved, the level of contamination or exposure and the immune status of the animals, determine the severity of the infection (Keeton and Navarre 2018; Macrelli et al. 2019; Alcala-Canto et al. 2020; Silva et al. 2020). An optimal immune response of affected animals is directly influenced by the general health status of the animals, which is related to age, physiological status and nutrition. External factors can be triggers because they can be a stressor, such as sudden changes in weather, the maintenance of hot and humid climates or the movement of livestock. The amount of vaccination and deworming status, as well as the presence of other concomitant diseases in the herd are also related to the occurrence of coccidiosis (Keeton and Navarre 2018; Alcala-Canto et al. 2020; Silva et al. 2020; Gondipon and Malaka 2021).

Diagnosis

To establish a diagnosis of coccidiosis, attention should be paid to the clinical signs of the affected animals and the herd/flock history (Keeton and Navarre 2018; Bangoura and Bardsley 2020). The most representative and relevant sign is diarrhea (which may be mucoid or bloody and some of the calves/lambs may have perineal areas stained with feces), followed by abdominal pain, straining to defecate and subsequent rectal prolapse, dehydration, anorexia, weakness, depression, pale mucous membranes and acute weight loss (Sudhakara-Reddy et al. 2015; Constable et al. 2016; Keeton and Navarre 2018; Gondipon and Malaka 2021). Nervous coccidiosis may occur as result of severe infection, producing among other nervous signs, muscle tremors, convulsions and nystagmus. Nervous coccidiosis is associated with a mortality rate of more than 80% (Sudhakara-Reddy et al. 2015; Keeton and Navarre 2018; Bangoura and Bardsley 2020).

Subclinical conditions often cause decreased appetite and reduced weight gains, due to intestinal damage, leading to poor growth rates and decreased feed efficiency, which in turn results in lost productivity and poor economic performance (Keeton and Navarre 2018; Squire et al. 2019).

One method of identification of the causal agent is the observation of oocysts by examination of fecal samples, however, the mere observation of oocysts does not ensure a definitive diagnosis, although it is ideal for identifying the species and obtaining the oocyst count (Constable et al. 2016; Keeton and Navarre 2018; Bangoura and Bardsley 2020). Some animals infected with other pathogens may present a high number of oocysts of non-pathogenic species (Keeton and Navarre 2018). Therefore, only the combination of history and clinical signs, as well as oocyst counts higher than 500 oocysts per gram will be suggestive of coccidiosis (Keeton and Navarre 2018; Bangoura and Bardsley 2020).

Common laboratory diagnosis is performed by direct smear, fecal flotation or McMaster's technique; quantitative fecal analysis is preferable to non-quantitative tests (Constable et al. 2016; Keeton and Navarre 2018; Alcala-Canto et al. 2020). Given that samples collected at the first days or days after the clinical phase of the disease may contain minimal numbers of oocysts (Constable et al. 2016), at least five individual rectal samples from a flock or herd must have significant oocyst counts to confirm coccidiosis (Constable et al. 2016).

When morphologic differentiation is difficult, is necessary to use molecular characterization to clarify species classification, although is not used routinely since it is much more expensive than fecal flotation (Silva et al. 2017; Bangoura and Bardsley 2020). Serologic diagnostic test is not available for routine use, although its potential to allow monitoring of herds or flocks; however, antibodies may persist after a self-limiting infection (Bangoura and Bardsley 2020).

Differential diagnoses include other intestinal parasites,

malnutrition, salmonellosis, toxins, or viral diseases (Constable et al. 2016). Because coccidiosis can occur as a co-infection, it is necessary to take a proper clinical history and identify clinical signs to eliminate other pathogens as additional causes of diarrhea (Constable et al. 2016; Macrelli et al. 2019).

When animals do not survive, necropsy may help confirm coccidiosis in the other ruminants affected (Wäsle et al. 2017; Bangoura and Bardsley 2020). Some of the changes observed during necropsy are intestinal hemorrhage as well as white/gray spots or lines in the mucosa; histopathological examination is necessary to confirm this (Wäsle et al. 2017; Keeton and Navarre 2018; Bangoura and Bardsley 2020).

Conventional Control

Coccidiosis is a self-limiting disease (Bangoura and Bardsley 2020). A controlled, limited or gradual exposure is necessary to develop a protective immune response (Keeton and Navarre 2018; Macrelli et al. 2019; Bangoura and Bardsley 2020; Silva et al. 2020).

Reduction of environmental contamination is important and reachable by limiting overcrowding; adequate sanitation of all areas and equipment, exposure to sunlight for desiccation are very efficient ways to reduce the count of oocysts in the environment (Constable et al. 2016; Keeton and Navarre 2018; Silva et al. 2020; Gondipon and Malaka 2021).

Control should include correction of management factors that contribute to development of clinical disease such as poor housing conditions and low or minimal ventilation (Gondipon and Malaka 2021). Other forms of control include the adoption of feeding practices that avoid fecal contamination of feed and water, grouping animals by size, and moving young animals from pen to pen (Constable et al. 2016; Silva et al. 2020).

Strengthening of the immune system is necessary to maintain a protective immune response (Bangoura and Bardsley 2020). To this end, it is highly recommended to reduce overcrowding, optimize feeding and minimize stress factors and other diseases through an adequate and effective health program (Bangoura and Bardsley 2020). Fecal examinations over time in the flock/herd may be helpful to maintain animal health and to control outbreaks (Keeton and Navarre 2018; Macrelli et al. 2019; Silva et al. 2020).

Preventive is preferable to corrective treatment due to the risk of subclinical infections that would lead to chronic disease and wide distribution within the herd or flock (Keeton and Navarre 2018; Silva et al. 2020).

There are several anticoccidial drugs available for treatment and prevention of coccidiosis in ruminants; these drugs work by impeding the growth and reproduction of coccidian parasites (Keeton and Navarre 2018). During an outbreak, affected animals should be isolated and given oral and parental fluid therapy; mass medication of water and feed may help to minimize the effects in the animals without clinical signs (Constable et al. 2016).

Sulfaquinoxaline (10-20 mg/kg/day for 3-5 days) and amprolium (10 mg/kg/day for 5 days) are drugs that can be used to treat clinically affected animals (Keeton and Navarre 2018). The first is particularly useful for weaned animals that develop bloody diarrhea (Constable et al. 2016).

Sulfonamides in the feed at 25–35 mg/kg for ≥15 days are effective to control coccidiosis in calves (Bangoura and Bardsley 2020), corticosteroids are contraindicated (Constable et al. 2016).

Amprolium (5 mg/kg/day for 21 days), decoquinate (0.5 mg/kg/day for 28 days) and lasalocid (1 mg/kg/day to a maximum of 360 mg/head/day), or monensin (100–360 mg/head/day) can be used for prevention (Constable et al. 2016; Keeton and Navarre 2018; Bangoura and Bardsley 2020).

Toltrazuril administered at 15 mg/kg as a single oral dose, 14 days after animals are moved into group housing, effectively prevents diarrhea due to coccidiosis (Constable et al. 2016).

The major benefits of coccidiostats are through improved feed efficiency and rate of gain, because allows immunity to develop (Constable et al. 2016).

To minimize risk of resistance to coccidiostats development its long-term preventive uses most be limited; once clinical signs appear, the apparent ineffectiveness of a treatment is due to intestinal damage that has already occurred (Keeton and Navarre 2018; Bangoura and Bardsley 2020).

Advantages and Disadvantages of Conventional Control

Conventional or chemical control for coccidiosis disease in bovines uses medications such as coccidiostats and coccidioicides; which reduce the parasitic loads of treated animals. It also reinforces natural defenses indirectly; however, they do not allow to eliminate definitively coccidia from a herd in the long term, since the coccidiosis disease persists due to the continuous reinfections of the treated animals and the infection of the healthy ones (Keeton and Navarre 2018).

Currently, the most commonly used sulfas for such effects are five and they are described below: 1) sulfaguanidine, 2) sulfaquinoxaline, 3) sulfamerazine, 4) sulfabromomethazine and 5) sulfamethazine. It is worth mentioning that in several studies it has been observed that if treatment with sulfa drugs is started 13 days after the start of the infection, the results are satisfactory; this is due to the fact that sulfas act on the merozoites, preventing the development of gametes that are the most harmful for cattle (Rodríguez-Vivas et al. 1996).

The advantages of using this type of chemical control is swiftness and dosage, as shown in the studies described below. In a study, by Mundt et al. (2005), carried out in Germany with cattle naturally infected with *E. bovis* and *E. zuernii*, it was found that treatment with toltrazuril (15 mg/kg b.w.) orally, applied in a single dose, controlled coccidiosis in calves housed in different conditions.

In another study, Mundt et al. (2007) evaluated the efficacy of toltrazuril (15 mg/kg b.w.) and diclazuril (1 mg/kg b.w.), in a single oral dose, observing that there was a greater efficacy of toltrazuril compared to diclazuril, since the animals treated with toltrazuril had a lower number of excreted oocysts and the duration of the excretion period was also significantly reduced compared to animals treated with diclazuril and the control group.

The disadvantages of the use of chemical products previously described for the control of coccidiosis in ruminants is the resistance to these products due to the use and abuse of these molecules (Stephan et al. 1997); Another very important factor is the residuality of these chemical products in the soil (Cruz et al. 2004) and damage to beneficial organisms (beetles and mites), which has been described in the use of antiparasitic agents, mainly anthelmintics (Quintero-elena et al., 2022).

In general, other recommendations to control coccidiosis infections are to carry out proper management, avoid mixing animals of different ages and thus control the infection in calves;

on the other hand, in the farms the feeders and drinkers must be placed on a base to avoid fecal contamination; During grazing, calves should be prevented from consuming dirty and stagnant water and, finally, overcrowding of animals in pens should be avoided.

Sustainable Alternatives to Control the Coccidiosis in Ruminants

Livestock provides the main source of protein for the human population throughout the world and is the most widespread form of land use worldwide and particularly in Mexico, for this reason this activity has great importance in several aspects: 1) economic, 2) social and 3) environmental (SIAP 2022). It should be noted that, in particular, Veracruz is one of the states in Mexico where livestock is one of the main economic activities, ranking first nationally in meat production and sixth in milk production. However, this apparent success of livestock activity throughout the State has had a great environmental impact and its expansion represents a complex ecological problem mainly due to the use of chemical products for different pests (Barrera-Bassols et al. 1993).

Derived from the frequent and indiscriminate use of chemical products for the control of coccidiosis in ruminants, sustainable alternatives with the environment have been sought. Among these alternatives are the use of ethno-veterinary medicine, plants with medicinal properties such as *Artemisia annua* (secondary metabolites), and the tannins obtained from "Quebracho" against *Eimeria* spp. and gastrointestinal nematodes in small ruminants. The clinical signs caused by coccidiosis decreased in animals that consumed the former plant and improved body condition and weight compared to animals that were not fed this plant (Acharya et al. 2018).

Another type of sustainable alternative is the use of edible mushrooms (*Lentinula edodes* and *Ganoderma lucidum*), secondary metabolites such as fatty acids (linoleic acid), antioxidants, an example is the secondary metabolite "Curcumin", present in the medicinal plant *Curcuma longa*, could reduce the severity of an infection of the upper and middle part of the small intestine caused by *E. acervulina* and *E. maxima* pathogens) (Quiroz-Castañeda and Dantán-González 2015).

Another type of control is essential oils, particularly used for formulations or diets to control coccidiosis (Quiroz-Castañeda and Dantán-González 2015).

Conclusions and Perspectives

The objective of this chapter was to present updated information on the epidemiology, transmission mechanisms, immunological aspects, diagnosis and conventional control of coccidiosis in ruminants. As a perspective, the use of sustainable coccidiosis control measures that do not damage the environment must be considered.

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CHAPTER 12

THE USE OF ESSENTIAL OILS AGAINST SHEEP GASTROINTESTINAL NEMATODES

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INTRODUCTION

Gastrointestinal nematodes (GINs) nowadays present a serious threat to sustainable sheep breeding. Various classes of commercial anthelmintics were used to control these parasites. However, due to the development of anthelmintic resistance (AR), the efficacy of these drugs has decreased, which in turn resulted in high economic losses. For these reasons, researchers are focused now on designing sustainable strategies for GIN control, based on the use of a combination of a bunch of options including the wise application of anthelmintic drugs, as well as applying alternative strategies. Within this context, phytotherapy (the use of plants or their products) presents one of the most promising alternatives. Essential oils (EOs) are natural, volatile and complex compounds characterized by a strong odor and extracted from aromatic plants. In various studies so far, these plant products showed high in vitro and, in some cases, in vivo efficacy against sheep GINs. The aim of this chapter is to review so far conducted studies based on the use of EOs against these parasites and to discuss results, as well as advantages of their use compared to commercial anthelmintics. On the other hand, current obstacles in the use of EOs and possible solutions on how to overcome them will be also discussed in this chapter. In this way, current and future perspectives of the use of EOs against sheep GINs are discussed here.

Sheep Gastrointestinal Nematodes and the Problem of Anthelmintic Resistance

Infections caused by gastrointestinal nematodes (GINs) are currently considered as one of the main obstacles for breeders of grazing sheep worldwide (Hammer et al. 2019). Although these infections are most commonly subclinical, manifested as impaired weight gain and lowered milk yields but in some cases they can lead to serious conditions such as anaemia, diarrhea, digestive problems, protein loss, lowered immunity and fertility and even death (Giovanelli et al. 2018; Bosco et al. 2020; Beleckė et al. 2021). Therefore, the negative effect of these parasites is reflected in various ways, from impaired animal health and welfare and reduced growth to a decrease in animal productivity and farm profitability (Velde et al. 2018). The economic losses caused by gastrointestinal parasitism are huge and difficult to estimate, although some reports indicate that these are estimated to be 17.94% of the total economic cost in animals (Abbas et al. 2020).

Nowadays, these parasites are widely distributed in many parts of the world. Generally, Haemonchus spp. and Cooperia spp. are more prevalent in sub-tropical/tropical environments, Ostertagia and Nematodirus spp. in the temperate regions, while Trichostrongylus spp. are prevalent throughout the world (Waller 2006). The prevalence of sheep GINs in Serbia is also high, with the following genera identified: Nematodirus spp. 71.22%, Ostertagia spp. 69.22%, Trichostrongylus spp. 66.55%, Haemonchus spp. 64.44% and Chabertia spp. 60.11% in Vojvodina, lowland landscape (Pavlović et al. 2017) as well as Haemonchus spp. (46.91%), Oesophagostomum spp. (40.73%), Trichostrongylus spp. (39.85%), Nematodirus spp. (35.88%) and Chabertia spp. (32.79%) in Eastern Serbia, predominantly mountainous (Kulišić et al. 2013). In southern Italy, the prevalence of sheep GIN genera varies but includes Haemonchus spp. (21-83%), Trichostrongylus spp. (2-59%), Chabertia spp. (0-48%), Teladorsagia spp. (0-25%) and Cooperia spp. (0-5%) (Bosco et al. 2020).

The control of sheep GINs is currently nearly exclusively reliant on commercial anthelmintic drugs (Bosco et al. 2020, Castagna et al. 2021). These include benzimidazoles (eg. albendazole, fenbendazole, mebendazole), macrocyclic lactones (eg. ivermectin, moxidectin, eprinomectin) and imidazothiazoles (eg. levamisole) (Dyary 2018; Velde et al. 2018). However, their improper use that refers to overfrequent treatments, miss-use or dose as well as continued use of one drug, has led to the development of anthelmintic resistance (AR) in different nematodes species and strains (Dyary 2018; Pinto et al. 2019; Bosco et al. 2020; Beleckė et al. 2021), which is now reported worldwide. This has also been reported against even newly developed drugs such as monepantel (Mederos et al. 2014), whereby AR to a new drug has been reported in less than 10 years after introduction to the market. Furthermore, widespread incidence of multidrug-

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resistant populations of Haemonchus contortus, Teladorsagia and Trichostrongylus to benzimidazoles, imidazothiazoles and macrocyclic lactones in sheep throughout Europe has also been reported (Fissiha and Kinde 2021). Therefore, the reduced susceptibility of drugs in nematodes has resulted in even higher economic losses, which in turn endangers the sustainability of livestock (Vineer et al. 2020).

Sustainable Strategies for the Control of Sheep Gastrointestinal Nematodes

Modern sustainable strategies in sheep GINs management are based on rational use of anthelmintics along with the use of alternative strategies. Within the use of commercial anthelmintics, incorporation of refugia is suggested which is based on the treatment of only a proportion of animals instead of the whole group. In such conditions, one part of the parasite population remains untreated, which allows the maintenance of drug-sensitive parasites (Hodgkinson et al. 2019). The best-recommended practices for preserving refugia are targeted treatments (TT), that are related to the treatment of the whole flock based on knowledge of the risk or parameters that quantify the severity of infection as well as target selective treatments (TST), which are based on the treatment of only individual animals within the group to provide epidemiological and/or production benefits (Calvete et al. 2020). In essence, both TT and TST signify the rational use of drugs, i.e. on animals that require treatment due to health, productivity and welfare reasons, whereby single or combined pathophysiological and/or production-based treatment indicators are used for the selection of animals. These include clinical signs, faecal egg count (FEC), FAMACHA© scores, weight gain, milk production, body condition score (BCS), breech soiling and the diarrhoea score (DAG) (Soto-Barrientos et al. 2018; Calvete et al. 2020). Combining anthelmintics which have a related spectrum of activity but different modes of action, as well as the rotation of used anthelmintic classes, are also recommended as a way of slowing down the development of AR (Fissiha and Kinde 2021). On the other hand, these strategies could be complemented or even replaced with alternative solutions for controlling sheep GINs. Genetic control methods involve the selection of animals resistant to GIN, which as a process relies on the existence of genetic variation in the host and the predominant environmental conditions (Zvinorova et al. 2016). The objective of pasture management is to minimize the use of anthelmintics by avoiding exposure to parasite burden that would lead to clinical disease and loss of production, while simultaneously aiming to allow sheep to build up immunity (Abbott et al. 2012). Nutritional manipulation relies on a balanced grazing system that provides an adequate source of nutrients and an acceptable GIN burden, which in turn should allow an optimum level of productivity (Torres-Acosta et al. 2012). Biological control measures include the use of natural enemies against GINs, which mostly refers to different nematophagous fungal species such as Duddingtonia spp. (Szewc et al. 2021). In the end, the development of efficient vaccines against intestinal parasites will allow antiparasitic medications to be used less frequently (Fissiha and Kinde 2021).

Among the proposed alternative strategies for the control of sheep GINs, phytotherapy is considered particularly promising. Phytotherapy is defined as the use of plants to treat ailments, which as a healthcare practice is recorded since ancient times and transferred from generation to generation (Borges and Borges 2016; Castagna et al. 2021). In veterinary medicine, there is an increasing interest in validating ethnoveterinary practices, judging by the high number of studies and articles on the topic (Eshetu et al. 2015). Antiparasitic properties are a common point of focus, whereby a wide range of plants or their products are considered suitable for the treatment of almost every parasitic disease in livestock (Athanasiadou et al. 2007). For this purpose, whole plants (e.g. rich in tannins), their parts or products such as extracts (e.g. aqueous and ethanol) and essential oils may be used. Within this context, plant based antiparasitic preparations may provide successful alternative remedies to synthetic drugs and be used in ethnoveterinary practices against sheep GINs (Castagna et al. 2021).

Properties and Chemical Composition of Essential Oils

Essential oils (EOs) are natural, volatile and complex mixtures of compounds characterized by a strong odor and extracted from aromatic plants (Bakkali 2008). As secondary metabolites, they are present in the specialized cells or glands and serve to protect plants from predators and pests, while also attract pollinators. These cells are present in all sections of these plants including flowers, leaves, buds, stems, twigs, seeds, fruits, roots, wood or bark (Bakkali 2008; Butnariu and Sarac 2018; Fokou et al. 2020). Therefore, EOs are considered as part of the plant immune system (Butnariu and Sarac 2018). As far as physical and chemical properties are concerned, EOs are liquid, volatile, and rarely colored, lipid-soluble and soluble in organic solvents with a generally lower density than that of water. These plant products are mostly extracted from flowers and leaves of various aromatic plants growing in temperate and warm regions such as the Mediterranean, the Amazon or tropical countries, where they represent an important part of traditional pharmacopoeia (Bakkali 2008).

The medicinal properties of EOs are recognized since ancient times which include antiviral, antibacterial, antifungal, antiparasitic, anti-inflammatory, antiseptic, anticancer and antispasmodic properties among others (Bakkali 2008; Mancianti and Ebani 2020; Zaman et al. 2020). In veterinary medicine, EOs are increasingly used for the prevention and treatment of various animal diseases. Although this still mostly refers to monogastric animals such as pigs and poultry (Mucha and Witkowska 2021), some implications and reports suggest their possible use as anthelmintic agents in ruminants as well (André et al. 2018). EOs may be obtained from plants by different methods of extraction, but the most common industrial methods are steam distillation and extraction with different solvents (Butnariu and Sarac 2018).

The active ingredients of EOs are responsible for their pharmaceutical effects. To date, approximately 5000-7000 different constituents of EOs are identified and described in which mono- and sesquiterpenes predominated, along with aromatic compounds such as phenylpropane derivates (Morsy 2017; Butnariu and Sarac 2018) as mentioned in Table 1. Terpenes present polymers of isoprene (C5H8) and may be divided into hydrocarbons or oxygenated derivatives (oxides, alcohols, aldehydes, ketones, acids) or reaction products thereof (esters, ethers) (Butnariu and Sarac 2018). On the other hand, the group of phenylpropenes comprises constituents derived from n-propyl benzene (Morsy 2017). EOs have a very high variability of their composition, both in qualitative and quantitative terms (Dhifi et al. 2016).

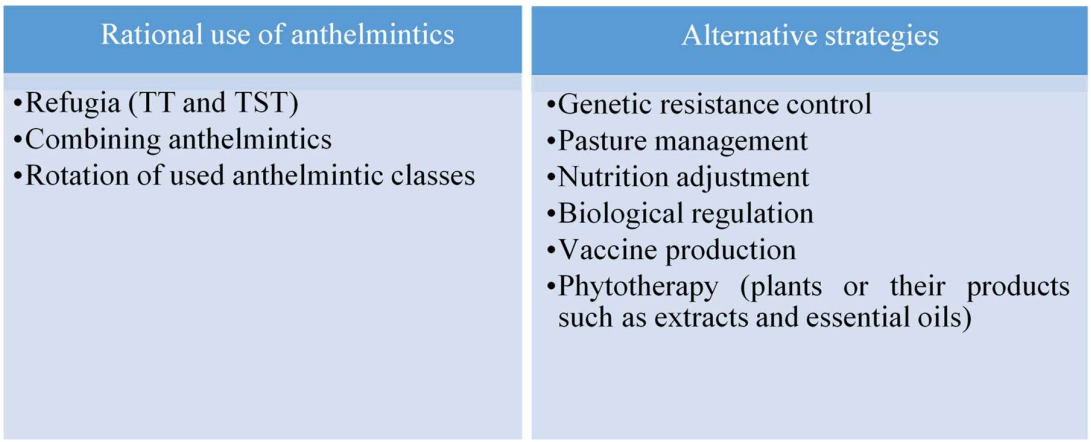

Fig. 1: Strategies for sustainable control of sheep GINs.

Table 1: Constituents of essential oils and their division by chemical groups and examples (Dhifi et al. 2016; Morsy 2017; Butnariu and Sarac 2018)

Chemical group	Examples
Hydrocarbon terpenes	Limonene, α- and β-pinene, camphene, α- and γ-terpinene, sabinene, myrcene, β-caryophyllene, germacrene B and D, o and p-cymene etc.
Oxygenated derivates (terpenoids)	a) Phenols - thymol, carvacrol b) Alcohols - linalool, citronellol, geraniol, menthol, α-terpineol, terpinen-4-ol, borneol c) Aldehydes - citral, citronellal, sinensal d) Ketones - α i β-tujon, camphor (2-bornenon), menton, carvone e) Oxydes - eucalyptol (1,8-cineole) f) Esters - linalyl-acetate, geraniol-acetate,
Phenylpropenes	Anethole, methyl chavicol (estragole), eugenol, vanillin, safrole, myristicin, cinnamaldehyde
Miscellaneous (sulfur- and nitrogen-containing compounds)	allyl sulfide, allicin, methyl anthranilate, indole, pyridine, pyrazine

Many factors such as light, precipitation, growing site (altitude, latitude), nature of the soil (pH, constituents), site of production and accumulation of the EOs in the plant, the age of the plant, the presence of soil organisms and microorganisms, predators and pollinators as well as postharvest treatment of EOs (Barra 2009; Fokou et al. 2020) may affect their chemical composition, which ultimately lead to variation in their pharmacological properties.

The Potential use of Essential Oils against Sheep Gastrointestinal Nematodes

In Vitro Tests

The interest of the use of EOs against sheep gastrointestinal nematodes, as well the number of studies upon that are in increasing trend over years. Within that context, different EOs showed anthelmintic potential so far. In vitro tests present the first step in the process of validating phytotherapy substances and are used for the initial evaluation and selection of plant species and their secondary metabolites that exhibit anthelmintic activity (Borges and Borges 2016; André et al. 2017; Štrbac et al. 2021a). Among in vitro tests, the most reliable and most common used tests are egg hatch assay (EHA) and larval development assay (LDA) that reflect ovicidal and larvicidal potential of EOs, as well as different larval and adult motility assays that suggest the effect of EOs on the motility of larva's or adults (Table 2). The advantages of the use of in vitro tests are ease of application, low cost, speedy, high reproducibility and no need for experimental animals (protection of animal welfare), and thus have been widely used in the screening of medicinal plants, often rather than in vivo tests (Ferreira et al. 2016).

EOs listed in Table 2. showed anthelmintic potential against sheep GINs (mostly against H. contortus), but it differed depending on the oil used. The highest ovicidal activity, expressed as IC50 values, was recorded for Cymbopogon schoenanthus and Cymbopogon martinii (0.04 and 0.1 mg/mL, respectively, Katiki et al. 2011), Thymus vulgaris (0.098 mg/mL, Štrbac et al. 2021a), Ruta chalepensis (0.1 mg/mL, Akkari et al. 2015), and Mentha arvensis (0.1 mg/mL, Chagas et al. 2018). Different EOs of Lippia spp. showed great larvicidal activity with IC50 less than 0.01 mg/mL (Chagas et al. 2018), followed by Thymus vulgaris with IC50 as 0.062 mg/mL (Ferreira et al. 2016), Hesperozygis myrtoides with IC50 as 0.07 mg/mL (Castilho et al. 2017), Piper aduncum with IC50 as 0.1 mg/mL (Gaínza et al. 2016) and Mentha piperita with IC50 as 0.2 mg/mL (Katiki et al. 2011). Cymbopogon schoenanthus exhibited a very high activity on the larval motility with IC50 as 0.009 mg/mL (Katiki et al. 2012), whereby Ruta chalepensis induced 87.5% inhibition of motility of adults 8 h after exposure at the dose of 1 mg/mL (Akkari et al. 2015). Along with Thymus vulgaris, Origanum vulgare, Foeniculum vulgare and Satureja montana showed a high ovicidal effect in our studies with inhibition of egg hatchability up to 100% for each concentration tested, 0.049-50 mg/mL (Štrbac et al. 2021a; Štrbac et al. 2022). In certain experiments, bioactive compounds of EOs are also evaluated for anthelmintic activity, mostly against H. contortus and with the same tests. The list is also wide and includes anethole, B-elemene, borneol, camphor, carvacrol, carvone,

Table 2: Essential oils that have shown *in vitro* activity against sheep gastrointestinal nematodes, assays and references

Essential oil(s)	Assays	GIN species used	Reference
Croton Zehtneri (two samples), *Lippia sidoides*	EHA, LDA	*Haemonchus contortus*	Camurça-Vasconcelos et al. 2007
Eucalyptus globulus	EHA, LDA	*H. contortus*	Macedo et al. 2009
Cymbopogon schoenanthus, Cymbopogon martinii, Mentha piperita	EHA, LDA, LFIA, LEA	*H. contortus and Trichostrongylus spp*	Katiki et al. 2011
Arisaema lobatum, Arisaema franchetianum	EHA, LDA, LMIA	*H. contortus*	Zhu et al. 2013a
Artemisia lancea	EHA, LDA, LMIA	*H. contortus*	Zhu et al. 2013b
Tagetes minuta, Coriandrum sativum Alpinia zerumbet, Lantana camara	EHA, LDA	*H. contortus*	Macedo et al. 2013
Eucalyptus citriodra	EHA, LDA	*H. contortus*	Ribeiro et al. 2014
Melaleuca alternifolia	EHA, LMIA	*H. contortus*	Grando et al. 2015
Zanthoxylum simulans	EHA, LDA, LMIA	*H. contortus*	Qi et al. 2015
Cymbopogon citratus	EHA, LDA	*H. contortus*	Macedo et al. 2015
Ruta chalepensis	EHA, AWMA	*H. contortus*	Akkari et al. 2015
Citrus sinensis, Melaleuca quinquenervia	EHA, LDA	*H. contortus*	Gaínza et al. 2015
Thymus vulgaris	EHA, LDA, LMIA, AWMA	*H. contortus*	Ferreira et al. 2016
Piper aduncum	EHA, LDA	*H. contortus*	Gaínza et al. 2016
Hesperozygis myrtoides	EHA, LDA	*H. contortus*	Castilho et al. 2017
Lavandula officinalis, Citrus aurantifolia, Anthemis nobile	EHA, LDA, AWMA	*H. contortus*	Ferreira et al. 2018
Mentha arvensis, Zingiber officinale, Lippia sidodes, Lippia alba, Lippia origanoides, Lippia gracilis, Curcuma longa, Mentha piperita	EHA, LDA	*H. contortus*	Chagas et al. 2018
Rosmarinus officinalis	EHA, LMIA	natural-mixed infection	Pinto et al. 2019
Eucalyptus citriodra	AWMA	*H. contortus*	de Araújo-Filho et al. 2019
Origanum majorana	EHA, AWMA	*H. contortus*	Abidi et al. 2020
Juniperus communis	EHA	natural-mixed infection: *Haemonchus* spp, *Trichostrongylus* spp, *Teladorsagia* spp and *Chabertia* spp	Štrbac et al. 2020a
Coriandrum sativum	LMIA	H. contortus, *Trichostrongylus axei*, *T. colubriformis*, *T. vitrinus Teladorsagia circumcincta*, and *Cooperia oncophora*	Helal et al. 2020
Achillea millefolium, two chemotypes	EHA	natural-mixed infection: *Haemonchus* spp, *Trichostrongylus* spp, *Teladorsagia* spp and *Chabertia* spp	Štrbac et al. 2020b
Origanum vulgare, Satureja hortensis, Thymus vulgaris, Mentha piperita, Helichrysum arenarium	EHA	natural-mixed infection: *Haemonchus* spp, *Trichostrongylus* spp, *Teladorsagia* spp and *Chabertia* spp	Štrbac et al. 2021a
Cinnamomum verum, Syzygium aromaticum	Mortality of nematode larvae in plant oil solution	*H. contortus*	Boyko and Brygadyrenko 2021
Cinnamomum verum, Syzygium aromaticum, Melaleuca alternifolia, Piper cubeba, Citrus aurantiifolia, Lavandula angustifolia	Mortality of nematode larvae in plant oil solution	*S. papillosus*	Boyko and Brygadyrenko 2021
Ocimum basilicum, 16 cultivares	EHA	*H. contortus*	Sousa et al. 2021
Origanum vulgare, Pimienta dioica	EHA, larval mobility	*H. contortus* and *Cooperia* spp.	Jiménez-Penago et al. 2021

**EHA - egg hatch assay; LDA - larval development assay; LMIA - larval motility inhibition assay; AWMA - adult worm motility assay, LFIA - larval feeding inhibition assay, LEA - larval exsheathment assay*

citral, cinnamaldehyde, eucalyptol, eugenol, linalool, thymol, terpinen-4-ol and vanillin among the others (Katiki et al. 2017; André et al. 2018). In a study of Katiki et al. (2017), the highest ovicidal activity was shown by cinnamaldehyde, anethole, carvone, carvacrol and thymol with IC50 of 0.018, 0.07, 0.085, 0.11 and 0.13 mg/mL, respectively. The high larvicidal effect of carvacrol and thymol was demonstrated in studies of André et al. (2016) and Ferreira et al. (2016) with IC50 values of 0.2 and 0.06 mg/mL, respectively. The activity of these phenolic compounds may be associated with damage caused to the cuticle and digestive apparatus on nematode larva's and neurotoxic effects on the free-living nematodes (interaction with SER-2 tyramine receptor) (André et al. 2016; 2017). Citral was also one of the most potent EO ingredients with an IC50 value of 0,13 mg/mL in EHA (Macedo et al. 2015).

In some cases, the efficacy of binary, ternary and quaternary combination of EO isolated compounds was evaluated as well, whereby the highest ovicidal activities were shown by cinnamaldehyde:carvacrol (1:1), anethole:carvone (1:1) and anethole + carvone + cinnamaldehyde + carvone (1:1:1:1) with

Table 3: *In vivo* efficacy of essential oils against sheep gastrointestinal nematodes

Essential oil	Test, the time of evaluation and GIN species	Dose, routes of administration and duration of use	Efficacy	Reference
Lippia sidoides	FECRT; Days 0, 7, 14 and 21 a.t.	230 mg/Kg, oral, during 5 days 283 mg/Kg oral, during 5 days	38.0% at D7; 30.0% at D14; 29.8% at D21 45.9 at D7;-54.0% at D14; 22.9% at D21	Camurça-Vasconcelos et al. 2008
Lippia sidoides	Controlled test; Day 7 a.t.; *Haemonchus* spp. and *Trichostrongylus* spp.	283 mg/Kg, oral, during 5 days	*Haemonchus spp.* 56.9% *Trichostrongylus* spp. 39.3%	Camurça-Vasconcelos et al. 2008
Orange oil emulsion	FECRT; Days 0 and 14 a.t.; *H. contortus*	600 mg/Kg, single 600 mg/Kg during 3 days	97.4% 94.9%	Squires et al. 2010
Cymbopogon schoenanthus	FECRT; Days 0, 1, 5, 10, 15 and 20 a.t.; *H. contortus*	180 and 360 mg/Kg during 3 days, oral	n.e.	Katiki et al. 2012
Cymbopogon schoenanthus	Controlled test; Day 20 a.t.; *H. contortus*	180 and 360 mg/Kg during 3 days, oral	n.e.	Katiki et al. 2012
Eucalyptus citriodora	FECRT; Days 0,10 and 17 a.t.; *Haemonchus* spp., *Trichostrongylus* spp., *Oesophagostomum* spp.	250 mg/Kg	55.9% at day 10; 34.5% at day 17	Ribeiro et al. 2014
Thymus vulgaris	FECRT; Days 0, 2, 4, 6, 8, 10, 12 a.t.; *H. contortus*	75, 150 and 300 mg/Kg oral on Days 0, 6 and 12 a.t.	n.e.	Ferreira et al. 2016
Mentha arvensis	FECRT; Days 0, 1, 3, 7, 14 and 21 a.t.; *H. contortus* and *Trichostrongylus* spp.	200 mg/Kg, single dose	61.6% at D1; 48.1% at D14; 44.9% at D21	Chagas et al. 2018
Cymbopogon citratus	FECRT; Days 0, 8 and 15 a.t.; *Haemonchus spp, Trichostrongylus spp and Oesophagostomum spp.*	500 mg/Kg, oral, during 3 days	19.6% at D8; 23.9% at D15	Macedo et al. 2019
Cymbopogon citratus	Controlled test; Day 15 a.t. *H. contortus, T. colubriformis, O. columbianum, T. ovis*	500 mg/Kg, oral, during 3 days	*H. contortus* 66.4% *T. colubriformis* 38.4%	Macedo et al. 2019
Eucalyptus citriodora	FECRT, Days 0, 7 and 14 a.t. *Haemonchus* spp. *Trichostrongylus* spp., *Oesophagostomum* spp. and *Strongyloides* spp.	500 mg/Kg, oral, single dose	41.8% at D7; 69.5% at D14	de Araújo-Filho et al. 2019
Thymus vulgaris	FECRT; Days 0, 7 and 14 a.t.; *Haemonchus* spp.; *Trichostrongylus* spp.; *Teladorsagia* spp.; *Chabertia* spp.	100 mg/Kg, oral, single dose	25.23% at D7; 24.42% at D14	Štrbac et al. 2021b

* *FECRT - faecal egg count reduction test; a.t. - after treatment; D - certain day after treatment; n.e. - not effective.*

IC50 values of 0.012, 0.013 and 0.02 mg/mL, respectively (Katiki et al. 2017). In our study, the activity of linalool:estragole binary combination at a ratio 19%:81% exhibited ovicidal activity with IC50 of 0.98 mg/mL (Štrbac et al. 2021c). However, as many studies have demonstrated so far, an EO often shows higher anthelmintic activity in comparison with the single isolated compound, due to the synergistic effect among many different constituents of the whole EO, although it should be stressed that a wide number of compounds is not crucial for high efficacy (Štrbac et al. 2022).

In Vivo Tests

The results obtained through in vitro tests must be confirmed in field condition trials. For this purpose, various in vivo studies are used to obtain the most authentic results of the efficacy of plant-based formulations (Table 3). Although these studies can be intensive, expensive and require time and animals for testing, these are essential as a further step in developing anthelmintic agents as they offer a clear picture of the possibility of using EOs and their ingredients against sheep GINs in everyday clinical practice. The most commonly used in vivo test is the faecal egg count reduction test (FECRT) which measures the percentage reduction in the number of nematode eggs excreted through faeces after administration of an active substance, and is confirmed by the controlled test that is based on the quantification of the worm burden after sacrificing animals which have previously been artificially inoculated with nematodes and treated (Kebede 2019).

As shown in Table 3, various EOs were found effective in different in vivo studies, whereby some were highly effective and some did not show any effect. Sometimes differences were found in efficacy of EOs of the same plant in different studies. This may be attributed to differences in chemical composition owing to variation in climate parameters, harvesting time, plant

parts used, solvents used for extraction etc. Thus, EO of Thymus vulgaris showed some anthelmintic effects in our study (Štrbac et al. 2021b), although it failed to reduce FEC of GINs in a study of Ferreira et al. (2016) at even greater doses. Those differences may be related to the different compositions and the isolate of EO used, which was confirmed in our study with in vitro tested Achillea millefolium EO (Štrbac et al. 2020b) or to the even other factors. The dependence of EO efficacy on the method of application (single or multiple uses) was also contradictory.

The in vivo efficacy of the isolated EO compounds or their combination was also evaluated in some cases. Some of them showed a high effect on the reduction of FEC, such as carvacrol-acetate and thymol-acetate with the efficacy of 65.9 % and 76.2 % on Day 14 a.t., respectively (doses of 250 mg/kG) (André et al. 2016; 2017). In a study of Chagas et al. (2018), pure menthol, at the dose of 160 mg/Kg, did not express in vivo efficacy unlike the whole oil whose main ingredient is, i.e. Mentha arvensis that reduced FEC by approximately 50% on Days 1, 7 and 14 at a similar dose tested, 200 mg/Kg. In our study, the efficacy of the binary combination of linalool: estragole (19:81%) in the FECRT at the single dose of 100 mg/kG was evaluated, whereby efficacy was found to be 24.21% and 25.90% on Days 7 and 14, respectively (Štrbac et al. 2021b).

Toxicity Studies

Rarely, toxicity studies of the use of EOs or their ingredients against sheep gastrointestinal nematodes have been conducted. In two studies on mice, LD50 values determined for carvacrol and thymol were 919 mg/Kg and 1350.9 mg/Kg, whereby for their acetylated derivates carvacrol acetate (CA) and thymol acetate (TA), these values were 1544.4 mg/Kg and 4144.4 mg/Kg with no changes observed in the mice behavior (André et al. 2016; 2017). According to the guidelines proposed by Clark and Clarke (1977), orally administered substances with an LD50 value above 1000 mg/Kg are safe or considered as low-level toxic substances. So, CA, thymol and TA can be considered as non-toxic, while further studies should be performed for carvacrol. In a study of Ribeiro et al. (2014), the EO of Eucalyptus citriodora was classified as safe with an LD50 value of 2653.0 mg/Kg for mice. Some EO compounds such as menthol are of very low acute oral toxicity (LD50 > 2000 mg/Kg) (Chagas et al. 2018). Oral administration of the EO of Origanum majorana at different doses of 1000-5000 mg/Kg displayed no signs of toxicity, nor caused fatal effects in any of the treated mice during an observation period of 24 hours (Abidi et al. 2020). Katiki et al. (2012) concluded that Cymbopogon schoenanthus is safe for sheep at the doses of 180 mg/Kg and 360 mg/Kg, since no significant differences among group means for the hepatic (enzymes) or kidney (urea and creatinine) parameters were recorded after treatment with EO. In our in vivo studies, no toxic effects were observed on sheep, neither after oral administration of Thymus vulgaris (100 mg/mL) nor linalol:estragole (100 mg/mL) (Štrbac et al. 2021b).

Advantages and the Barriers of the use of Essential Oils to Control of Gastrointestinal Nematodes in Sheep

To date, EOs from various plants have shown efficacy against sheep GINs. As discussed above, their high anthelmintic potential is owed to various compounds that make up their composition, of which the primary component is most important (Dhifi et al. 2016). These compounds belong to different chemical groups, which impart antiparasitic activity through different mechanisms of action and synergism. These involve interruption of the nematode nervous system, interference with the neuromodulator octopamine or GABA-gated chloride channels, the inhibition of AChE activity, disruption of the cell membrane of the nematode thereby changing its permeability, membrane and ion channel perturbations modifying membrane-bound protein activity and the intracellular signaling pathways inducing different neurological and structural changes leading to nematode paralysis and death (Andrés et al. 2012). Apart from the high efficacy, different chemical origins of their ingredients may contribute to less susceptibility of EOs to anthelmintic resistance (Macedo et al. 2010; Borges and Borges 2016).

Moreover, the natural origin of plant-based formulations may contribute to less toxicity to hosts, as well as to fewer residues in meat and milk compared to synthetic drugs (Ferreira et al. 2018). Although this still needs to be confirmed, natural-based drugs are certainly much more environmentally acceptable. Finally, the use of chemical drugs is less and less sustainable not only due to AR, yet from the financial aspects, as drug prices continue to rise (Prakash et al. 2021). Also from that point of view, the use of different plant formulations could be a more sustainable and acceptable option given their low prices and ease of acquisition, especially in countries with developed biodiversity (Ferreira et al. 2018).

The main barriers in the use of EOs against sheep GINs as the widespread practice may be the lack of scientific data and trials aimed at verifying their efficacy against these parasites, which especially refers to in vivo trials. As discussed earlier, efficacy in field conditions must be proven before the use of some active substances in practice. Furthermore, toxicity studies should be conducted on the host animals. However, this field is relatively new and there is an increasing number of various studies aimed to confirm the efficacy and sustainable use of EOs against sheep GINs (Muthee 2018). Worsening of the situation due to AR forced many researchers worldwide to search for effective antiparasitic herbal formulations as a promising alternative to synthetic drugs. Our research group is actively engaged in the evaluation of new EOs for any anthelmintic efficacy through in vitro, in vivo and toxicity studies (data not shown).

The second problem about the current potential use of EOs in the practice is related to the low efficacy shown in field condition trials, which is still not comparable to commercially available anthelmintics (Macedo et al. 2010). Low in vivo efficacy is attributed to less bioavailability of active ingredients of EOs. This fact may be explained on the one hand by the anatomical and physiological specificity of the ruminant gastrointestinal tract (Hoste et al. 2008), and on the other hand by the unstable nature of EOs (Maes et al. 2019). Active ingredients of EOs are prone to evaporation and reaction with various factors inside the gastrointestinal tract. That leads to their partial or total inactivation before reaching the target place in abomasum or intestine. Thus, they usually show lower anthelmintic activity compared to that showed in different in vitro studies. Keeping in mind these factors, finding of plant species, the dose and route of administration effective in vivo is a challenge for ethnobotanists to be addressed. However, it also seems that increasing interest and the number of studies within this topic can contribute to overcoming this problem. Nevertheless, so far showed efficacy in different in vivo studies suggest that EOs and their active ingredients may be used as a valuable additional source in a nematode control along with

other measures, if not capable to be used independently (Macedo et al. 2010).

Encapsulation as a Novel Approach

Encapsulation is the method of protecting active components via physical or chemical processes. In this way, the active substance is physically separated from the environment by the creation of a protective shell, often referred to as the active carrier component or matrix (Lević et al. 2014). Nowadays different encapsulation techniques are used such as emulsification, nanoprecipitation and coacervation with chitosan, alginate or cyclodextrin as matrices (Maes et al. 2019). Given the instability and volatility of EOs, encapsulation could be of great importance when it comes to their application against GINs in sheep. Encapsulation reduces the interaction of the active substances with various factors in the environment (Radunz et al. 2018) and leads to reduced inactivation of active ingredients of EOs in the animal which ultimately results in increased bioavailability. Also, encapsulation represents a sustainable and efficient approach to increasing physical stability and protection against evaporation, enabling longer retaining properties and shelf life of EOs (Majeed et al. 2015). The other advantages of encapsulation include the increase the ease of handling active substances, reduction of odor and unpleasant taste (may be important for oral administration), as well as controlled release of the active substance (Radunz et al. 2018).

In a study of Mesquita et al. (2013), emulsified EO of (italic) given orally to the sheep at the dose of 365 mg/Kg once, reduced the total number of nematodes in sheep gastrointestinal tract by 60.79%, which was better than ivermectin that reduced the number up to 48.70%. The reduction of abomasal nematodes was significantly higher in the group treated with EO (83.75% and 35.00%, respectively). Similarly, the nanoemulsion of the same oil at the dose of 250 mg/Kg once, reduced the FEC of GINs similarly to levamisole ($p>0.05$) in 8 of 10 days observed (Ribeiro et al. 2017). The dose of 250 mg/Kg of the encapsulated formulation of anethole:carvone (10% each and 80% of lipid matrix) given in food to lambs for 45 days significantly reduced FEC at the days 43 and 45, whereby the effect was attributed to a decrease in the size of males and a decrease in the fecundity of female nematodes (Katiki et al. 2019). At the same time, the formulation did not affect liver or kidney function of the lambs. When compared to free EO, nanoencapsulated oil of E. citriodora showed higher ovicidal (0.5 compared to 1.3 mg/mL) and similar larvicidal (both 1.7 mg/mL) in vitro, but slightly lower in vivo effect measured through faecal egg count reduction test at the same dose of 250 mg/Kg (40.5% compared to 55.9% at Day 10 a.t.) (Ribeiro et al. 2014). However, nanoemulsion of C. citratus showed clearly higher in vivo effect in the reduction of FEC compared to free EO, given orally for three days at the doses 450 and 500 mg/Kg, respectively (51.7% to 19.6% at Day 8 a.t.), whereby at the same time exhibited lower toxicity (Macedo et al. 2019). In most of these studies, chitosan was used as a carrier. However, studies aimed to confirm the positive impact of encapsulation techniques for the use of EOs against sheep GINs are needed.

Conclusion

In the era of AR, novel strategies for sustainable control of GINs in sheep farms should be designed. The use of EOs as an alternative method show great potential due to their high efficacy originating from rich chemical composition, their affordable price and easy acquisition, especially in countries with developed biodiversity. Along with this, EOs possess significantly less susceptibility to resistance and better host and environmental acceptability from the toxicity aspect in comparison with synthetic drugs. The major obstacles are reflected in the lack of trials conducted in field conditions, as well as still usually lower in vivo effects than commercial drugs. However, these obstacles may be overcome with an increasing number of field studies in different conditions, especially with EOs and their ingredients that showed great in vitro potential. Moreover, applying novel methods such as encapsulation offers an opportunity to protect active EO ingredients from degradation and inactivation and thus allow a higher in vivo efficacy. From all the above, the use of these plant products may significantly contribute to the sustainable control of sheep GINs in the near future.

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CHAPTER 13

MOLECULAR CHARACTERIZATION OF CYSTIC HYDATIDOSIS IN BASRAH GOVERNORATE- SOUTHERN IRAQ

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INTRODUCTION

Hydatid disease is considered as one of the most dangerous diseases that spread in all parts of the world, and Iraq is one of the countries where this disease is present in a large and widespread among ruminants, human and stray dogs (Lazim, 2019). Typically, hydatidosis can be classified according to the larval stage and divided into two types: Cystic Echinococcosis (CE) and Alveolar Echinococcosis (AE), Cystic Echinococcosis is a zoonotic disease and occurs by the larval stage of *Echinococcus granulosus* that has a cosmopolitan allocation and it is one of the most significant zoonotic diseases around the world (Thompson 2008). CE cause huge economic losses and has great public health significance worldwide (Romig et al. 2011). While, alveolar Echinococcosis is caused by *E. multilocularis* (Wen and New 1993), and this disease has a serious impact on human and animal health (Snabel et al. 2009). It also results in a significant economic and public health problem in many parts of the world especially in rural areas where dogs and livestock are raised together (Groeneveld et al. 2010; Sikó et al. 2011).

Types of Echinococcus

The genus *Echinococcus* is known to include nine recognized species according to (Thompson and McManus 2002):
1- *Echinococcus granulosus* has two types of hosts: definitive hosts (dogs and other canids) and intermediate hosts (sheep, goats, cattle, pigs and human).
2- *Echinococcus equines*: definitive hosts (dogs and other canids), intermediate hosts (horses and donkeys).
3-*Echinococcus canadensis*: definitive hosts (dogs and other canids),intermediate hosts (human as well as domestic and wild animal).
4-*Echinococcus felidis*: definitive hosts (dogs and other members of family canidae), intermediate hosts (lions).
5-*Echinococcus ortleppi*: definitive hosts (dogs and other canids),intermediate hosts (cattle and human) which cause cystic Echinococcosis (CE).
6-*Echinococcus multiocularis*: definitive hosts (dogs and other canids)intermediate hosts(human) which causes alveolar Echinococcosis.
7-*Echinococcus oligarthrus* and *Echinococcus vogeli*: definitive hosts (dogs and other canids), intermediate hosts are rodents and ungulates and accidentally, human, causes Polycystic Echinococcosis (PE).
8-*Echinococcus shiquicus*: definitive hosts (dogs and other canids), intermediate hosts (small mammals) with cysts similar to CE or PE but of unknown zoonotic status (Nakao et al. 2007; Badaraco et. al. 2008).

Clinical Diagnosis

The clinical diagnosis of (CE) in human and animals were difficult because the disease continues without symptoms and the morbid identification of the causative species was difficult in the cases of irregular forms (Eckert and Depalazes 2004). Also, Nakao et al. (2010) noticed that *Echinococcus* spp. Must be subjected to molecular diagnosis for species identification. Now a day, clinical samples taken at biopsy are subjected to PCR, and the amplified the fragments of mitochondrial and nuclear DNA are subsequently sequenced and strains are determined.

Clinical Sings of Hydatidosis in Animals

Clinical sings depend on location and size of hydatid cyst in their intermediate hosts (David and Petri 2006). Infection remains asymptomatic for many years before increasing the number and size of cysts, which are able to cause symptoms in the infected organs, However, this disease may be in progress and result in obstructive symptoms (Paniker 2013). Sometimes, hosts show clinical symptoms, such as slow growth, weakness and lameness (OIE 2008). The degree of symptoms varies depending on the severity of the disease and the location of the hydatid cyst. Clinical indicators in the affected animal include decreased milk production, poor wool, and organ damage in the affected area (Eckert and Deplazes 2004; Eddi et al. 2006).

Clinical Sings of Hydatidosis in Human

The symptoms in humans stay reliant on the involvement of precious organ and the liver is the most vulnerable organ, with a rate of infectivity roughly around 60-70 percent, followed by the lungs (20-22%), spleen, heart, muscles, eye, and thyroid gland (6%), kidneys, brain and bones (1%), Also,

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there is no organ in the body that is free of hydatid cysts to preserve teeth, nails and hair (6%) (Marquardt et al. 2000). Even if the cyst is tiny, symptoms appear, and the majority of instances of brain cyst illness are discovered in youngsters. This infection is dangerous enough to cause death in certain circumstances (Moro and Schantz 2009). Also, there is inflammation and sensitivity on palpation of liver abscesses, as well as discomfort in the stomach, vomiting, nausea, as well as increased hepatic blood pressure and in the lumen of the inferior vena cava, are indicators of the liver. The bile ducts have secondary fibrosis, and the hydatid cyst puts a lot of strain on the diaphragm (Brunetti 2015).

Diagnosis of Hydatid Cysts

Hydatidosis can go unnoticed for years until the quantity and size of cysts grow large enough to produce symptoms in the afflicted organs. The diagnosis of *Echinococcus granulosus* in the intermediate host is based on the detection of a hydatid cyst, which can occur in almost any organ but is most common in the liver and lungs. Ultrasound and computed tomography examinations for abdominal Echinococcosis, X-ray for lung Echinococcosis, and immunodiagnostic tests are used (Pawlowski et al. 2001). While the discovery of adult Echinococcus spp. in feces or the small intestine, or the detection of particular coproantigens or coproDNA, is required for the diagnosis of Echinococcosis in dogs or other carnivores (OIE2008).

Distribution of Echinococcosis

Echinococcosis is one of the serious public health problems in all the parts of Iraq in both human and animals. This is because of distribution of stray dogs in all parts of Iraq. Also, hydatid cysts have been frequently reported in livestock slaughtered at slaughterhouses in most of the Iraqi provinces and human. For example, a case of AE has been reported from the north of Iraq, in Zakho (Al-Attar et al. 1983) in human. Amr et al. (1994) recorded that CE was prevalent 60% female and 40% male. With major affect in liver 60% and lung 26.4% respectively. Another investigation found a high prevalence of hydatid cysts in sheep (14.75) prevalence of 1.5%, 5.9%, and 13.7% in North, Middle and South of Iraq Mohamad et al. (2008). In the north of Iraq, the prevalence of hydatid cysts was reported at 12.7% in sheep, 4.8% in goats and 4.3% in cattle (Mero et al. 2013), while, in Al-Najaf the prevalence of hydatidosis was 0.62% in sheep (Al-Shabbani 2014). In Arbil province, the rate of infection in sheep was 15.0% (Saeed et al. 2000), and in Mosul 3.16 % (Jarjees and Al-Bakri 2012), in Thi-Qar the rate was 15.15% (Al-abady et al. 2010) and hydatid cysts were found in 42% of sheep in Baghdad (Imari 1962). By the other hand, hydatid disease is one of the common zoonotic parasitic infection in human, in Sulaimaniya province for example the rates was 5%, 7.3%, 3.5% and 7.7% among veterinarians, assistant veterinarian, slaughterhouse workers and animal breeders (Abdulla et al. 2014). In Baghdad a total of sixty cases of human hydatidosis were collected, with (73%) were liver cysts, (20%) were lung cysts (Khalf et al. 2014). However, most of the scientific research conducted on hydatidosis in Iraq has not investigated risk factors for infection or evaluated the impact, including economic burden, of the disease on the community and only one study performed has evaluated economic losses from the condemnation of affected viscera of sheep, cattle and goats. In that study, conducted in Kirkuk, an overall annual economic lossof 10,430,000 Iraqi dinars (approximately US$ 8,800) was estimated (Kadir et al. 2012).

In Basrah province- southern Iraq, a study recorded by Al-Shalabbi (2007) showed the rate of infection in sheep 4.2%, also a study showed the effect on killing of the protoscolices of *E. granulosus* in vitro and in vivo in the laboratory mice. A case with *E. multilocularis* has been recovered from the liver of a woman in Basrah Southern Iraq from 55 years-old living in Al-Hartha region, southern that was in contact with sheep and dogs (Benyan et al. 2013).

The prevalence of hydatid cysts in slaughtered sheep found to be 14.75%. The female sheep 22.9% (123/536) was observed to be more infected with hydatid cysts than male's sheep7.5% (46/609) (Mutar et al. 2017), the prevalence in liver and lungs constituted 61.6% (104/169) and the lungs 38.4% (65/169) were recorded by Abdulhameed et al. (2018). Other study in Basrah province recorded the infection with hydatidosis in sheep, donkey and human with prevalence rate 43.15, 28.5 and 8.8% respectively (Lazim 2019). According to the Iraqi CDC (2012), the number of cases of hydatidosis in humans has increased dramatically since 2000 and from 2011 to 2015 4,769 human CE cases were recorded by the Communicable Diseases Control Center (Parasitology and Helminthology Units) in Iraq (Saheb and Noori 2019). The strategic implementation of a control programme to eliminate or reduce the number of free roaming dogs, as well as owned domesticated dogs, has not been implemented in Iraq (Al-Shabbani 2014).

Molecular Study

Molecular diagnosis of Echinococcus is important for understanding the genetic structure and status of genetic variation of the parasite which contains important suggestions for epidemiology and effective control of Echinococcosis in different regions and countries.

Ten genotypes of *E. granulosus* are known worldwide and categorized as G1-G10 (McManus et al.2003), but just five strains affected humans like sheep (G1), Tasmanian sheep (G2), cattle (G5), camel (G6), and pig (G7) strains (Bart et al.2006). Also, the buffalo strain(G3) and equine strain (G4) have been recorded from Spain, Italy, Lebanon and Syria (Harandi et al. 2002).

Molecular Diagnosis of *Echinococcus* spp.

Human

The results of molecular diagnosis of human samples collected in Basrah suspected to be infected with Echinococcosis are in agreement with those of Hama et al. (2012); Barak (2014) and AL-Nakeeb et al. (2015). The results of this study are consistent with studies in different parts of the world showing that the sheep breed represents the most prevalent form of Echinococcosis responsible for human infection and disease in a wide range of intermediate hosts (Busi et al. 2007; Andresiuk et al. 2009; Guoa et al.2011). Pezeshki et al. (2013) reported that the sheep breed is more prevalent in humans, sheep and goats of Iran. Utuk et al. (2008) concluded that the sheep breed is the dominant phenotype in humans, cattle, sheep, goats and camels. Though, the species status of the

four *E. granulosus* genotypes G6, G7, G8 and G10 remains unsure (Romig et al. 2017). The G6, G7, and G8 genotypes have been isolated from humans. The human cases with the G9 genotype that was described in 1997 are now considered to be the G7 genotype (Cucher et al.2016). Also, camels are significant on the topic of the epidemiology *E. intermedius* (G6), which can be transmitted to human (Thompson 2008).

Molecular studies of *E. granulosus* genotypes in special parts of the world can create a useful place of data about the parasites' epidemiology, ecology, transmission and the sources of human infection. The newest organization indicates that *E. granulosus* includes G1, G2 and G3 genotypes in sheep, human and also in cats (G1) (Cucher et al. 2016). Unusual molecular strategies and genetic targets have been useful for the identification of *E. granulosus* which includes*cox1* (M'Rad et al. 2005), *nad1* and *cox1* (Abushhewa et al.2010). In addition, NADH dehydrogenase 1 gene (*nad1*) is most preserved nucleotide sequence among different genotypes (Bowles and McManus 1993). So, the polymerase chain reaction (PCR)it is basically accurate and responsive scan Bowles et al. (1992) and is performed using gene for cytochrome oxidase subunit I (*cox1*) gene (Osman et al.2009). The pleural cyst showed a difference in epidemiology, developmental biology, morphology and genetics. They are subjected to PCR and amplification of mitochondrial and DNA fragments and then sequenced and strained (Nakao et al. 2010). Molecular study showed that the specific gene for *E. granulosus* G1 sheep and human strain NADH dehydrogenase subunit 1 as dominant (Sanchez et al. 2012). Also, more than a few molecular studies have identified the presence of two genotypes including the common sheep strains G1 and camel strain G6 in Iran (Harandi et al. 2002).

Human cyst occurs generally as two forms which differ in pathology, morphology and epidemiology. Cystic hydatid disease (CHD) is caused by the larval stage of *E. granulosus* and alveolar hydatid disease (AHD) is caused by *E. multilocularis*. More responsive molecular techniques are applied for discriminating species (Wen and New 1993).

Some Animals (Sheep, Camels, Horses and Donkeys)

Sheep breed-specific genes (*G6, sh4-1, cox1*) were identified in Basrah, Iraq (Lazim 2019) which are in agreement with Hosseinzadeh et al. (2012) who extracted DNA and used the *G7-6* and *sh-1* genes in order to detect *E. granulosus* in sheep. The results of amplification of these genes were (234 bp) and (294 bp) for the sheep strain, while the aligned sequence array (792 bp) for the partial *cox1* gene contained 124 variable loci (Junying et al. 2012). It was used to determine the species identification of Echinococcus (Pour et al. 2011). While, these genes (*nad1*, *cox1*) were used for the human strain, but in this study, three of the extracted DNA showed positive results for the *nad1* gene and G6 for the *cox1* gene.

It was found that there are many reasons for sheep to be infected with the human race. The first reason is related to stray dogs, which are considered one of the important reasons for the distribution of the human race in sheep, because stray dogs are infected with multiple strains of *E. granulosus* and *E. multilocularis*, at the same time, then these organisms transfer information between each other and are then eliminated by defecation and then infect the sheep with feces. The important source of spread of this disease is the occasion of offering sacrifices Basrah. The last reason is related to foxes, jackals or any other migratory animals because the borders are open with other countries and other governorates which has diverted this infection to Basrah.

The results of donkey samples detected through *cox1*genes agree with those of (Blutke et al. 2010). In this study the *cox1*gene is used to identify *E. equnis*. In the molecular diagnosis of Echinococcus spp. in Basrah, Iraq (Lazim 2019) representative of the sheep breed (*G6-7, sh4-1, cox1*) are in agreement with Hosseinzadeh et al. (2012) who extracted DNA and used *G7-6* and *sh4-1* genes in order to detect *E. granulosus* in sheep. The results of amplification of these genes were (234 pixels) and (294 pixels) for the sheep strain, while the aligned sequence array (792 bp) for the *cox1* partial gene contained 124 variable loci (Junying et al. 2012). This gene was used to determine the species identification of Echinococcus (Pour et al.2011).

The results of Iraq, Basrah are consistent with studies conducted in different parts of the world showing that the sheep breed represents the most important source for human infection and a wide range of intermediate hosts (Busi et al. 2007; Andresiuk et al. 2009; Guoa et al. 2011). Pezeshki et al. (2013) reported that the sheep breed is more prevalent in humans, sheep and goats in Iran. Utuk et al. (2008) reported that the sheep breed is the dominant phenotype in humans, cattle, sheep, goats and camels. *Cox1* is a partial gene of three of the DNA extracted from human show positive results for this gene due to multiple infections.

The sequencing results recorded in Basrah showed that the *G6-7* gene was an identification (96%) of an Estonia isolate that was recorded in GenBank at the entry number (KX039965.1), the partial gene registered by Laurimae et al. (2016). However, the sequencing result for the sh4-1 gene in the compartment with the database in GenBank shows that there was 99% similarity with a single isolate registered in accession number (HM563031.1) as recorded by (Harandi et al. 2011) in southern Iran. The sequencing result for the *cox1* gene compared to the database in GenBank shows that there was 99%similarity with the isolate recorded in the accession number (MF281540.1) as recorded by Yan et al. (2018), while in the *coxl* gene, the sequencing results show that there was 99% identification with Estonia isolates (Kinkar et al. 2018).

A study by Al- Ataby (2022) was done in Basrah province, isolated *nad1* gene from sheep samples, and found that there is 100% identification with the MG672293.1 strain submitted by Kinkar et al. (2018), while, the sequencing results for *cox1* from Donkey samples when compared with the database in GenBank found 100% matched with Estonia isolate strain number KY766905.1 registered by Kinkar et al. (2017).

The results of genetic analysis of the G6-7 gene in a study conducted in Iraq, Basra showed 100% identification compared with several other countries such as Iran, Turkey, Algeria and India, while the sh4-1 gene in the same study showed that the homology (99%) with isolate from Iran (Harandi et al. 2011) and the percentage was 100% with Iraqi isolation, and that the percentage was 100% identity with many countries such as Tunisia, Brazil, Turkey, India and Australia. One of the new studies (Al-Ataby 2022) in Iraq, Basrah recorded a molecular study using polymerase chain reaction (PCR) technology where four genes were used which are *nad1*gene (418 bp), *cox1*gene (370 bp), *nad1* gene subunit gene (674 bp) Basis) for *E. granulosus* and *nad2* (551bp) of Echinococcus in three hosts four strains, sheep scored five strains and camel scored two strains. And

studying the genetic sequences of those strains and comparing them with the strains registered in the gene bank by analyzing the phylogenetic tree. Isolates were scored in NCBI, under accession numbers (MW084709.1, MW077506.1, MW080539.1, MW093745.1, LC600749.1, LC600747.1, LC600748.1, LC600745.1, LC600746.1, LC600751. 1 and LC600750.1). The sequencing result showed that there is (99%) homology with the Estonian isolate in *cox1* while in the *nad1* gene, this sample identified 100% with a sample isolated from human, camel in Nigeria and from sheep in China. While in the gene *nad1* (674bp) some strains have 100% similarity in the genebank with the strain isolated from human and Iraq and in the identification of camels in an isolate sample of sheep in Nigeria and China. Finally, about (551bp) *nad2*of Echinococcus, sequencing result compared to five strains of *E. equines* recorded in the NCBI World database.

We can classify the genes detected in Basrah, Iraq from human, sheep and camel to three parts those are as below:

cox1 Gene in Human, Sheep and Camel

The *cox1* gene was responsible for encoding the mitochondrial cytochrome c oxidase subunit I (*cox1*) gene. To 95% of the energy of living eukaryotic cells (Johnston, 2006), it directly affects metabolic performance. The *cox1* subunit I is the most conserved of the 3 genes coding for cytochrome oxidase, so it has been used in many genetic studies (Traversa et al. 2007).

When comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences from GenBank found matched with the isolate (GenBank acc. NC_044548.1). A phylogenetic tree was established in study of Al-Ataby 2022 (Fig. 1), which was based on the observed differences in DNA. This genetic tree contained samples (A1, A3, A4 and A5) along with other relative DNA sequences. The total number of DNA sequences aligned in this neighbor-binding method (Saitou and Nei 1987), was 10. Remarkably, the examined samples were grouped into two adjacent blocks within the *E. granulosus* sequences. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987).

However, there was a genetic polymorphism that could be detected in sample (A1) identified in the samples examined in the study, this tree provided many informative data about all samples analyzed whether in terms of their actual location, phylogenetic tree or distances. The genetic proximity between these samples and their close sequences on one side and other sequences incorporated within the same tree forming the other side, resided all examined samples in the immediate vicinity of GenBank entry number MN787551.1, which belongs to the isolated human city of Kyrgyzstan. Interestingly, the examined samples were grouped into two phyla within the *E. granulosus* sequences. One of these branches was made of three samples (A3, A4 and A5).

While the other clade consists of other comparison samples; As well as sample (A1) in a separate branch. In fact, the positioning of (A1) is due to the presence of a mutation in this sample. In addition to the proximal position of this sample (A3, A4 and A5), it was also positioned near several reference sequences embedded in the same tree.

The three samples (A3, A4, and A5) were found in close proximity to the GenBank entry number MN787558.1, MN787556.1, MT537158.1, MT537159.1, MT537162.1 and MT537165.1, both belonging to *E. granulosus* sequences. In addition, another mode of this branch is also observed with four GenBank entry numbers MN787550.1, MN787552.1, MN787541.1 and MN787540.1, which belong to the same organism.

All examined samples settled in the immediate vicinity of GenBank entry number MN787551. Comparison with the database in GenBank showed that there was (99%) identification with the isolate recorded in the accession number (MF281540.1) as recorded by Yan et al. (2018), while in the *cox1* gene, the sequencing results showed that there was (99%) Identification with Estonia Isolate (Kinkar et al. 2018).

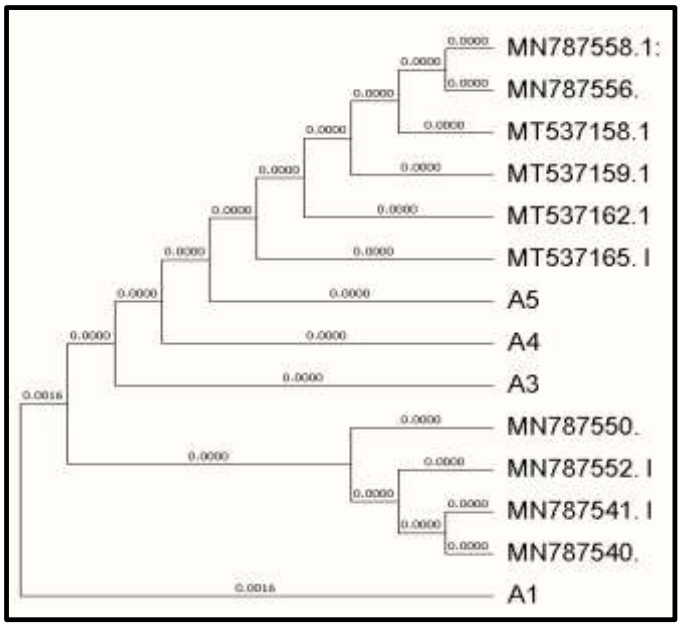

Fig. 1: Phylogenetic tree of genetic variants of the *Cox1* fragment of four *E. granulosus* local samples (Al-Ataby 2022): A1, A2 and A3(Samples showed genetic variation by sequences when used *Cox1*).

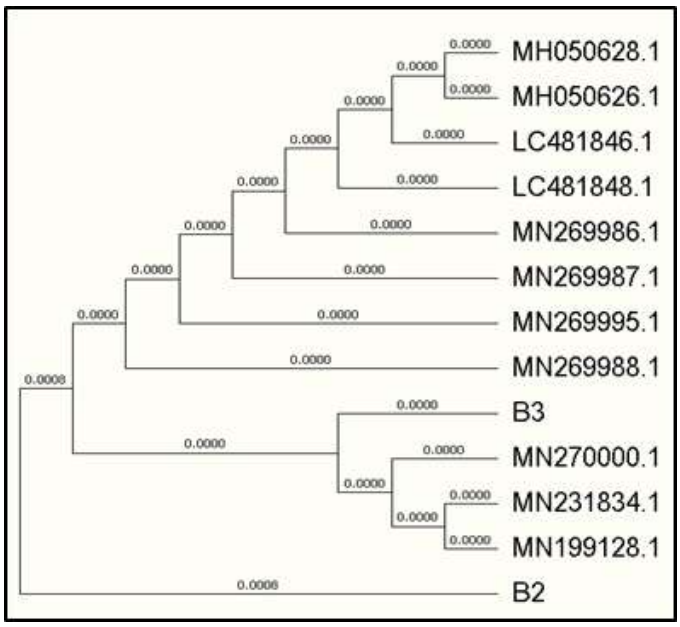

Fig. 2: Phylogenetic tree of genetic variants of the *Nad1* fragment of four *E. granulosus* local samples. (Al-Ataby 2022): B2 and B3 (Samples showed genetic variation by sequences when used *Nad1*).

Another study in the sequencing result of the *cox1* gene in human showed that there was (100%) with isolate recorded by Shafiei et al. (2018) in entry number MH010310.1. Whereas, the sequencing results compared with the database in GenBank showed that there was (99%) isolate identification recorded in strain number (FJ608748.1) as recorded by Calderini et al. (2018) in Italy, while other isolates from sheep showed that (100%) of Turkey isolate was recorded in the strain number (MF544127.1) recorded by Oguz et al. (2018).

Nad1 Subunit Dehydrogenase Gene in Human, Sheep and Camel

The study recorded by Al-Mohammad (2011), which showed three common genotypes found in Iraq based on *cox1* and *nad1* genetic sequence analysis including sheep (G1) breed, buffalo breed (G3) and camel breed (G6). (G6) did not appear in human and sheep hydatid isolates. Two strains in human isolates are sheep (G1) and buffalo (G3) with 92% matching for *cox1* and 99% matching for *nad1* in strain (G1) and 99-100% matching for *cox1* as well. At the same rate as *nad1* in strain (G3). These results did not agree with the study recorded by Lahmar et al. (2004) in Tunisia who discovered most infected camels of the sheep breed (G1) while Rahimi et al. (2011) detected (G1, G3, and G6) in camel isolates in Iran. The strain was the type dominant genetic.

Two samples were included in the Al-Ataby study (2022), these samples were screened for amplification of the *nad1* gene sequence in the genetic sequence of *E. granulosus*. The *nad1* gene is responsible for encoding the NADH dehydrogenase sub-gene1 (*nad1*). The ND1 protein is a subunit of NADH dehydrogenase, which is located in the inner mitochondrial membrane and is the largest of the five complexes of the electron transport chain (Voet et al. 2013). So far, ten remarkable genotypes (G1-G10 strains) of *E. granulosus* have been qualified in the world according to nucleotide sequence analysis of some genes such as NADH dehydrogenase 1 (*nad1*) gene and other genes such as (*cox1*) gene and transcribed spacer 1 (*ITS1*). These lineages are related to notable intermediate hosts including: sheep, goats, horses, swine, sardines, cattle and camels (Sánchez et al. 2010). Sequencing reactions indicated the exact identity after performing an NCBI blast of these PCR amplicon (Zhang et al. 2000). Regarding the 674bp amplicon, the NCBI blast engine showed about 100% sequence similarity of the B3 sample but 99% of the B2 sample between the sequenced samples and the intended reference target sequence. And when comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences from GenBank found matched with GenBank acc. MN_199128.1.

The alignment results of the 674 bp Basrah samples (Figure 2) revealed the presence of single mutations of interest in all samples analyzed compared to the reference DNA sequence, however, the majority of the mutations were localized in sample (B2) that matched 100% with MN231834.1 strain isolated from human, and MN199128.1 strain isolated from camel, while the strain isolated from sheep MN270000.1 which belongs to *E. granulosus*.

This similarity resulted from a mutant (B2) sample. Genotype (G1) was the most common infectious sheep strain of *E. granulosus* worldwide with a variety of hosts (Craig et al. 2003). In these areas, dogs were usually fed with the intestines of cattle which may have given rise to infection with *E. granulosus* (Sánchez et al. 2010). This efficiency may be sufficient for the reproduction of the current endemic state.

A phylogenetic tree was established in the Al-Ataby study, which was based on the observed differences in DNA. This genetic tree contained samples (B2 and B3) along with other relative DNA sequences. The total number of DNA sequences aligned in this neighbor-linking method was 11. Remarkably, the examined samples were grouped into contiguous groups within the *E. granulosus* sequences.

However, there was a genetic polymorphism that could be detected in the (B2) sample identified in the samples currently examined, this tree provided many informative data about all samples analyzed both in terms of their actual position in the phylogenetic tree or genetic distances between these samples and her samples. Sequences close to one side and other sequences combined in the same tree form the other side. Interestingly, the examined samples were grouped into two phyla within the *E. granulosus* sequences. One of these interfaces was made from sample (B3), while the other close layer was made from other reference spots; while (B2) is located in a separate branch.

In fact, the localization of (B2) in the branch is due to the presence of a mutation in this sample. A sample (B2) was found near the clade containing some strains with 100% similarity to (B2), and was erected near GenBank entry numbers MN231834.1 isolated from human, Iraq; MN199128.1 isolate from camel, Nigeria"; MN269987.1 isolate from sheep, China.

The reason may be attributed to the fact that sheep are clearly sensitive to the sheep strain (G1) of *E. granulosus* and the hydatid cysts in this intermediate host are mostly fertile, so sheep are a primary source of canine Echinococcosis. This sample was 100% identified with sample isolated from human, Iraq, camel, Nigeria and from sheep, China. (Rahimi et al.2011).

Nad2 of *Echinococcus equinus* Gene in Human, Sheep and Camels

The PCR product of the *nad2* gene in samples isolated from Basrah in Iraq showed moderation for four samples of 551 bp. This sample was isolated from humans, sheep and camels. Compare the current sequencing result with the identity of *E. equinus* isolated FSJ01 with five strains of *E. equinus* recorded in the NCBI Worldwide database found that completely different from five strains of *E. equinus* in the world.

Some studies of *E. equinus* in the world such as the study in Turkey is the first study of *E. equinus* isolated from the human host in Turkey and this study of 82 samples was identified as *E. granulosus*. The sequence obtained from *E. equinus* is submitted to the GenBank accession number (MT621047). These results are in agreement with the results recorded in Basrah, Iraq in isolated samples from humans, and the sequencing result is considered the first report on *E. equinus* and it is called FSJ01. To the present, *E. equinus* has been accepted as being specific to the subfamily Equidae, although there has been a study conducted on molecular characterization of *E. granulosus* by PCR-RFLP technology and it has been reported by Nakao et al. (2013) and Romig et al. (2017).

Another research found *E. equinus* in a horse and described the first case of a molecularly verified *E. equinus* infection. Restriction and sequencing analysis of gene 1 in subunit 1 of

the nicotinamide adenine dinucleotide gene verified the diagnosis of *E. equinus* in Germany (Andreas et al. 2010).

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CHAPTER 14

IMMUNOLOGICAL ASPECTS OF CYSTIC ECHINOCOCCOSIS: AN OVERVIEW

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ABSTRACT

Cystic Echinococcosis (CE) is one of the most important zoonotic diseases that cause a serious threat to human life, both healthily and economically. Herein, we aimed to update information and highlight the immune response against cystic echinococcosis. The evading mechanisms and the modulation of the immune system by *E.granulosus* metacestode are also addressed. Several investigations demonstrated that *E. granulosus* is immunogenic, inducing pro-inflammatory cellular stimuli, including the production of antibodies/T cell- and other cell-mediated responses in different intermediate hosts, including humans. Recently, several studies have characterized the protective antigens of *E. granulosus* and their role in immunizing various animal host species. Recent results proved that DNA vaccines with antigen B and recombinant protein vaccines produced from EG95 antigen possess the best outcome and elicit protective immunity. This chapter summarizes the consensus on CE's immunology and strengthens previous findings with results from relevant and recent investigations.

Introduction to Cystic Echinococcosis

The Parasite, its Characteristics, and Life Cycle

Cystic Echinococcosis is a life-threatening and neglected zoonotic parasitic infection resulting from the larval stage of the cestode species complex *Echinococcus granulosus* (sensu lato). It confers considerable worldwide health and economic burden. Among the complex, *E. granulosus* sensu stricto of G1 and G3 genotypes is the most common cause of human CE (Kinkar et al. 2018). Hydatid cyst disease is well-known as one of the most common cosmopolitan parasitic infections, infecting different hosts such as humans, wild animals, and domestic livestock (Casulli et al. 2019).

The life cycle of Echinococcus is complicated as it includes two mammalian hosts, the definitive host, which is represented by canines (e.g. dogs, wolves, coyotes) while the intermediate host includes other mammals, for instance, sheep, goats, cattle, buffaloes, pigs, horses, camels, besides humans. Hydatid cyst occurs in humans as dead-end hosts via accidental infection by the larvae after ingesting eggs of *E. granulosus* defecated from the dog as the final host. The larval stage develops as a cyst, mainly in the liver (about 70%), followed by the lungs, spleen, kidneys, and brain (Brunetti et al. 2010). After their consumption by the appropriate intermediate host. The eggs hatch, releasing the embryos that breach the mucosa and then find their way via blood or lymph to the liver, lungs, or other sites to produce unilocular fluid-filled hydatid cysts. When the definitive hosts consume the infected viscera of animals with the hydatid cysts, the protoscoleces evaginate, attach to the mucosa of their intestine and develop into worms (Shnawa et al. 2021a) (Fig. 1).

The Biology of Intermediate Hosts Infection with Cystic Echinococcosis

The oncospheres hatched from the swallowed eggs and stimulated in the small intestine of an appropriate intermediate host. Lytic enzymes of the parasite facilitate the invading of the oncosphere. Therefore the parasite finds its way through the intestinal mucosa to the host blood; then, they are circulated to hepatic and pulmonary tissues and other organs. Oncospheres reach the favourite tissue where the hydatid cyst development begins (Siracusano et al. 2009).

The hydatid cyst of *E. granulosus* sensu lato consists of an internal cellular germinal layer and an external non-cellular laminated layer of a carbohydrate-rich structure. The cyst of CE grows gradually, and host-derived adventitial (fibrous) layers protect the cyst. This fibrous capsule is a resultant of the local tissue response of the host against the parasite (Díaz 2017; Thompson 2017). The laminated layer is a multi-laminated building produced originally by the developing hexacanth embryo and later by the germinative layer. It consists of glycosylated glycoproteins. Also, it is vital for the metacestode's immune evasion within the infected host (Díaz et al. 2011a; Díaz et al. 2011b; Tamarozzi et al. 2016). The germinal layer consists of embryonic cells involved in the formation of brood capsules and protoscoleces. A fully developed fertile cyst comprises brood capsules with protoscoleces. It is filled with clear hydatid fluid and rich in daughter cysts (Eriksen and Agopian 2017). Hydatid cysts are shown in Fig. 1, 2, and 3.

It has been suggested that the two layers of hydatid cyst possess a significant role in inducing the innate immunity in the host-parasite attachment tissue because of gathering different immunogenic antigens (Siracusano et al. 2008; Díaz et al. 2011b). Recurrence of CE may result from cyst fluid spillage during surgery or an accident, leading to secondary infection.

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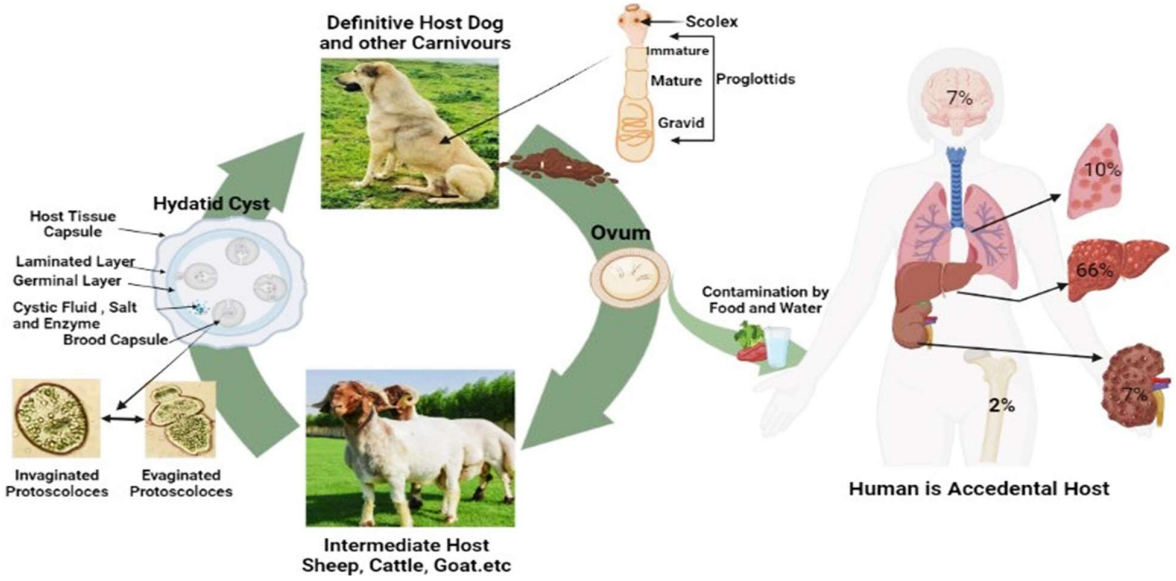

Fig. 1: The life cycle of *Echinococcus granulosus.*

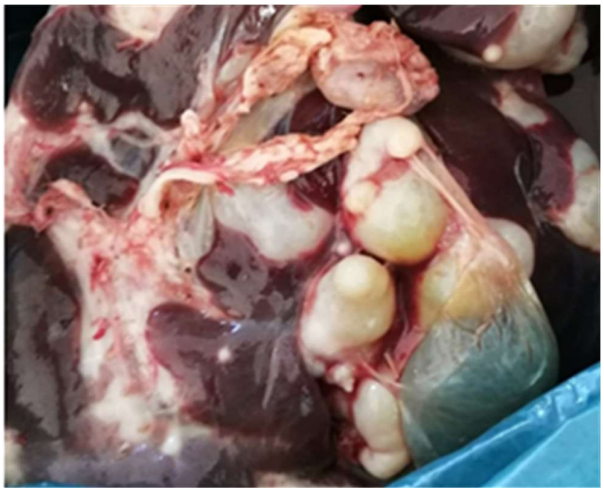

Fig. 2: Heavy naturally infected sheep's liver with CE (Jalil et al. 2021).

Therefore, this disease can be considered a significant challenge both from medical and economic points of view (Eckert and Deplazes 2004). Also, experimental secondary infection can be produced in Balb-c mice, which may help with novel medication and vaccination attempts, as shown in Fig. 4.

The clinical symptoms of hydatid cysts show no specific signs, whereas depending on the number, location, and size, it has variable effects ranging from mild to deadly (Junghanss et al. 2008).

In the aspect of serological diagnosis, the proteome of the metacestode fluids of *E. granulosus* and other ovine cestodes cysts like *Taenia hydatigena* and *Taenia multiceps* were studied via a shotgun proteomic method. Parasite and host molecule shapes among the three hydatid fluids were distinguished and compared. Recognized proteins include different parasitic markers of serodiagnosis importance because of their immunoreactivity in humans. This comprised Ag5, AgB antigens, 8-kDa glycoproteins, hydatid cyst diagnostic antigen P29, and egg antigen P40. Especially, seven proteoforms of AgB and 8-kDa glycoprotein showed promised diagnostic biomarkers due to their ability to predict CE in sheep and differentiate among diverse types of parasites (Biosa et al. 2021). Ahn et al. (2015) mentioned that recognizing a single distinct molecule might not accurately diagnose this disease due to the alteration of specific immunodominant epitopes with the progression of the infection. The immunoproteomic technique supported by imaging investigation may help diagnose both CE and AE and for identifying the stage of CE, which are appropriate for patient follow-up. Their results also emphasized the probable biological roles of HF proteins that might be shared in the homeostatic preservation besides pathophysiological adaptation of the *E. granulosus* through long-term disease.

There is no potential, efficient vaccine against CE, and treatment is the option against CE. The therapeutic methods for hydatid cyst treatment are medical treatment, surgery, endoscopic interventional, and percutaneous procedures (puncture, aspiration, injection, and re-aspiration (PAIR) (WHO 1996). Therefore, in small and inactive cysts, the selected treatment is chemotherapy with benzimidazole derivatives (mebendazole and albendazole). Still, a surgical operation is a single-choice treatment for large cysts (Dehkordi et al. 2019). Recent works have mentioned chemotherapy with benzimidazole derivatives which may cause many side effects, like hepatotoxicity, teratogenicity, Hematuria, leukopenia, thrombocytopenia, and osteoporosis (Moro and Schantz 2009; Eriksen and Agopian 2017). Nowadays, medication is motivated to use green biosynthesized nanoparticles as novel protoscolicidal agents, such as silver, zinc oxide, and others within in vitro and in vivo models (Shnawa et al. 2021b; Hamad et al. 2022). In previous works, we reviewed the publication regarding the possibility of using nanoparticles as a new approach for CE treatment (Shnawa 2018; Shnawa et al. 2021a).

Parasite Antigens and Metabolites

Echinococcus is a very complex multicellular parasite. It is highly immunogenic, provoking or inducing pro-inflammatory responses, producing specific antibodies, cell-mediated immunity, specifically T-cells in patients and other infected

Fig. 3: Histological sections of hydatid cysts of intraperitoneally administered mice with *E. granulosus* protoscoleces show the GL.: germinal layer; LL.: laminated layer; AV.: adventitious tissue with dense inflammatory cells. They were treated with hematoxylin-eosin stains. Scale bar of A and B = 5 µm , & C= 2 µm (Hamad et al. 2022).

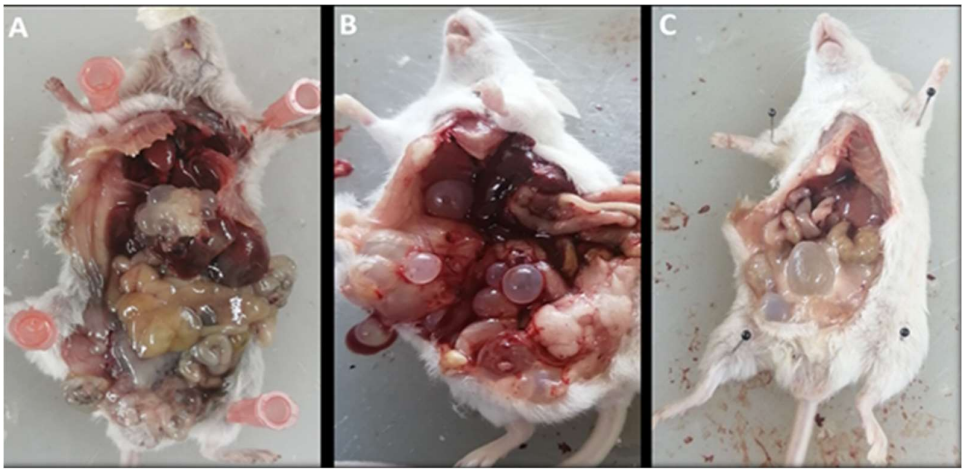

Fig. 4: A- Several hepatic hydatid cysts in the experimentally infected mouse, B, and C multiple hydatid cysts in the peritoneal cavity of mice (Hamad et al. 2022).

hosts (Zhang et al. 2008b). Immunity has a maximum impact on the host-parasite association in hydatid disease. This immunity is related to the production of protective antibodies. This character depends on to discover an efficient EG95 vaccine against CE parasite (Zhang et al. 2008b). In the first experiment, AgB subunit structure was studied in specimens of single hydatid cysts, giving qualitative and quantitative variances among the tested specimens. They found diverse subunits of AgB oligomers with dissimilar abundances and oligomerization characteristics are formed (Monteiro et al. 2012).

Ten B cell epitopes and seven cell epitopes were recognized on Ag5, suggesting the robust immunogenicity of this protein, which could help in designing antigens as peptide vaccines for hydatid cyst disease (Pan et al. 2017). The recent findings proved that DNA vaccines with antigen B and recombinant protein vaccines created from EG95 antigen exhibited the best results and provoked protective immune responses (Anvari et al. 2021). Recent findings confirmed that Eg mefAg-1 had significant serodiagnostic importance, which may be a candidate for CE diagnosis (Tianli et al.2019). Characterized antigens, and molecules of E.granulosus are shown in Table.1.

Immune Response against Cystic Echinococcosis

As it is well known, the arms of human immunity are innate besides acquired or adaptive immune responses. Despite the presence of the laminated layer, the cysts are not entirely insensitive to the different mechanisms of the host immune effectors. During infection, the humoral immune response represented by the production of immunoglobulins (antibodies) will be activated, which will activate the complement system. Both antibodies and complement will be directed against the laminated layer and, in some circumstances against the germinal layer causing direct damage to the cyst and triggering inflammation in the surrounding area. Usually, continuous inflammation is associated with the low viability of the parasite; in contrast, the parasitic larvae will thrive in the case of declined inflammation (Zhang et al. 2012). Even in protozoal infection, inflammatory responses are also documented clearly in Sarcocystis infection of sheep and goat's esophagus (Swar and Shnawa 2021).

Innate Immune Response

It is well-thought that the innate immune response is the first line of protection against different parasitic infections, distinguishing pathogen-associated molecule patterns (PAMPs). The recognition process is performed by pattern-recognition receptors (PRRs), like Toll-like receptors (TLR), in addition to nucleotide-binding oligomerization domain-like receptors (NLRs). The mentioned receptors are located on the surface of immune cells, like macrophages, neutrophils, endothelial cells, dendritic cells, and lymphocytes. TLRs might trigger apoptotic pathways during their course of action (Akira et al.

2006). The stimulation of type 2 immune response by parasites, especially helminths, is well known.

It has been suggested that the two layers of hydatid cyst have a significant role in motivating the innate immunity in the host-parasite interaction because of different antigens accumulation (Siracusano et al. 2008; Díaz et al. 2011a). Amri and Touil-Boukoffa (2015) have observed that the laminated layer protects the parasite from the nitric oxide (NO) synthase activity via upregulation of the host arginase route. Moreover, another study has mentioned that the creation of NO species of activated macrophages is vital in preventing the spread of CE disease. Also, their findings proposed the possible in vivo production of peroxynitrite (ONOO−) and its role against the hydatid cyst. Therefore, they pointed out the efficiency of these metabolites as scolicidal materials (Zeghir-Bouteldja et al. 2009).

As mentioned, the laminated layer provides a physical shield to protect the cyst, but this protection is usually not enough. Therefore, the parasite also involves the regulation of its host's immunity. As recorded, the immune responses against these parasites involve innate and adaptive mechanisms.

The scheme of innate immunity is based on the stimulation of the immunological defense mechanisms of the host and the host-parasite cross-talk that results in triggering suppressive mechanisms to the host defenses. One of the effects of innate immunity on CE is the production of reactive oxygen species (ROS), and nitrogen (RNS) found on the adventitial layer of hydatid cysts, which cause the infertility of the cyst (Cabrera et al. 2008). In addition, it has been reported that during CE, the host's immunity will be suppressed via the regulation of anti-inflammatory cytokines by TLRs, especially TLR-2 and TLR-4 (Fig. 5) (Apaer et al. 2017; Bakhtiar et al. 2020).

The leading role of neutrophils and macrophages is detecting and eliminating the parasite, but it can evade it via its metabolites. For instance, E. *granulosus* secrete antigen B, which can neutralized the action of neutrophilic elastase, allowing the parasite to escape from the effect of neutrophils. Moreover, antigen B plays a role in the modification of macrophage activities as well as suppressing the action of cytokines. Eosinophils proved their effectiveness in innate immunity against *E. granulosus* metacestodes (Silva-Álvarez et al. 2016; Zheng et al. 2017).

Table 1: Antigen and immunogenic molecules of *E. granulosus*

Antigen	Name	References
Antigen 5	Ag5	Capron et al. (1967)
The composition of the subunit and specificity of the cyst fluid antigens of *E. granulosus*	AgB	Lightowlers et al. (1989)
Paramyosin of *E.granulosus*, tegumental antigen	EG36	Mühlschlegel et al. (1993)
Sheep-vaccinated with the antigen EG95,	EG95	Lightowlers et al. (1996)
Thioredoxin peroxidase	TPx	Salinas et al. (1998)
The recombinant EgA31 (rEgA31) was tested as a protective vaccine	EgA31	Fu et al. (1999)
Elongation factor 1β/δ	EgEF-1 *β/δ*	Margutti et al. (1999)
The p176 could be valid as a standard antigen in diagnosing hydatid disease.	p176	González-Sapienza et al. (2000)
E. granulosus cyclophilin	EA21	Ortona et al. (2002)
The rEpC1-GST fusion protein for CE diagnosis	rEpC1-GST	Li et al. (2003)
Tropomyosin (EgTrp) is expressed in metacestode and adult *E. granulosus.*	EgTrp	Esteves et al. (2003)
Eg2HSP70 is a novel molecule of antigenic that provokes B and T cell immunity.	HSP70	Ortona et al. (2003)
A new tegumental antigenic molecule EgTeg of *E. granulosus*	EgTeg	Ortona et al. (2005)
A new 19 kDa *E. granulosus* antigen	Eg19	Delunardo et al. (2010)
Heat shock antigen 20 (HSP20) as a detectable marker of acute CE.	HSP20	Vacirca et al. (2011)
Four recombinant antigens from *E granulosus*	B1t, B2t, E14t, and C317,	Hernández-González et al. (2008)
Recombinant glutathione transferase from the *E.granulosus*	rEgGST	Harispe et al. (2010)
The EG95 antigen was cloned from the G1 genotype of *E. granulosus*	EG95−5G1	Rojas et al. (2012)
	Differences between EG95 proteins from the G6 genotype and EG95 protein from the G1 genotype.	
The structure Antigen B: The Composition of Subunit and Oligomers	rAgB8/1, rAgB8/2 and rAgB8/3	Monteiro et al. (2012)
14-3-3 protein	14-3-3	Teichmann et al. (2015)
8-kDa subunit of antigen B (HydI) of *E. granulosus*	8-Antigen B (HydI)	Azizi et al. (2016)
Immunization of sheep with rEg.myophilin could reduce the hydatid cysts development of *E. granulosus*	rEg.myophilin	Zhu et al. (2016)
In sheep, E. granulosus rEg.P29 showed immunoprotection and induced Th1 and Th2 immune responses.	rEg.P29	Wang et al. (2016)
Ten B cell epitopes and seven T cell epitopes were identified on Antigen 5	Ag5	Pan et al. (2017)
Protoscolex tegumental surface antigens	PSTSA	Valizadeh et al. (2017)
Protoscoleces proteins of *E. granulosus*	egM9, and egM123	Zhang et al. (2018)
EgA31 and EgG1Y162 proteins as multi-epitope vaccines against *E. granulosus.*	EgA31&EgG1Y162	Zhao et al. (2019)
Multi-epitope fusion protein	Eg mefAg-1	Tianli et al. (2019)
Glycosylphosphatydilinositol (GPI)-anchored protein	EG95	Haag et al. (2009)
E. granulosus enolase is several -epitope vaccines utilized as a novel preventable anti-hydatid cyst material.	EgEnolase	Pourseif et al. (2019)

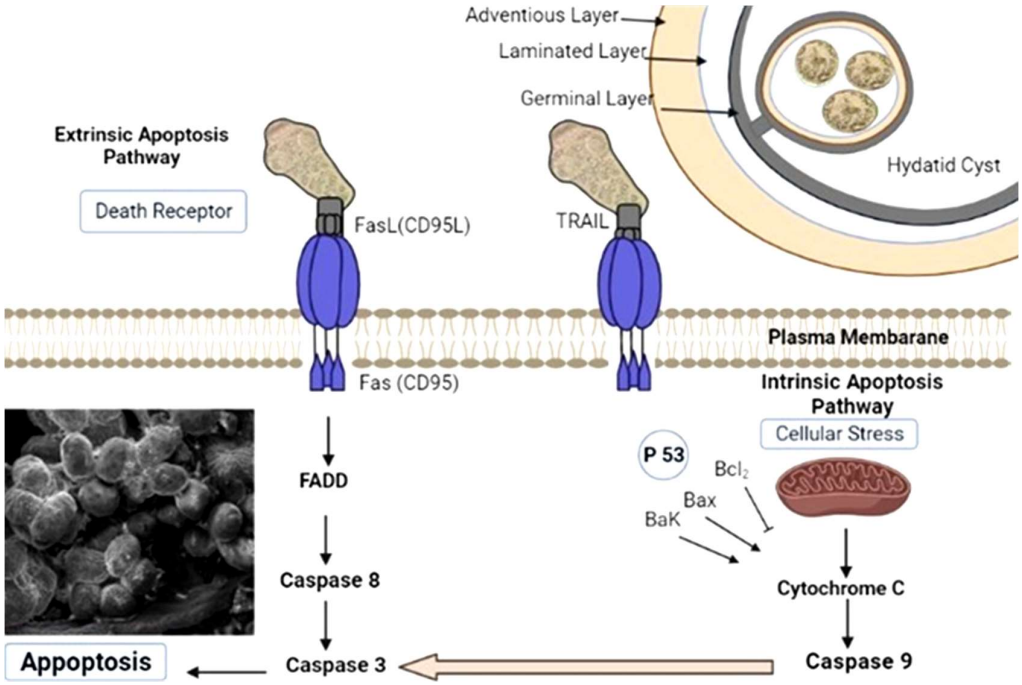

Fig. 5: Host innate immune response (inflammasome, TLRs and apoptosis) against CE (Modified from Bakhtiar et al. 2020) (SEM image of protoscolices from (Shnawa et al. 2017).

One of the essential innate immunity mechanisms is inflammation. The inflammation has a reverse correlation to the viability of the parasite. The explanation of this correlation is still not clear. Still, some toxic materials such as nitric oxide can infiltrate the laminated layer, in addition to the effect of eosinophils. In infected persons, the inflammation's severity is mild compared to what is probably predictable when a pathogen like *E. granulosus* enters the body. The parasite's behaviour might explain this as it surrounds itself with a non-infiltrated layer of collagen (Meeusen and Balic 2000).

The parasitic metabolites can impair the natural killer cells (NKs) and the DCs, altering these cells' differentiation, maturation, and function. Furthermore, it has been documented that these metabolites are represented by the excretory/secretory (E/S) antigens of CE, which have an immunosuppressive effect on DCs (Casaravilla et al. 2014; Wang et al. 2015).

Moreover, the participation of inflammasomes was also reported as a part of the host defense mechanism. The inflammasomes, which are platforms of NLRs, have a high affinity to various PAMPs and regulate different immune responses. Inflammasomes may cause the activation of caspase-1, and through this activation, it will regulate IL-1β and IL-18 as pro-inflammatory cytokines. However, inflammasomes involvement is still unclear in CE or Alveolar echinococcosis, but reports indicated that helminths metabolites might trigger them (Jin et al. 2014, Meng et al. 2016; Gottstein et al. 2017).

Some studies investigated NK cells' role in Echinococcosis. In contrast, these cells are involved in innate immune responses against viral, bacterial, and protozoal infections, besides complement and other innate immunity components (Shnawa 1995; Zhang et al. 2008a; Shani et al. 2012). The analysis of the blood of humans suffering from active CE cysts revealed a large number of NK cells (CD56CD8) when compared with healthy control (Hernández et al. 1999), but no information regarding the cells at the margin of the hydatid cysts was achieved; therefore, their action in the CE disease was unclarified. Dissimilarity, patients with alveolar echinococcosis had a lesser peripheral blood mononuclear cells (PBMC) NK level than controls and persons of non-parasitic biliary disease. This may be attributed to a minor number of NK present in peripheral blood of those infected with *E. multilocularis* or related to sera inhibitory materials, for example, immune complexes and antibodies (Vuitton et al. 1989). It assumed that in AE, the MHC class I chain-related molecules A and B, stimulated by *E. multilocularis*, twist the NKG2D stimulation on NK and CD8, preventing NKG2D-dependent cytotoxicity and thus causative to the long life of this parasitic infection (Zhang et al. 2008a). Controlling action of IL-12 in the natural resistance of some intermediate hosts to Echinococcosis was proposed. Mice inoculated with vector encoding IL-12 or rIL-12 were protected from secondary cystic and alveolar echinococcosis due to Th1 mechanisms (Emery et al. 1998; Al-Qaoud and Abdel-Hafez 2008). TLR has been proved to possess a vital action in innate immunity mechanisms. Nonetheless, there is restricted research regarding its participation in natural resistance to Echinococcosis. *E. granulosus* antigens can modify dendritic cells maturation through TLR, leading to weak antiparasitic immune responses (Rigano et al. 2007).

Adaptive Immune Response

Since the hydatid cyst can survive for a long time in its intermediate host, the chronic disease depends on host existence for parasite spread and includes a cross-talk between the parasite and its host (Zhang and McManus 2006). The importance of secretory metabolites of parasitic worms as immunomodulators was mentioned by Harnett (2014) previously, as immunomodulatory signals from the parasite and the host are recognized. The parasite produces immunomodulatory molecules that change the function of the host enzymes and the immune system mechanisms (Maizels et al. 2004).

In the adaptive immune response, the T-cell immunological status of cystic hydatid cyst patients was investigated to detect blood cytokines, cell surface, and intracellular markers in (PBMCs) by flow cytometry and cytokines provoked from PBMCs by antigens of the hydatid fluid. The remarkable findings are the changes in factors related to the Th2 mechanisms. Nevertheless, in several cases, elevation in Th1- components are recorded compared to healthy subjects.

The immunoglobulins concentration of IgG4 and IgE and common eosinophilia during hydatidosis revealed the immune mechanisms against CE which are thought to be Th2 dominated, and the parasite antigens modulate the T-cells. Previous results of immunological studies showed significant in vitro production of parasite antigen-induced IL-4, IL-5, IL-6, IL-10, and IFN-γ by (PBMC) prepared from the blood of persons infected with hydatid disease, confirming the fact that immune mechanisms against this disease are mainly controlled by Th2 cell stimulation, beside the cell subset of Th1 (or Th0) (Riganò et al. 1995). It has been proposed that Th1 cytokines are associated with protective immunity while Th2 cytokines are correlated to disease susceptibility and associated with the chronic stage (Mezioug and Touil-Boukoffa 2009; Petrone et al. 2015). In experimental infection with CE, previous findings confirmed the suggestion that earlier production of IL-10 by B cells in response to antigens may favour parasite-survival as well as the activated type-2 cytokine action establishment (Baz et al. 2006). Other researchers proposed that IL-4/IL-10 weakens the Th1 immune activity and permits the parasite to live in CE hosts (Amri et al. 2009). Secondary experimental infection of mice documented the probable immuno-suppression of IL-10 and TGF-β as a method that assists the parasite in escaping the cell-mediated action of the host (Mondragón-De-La-Peña et al. 2002). Furthermore, the possible immune-suppressing role of TGF-β (and regulatory T cells) was proved in *E. multilocularis* experimentally infected mice. These alveolar cysts induce the differentiation of dendritic cells (DCs), which express TGF-β-, which persist immaturely and modify CD4+ and CD8+ regulatory T-cell expansion (Mejri et al. 2011).

The characteristic immune response against the hydatid cyst of humans and animals' echinococcosis related to Th2 type. It includes Interleukins, IL-4, IL-5, IL-10, and IL-13, antibodies IgG1, IgG4, besides IgE, in addition to a massive number of eosinophils and mast cells. Also, on the other hand, stimulated macrophages are incorporated into this process (Zhang et al. 2008b; Zhang et al. 2012).

The cellular immune response plays a prominent role during hydatid cyst formation in the acute stage, as inflammatory cells infiltrate and activate, primarily macrophages and neutrophils (Zhang et al. 2008b). Proteases enzymes, for instance, neutrophil elastase, produced by triggered neutrophils, can overcome the parasite and provoke neutrophil chemotaxis. The effective secretory enzyme inhibitor, EgKI-1 of the *E. granulosus* oncospheres, can protect the parasite from the immune challenge of the host (Ranasinghe et al. 2015). Additionally, if the hydatid cyst is damaged during the chronic phase of the disease, neutrophils are attracted to fight the protoscoleces, which may be result in rise of Antigen B (AgB) in the hydatid cyst fluid (Shepherd et al. 1991). AgB as an effective protease inhibitor can decrease neutrophil recruitment, which leads to postponing of protoscoleces elimination by neutrophils until the parasite develops into large hydatid cysts and produce secondary hydatidosis. Therefore, inhibition of neutrophil elastase secretion and neutrophil chemoattraction is essential for the parasite thriving through acute and chronic stages of cystic echinococcosis (Fig. 6).

During the acute phase of this disease, both macrophages and neutrophils migrate towards the small intestine's mucosa to fight and overcome the oncospheres. Also, neutrophils are attracted if hydatid fluid is disseminated from a damaged cyst during the chronic phase of the disease. Still, both neutrophil elastase and neutrophil chemotaxis are inhibited by antigen B to protect protoscoleces from their effects; therefore, the protoscoleces continue to grow to produce secondary hydatid cysts, as in Fig. 6 (Ranasinghe and McManus 2018).

Recent studies proposed that specific Echinococcus parasite antigens can induce an adaptive immune response against cancer. EgKI-1, one of *E. granulosus* secretions, displays direct anti-cancer effects. Consequently, this canine cestode may offer some aim as a probable therapy against some types of cancer.

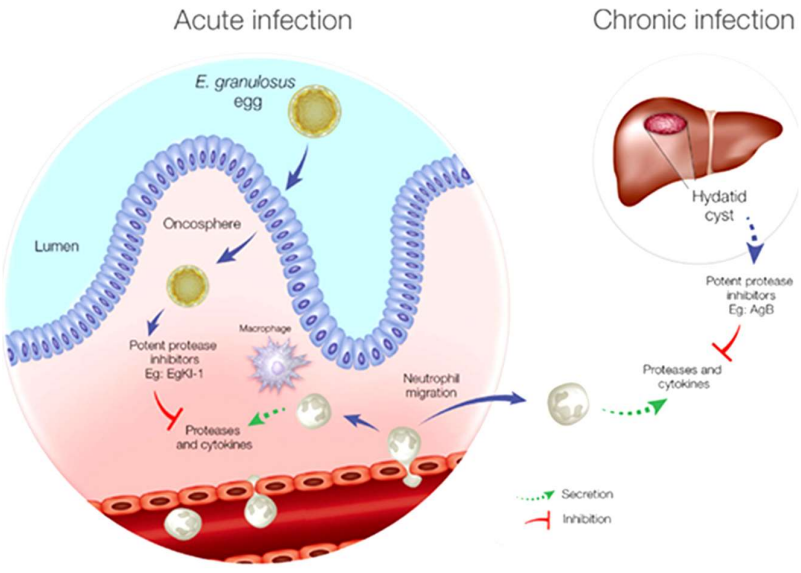

Fig. 6: Neutrophil role through acute and chronic hydatid cyst disease (Ranasinghe and McManus 2018).

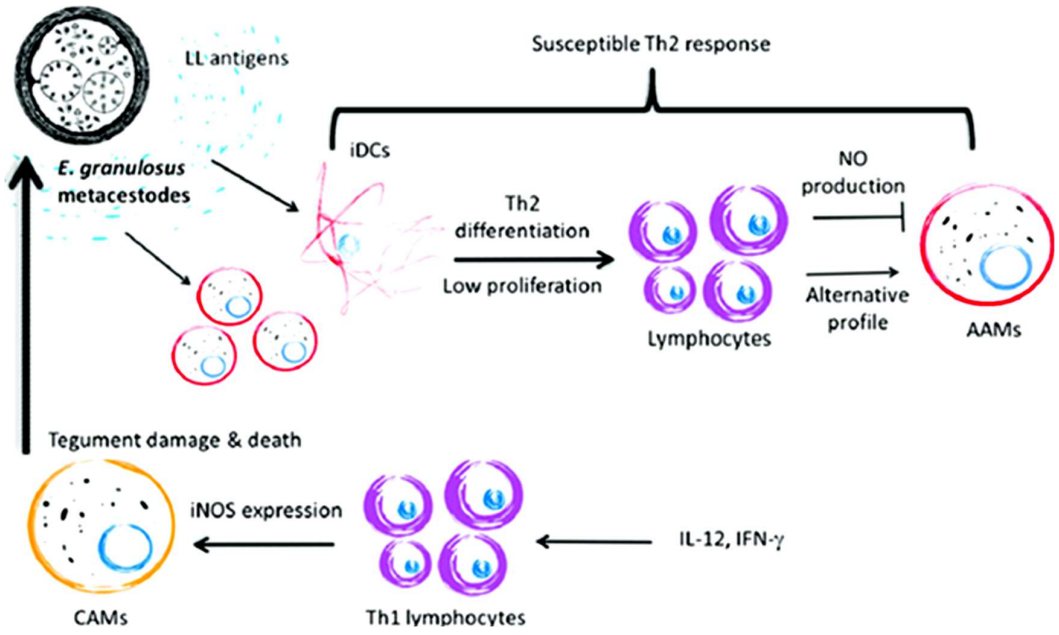

Fig. 7: *E.granulosus* survival mechanisms. Laminated layer antigens can stop the maturation of DCs, thus starting a tolerogenic Th2-response that will block NO-releasing by the induction of an alternative profile in macrophages (AAMs). If the pro-inflammatory cytokine injection induces a Th1 response, CAMs will activate to eradicate the parasite (Peón et al. 2016).

Further investigations are mandatory to progress this study and categorize other *Echinococcus*-specific immunogens for anti-cancer treatment (Ranasinghe and McManus 2018).

Immunomodulation and Immune Escaping Strategies *of E. granulosus* Parasites that Leads to Cystic Echinococcosis Disease

It is necessary to understand how the hydatid cyst of the *E. granulosus* parasite can evade the human immune response? Echinococcus parasites practice several immune evasion mechanisms during their life cycle. It comprises antigenic variation, detaching of tegument protein, and protease creation. In addition to active modulation like immunosuppression, twisting the Th1/Th2 cytokine patterns, molecular mimicry, T cell suppression, minimizing the chemotaxis of effector immune cells, and production of immunogenic molecules (Rigano et al. 2007; Zhang et al. 2008a; Zhang et al. 2008b). Previous investigations mentioned that *E. granulosus* produce many antigenic molecules in its protoscoleces and hydatid cyst fluid that can modify the immune mechanisms of the hosts. Therefore, the cytokine production changes to Th2, which assists immune evasion, leading to a long period of survival of parasites (Rigano et al. 2004; Siracusano et al. 2008). These molecules interact with the process of antigen presentation, proliferation, activation of the cell, and production of antibodies (Fig. 7), which may result in cell death and excite regulatory responses. AgB, a 12-kDa subunit protease inhibitor, modulates the host immune response as it inhibits the recruitment of neutrophils and helps the parasite escape immune responses (Shepherd et al.1991).
AgB can moderate the innate and adaptive immunity of the host; it has an essential action in the immunomodulatory process on which hydatid cysts depend for growth progression and finally result in chronic infection (Siracusano et al. 2008).
E. granulosus involves a couple of mechanisms to undermine the host's immune attack: Firstly, via passive escape, by developing into the hydatid cyst, by which it can avoid the destructive effects or secondly, by the immunomodulation of the host immunity, which is achieved by the active interrelation of the parasite with the immune system of the host leading to the reduction of the response impact (Siracusano et al. 2009).
Recently, a new immunomodulating antigen has been obtained from the cDNA library of the *E. granulosus* genome and its reaction with IgG4 from a patient with cystic echinococcosis. Antigen EgTeg is a protein of the protoscolex tegument (from which the name was derived) localized on the germinal layer of the hydatid cyst wall. Similar to AgB, EgTeg hinders chemotaxis and provokes IL-4-positive T lymphocytes as well as non-complement-fixing immunoglobulin (IgG4). Like other organisms, CE shares similar protein molecules or antigens with other helminths; Similar to the EGTeg, other antigens were discovered by screening the cDNA library of *E. granulosus* genome through their reaction with host IgE. These antigens are associated with acute cutaneous allergic symptoms. The antigens are EgEF-1 β/δ, EA21, and Eg2HSP70 and are related to the allergic disorders associated with CE (Siracusano et al. 2009).
Novel therapeutic developments for CE result from several studies regarding its immunological aspect. For example, immunomodulation via interferon-alpha, Th2-mediate immunological mechanisms, and IL-10-associated tolerance are standard features of echinococcosis and atopic allergy. Echinococcus stimulates IgE in most human infections and is related to the severity of the disease. Histamine production from circulating basophils enhanced by *E. granulosus* allergens is detectable in all sera of patients with both types of echinococcosis. Echinococcosis stresses the unclear associations between parasitic and allergic diseases. It concludes that investigating these diseases can assist in understanding how immune variation produces the pathological effects in infected persons, which resultantly may assist in discovering therapeutic or preventive medications (Vuitton 2004).

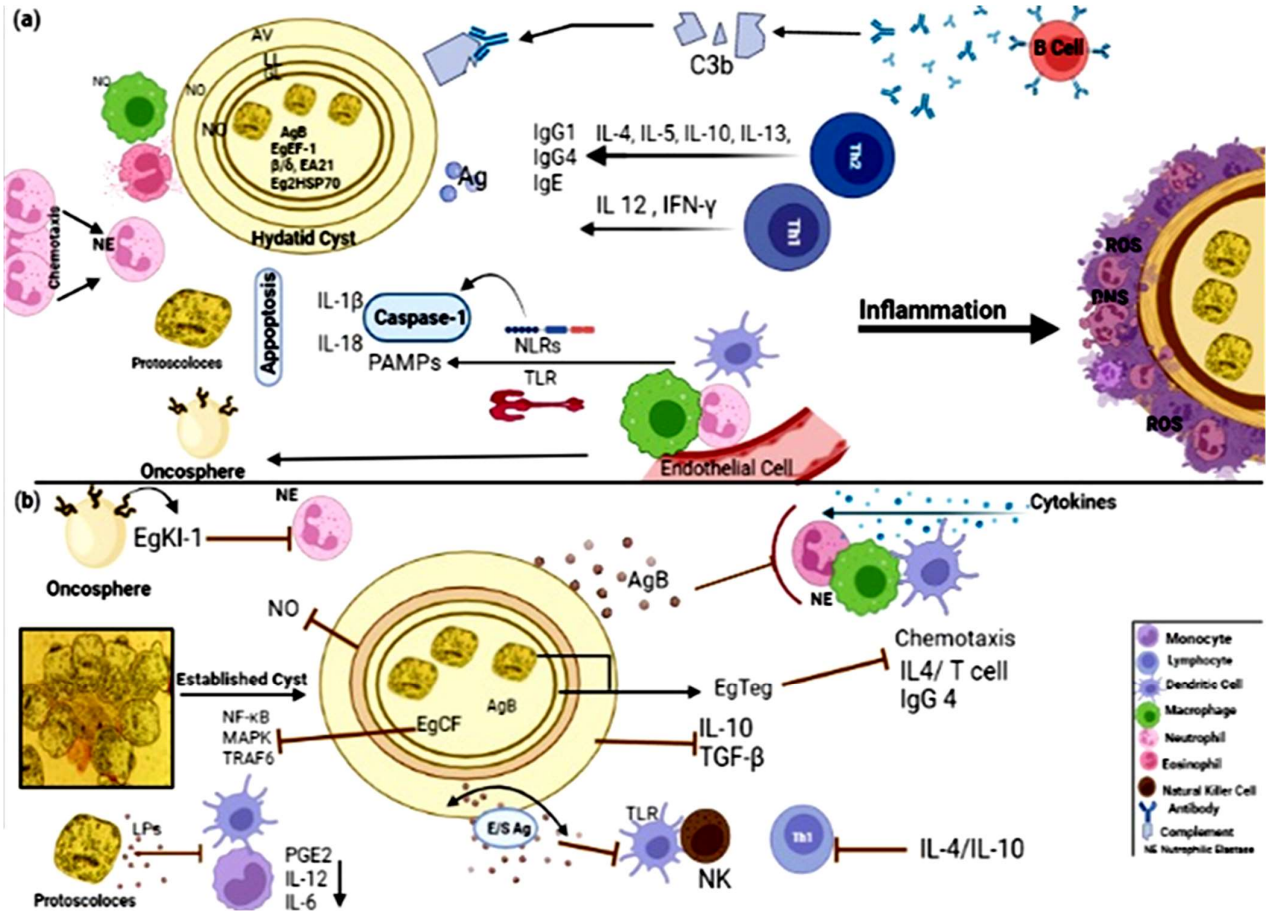

Fig. 8 (a): Expected immune response against cystic echinococcosis. (b) Proposed modulation and evading mechanisms of host defense by *E. granulosus*. Black arrows represent events indued immunity against the parasite, while brown lines represent the parasite antigens that inhibit the immune responses.

Previous findings pointed out that CE escapes the host immunosurveillance by altering the differentiation and maturation of monocyte and DCs (Rigano et al. 2007). *E. granulosus* has elaborated several defense mechanisms for protection from the host's immune attack (Siracusano et al.2012). In the frame of the variation of DCs differentiation and cytokine secretion by the hydatid cyst fluid of E.granulosus showed that *E. granulosus* metabolic product induced the production of prostaglandin E2 (PGE2) and IL-6 during treatment with human adherent (PBMCs) cultivated in GM-CSF/IL-4. The identical cultivation decreased the capability of these cells to produce IL-12, IL-6 or PGE2 as a response to lipopolysaccharides (Kanan and Chain 2006). AgB interferes with host DCs function via a couple of strategies (Rigano et al. 2007):

1. It weakens the differentiation of monocyte precursors to immature DCs, making them incapable of responding to Lipopolysaccharide stimulation.
2. AgB modulates the maturation of sentinel dendritic cells, allowing the polarization of lymphocytes into Th2 cells that benefit the *Echinococcus*.

Furthermore, antigen B secreted by the CE parasite can affect the neutrophil function by the elastase released from neutrophils and permit the hydatid cyst to escape from the immune mechanisms of the host (Silva-Álvarez et al. 2016).

More recently, Lin et al. (2021) pointed out that the E.granulosus cyst fluid (Eg CF) suppressed the stimulation of nuclear factor (NF)-κB p65 and the mitogen-activated protein kinase (MAPK) signaling pathways and the pro-inflammatory responses. This anti-inflammatory action is achieved by helping the proteasomal degradation of TRAF6 in macrophages of humans and mice.

Conclusion

Hydatid cyst disease is a parasitic life-threatening zoonotic infection. The life cycle ofE.granulosus includes the definitive and intermediate host. The hydatid cyst grows slowly and consists of germinal, laminated layers and host-derived adventitial (fibrous) layers. The first-choice treatment for hydatid cysts is a surgical operation. Chemotherapy with benzimidazole is recommended against CE. Nowadays, medication is motivated to use biosynthesized nanoparticles as novel protoscolicidal agents. Several studies used different nanoparticles within in vitro and in vivo models with acceptable results.

Echinococcus is highly immunogenic and possesses various antigens within the layers of hydatid cyst/ hydatid fluid, inducing pro-inflammatory cellular responses, specific Ab production, T cell- and other cell-mediated immune responses in humans. However, the parasite can survive in its mammalian hosts for several years, suggesting modulating the antiparasitic immune responses.

The laminated layer of the hydatid cyst is considered a first barrier against the intermediate host's immune mechanisms.

The function of this layer is to maintain the physical integrity of metacestodes and to shelter the embryonic cells of the germinal layer from host immune responses.

In CE of humans and animals, the typical response is the Th2 type which contains several interleukins like IL-4, IL-5, IL-10, and IL-13. Also, the immunoglobulins IgG1, IgG4, IgE and eosinophils, mast cells, and activated macrophages are all involved. *E. granulosus* probably manages the dialogue among the immune cells by releasing antigens that induce Th2 responses and inhibit the other regulatory T and B cells. The Th2 is related to chronic cases and might standardize parasite development. More information is required on the role of Th2 cytokines on immunoglobulins production, hydatid cyst development, and treatment efficacy. Fig. 8 summarizes the events involved in the immune response against CE and the immune evading mechanisms by the parasite.

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CHAPTER 15

IMMUNOREACTIVE PROTEINS: THEIR PRODUCTION AND IMPORTANCE IN HAEMOPROTOZOAN DISEASES

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INTRODUCTION

Most epidemiological studies on haemoparasitic infections in veterinary medicine are concerned with the prevalence. Serological methods have been used to diagnose haemoparasitic infections, which have been mainly diagnosed with traditional methods for many years, since the 1990s. In recent years, the prevalence of haemoparasites can be precisely determined by DNA-based methods. These methods are valuable research tools, but costly and time-consuming for large-scale epidemiological studies. Therefore, it is necessary to develop sensitive and cost-effective diagnostic methods, even applicable in the field, to implement effective control strategies in protozoan infections. Since parasite antigens prepared from infected tissues contain some host proteins, false-positive reactions are encountered in serological tests using these antigens. Immunoreactive proteins obtained using recombinant DNA technology are more purified and do not carry host factors, so the speed, specificity, and sensitivity of tests using these proteins are very high. These proteins can be easily used as antigens in a quantitative test such as ELISA or an immunochromatographic test that can be applied even in the field. Immunoreactive proteins are also significant as vaccine candidates in controlling infections (Kumar et al. 2002; Huang et al. 2006; Terkawi et al. 2007; Ooka et al. 2012).

Serological methods are widely used in epidemiology. However, no commercial product is used for the serodiagnosis of all parasitic diseases. ELISA and IFAT are the most widely used serological methods in the diagnosis of protozoan diseases. The antigens used in the IFAT are prepared with parasites grown in culture or obtained from an infected host. The disadvantages of the IFAT include the need for an expert and a fluorescent microscope during the evaluation phase, the observation of cross-reactions between some species, and the long evaluation phase. Therefore, it is essential to develop parasite-specific antigens and to use them in diagnostic methods suitable for large-scale epidemiological studies. ELISA finds more widespread use in epidemiological studies due to its automation, no need for an expert, objective and quantitative evaluation of the results, the possibility of examining many samples together, and its higher specificity compared to the IFA test. Various types of ELISA are used to diagnose protozoan species such as *Babesia ovis, Toxoplasma gondii, Neospora caninum, Cryptosporidium parvum.* In the past, crude antigens prepared from infected animal blood were used in ELISA for the indirect diagnosis of haemoprotozoan infections. Accordingly, high cross-reactions with serum proteins were observed during the test. This situation has recently disappeared with the use of recombinant immunoreactive proteins as antigens in ELISA tests, and the specificity and reliability of ELISA have been increased (Duzgun et al. 1991; Sevinc et al. 2015a; 2015b; Ceylan and Sevinc 2020).

Studies on *Plasmodium* and some *Babesia* species have revealed the host immune system reaction against the merozoite proteins of these protozoa. Based on this, great importance has been given to studies to determine the immunoreactive proteins of parasites. Immunoreactive proteins are of great importance both as antigens in indirect diagnosis and as vaccine candidates that can be used in protection against the disease since they are the parts of the parasite stimulating the host immune system. Recombinant proteins are synthesized in vitro with the help of recombinant DNA technology. Parasite's immunoreactive proteins are being replicated by inserting genes into suitable vectors and transferring these vectors to a suitable host (Brown et al. 2006; Terkawi et al. 2007; Temizkan and Gozukirmizi 2018).

The development of new and practicable diagnostic methods and protective vaccines against diseases is one of the priority areas in veterinary research. This book chapter discusses recent developments in recombinant proteins and their role in diagnosing protozoan diseases and vaccine development. The first and most important step in this area is the selection of proteins or antigens to be used during the serological examination or in the vaccine development. For this purpose, various proteomic, genomic, and immunological screening protocols are used. Sometimes this selection is more functional or requires more biological data. Once the candidate gene encoding a particular protein is identified, several key steps are initiated, including cloning, production, purification, validation, and assay design. The selected protein can be produced in a heterologous expression system or host cells grown under certain laboratory conditions. Among the alternatives, the most frequently used heterologous expression system is *Escherichia coli* (Brondyk et al. 2009; Sözen et al. 2010).

The usage of immunoreactive proteins in different fields

In parallel with the advances in molecular biology, additional diagnosis, treatment, and prevention methods can be developed in the field of health by various changes which have

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recently been made on micromolecules such as DNA, RNA, protein, and antibodies. Using recombinant DNA technology, molecules of a pathogenic microorganism can be synthesized at the desired time and amount. These can be used for both diagnostic methods and vaccine development. Recombinant antigens in diagnosing diseases have increased the speed, specificity, and sensitivity of diagnostic tests. There are many recombinant proteins used in veterinary medicine to diagnose diseases. Recombinant antigens determined as a result of immunological screening of cDNA libraries are utilized in most of the methods used in the diagnosis of diseases such as malaria, trypanosomiasis, babesiosis, and leishmaniosis (Lodish et al. 2004; Fernandez-Robledo and Vasta 2010).

Proteins are encoded by genes to carry out the life activities of living creatures. In the case of disease, the protein density of the organism changes, depending on the biological stages of the disease-causing agent in the host body. In such cases, it may be possible to solve the problems related to diseases by analyzing the structural properties, functions, and interactions of the synthesized proteins with other molecules. While parasites continue to develop after entering the host body, many immunogenic proteins are released by the parasite into the host body. Accordingly, the host immune system tries to inhibit the development of the parasite by activating defense factors against these proteins (Lodish et al. 2004; Konuk 2004; Brown 2009; Sözen et al. 2010).

The vast majority of vaccines used to protect against various organisms are live vaccines obtained by attenuation of pathogenic microorganisms or inactivated vaccines. Due to the risk of active infection in attenuated live vaccines or live vaccines with incomplete inactivation, immunoreactive proteins have been used as immune system stimulating factors in vaccination studies in recent years. When a protein vaccine is administered to an individual, the immune mechanism of the host becomes active against the epitopes in the protein's structure that makes the protein antigenic or immunogenic. The development of the infectious agent in the host is prevented by the antibodies formed against the protein antigens and the developing cellular immune reactions in the body of the vaccinated individual (Lodish et al. 2004; Konuk 2004; Brown 2009; Sözen et al. 2010).

Immunoreactive proteins of any pathogen are valuable products of medicine and veterinary health. They are used both as a specific antigen in the serological diagnosis and as immunogens activating the host's immune system. Proteins are expressed in low concentrations and are therefore difficult to isolate and purify in large quantities by standard biochemical techniques. To produce these proteins in sufficient quantities and carry out basic research on their structure and function, systems that can produce them in large quantities at a reasonable cost are required. Recombinant DNA techniques, which transform *E. coli* cells into factories synthesizing such proteins, are now utilized to produce human proteins used for therapeutic purposes, such as blood coagulation factor, granulocyte colony-stimulating factor, insulin and growth hormone. In veterinary medicine, they are primarily used to diagnose diseases and develop vaccines (Jenkins 2001; Brown et al. 2006; Ferrer-Miralles et al. 2009).

Identification of immunoreactive proteins of parasites

Producing immunoreactive proteins in sufficient quantity and purity is possible by cloning the genes encoding these proteins using recombinant DNA technology and synthesizing them in a heterologous expression system. The most important resources used to identify genes encoding immunoreactive proteins are DNA libraries (Temizkan and Gozukirmizi 2018).

DNA libraries

A researcher who uses recombinant DNA technology to clone a unique gene or DNA fragment must have sufficient knowledge of the gene of interest to achieve the goal successfully. Therefore, DNA libraries are needed. Firstly, DNA is isolated from the studied microorganism and subjected to the cutting process with restriction enzymes. DNA fragments obtained as a result of cutting are cloned in various vectors (λ cloning vector, BAC, YAC, etc.) to reveal many colonies carrying certain genome regions. All clones contain at least one copy of each gene in the genome from a DNA library. DNA libraries can be screened to detect a specific gene or clone containing DNA fragments. DNA libraries are also used to isolate some specific genes and genome organization. DNA libraries are separated into two groups, as genomic DNA (gDNA) library and complementary DNA (cDNA) library, according to the type of DNA prepared in these libraries. Genomic DNA libraries are assumed to represent the entire DNA sequences in an organism, whereas cDNA libraries contain only the transcribed parts of the genome (Sözen et al. 2010).

A specific gene can be removed from the libraries and can be studied. These libraries can be reproduced in need, used for many different purposes, and preserved for years. Since only the expressed genes in an organism are transcribed into mRNA, if the material cloned in the preparation of the DNA library is cDNA obtained from mRNA, the clones formed only cover a group of the total number of genes in the cell. The mRNA cloning method is more beneficial than gDNA if the desired gene is expressed in a particular cell type at high levels. mRNA cannot bind to the cloning vector. Therefore, after it has been converted into a double-stranded cDNA molecule, it can be ligated into a vector and cloned. The resulting cDNA clones represent mRNA. If the mRNA is prepared from a single-celled protozoan, the genes encoding all the proteins synthesized by the protozoan during development are largely contained in the cDNA library. It is a much more laborious process to detect the equivalent of a gene of cDNA library in the gDNA library (Brown 2009; Sözen et al. 2010).

Genomic DNA libraries

A gDNA library is a collection of clones thought to contain one copy of all DNA sequences in the genome. These libraries can be built in a variety of ways. At first, gDNA is cut with various restriction enzymes, and the resulting fragments are cloned. However, this method has several disadvantages. For example, the gene to be studied may have recognition sites for the restriction enzyme, and as a result, the gene may be undesirably fragmented and cloned separately. Another disadvantage is that the fragments obtained by cutting with the enzyme are small. This means that when large eukaryotic genomes are considered, the library to be created will contain many clones. This increases the workload of scanning the library for a specific gene and is not economical. To overcome such drawbacks, there is need to obtain longer DNA fragments to create a library with few clones, in which genes are placed in a

single clone as much as possible. It is possible to achieve this by partially cutting the gDNA with various enzymes (Sau3A, Mob1) with a 4-base pair recognition region and reducing the amount of enzyme placed in the medium and incubation time during this process. After cutting, DNA fragments representing the whole genome and having the desired size are collected by agarose gel electrophoresis and cloned directly. Various λ displacement vectors (EMBL4), cosmid vectors (pJ88, c2RB), artificial yeast chromosomes (YAC) and artificial bacterial chromosomes (BAC) vectors are used for cloning. Generally, bacteriophage and cosmid vectors are used to create gDNA libraries. After the DNA fragments are transferred into the selected vector, they are transferred to *E. coli* and amplified in a selective medium. It is assumed that each resulting colony contains a different DNA fragment clone. Then, by using various methods, studies can be carried out to reach the targeted gene from these gDNA libraries (Sözen et al. 2010; Allison 2014; Temizkan and Gozukirmizi 2018).

cDNA libraries

cDNA libraries are constructed using the parasite's mRNA molecules. These libraries are the most critical resource for detecting genes encoding immunoreactive proteins. When cDNA libraries are subjected to immunological screening, it can be determined that many genes in the library encode immunoreactive proteins. Once a specific gene is obtained, its molecular, genetic and immunological properties can be examined, and solutions can be developed for diseases (Brown et al. 2006). Since the RNase enzyme rapidly degrades single-stranded mRNAs, mRNA isolation in vitro is tough. Therefore, single-stranded mRNA molecules are converted to their DNA counterparts, cDNAs. cDNA libraries can be created by cloning cDNAs synthesized from mRNA. cDNA libraries allow us to have information about the active genes at the time of mRNA isolation from the studied cell type (Sözen et al. 2010; Temizkan and Gozukirmizi 2018).

To prepare a cDNA library, total RNA is isolated from tissues or cells of interest, followed by mRNA isolation. Various essential elements such as reverse transcriptase enzyme and oligo-dT primers are required to convert the isolated mRNA to cDNA. The restriction sites on the cDNA are methylated to protect them from cleavage by the restriction enzyme. Then the recognition site sequence of a restriction enzyme, which can form sticky ends, is added to both ends by the DNA ligase enzyme. Then, the ends of this cDNA double strand are cut with a restriction enzyme to obtain sticky ends. Meanwhile, the region of the cloning vector bacteriophage λ to which foreign DNA is inserted, is also cut with the same enzyme and the same sticky ends are obtained. Complementary λ-phage and cDNA assembly with sticky ends are mixed under appropriate conditions and covalently coupled with DNA ligase. Each of the resulting recombinant DNA molecules contains a piece of cDNA located between the two arms of the DNA of the λ vector. The virions containing the recombinant DNA are then packaged in vitro. After packaging, the recombinant λ-phages are transferred onto petri dishes coated with *E. coli* cells. Phages adhere to the membrane of tail *E. coli* cells, transfer the DNA molecule in the head region into the cell, and many new phages are formed inside the cell. The newly formed phages are released by dividing the cell and continue to multiply in the same way by entering other cells. Meanwhile, numerous individual plaques of transparent appearance are formed due to the disintegration of the cells. Since each plaque is derived from a single recombinant phage, all new phages formed in a plaque are genetically identical and form a clone carrying a cDNA derived from a single mRNA. The plaques formed are called a λ-cDNA library (Mullinax and Sorge 2003; Lodish et al. 2004; Brown 2009; Temizkan and Gozukirmizi 2018). The steps of constructing a cDNA library are shown in Figure 1.

Immunoscreening

After the cDNA library is created, it can be immunologically screened to identify various antigenic proteins. Serological screening of libraries from microbial pathogens plays an essential role in the development of new drugs and recombinant vaccines. Identification of a portion of the pathogen proteome recognized by the host immune system is essential in identifying epitopes of the pathogen that trigger protective immunity. Systematic mapping of complex parasite immunome is faster and more efficient than the methods initially developed for serological screening of expression libraries. Genomic and cDNA libraries of various organisms contain thousands of clones. Two general approaches are used to screen libraries to detect clones carrying a DNA region or gene region of interest. The first of these is the detection with the help of oligonucleotide probes that can bind to the clone; the second is the detection based on the expression of the encoded protein (Ardeshir et al. 1985; Sözen et al. 2010).

In screening a λ cDNA library, a nitrocellulose membrane is firstly placed on the petri dish containing many λ clones, and the clones then adhere to the membrane. The membrane is then tested using a radioactive-labeled probe specific for recombinant DNA containing the DNA fragment of interest. Once a cDNA clone encoding a specific protein is found, the full-length cDNA can be labeled with radioactive substances and used as probes to locate clones containing fragments of the gene of interest from the genomic library. The appearance of a spot on the autoradiogram indicates a recombinant clone λ containing the DNA complementary to the probe. The position of the spot in the autoradiogram is the mirror symmetry of the particular clone in the petri dish. Well-separated plaques can be formed by recoating the detected phage particles. Finally, pure isolates are obtained by repeating the hybridization method (Lodish et al. 2004).

Nowadays, various immunodominant or immunoreactive proteins belonging to protozoa can be obtained by scanning cDNA libraries prepared from many parasitic protozoa causing diseases in humans and animals. These proteins are then used in diagnostic studies and vaccine studies (Bose et al. 1990; Kappmeyer et al. 1999; Ikadai et al. 2000; Fukumoto et al. 2001; Boonchit et al. 2002; Goff et al. 2003; 2006; 2008).

Screening of cDNA library using specific antibodies

Recombinant protein production is complicated in practice. To produce large quantities of a protein, the cDNA clone encoding this protein must first be removed from the library. One of the procedures that enable the detection of the desired clone from the cDNA library is the hybridization-probing technique that directly identifies the recombinant DNA molecule. The main alternative to this technique, which has limited use, is immunological screening. The proteins encoded by the cloned gene are detected in the immunological screening method. Specific antibodies are

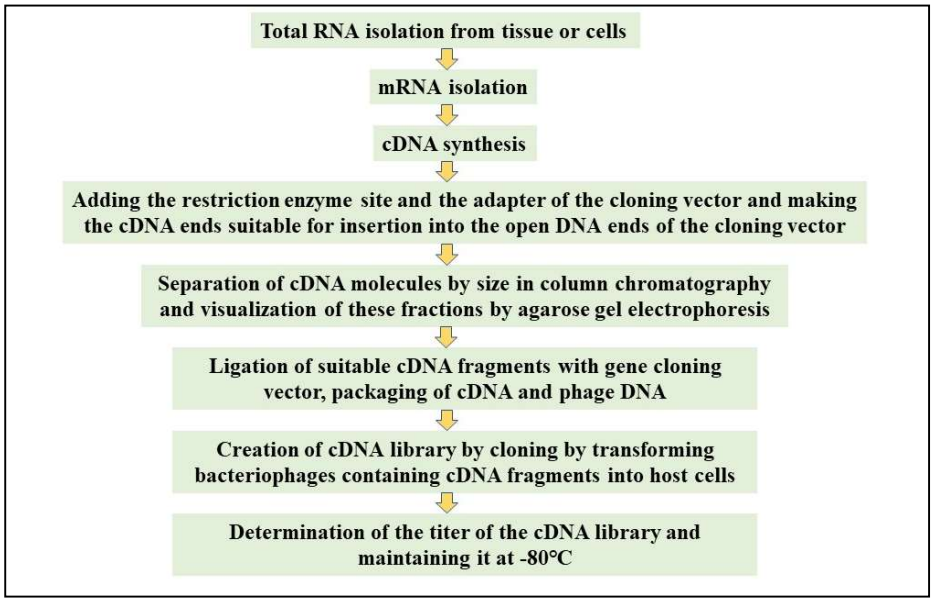

Figure 1. The steps of constructing a cDNA library.

needed to detect protein in recombinant colonies. After transferring the recombinant colonies to the polyvinyl or nitrocellulose membrane, they are treated first with the primary antibody solution and the enzyme-labeled secondary antibody. Positive colonies are then highlighted by using a substrate to express the enzyme. After the positive colonies in the medium are removed from the medium and cloned again in a suitable host, the relevant gene is extracted, the sequence information is determined and then it is possible to produce it in a bacterial expression system at any time and in desired quantities by placing it in an expression vector to produce a large amount of immunoreactive protein (Mullinax and Sorge 2003; Lodish et al. 2004; Brown 2009; Sözen et al. 2010; Bayraç et al. 2012; Temizkan and Gozukirmizi 2018).

Synthesis of proteins from genes encoding immunoreactive proteins

To produce recombinant proteins in large quantities, expression vectors must be constructed to synthesize the encoded protein when transferred into *E. coli* cells. Expression vectors, unlike cloning vectors, contain a promoter and a terminator region that can initiate protein synthesis. Transcription of the target gene begins at the promoter region and ends at the terminator sequence. The most abundant promoters in expression vectors are the Lac, Tac and T7 promoters. Most expression vectors have multiple cloning sites containing the ribosome binding sequence that provides efficient translation initiation and restriction enzyme cleavage sites. For example, a plasmid expression vector producing the β-galactosidase protein has a fragment of the *E. coli* chromosome containing the lac-promoter and the lac-z gene inserted in its DNA. When lactose or its analog isopropyl thiogalactoside (IPTG) is present in the culture medium, the RNA polymerase enzyme transcribes the lac-z gene starting from the promoter region, and lac-z-mRNAs are produced. The β-galactosidase protein is produced from these mRNAs. Protein production continues as *E. coli* cells proliferate in a culture medium containing IPTG. Thus, some eukaryotic proteins can be produced in *E. coli* cells using plasmid vectors containing promoter regions (Brondyk et al. 2009; Zerbs et al. 2009; Temizkan and Gozukirmizi 2018).

Product purity and high yield are critical in recombinant protein production. Therefore, eukaryotic proteins produced in *E. coli* expression systems are often synthesized as a fusion protein to facilitate differentiation from endogenous *E. coli* proteins. Most expression vectors produced in biotechnology carry expression tags such as glutathione S transferase (GST), maltose-binding protein, and histidine. Expression tags are required for the purification of the protein after translation. GST is one of the most commonly used expression tags in recombinant protein production. GST-labeled fusion proteins are expressed at high levels in the *E. coli* expression system. Recombinant proteins can be easily purified by glutathione affinity chromatography at the end of production (Hunt 2005; Malhotra 2009; Graslund et al. 2008).

The pGEX series plasmid vectors produced by different companies have been successfully used to produce proteins encoded by specific genes detected by immunological screening from cDNA libraries in *E. coli* expression systems. pGEX expression vectors carrying ampicillin resistance gene, tac promoter, lac suppressor gene region, and GST tag are among the most widely used plasmids for expressing proteins. When IPTG is added to the culture medium, the tac promoter region in these vectors is induced, and protein expression then begins (Ikadai et al. 1999; Fukumoto et al. 2001; Liu et al. 2010; Ooka et al. 2011; Luo et al. 2011; Cao et al. 2013).

Cloning vectors

In order to molecularly investigate the structure and functions of a gene, that gene must be purified in large quantities. The most important part of cloning a specific DNA is combining it with a vector DNA molecule. Vectors are intermediary molecules commonly used for gene cloning. The vector should have the ability to make copies in a suitable host cell. Recombinant DNA molecule consisting of vector DNA and a foreign DNA fragment attached to it, is transferred into a host

cell. This recombinant DNA replicates in the host cell, producing multiple identical copies called clones. As host cells proliferate and increase in number, recombinant DNA is also passed on to newly formed offspring, creating a population of identical cells carrying the cloned sequences. Then, the cloned DNA fragment can be retrieved from the host cell, purified, and used for various purposes such as gene expression, immunological screenings and sequence analysis (Sözen et al. 2010; Allison 2014).

DNA fragments obtained after cutting with restriction enzymes, cannot enter the bacterial cell directly under normal conditions. Therefore, they must be inserted into a vector DNA that can replicate in bacteria. The vectors used in cloning must have some properties. The vector should have small molecule structure. It should be easily isolated from bacteria and carry a suitable replication center for the host. Many enzymes must have only one recognition site in vector DNA. When this region is cut with an enzyme, it is used to insert the gene fragment cut with the same enzyme. Unlike these, vector DNA must contain a selective marker gene to distinguish between host cells with and without the vector, such as a gene responsible for antibiotic resistance or a non-host enzyme gene (Allison 2014).

The majority of cloning vectors have been developed for use in *E. coli* cells. In recombinant DNA technology, many cloning vectors are used, depending on the size of the DNA to be cloned and the purpose of the study. Although plasmid and bacteriophage-λ vectors are generally used as vectors for cloning purposes, various cloning vectors such as cosmid vectors, bacterial artificial chromosomes (BAC), yeast artificial chromosomes (YAC), human artificial chromosomes (HAC), and mammalian artificial chromosomes (MAC) are also used (Sözen et al. 2010; Temizkan and Gozukirmizi 2018).

Expression systems

Many proteins produced for research purposes are generally expressed at low concentrations. These proteins can be produced in large quantities with recombinant DNA technology. Recombinant protein production requires cDNA cloning encoding the protein of interest in the expression vector. The choice of an appropriate method to express a recombinant protein is an essential factor in obtaining the desired quantity and quality of the recombinant protein within a reasonable time. Misfolding, deficiency in posttranslational modifications, or inappropriate modifications may occur due to the selection of the wrong expression host. Factors such as the size of the protein and the number of disulfide bonds must be considered when choosing an expression system (Brondyk et al. 2009; Sözen et al. 2010; Temizkan and Gozukirmizi 2018).

Expression vectors, which have all the features of cloning vectors, also carry transcription-initiating promoter and terminating regulatory sequences, which will ensure the desired level of transcription of the gene. Transcription of the gene begins in the promoter region and ends when the terminator sequence is reached. Some promoters are found in expression vectors; lac, tac, phage λPL, and phage T7. Most of the current expression vectors have a ribosome binding site above the start codon, which provides an efficient translation initiation in bacteria. Expression vectors have fewer restriction enzyme cut sites in the multiple cloning region than cloning vectors (Sözen et al. 2010). *E. coli* is one of the most preferred systems as expression systems. Evaluation of recombinant gene expression in *E. coli* takes less than a week. The culture media are inexpensive and relatively easy to understand to increase bioproduction. Recently, *E. coli* systems with T7 promoters are being widely used to express proteins. In addition, *Bacillus subtilis*, *Pichia pastoris*, *Baculovirus*/insect cell, and mammalian expression systems are utilized (Brondyk et al. 2009; Sözen et al. 2010; Temizkan and Gozukirmizi 2018).

Brewer's yeast also reproduces rapidly like bacteria and is inexpensive to produce. They are used in protein expression because they have enzymes to make posttranslational changes. However, since they have many active proteases, protein production can yield reductions. Baculovirus expression vectors in insect cell cultures can also be preferred as protein expression systems because they provide high amounts of protein and allow many of the correct folding and posttranslational changes in proteins. Yeasts have also been used successfully to express proteins. The yeast *Saccharomyces cerevisiae* was the first to be routinely used to express recombinant proteins. However, *Pichia pastoris* has recently become the yeast of choice as it allows higher levels of recombinant protein expression (Cregg et al. 1985; Brondyk et al. 2009; Sözen et al. 2010).

Viruses are one of the most frequently used expression systems in vaccine development studies. Using the baculovirus expression vector system, various proteins used for treatment against many diseases in humans are produced. It is widely used to eliminate drug raw material deficiencies and describe genes belonging to some organisms (Demirbag et al. 1998; Ikonomou et al. 2003; Brondyk et al. 2009; Fernandez-Robledo and Vasta 2010; Temizkan and Gozukirmizi 2018).

Purification of proteins

In order to study the structure and functioning of a protein, it must first be purified. Since proteins differ in some properties such as charge, size and solubility in water, many methods are used to isolate proteins. The two properties mainly utilized in separating proteins are the size of the protein and its binding affinity for a particular binding molecule. Various centrifugation methods, electrophoresis, and liquid chromatography methods are used to separate proteins. The basic principle of centrifugation is the precipitation of particles in a mixed suspension at different rates. There are varieties such as differential centrifugation and velocity-zonal centrifugation. In electrophoresis, which is one of the most commonly used techniques, the molecules in the mixture are separated under the influence of the electric field. SDS-Polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis are commonly used electrophoresis types to separate proteins by molecular weight (Lodish et al. 2011).

Another method used to separate proteins is liquid chromatography. The principle of liquid chromatography is that dissolved molecules interact with a particular solid surface in different ways, such as bonding or separating according to the chemical properties of the surface. If the solution is allowed to flow across the surface, molecules interacting with the surface bind to specific areas, while other molecules do not interact with the surface flow. The sample used in liquid chromatography is placed on spherical particles compressed into a cylindrical column made of glass or plastic. It is then sent down by gravity, pump, or hydrostatic pressure. The fraction leaving the column is collected to analyze the presence of the desired protein. The structure of the filler particles in the

column determines the separation of proteins according to charge, mass, and binding affinity. There are various liquid chromatography methods. These are gel filtration chromatography, ion-exchange chromatography, and affinity chromatography. Among these, affinity chromatography is the most widely used in studies on protozoa (Lodish et al. 2011; Allison 2014).

Some recombinant immunoreactive proteins belonging to protozoa and their usage areas

There are many studies on protozoan diseases using molecular and biotechnological methods, some of which are as follows. Studies detecting immunodominant proteins of *Theileria uilenbergi*, one of the blood protozoa of sheep, stated that these proteins could be used both in developing diagnostic methods and in designing vaccines (Gao et al. 2002; Abdo et al. 2010; Liu et al. 2010). The merozoite antigens of a *Babesia* strain (*Babesia* sp. BQ1-Lintan) genetically similar to *Babesia motasi* in sheep were purified and used in the ELISA test in China (Guan et al. 2010).

Harning et al. (1996) synthesized the SAG1 (surface antigen1) protein, the immunodominant surface antigen of *Toxoplasma gondii*, as a histidine-labeled fusion protein in *E. coli* cells. The recombinant protein was detected by immunoscreening using *T. gondii* specific human IgG and IgM antibodies and mouse monoclonal antibody (S13). These recombinant fusion proteins purified in Ni-chelate column and liquid chromatography were used as antigens in diagnostic methods to detect anti-SAG1 specific IgG and IgM antibodies.

Ikadai et al. (1999) prepared a cDNA expression library from *Babesia caballi* merozoite mRNAs and immunologically screened this library with BC11D, a monoclonal antibody against *B. caballi* merozoite rhoptry protein. As a result, a cDNA fragment encoding a 48 kDa protein of *B. caballi* was cloned and synthesized as a GST-tagged fusion protein in *E. coli* cells by the pGEX4T expression vector. This recombinant protein was used as antigen in the ELISA test. Specific antibodies were determined in the serum of horses infected with *B. caballi*, and no cross-reactions were observed when examined with ELISA using this antigen. As a result, it was stated that this simple and highly sensitive test could be used in the field to diagnose horses infected with *B. caballi*.

Erster et al. (2015) identified the gene encoding *Babesia ovis* surface protein D (BoSPD) from the cDNA library prepared from *B. ovis* merozoites and then cloned this gene in a plasmid vector. They developed a PCR in which this gene was selected as the target gene to detect *B. ovis* in field samples, experimentally infected sheep, and *Rhipicephalus bursa* ticks.

Several genes of *Theileria annulata* have been cloned, sequenced, and recombinant proteins with various names such as TaD, TaSE, TaSP and TamtHSP70 have been expressed (Schnittger et al. 2002; Schneider et al. 2004; Schneider et al. 2007). Among these, the TaSP recombinant protein is suitable for diagnosing tropical theileriosis (Seitzer et al. 2008).

Toxoplasma gondii matrix antigen (MAG1) was cloned, purified, and used in serological techniques by Holec et al. (2007).

Zhou et al. (2007) obtained a cDNA encoding the RAP-1 (rhoptry-associated protein 1) homolog by immunoscreening from a library, they prepared from *Babesia gibsoni* merozoite mRNAs. The whole nucleotide sequence of the gene was found as 1740 bp. Computer analyzes showed that the sequence contains a 1425 bp ORF (open reading frame), which encodes a protein with a molecular weight of 52 kDa. This protein was identified as *Babesia gibsoni* RAP-1 (BgRAP-1) based on sequence similarity. BgRAP-1 gene was expressed in *E. coli* BL21 strain and used as antigen in recombinant BgRAP-1 ELISA assay. It was determined that recombinant BgRAP-1 protein might be useful as a diagnostic antigen for detecting antibodies in dogs infected with *B. gibsoni*.

Pathogen-specific immunoreactive proteins used in the serological diagnosis of *B. ovis* infections in sheep have also been produced. Two immunoreactive proteins were obtained from *B. ovis* by recombinant methods. To detect immunoreactive proteins, a lambda phage cDNA library was constructed from mRNA molecules of *B. ovis* merozoites. As a result of the immunological screening of this library using immune serum, cDNA fragments encode two important proteins named *Babesia ovis*-secreted antigen 1 (BoSA1) and *Babesia ovis*-secreted antigen 2 (BoSA2) were determined. The BoSA1 cDNA fragment consists of an open reading frame of 1137 base pairs, and this open reading frame encodes the BoSA1 protein consisting of 378 amino acids with two internal repeat domains. The length of the BoSA2 cDNA fragment consists of 1161 base pairs and encodes the BoSA2 protein consisting of 385 amino acids with two internal repeat domains. These fragments cloned in pGEX expression vectors were amplified in competent *E. coli* DH5α cells and the proteins encoded by these fragments were synthesized as GST-fusion proteins by IPTG induction. The antigenicity of the proteins purified by glutathione affinity chromatography after synthesis was investigated by Western blot and ELISA methods. The results showed that these recombinant proteins could detect *B. ovis*-specific antibodies in both experimental and natural infected sheep. Therefore, these proteins appear promising as antigens that can be used to develop serological methods for the diagnosis of *B. ovis* infections (Sevinc et al. 2015a; 2015b). In addition, a recombinant protein named ovipain-2 obtained from *B. ovis* seems promising in subunit vaccine and chemotherapeutic drug development studies (Carletti et al. 2016).

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CHAPTER 16

BABESIOSIS- A THREAT TO LIVESTOCK AND COMPANION ANIMALS

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INTRODUCTION

Parasitism is a biological process in which one partner (parasite) is dependent upon another host for its physiological needs by causing deleterious effects on its health. It is one of the major global issues that can encounter every single species living on the planet. There are many diseases related to ectoparasites that are of zoonotic and economic importance like leishmaniasis, babesiosis, hydatidosis and toxoplasmosis (Hussain et al. 2021). Babesiosis is an important parasitic zoonotic disease that can be very hard to eradicate due to stubborn ticks.

It is the most lethal tick-transmitted disease which is caused by haemoparasites of the protozoan genus Babesia, phylum Apicomplexa, order Eucoccridiorida, suborder Prioplasmorina, and family Babesiidae (Telford et al. 1993; Radostits et al. 2006). These are the second most common blood-borne parasites of mammals after the trypanosomes (Telford et al. 1993). This disease is epidemic in almost all regions of the world, thus affecting many species of mammals with a foremost impact on cattle and man. After trypanosomes, babesia species are the second most abundant species found in the blood of mammals and have been reported to infect all kinds of domestic and wild animals, including marsupials and humans, as well as some birds (Schnittger et al. 2012). This disease is primarily characterized by fever, hemolytic anemia with frequent hemoglobinuria, and death (Zintl et al. 2003; Bock et al. 2004; Brayton et al. 2007).

In cattle, babesiosis is mainly caused by ixodid ticks and is majorly part of the tropical and sub-tropical climate. *Babesia bovis* and *Babesia bigemina* are the two most important species in cattle that have heavy economic losses in the livestock sector (Mosqueda et al. 2012). Parasites usually reproduce in the red blood cells and form a pear-shaped appearance called piroplasms. Infected erythrocytes may cause cerebral babesiosis as the piroplasms travel into the brain capillaries. (Radostits et al. 2006).

Host immune response plays an important role to cease the further pathogenesis of babesiosis by doing immune-mediated erythrocyte lysis which starts cytokines activation. Eventually, this will lead to vasodilation, hypotension, increased capillary permeability, endothelial damage vascular stasis, lower blood pressure, oedema, and intravascular coagulation (Ahmed 2002). Infected erythrocytes are usually induced due to the presence of piroplasms in capillary beds which cause deleterious pathophysiological effects in brain and lung. Hence, it will create hemolytic anemia during the infections caused by B. bovis (Brown et al. 1999; Bock et al. 2004).

Overview

Babesiosis, commonly known in cattle as tick fever or cattle fever which is caused by protozoan parasites belonging to genus Babesia. These are intraerythrocytic parasites that cause the lysis of red blood cells, leading to anemia, jaundice and hemoglobinuria. It may cause severe complications to livestock, including production and economic losses (Mosqueda et al. 2012). The primary source of its transmission is ticks, belonging to Rhipicephalus spp., which is a single host tick. The other domestic animals are at risk like goats, sheep, pigs, horses, cats, and dogs (Ahmed 2002). According to a study, there is an association between Babesiosis and rational use of antiprotozoal drugs (Rizk et al. 2019). The ecology of area and management practice also play important role in disease spread (Rizk et al. 2017).

History

Babesia was previously characterized through the host and presence of trophozoites in RBCs having different size, shape, and quantity of merozoites. On the bases of size, babesia is also divided into two groups, one is large, and the other is small. Babesia also divided into 5 groups based on piroplasms. The host-based division includes babesia from wild rodents, felids, canids called microti group and if Babesia from dogs and human is called western piroplasms, Duncani group or prototheilerids (Criado-Fornelio et al. 2003).

According to certain studies, unguilibabesids and babesids belong to same monophyletic group, on the other hand theilerids are divided into three groups (Nakajima et al. 2009). Regardless of divisions, multiple research show that the organisms now classified as Babesia are polyphyletic (Lack et al. 2012). There is uncertainty about the real number of species, even at the species level, for example, Babesia microti is the most common cause of babesiosis in the US, Europe, and Asia includes at least four 'named' types US, Munich, Kobe, and Hobetsu (Uilenberg 2006). According to many studies, Babesia infection is reported from many rodents and mammals (Nakajima et al. 2009).

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The white-footed mouse (Peromyscus leucopus) is the principal reservoir of human babesiosis in the US (Stafford et al. 1999). It is reported that many rodents have also shown babesiosis like meadow volves (Microtus pennsylvanicus), short-tailed shrews (Blarina brevicauda), brown rats (Rattus norvegicus), Eastern cottontail rabbits (Sylvilagus floridanus) (Anderson et al. 1991). By collecting engorged Ixodes (I.) scapularis larvae and evaluating the nymphs for B. microti, researchers were able to determine reservoir competence for a variety of possible hosts. The nymphs had two strains of B. microti, one of which was linked to human illnesses, while the other was genetically distinct and only found in opossums (Didelphis virginiana), raccoons (Procyon lotor), and a solitary wood thrush (Hylocichia mustelina). The white-footed mouse showed the highest reservoir competence for the B. microti strain linked to human illnesses (an average of 29.5 percent of ticks were infected), followed by short-tailed shrews and eastern chipmunks (averages of 21.9 percent and 17.6 percent, respectively) (Hersh et al. 2012).

Prevalence

Babesiosis was reported in the human population in Europe in 1957 which was caused by B. divergens and it showed that it had zoonotic importance (Hunfeld et al. 2008). Some infections of babesia go untreated in Sweden, Germany, and US, and after serological investigation, antibodies were found against B. divergens and B. microti (Hunfeld et al. 2002). In Asia, at least four zoonotic Babesia species or genotypes have been identified (Kim et al. 2007; Uhnoo et al. 1992).

B. divergens was responsible for the first human case of babesiosis, reported in Croatia. Human instances are usually severe, especially in those whom spleen had been removed. It has been documented roughly 40 instances, predominantly from France, Ireland, and the United Kingdom, with smaller occurrences recorded from Sweden, Switzerland, Spain, Portugal, and Croatia (Martinot et al. 2011). B. divergens infection was also reported in cattle from Europe and Africa through a vector, Ixodes ricinus (Zintl et al. 2003). Babesia infection was also reported in farmed reindeer (Rangifer tarandus) in the UK (Malandrin et al. 2010).

Babesia divergens, like B. capreoli and two other zoonotic Babesia in Europe, use the same vector (B. sp. EU1 and B. microti). Infections in I. ricinus have been found in Hungary, Austria, Belgium, the Netherlands, Switzerland, Germany, Norway, and Estonia (Øines et al. 2012). Some studies also show that B. divergens also transmit transovarially (Bonnet et al. 2007).

According to many studies, the infection caused by B. divergens is most likely with *B. capreoli*. Furthermore, unlike B. divergens, *B. capreoli* does not infect gerbils (Malandrin et al. 2010). There is a need for more work to validate *B. divergence* capacity to use non-spleenectomized cervids as reservoirs (Zintl et al. 2011). Various spleenectomized species, including chimps (*Pan troglodytes*), rhesus macaques (*Macaca mulatta*), laboratory rats, roe deer (*Capreolus capreolus*), fallow deer, red deer (*Cervus elaphus*), European mouflon (*Ovis orientalis musimon*), and domestic sheep, have been found infected with *B. divergens* (Malandrin et al. 2010).

Life cycle

The important species of Babesia which cause Babesiosis in cattle include B. bigemina and B. bovis (Smith et al. 1980). The most important and widely distributed species in tropical and subtropical regions are B. bigemina and B. bovis. Here is the life cycle of B. bigemina.

The life cycle of Babesia spp. comprises of three stages:

a) Schizogony- in vertebrate host- asexual reproduction stage.
b) Gametogony- in a tick- takes place in intestinal cells where gametes formation and fusion occur.
c) Sporogony- in the salivary glands of the tick where the asexual reproduction occurs and leads to the formation of transmittable, infectious sporozoites (Mehlhorn et al. 1998).

The life cycle starts when the tick takes a blood meal from the vertebrate host simply by sucking the blood, the gametes are also ingested. These gametes harbor the gut of the tick where the fertilization occurs. Furthermore, the process in which the gametes fuse is known as gametogony, and it occurs in the midgut of the tick (Mehlhorn and Schein 1985).

The next step is sporogony, in which the sporozoites are formed in the salivary glands of the tick by asexual reproduction. Moreover, these sporozoites tend to cause the infection to enter the final host, which is cattle. These sporozoites keep on multiplication in the salivary glands, and a stage comes when the gland is full of them (Mehlhorn and Schein 1985).

After that, when the tick takes a blood meal from the final host, the sporozoites present in saliva simultaneously find their new site of inoculation. This results in the introduction of sporozoites into the host circulatory system. These merozoites directly infect the red blood cells of the host and cause lysis. Furthermore, it is there where schizogony takes place (Mehlhorn and Schein 1985).

The invasion of sporozoites into the host erythrocytes results in hemolysis, both intravascular and extravascular. After that, there is rapid production of these parasites in the host red blood cells, which leads to fever, hemoglobinuria, and methemoglobinemia. This condition may be as acute as to cause the death of an animal within some days (Mehlhorn and Schein 1985).

During the whole process, the packed cell volume of the animal may fall to such a low extent, i.e., below 20%. The main consequence of it is anemia which is so fatal and causes emaciation, thus causes milk and meat production loss. In extreme cases with the immunocompromised animal, death may occur (Vishwakarma and Nandini 2019).

Host immune responses

When the bovines get affected with Babesia spp., the protective immune response helps them combat their survival. For this purpose, both the innate and adaptive immune responses play a crucial role according to the animal's age. In young calves, innate immunity is seen to do a favor by providing a safeguard from the deadly parasite and adaptive immunity in adult and vaccinated animals (Kim et al. 2007; Suarez et al. 2019).

In calves

Young calves are at a lower risk of getting this infection than adult animals. In young animals, the innate immunity acts by activating macrophages, releasing several chemical compounds like Nitric Oxide (NO) and Interferon (IFN) (Suarez et al. 2019). The young calves have a natural resistance against the Babesial infection and can easily survive the exposure whenever they face endemic. This process of natural immunity in young calves

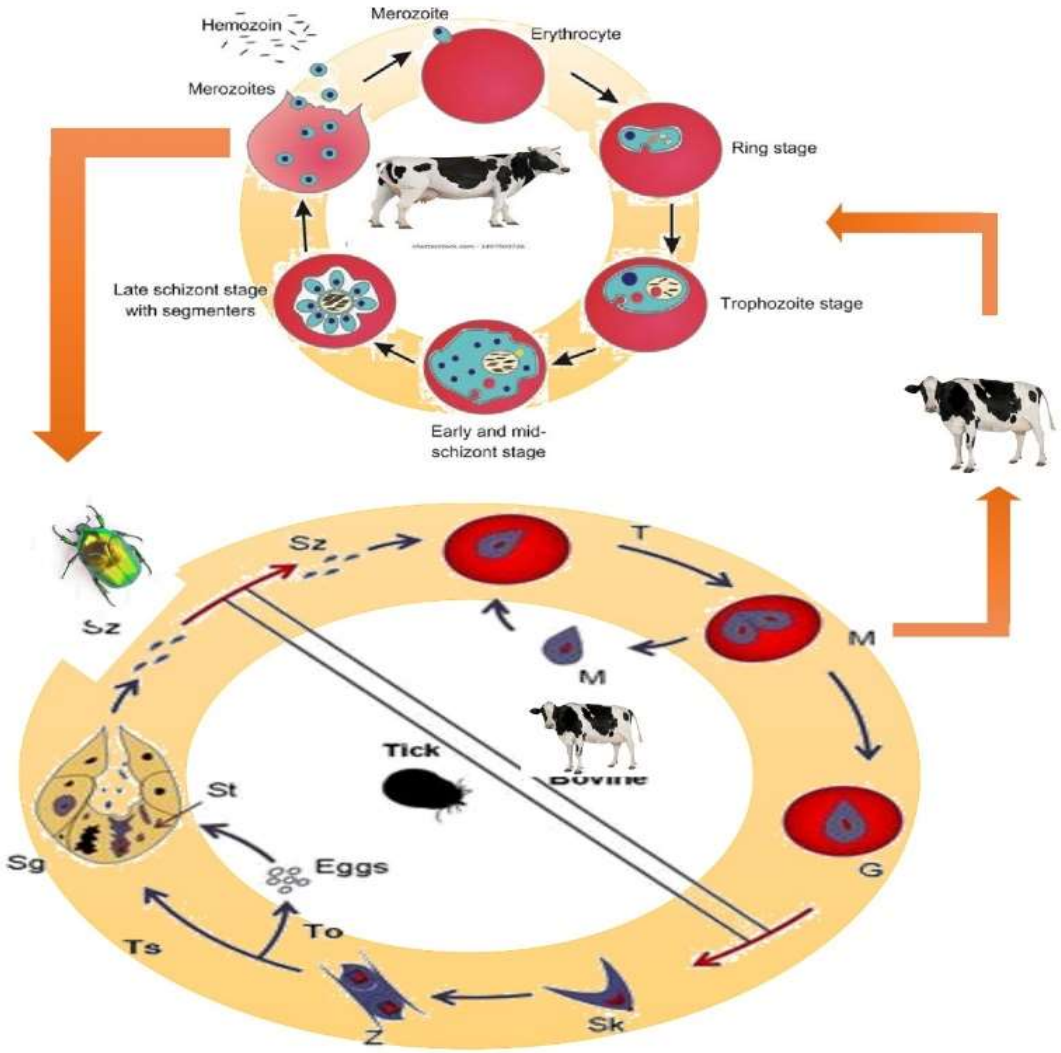

Fig. 1: Life Cycle of Babesias.

is known as Premunisation. Moreover, the young calves exhibit more pronounced innate immune responses than adult ones (Suarez et al. 2019).

In adult animals

On the other hand, the adult animals are at a greater risk of getting the more acute and fatal form of Babesiosis. There are chances that some animals survive this acute form of the disease. Those animals become immune by developing chronic Babesiosis, thus getting life-long protection (Suarez et al. 2019).

In vaccinated/persistently infected animals

When the animal gets vaccination against this deadly disease, the adaptive immune response is shown by macrophages and neutralizing antibodies. These neutralizing antibodies, along with macrophages, control the parasitemia (Suarez et al. 2019).

Canine, feline, caprine and ovine babesiosis

Along with the cattle, other domestic animals are also at the risk of getting Babesiosis like cats, dogs, goats, and sheep. Here we will discuss them one by one.

Canine Babesiosis

Babesiosis in canine is primarily caused by apicomplexan parasites that are either large (5*2.5mm) or very small (2*1.5mm). *Babesia canis* is further divided into three different species such as *Babesia canis*, *Babesia rossi*, and *Babesia vogeli* (Kuttler 1980; Schetters 2005). Babesia Coco is a newly discovered species of Babesia that is related to canines (Birkenheuer et al. 1999; Lehtinen et al. 2008).

These all species are morphologically identical but have prodigious variations in geographic distribution, vector specificity, genetic characteristics and the clinical signs they persuade in dogs, therefore now widely considered to be separate species (Carret 1999).

Just like the other domestic animals, dogs are at the same risk of developing Babesiosis which may lead to spleenomegaly, hemolytic anemia, and thrombocytopenia of varying degree. The severity of the disease depends on the type of Babesia species and the immune status, and the dog's age (Vishwakarma and Nandini 2019).

The important species are, *B. canis canis*, *B. canis rossi*, *B. canis vogeli*, *B. gibsoni*, *B. vulpes*. Among the three subtypes of B. canis, the most virulent form belongs to Southern Africa, *Babesia canis rossi*. The main source of their transmission is by hard

ticks, including *Rhipicephalus sanguineus*, etc. (Vishwakarma and Nandini 2019).

Feline Babesiosis

Four small Babesia species causes clinical disease in cats: *B. felis*, *B. cati*, *B. leo* and *B. microti*. *B. felis* among all is small with approximately (0.9*0.7mm) and considered as highly pathogenic piroplasm (Greene 2006; Bosman et al. 2007). *B. cati* results in milder clinical disease and morphologically as single or paired annular bodies within the erythrocytes (Lopez-Rebollar et al. 1999). Cats are usually affected by ticks of genus Ixodes, Dermacentor, Rhipicephalus, Amblyomma, and Haemophysalis and also vectors for transmission (Baneth et al. 2004).

Feline babesiosis is mainly the topic of discussion in South Africa but rarely in Europe and Asia. It is again the protozoal disease that causes severe circulatory diseases in cats. This disease is of great veterinary importance where there is high prevalence due to its economic losses regarding cats (Penzhorn et al. 2020). There are four majors Babesial spp associated with feline (Penzhorn et al. 2020) like; *B. felis*, *B. herpailuri*, *B. pantherae* and *B. cati*. The best treatment is available in the form of antiprotozoal drugs and supportive therapy. Mortality rates range from 15 to 20% in severe cases (Ayoob et al. 2010).

Caprine babesiosis

Goats may develop pre-hepatic jaundice along with Babesiosis. The symptoms associated include dyspnea, icteric sclera, mucopurulent nasal discharge, diarrhea, bloated abdomen, and coffee-colored urine. The animal in this condition is likely to develop the conditions like anemia, thrombocytopenia, hemoglobinuria, and hypoproteinemia, etc. (Ajith et al. 2017). The major Babesia species that can cause caprine babesiosis is *B. ovis*. Proper medical treatment is essential for the survival of the animal. If timely diagnosis is not made, the situation may get fatal and lead to the animal's death. This may be of significant economic loss on a large scale (Ajith et al. 2017).

Ovine Babesiosis

There is evidence of a close relationship between the disease and the distribution areas of the tick, Rhipicephalus bursa in ovine Babesiosis. Whereas the regions where the humidity level is low limit the distribution of vector tick. Packed cell volume decreased from 30 to 40% (Yeruham et al. 1998; Razmi et al. 2002).

Moreover, other significant complications during ovine Babesiosis include glomerulonephritis, vascular alteration, intravascular coagulation syndrome, and liver damage. Important species that cause ovine Babesiosis are *B. ovis*, *B. motasi* (Yeruham et al. 1998).

Treatment of Babesiosis

Anti-babesia drugs play a pivotal role in tick management and immunization. These approaches should be carried out in the area where the cases are always prevalent. Nowadays chemotherapy for prevention and control is emerging (Bork et al. 2003). But is always important to diagnose the disease early and prompt action should be taken accordingly. New drugs that are chemotherapeutic in nature are host friendly and have low level of drug toxicity which makes them potent to control the disease. (Abou Laila et al. 2010; Mosqueda et al. 2012).

Furthermore, severe cases of babesiosis need supportive therapy to save the life of animals. It may include transfusion of blood from a healthy donor, tick repellents, non-steroidal anti-inflammatory, combination of water and fat-soluble vitamins, electrolytes such as dextrose, purgatives, and iron supplements. Trypan blue was the first documented drug that was used for babesiosis. (Vial et al. 2006; Mosqueda et al. 2012). This drug host-specific which means it was only effective for *B. bigemina* infections, however, it was not that potent for *B. bovis* infections. This drug causes discoloration of the carcass that's why it was infrequently used (Kuttler 1980).

For the treatment of Babesiosis in Europe, several drugs were available, like amicarbalide isethionate, quinuronium sulfate, and diminazene aceturate. A fourth effective drug was introduced in Europe named imidocarb diproprionate, which became the drug of choice (Zintl et al. 2003). When the experimental trial of imidocarb was made, it was clear that it produced a toxic effect when given intravenously. That's why a subcutaneous or intramuscular route of administration is preferable (Zintl et al. 2003; Vial et al. 2006).

Along with the therapeutic effects, there are several side effects when high doses are given. These include muscular tremors, colic, excessive salivation, coughing, and irritation at the site of administration. Imidocarb shows slow action compared to quinuronium sulfate, but it is the only babesicide that shows consistency in clearing the parasites' host (Zintl et al. 2003). Animals that have encountered acute babesiosis will be subjected to blood transfusion that would be helpful for them to recover from anemic anoxia.

Commonly used drugs

Imidocarb dipropionate

It is one of the most common babesicide. Over the course of the last 20 years, it is used as a treatment and prophylactic agent in almost all the animals. It has the potency to wipe out all the intracellular parasites related to babesiosis or anaplasmosis (Kuttler 1980; Suarez et al. 2011). Mostly it is administered by two important routes intramuscular and subcutaneous route, intravenous route is not practiced due to the high level of toxicity which causes sudden death in animals Recommended dose must not exceed 1-3 mg/kg. Many species of bovine babesiosis such as *B. bigemina*, *B. bovis*, *B. divergens*, and *B. caballi* (Kuttler 1980).

Mode of action and drug residues

Imidocarb mechanism of action has been proposed to have two ways.

I. Catabolism of polyamines by polyamine oxidase (PAO) as bovine serum contains significant levels of PAO. As a result, ROI (Reactive oxygen intermediates) are released. So, imidocarb causes interference in the function of polyamines (Gahl et al. 1982; Johnson et al. 1996).

II. Imidocarb interfere with the inositol production in the erythrocytes which prevent babesia from entering in the erythrocytes which causes starvation of the protozoan (Mchardy et al. 1986; Mintzer et al. 1988; Baneth 2018).

Imidocarb residues get into the edible tissues of ruminants after a spell of treatment, which causes deleterious effects on the

health of humans (Moore et al. 1996; Suarez et al. 2011) Imidocarb has the tendency to cross the blood-brain barrier as some of the researchers documented that during the first day of treatment (Belloli et al. 2006).

Diminazine Aceturate

It is one of the most extensively used anti-trypanosomal agents with the efficacy of treating bovine babesiosis and Trypanosoma species. Approximately more than six decades ago it was first marketed and until today it is extensively (Da Silva Oliveira et al. 2015). The recommended dose of diminazine ranges from 3 to 5mg/Kg that shows activity against *B. bigemina*, *B. bovis*, *B. caballi*, *B. gibsoni* and *B. canis* (Birkenheuer et al. 1999; Vial et al. 2006; Rashid et al. 2008; Singh et al. 2011). Diminazine is highly toxic and having many severe side effects with an unknown mechanism (Collett 2000).

Control and Prevention

Tick vector control strategy is best suitable for the eradication of Babesia and in the areas where tick control is not feasible intensive chemotherapeutic regimes must be adopted. Regular inspection of animals and premises for proper usage of tick repellent and acaricides could reduce the tick exposure of cattle. Acaricidal resistance development of the vector tick could be a concern in chemical control strategies (Singh et al. 2011).

For the destruction of the ticks and tick habitat ecologically desirable environmental modification can be considered. Natural endemic stability is unreliable as the sole control strategy, as it can be affected by climate, host factors and management.

Monitoring of endemic environment should be concerned before:

- Introduction of immuno-naïve animals
- Involvement of new species or strains of the disease agent
- Interruptions in exposure to ticks and disease due to changes in climate, host factors, and management

A strong immunity can be triggered against babesiosis in cattle immune system after the first infection with *B. bovis*, *B. divergens*, or *B. bigemina* (Baneth 2018).

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CHAPTER 17

CYSTIC ECHINOCOCCOSIS

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INTRODUCTION

Cystic echinococcosis (CE) is a helminthic zoonotic disease that affects various body organs, primarily the liver and then the lungs. This disease is caused by the tapeworm of genus Echinococcus (family Taeniidae) which is endemic in most regions of the world (Armua-Fernandez et al. 2014, Romig et al. 2015; Karamian et al. 2017). As the intermediate hosts ingest the eggs, they develop into a fluid-filled cysts (Cardona and Carmena 2013; Rojas et al. 2014).

The parasite needs two hosts to complete its life cycle. These hosts are the definitive host which are carnivores of the family Canidae and the second host is the intermediate host that includes many ruminants such as sheep, goats, cattle, camel etc. (Romig and Deplazes 2017). The adult worm lives in the small intestine of canids, mostly dogs and wolves (Karamian et al. 2017, Chaudhari et al. 2017; Mulinge et al. 2018). The adults produce eggs that pass with the feces of dogs and contaminate water, vegetables, fruits, etc. (Paniker and Ghosh 2013; Otero-Abad and Torgerson 2013). When ingested by the intermediate hosts, eggs hatch releasing the hexacanth embryo. These are then carried by the blood to various body organs where they develop into cysts characterized by long-term growth in the intermediate hosts (Deplazes et al. 2017).

The disease is endemic in rural sheep-raising areas, and in areas where there is close contact with the final host, in addition the dogs consume the infected intermediate host organs (Grosso et al. 2012 and Lett 2013). Cystic echinococcosis is hyperendemic in Iraq and is regarded as one of the most economically devastating zoonotic diseases affecting livestock and humans (Benyan et al. 2013).

Many studies have been performed on CE in Kurdistan region and Iraq. Some of them on the prevalence of cysts in domestic animals, such as goats, sheep and cattle (Saeed et al. 2000, Bajalan 2006, Ghaffar 2008, Saida and Nouraddin 2011, Meerkhan and Abdullah 2012, Al-Berwari 2012, Jarjees and Al-Bakri 2012, Sargali and Mero 2013, Al-Bosely 2013, Mero et al. 2014, Murtaza et al. 2017, Meerkhan et al. 2018, Abdulhameed et al. 2018, Alsaady and Al-Quzweeni 2019, Abdulla et al. 2020; Mohammed 2021). While other studies involved the molecular characterization of CE in domestic animals (Ahmed 2012, Hama et al. 2015, Fadhil and A'aiz 2016, Hassan et al. 2016, Hama et al. 2018; Mahdi et al. 2020).

History of Cystic Echinococcosis

Cystic Echinococcosis is one of the earliest known animal diseases. Its origin may be traced back to Hippocrates (377 BC), who wrote in his notes (Seventh, 55): "In those whose liver is filled with water open into the peritoneum, the belly is filled with water, and they die" (Thompson et al. 2017; Slimane et al. 2018). Galen regarded the liver to be the primary location of CE in slaughtered animals about 200 BC. The existence of the cysts in animals and humans was later on widely documented (Slimane et al. 2018). In the Bible Talmud of 1534, Egyptians and Babylonians characterized the cyst as a bladder filled with liquid (Sabau 2011).

The exact nature of CE remained unclear until the early modern period when Philip Hartmann described a small, spherical structure connected to the bladder in 1685. He confirmed the animal nature of cystcerci, in 1760. Then Peter Pallas classified the CE as a separate group (bladder worms) and described them as small bodies located on the inner wall of the bladders in his medical thesis (Thompson et al. 2017). In 1782, Goeze described the tapeworm scolex and the front end, naming it Taenia visceralis socialis granulosus (Alam-Eldin 2009). Batsch, on the other hand, termed it Hydatigena granulose in 1786 (Rausch 1967). The term Echinococcus was first used in zoology by Carl Asmund Rudolphi in 1801 (Thompson et al. 2017). Echinococcus is a Greek term that signifies hedgehog berry (echinos = spine and kokkos = grain) (Moringlane 2003). Echinococcus granulosus is the parasite's current scientific name.

Classifications of Echinococcus spp.

According to, Nakao et al. (2007) and Huttner et al. (2008) the genus Echinococcus, was classified as mentioned below:
Kingdom: Animalia
Subkingdom: Metazoa
Phylum: Platyhelminthes
Class: Cestoidea (Rudolphi 1808) / Cestoda
Subclass: Eucestoda (Southwell 1930)
Order: Cyclophillidea
Suborder: Taeniata
Family: Taeniidae
Subfamily: Echinococcinae
Genus: Echinococcus (Rudolphi 1801)

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Species: The genus Echinococcus has 9 species namely, Echinococcus granulosus (Batsch 1786), E. felidis (Ortlepp 1937 and Huttner et al. 2008), E. equinus (William and Sweatman 1963), E. ortleppi (Ortlepp 1934), E. canadensis (Sweatman and Williams 1963), E. multilocularis, E. shiquicus (Xiao et al. 2005), E. oligarthra (Diesing 1863) and E. vogeli (Rausch and Bernstein 1972, McManus 2013; Nakao et al. 2013). The most common type that develops in humans and animals is E. granulosus. Other uncommon species that infect humans are, E. multilocularis causing alveolar echinococcosis and the rare types E. vogeli and E. oligarthrus causing polycystic and unicystic echinococcosis, respectively (WHO 2013; Eckert et al. 2001).

Morphology

The adult worm (Fig. 1) is hermaphrodite, white in color, measures from 2-11 millimeter long (Torgerson and Budke 2003; Alvi et al. 2021). The scolex is spherical and provided with four oval suckers and a double row of 30-60 hooks with a non-retractable conspicuous rostellum, with a short neck. Strobila is segmented and consists of mainly 3 to 6 proglottids (Rahman et al. 2015; Thompson et al. 2017). Each of the proglottid has a single genital opening and the last proglottid is called gravid proglottid that contains a uterus filled with 1000-1500 eggs and is frequently more than half the length of the worm (Thompson et al. 2017; Alvi et al. 2021).

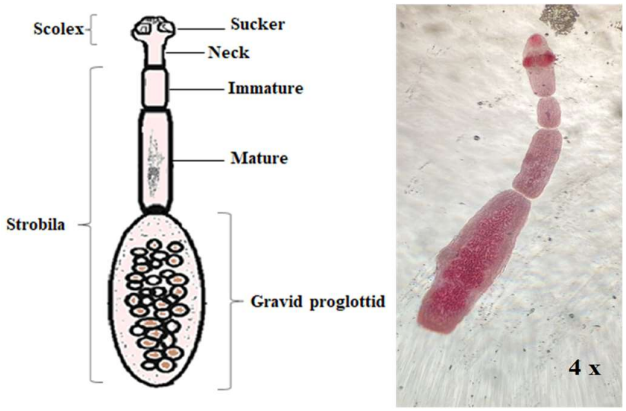

Fig. 1: Morphology of adult Echinococcus granulosus worm.

Fig. 2: The egg of E. granulosus in dog feces.

The eggs are spherical or ovoid in shape measuring 30 to 40 µm in diameter (Fig. 2). Eggs are similar in appearance to those of other Taeniid eggs, thick dark brown to yellow in color and contain hexacanth embryos (oncospheres) surrounded by several membranes and an external keratinized highly resistant layer (Torgerson 2014; Rahman et al. 2015).

The cyst is usually unilocular; mostly spherical in shape and differs in size according to the location. Structurally it is made up of three layers (Fig. 3) with hydatid fluid inside. First layer is the adventitial layer (pericyst) that originates from host cells and forms a dense fibrous protective layer surrounding the cyst, and inhibits the entry of large macrophages into the sac (Handa et al. 2005; Fritsche and Pritt 2017). The middle layer, the laminated membrane (ectocyst layer), which is white in color and non-cellular with a coarse elastic composition. This layer is produced by the parasite and consists of a polysaccharide-protein complex. The thickness of this layer ranges from 1-2 mm and increases in its thickness by age (Siracusano et al. 2009; Eriksen and Agopian 2017). The third layer is the inner germinal layer (endocyst) which is a living part of the cyst wall and is 20-25 micrometers thick. Its functions are controlling the permeability of the cyst, asexual reproduction and the production of the hydatid fluid that fills the cyst cavity (Schmidt and Roberts 2000; Galindo et al. 2003). This layer produces the brood capsules. A fully developed cyst contains brood capsules with protoscolices and is filled with clear fluid (Eriksen and Agopian 2017; Fritsche and Pritt 2017). The cysts which contain protoscolices are called fertile cysts

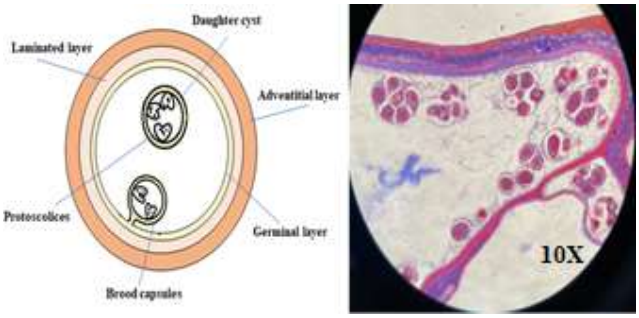

Fig. 3: The metacestode of Echinococcus granulosus.

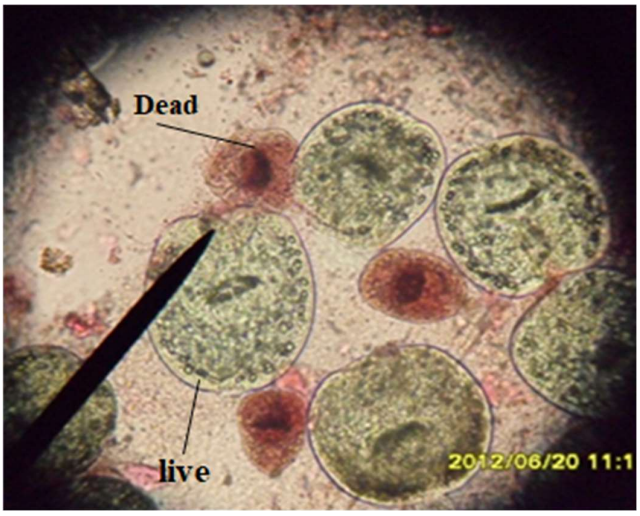

Fig. 4: Protoscolices of Echinococcus granulosus stained with aqueous eosin stain (40X).

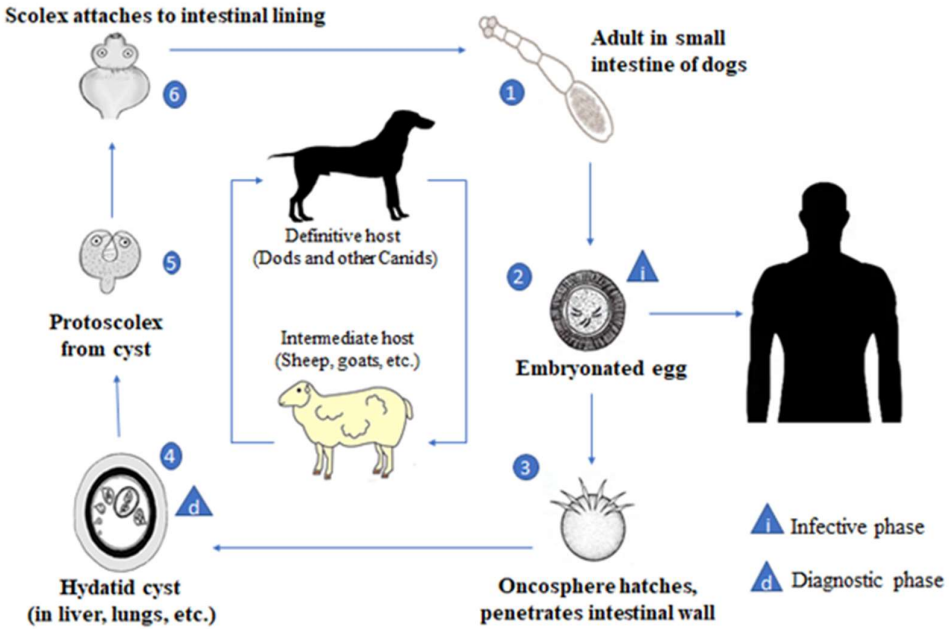

Fig. 5: Diagram of the domestic life cycle of *E. granulosus*..

(Fig. 4), while those without protoscolices are called sterile cysts (Kamentzk et al. 2000). The hydatid fluid is a colorless or slightly yellowish fluid which is odorless and sterile. It fills the cavity of the cyst. Specific gravity of the fluid is 1.007–1.015 and its pH is between 6.7 - 7.2. It contains a number of enzymes, proteins and salts (Muller and Waklelin 2001; Halezeroglu et al. 2012; Yakhchali et al. 2017).

The Biochemical Constituents of Echinococcal Cyst

The analysis of cyst layers, protoscolices, the hydatid fluid and surrounding host tissues of sheep, goats and cattle showed the presence of high levels of lipids, triglycerides and cholesterol in the protoscolex and infected tissue surrounding the cyst in cattle. On the other hand, high levels of glucose were found in the hydatid fluid and tissues surrounding cyst isolated from sheep (Meerkhan and Mero 2013). Also, the laminated layer of sheep, goats and cattle cysts contained high level of triglyceride, while the germinal layer of the cysts contains high levels of cholesterol (Merza and Mero 2013). With regards to enzymes, the activities of ACP, ALP, LDH, GOT and GPT were higher in the laminated layer than germinal layer of the same hosts (Mero and AL Bosely 2014).

Life cycle of Echinococcus Granulosus

The life cycle of E. granulosus is of two types, depending on the type of host. First one is the domestic life cycle, that involves mainly dog as a definitive host and a wide range of domestic ungulates (sheep, cattle, camel, goats, buffalo, pigs. etc.) and humans as intermediate hosts (WHO 2001, Cucher et al. 2016; Romig and Deplazes 2017). The second form is the sylvatic life cycle, which occurs in northern North America and Eurasia, and involves mainly wild animals like wolves, cervids, moose and reindeer (WHO 2001).

In the domestic life cycle (Fig. 5), the adult worms are attached to the mucosa of dog's small intestine and produce eggs (Santivanez and Garcia 2010). These eggs are released after the separation of the gravid proglottids into the external environment with the feces. They are resistant and remain infective for months outside the body of intermediate host (Morar et al. 2014; Slimane et al. 2018). After their ingestion by the intermediate hosts during grazing in the farms, eggs are hatched in the small intestine of these hosts releasing hexacanthus embryo. Within 12 hours of ingestion, the oncosphere (early-stage larva) penetrates the wall of the small intestine by the aid of hooks and finds its way via blood or lymph to the liver, lungs or other sites to develop into a unilocular fluid-filled cyst. This cyst keeps growing in size by time. The incubation period could be 12 months to years, depending on the number and location of the cysts, producing protoscolices in the bladders by asexual multiplication (Roelfsema et al. 2016, Thompson et al. 2017; Shnawa et al. 2021).

The liver is the first filter for embryos to develop, holding a high number of them while the remaining goes through the lungs, the second filter, which holds fewer embryos. A few of the embryos pass through the lungs' capillaries to enter the systemic circulation, where they are transported to various organs and tissues throughout the body, including the spleen, kidneys, heart, brain and bones (Gurbuz et al. 2006). The definitive host becomes infected by consuming the infected viscera of intermediate host having fertile cyst. The protoscolices evaginate, attach to the mucosal lining of their intestine and grow into the adult worms within 6 - 8 weeks depending on the parasite strain and host sensitivity (Lightowlers et al. 2003; Shnawa et al. 2021). Adult worms remain alive in the final host for 5-20 months, then begin to release eggs or gravid segment every 2 weeks (Rahman et al. 2015, Thompson et al. 2017, Mohamed et al. 2017; Tse et al. 2019).

Molecular Characterization of Echinococcus Granulosus

Echinococcus granulosus is a complex parasite that exhibits genetic diversity and it has been divided into a series of

genetically distinct strains/genotypes according to the hosts (Thompson et al. 1995; Lavikainen et al. 2003). Molecular genetic analysis, using mitochondrial Cox1 cytochrome, Nad1 dehydrogenase genes and intra transcribed spacer 1 (ITS1) have been used for the identification of Echinococcus genotypes from definitive and intermediate hosts (Sanchez et al. 2010, Lymbery and Thompson 2012; Chaligiannis et al. 2015). So far, 10 genotypes have been identified (G1-10) and the name E. granulosus sensu lato has been used as a general term for all of these species and strains. These include two sheep strains (G1 and G2) and buffalo strain (G3), both grouped together in the species E. granulosus sensu stricto, G4 genotype (horse strain) called Echinococcus equinus, G5 genotypes (cattle strain) named Echinococcus ortleppi, and G6 genotype (camel strain), G7 genotype (pig strain), G8 (cervid strain), G9 (a Poland swine strain) and G10 (a Eurasian reindeer strain) are grouped together in the species Echinococcus canadensis (Rahman et al. 2015, Romig et al. 2015; Roelfsema et al. 2016). Globally, the G1 genotype of E. granulosus is the most prevalent strain which infect humans and domestic animals such as sheep, goats, cattle and camel which acts as the intermediate host more frequently than other species or genotypes (Sharma et al. 2013, Boufana et al. 2014; Lymbery 2017).

Distribution of Echinococcus granulosus and its' Genotypes in Livestock

Cystic echinococcosis in livestock has a cosmopolitan distribution and is more frequent in rural areas, particularly in developing and under developed countries. However, the greater prevalence of cystic echinococcosis (CE) is found in agricultural regions particularly in Mediterranean region, South America, the Middle East, Central Asia, China, New Zealand, Canada, parts of Africa as well as Australia (Yang et al. 2005, Rojas et al. 2014; Ito and Budke 2017). The disease is more common in areas where sheep are reared together with the dogs. It is also prevalent in the areas where older animals are slaughtered such as cattle, sheep, and others (Arora and Arora 2011; WHO 2021).

Iraq is considered as one of the hyperendemic countries for CE infection which is widely distributed throughout most of its governorates and different infection rates were reported among both humans and livestock (Ahmed 2012; Benyan et al. 2013). For the first time CE was reported in Iraq by Babero et al. (1963). They observed echinococcal cysts in sheep, cows, buffaloes and camels in Baghdad.

Many epidemiological studies reported variable prevalence of CE in domestic animals from Kurdistan region and other parts of Iraq, as shown in Table (1).

Table 1: Published information concerning the prevalence of CE in livestock in different governorate of Kurdistan regain and Iraq.

Governorate	host	Prevalence (%)	References
Duhok	Sheep	24.63	Ghaffar 2008
	Goats	13.54	
	Cattle	1.68	
	Sheep	9.921	Meerkhan and Abdullah 2012
	Goats	6.245	
	Cattle	10.58	
	Sheep	19.46	Al-Berwari 2012
	Goats	15.52	
	Cattle	6.94	
	Sheep	24.63	AL-Bosely 2013
	Goats	13.56	
	Cattle	0.64	
	Sheep	4.25	Meerkhan et al. 2018
	Goats	0.64	
	Cattle	0.37	
Erbil	Sheep	15.0	Saeed et al. 2000
	Goats	6.2	
	Cattle	10.9	
	Sheep	11.1	Saida and Nouraddin 2011
	Goats	1.66	
	Cattle	7.77	
	Sheep	9.07	Hassan 2017
	Goats	0.54	
	Cattle	1.56	
Slemani	Sheep	0.27	Bajalan 2006
	Goats	0.11	
	Cattle	0.57	
	Sheep	12.7	Mero et al. 2014
	Goats	4.8	
	Cattle	4.3	
Al-Qadisia	Sheep, goats and cattle	20.59	Al-Fatalawei 2002
Mosul	Sheep	2	Jarjees and Al-Bakri 2012
	Goats	0.52	
	Cattle	0.55	
Basrah	Sheep	14.75	Murtaza et al. 2017
	Sheep	7.3	Abdulhameed et al. 2018
Baghdad	Sheep	2.28	Najim et al. 2020
	Sheep	2.0	Mohammed 2021
	Cattle	1.1	

The Genotypes Reported in Kurdistan Region and other Parts of Iraq

Some genotyping studies have been carried out in Kurdistan region and other parts of Iraq, such as, in Duhok governorate, Ahmed (2012) performed a molecular analysis of cysts isolated from sheep, goats and cattle using two mitochondrial genes (cox1 and nad1) and reported G1-G3 genotypes in all samples. In Erbil governorate, Hassan (2017) reported G1 and G1c in the cysts isolated from the above-mentioned hosts using the mitochondrial cox1 gene. In Koya city of Erbil governorate, Abdulla et al. (2020) reported the sheep strain (G1) among 19 sheep and one cow using the same gene region. In Slemani governorate, Hama et al. (2013, 2015 and 2018) studied the molecular identification of Echinococcus granulosus cysts isolated from sheep, goats and cattle in Slemani abattoir using cox1 gene and found the majority of the samples belonged to sheep strain (G1) and few with slight micro variations within G1. In Al-Qadisiya governorate, Fadhil and A'aiz (2016) performed a genotyping study of Echinococcus granulosus isolated from domestic animals using nad1 mitochondrial gene and reported G1 and G3 genotypes in sheep and G1, G3 and G6 in cattle and camels. In Kirkuk, Hassan et al. (2016) isolated the sheep strain (G1) of Echinococcus from sheep, goats and cattle using the ITS 1 fragment of rDNA. In Misan governorate, Alsaady and Al-Quzweeni (2019), reported G1 and G3 genotypes using cox1 mitochondrial gene in echinococcal cysts isolated from sheep, buffalo, camel, cow and goats collected from central slaughterhouse of Al-Amarah city. In Al- Najaf and Al-Diwaniyah provinces, Mahdi et al. (2020), using the same marker (cox1), reported G1, G3 and G6.

Symptoms of Cystic Echinococcosis

The clinical symptoms of CE in humans are variable and the disease is asymptomatic in the early stages due to the slow growth and development of the cyst. The incubation period is variable, until the cysts grow and begin to exert pressure on host tissues (Pakala et al. 2016, Kern et al. 2017; WHO 2021). The cysts can be found in any organ but most often cysts develop in liver and lungs, and less frequently in the bones, kidneys, spleen, muscles, abdominal cavities, brain, ovary, testis and central nervous system (Eckert et al. 2001; WHO 2021).
In livestock, the disease is not diagnosed, and the animals may be sacrificed before the appearance of the symptoms (Al-Khafaji 2006). The appearance of the symptoms depends on the number of the cysts and their location. The recognizable symptoms include reduced milk production, sluggish development, frailty, poor wool, jaundice, bronchopneumonia in the case of lung infection (Eckert and Deplazes 2004; Eddi et al. 2006). The complications related to cyst rupture, spread of protoscolices, and bacterial infection can affect the clinical symptoms (Thompson 2001).

Diagnosis and Detection of Cystic Echinococcosis in Livestock

Most of CE infections stay asymptomatic for many years before the cysts grow in size and impose pressure on the tissues to be able to cause symptoms in the affected organs. Different approaches have been employed for the diagnosis of CE, such during slaughtering the infected intermediate host can be diagnosed, while live livestock can be diagnosed by immunological techniques (Craig 1997). The most common method for detecting cysts in domestic intermediate hosts (sheep, goats, cattle, pigs etc.) is post-mortem examination. The results are based on inspection, palpation, and/or incision used to identify the parasite's larval stage (metacestode) during meat inspection (either in an abattoir or before consumption/sale) (OIE 2008 and Chihai et al. 2016). The only accurate way for detecting CE is a post-mortem (necropsy) examination of visceral organs (Craig et al. 2015). Although it is possible to use serological examinations to detect the parasite in livestock (Golassa et al. 2011; Bulashev et al. 2017). Hydatid fluid (hydatid sand) aspirated from a cyst through its opening may show the presence of protoscolices, which are normally invaginated. The presence of protoscolices is diagnostic and based on morphology of the cyst (Craig et al. 1995). Several studies involved the molecular methods (species and genotypes) of cysts found at slaughter inspection proved the usefulness of this approach (Casulli et al. 2008; Boufana et al. 2014). Also, the real time PCR has been used to distinguish E. granulosus genotypes using cyst material (Maurelli et al. 2009; Pestechian et al. 2014).

Control and Prevention of Cystic Echinococcosis in Livestock

According to WHO, cystic echinococcosis is a serious health concern that causes large economic losses in farming animals (WHO 2006). It is difficult to adjust the exposure to echinococcosis due to difficulty in staying aloof from the parasite eggs which are transmitted with feces of wild animals causing recurrence of CE. On the other hand, it's difficult to diagnose because the disease in animals is asymptomatic (Velasco-Tirado et al. 2017; CDC 2019).
Control of CE in endemic places relies on preventing definitive hosts (dogs) from ingesting infected intermediate host organs and preventing the intermediate hosts from consuming food contaminated by dog excreta (Mandal and Mandal 2012, Mohamed et al. 2017).
Control measures include regulation of livestock slaughtering, controlling the infection in dogs, deworming, treatment with praziquantel at a dose of 5 mg / kg every six weeks, to reduce the prevalence of E. granulosus below the levels favorable for continued transmission (Gemmell et al. 2001, Craig et al. 2017; Jiang et al. 2017). The eggs in the stool samples may be killed by boiling for 5 minutes, using autoclave or freezing at -80°C for 2 days (Eckert et al. 2001). Development of effective vaccines to prevent oncosphere development to cysts in sheep and other intermediate hosts, thus it prevents the development of adult gravid tapeworms in dogs (Ecklert and Deplazes 2004, Gauci et al. 2005, Morariu et al. 2010; Craig et al. 2017).

Conclusions

From this review it can be concluded that echinococcosis is a zoonotic parasitic disease and is endemic disease with worldwide prevalence including Kurdistan region and Iraq. The parasite has an indirect life cycle requiring two hosts, the first host is the intermediate host in which the cysts can develop in many organs and tissues of humans and domesticated mammals such as sheep, cattle, pigs, goats and camels. The second host is definitive host in which the parasite develops in the small intestine of carnivores such as dogs, wolves and foxes. Reducing the infection needs urgent public

awareness of disease and adopting preventive measures to reduce its spread.

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CHAPTER 18

EPIDEMIOLOGICAL AND MOLECULAR STUDIES OF ANIMAL FASCIOLIASIS IN IRAQ

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INTRODUCTION

Fascioliasis is considered as a neglected zoonotic disease of ruminants. This disease is caused by two species of Fasciola (F.) namely *Fasciola hepatica* and *F. gigantica*. They are distributed in more than 70 countries except Antarctica, in areas where sheep and cattle are reared (WHO 2016; CDC 2019). This disease was first recorded as early as 2000 BC. Fasciola spp. cause serious pathological effects to livestock (Abbas et al. 2019). The number of infected sheep exceeds 250 million and about 300 million cattle are infected globally with fascioliasis. The disease cause losses exceeding 3.2 billion $ yearly, due to decrease in milk and meat production and an increase in mortality rate in addition to liver condemnation and expenditure of anthelminthic (Hillyer and Apt 1997; Jaja et al. 2017).

Nowadays, attentions have been paid to fascioliasis due to its zoonotic feature, because it infects both humans and animals. Human cases are estimated to be around 2.6 million worldwide (Schweizer et al. 2005). Humans acquire fascioliasis incidentally through the ingestion of cercaria encysted on watercress or with drinking water. Human and animal infections have shown worldwide expansion which mainly related to climatic changes (Caravedo and Cabada 2020).

The geographic distribution of Fasciola spp. depends on the presence of the intermediate host, the snail, in an area which is responsible for spreading the disease and leading to the infection of new hosts (Mas-Coma et al., 2005). The intermediate host for *F. hepatica* is Lymnaea truncatula, that exists in cold and temperate zones, so it is prevalent in North America, Europe and Australia. While *F. gigantica* is common in sub-tropical and tropical zones of Asia and Africa (Mas-Coma et al. 1997).

Morphology

The both species of liver flukes; *F. hepatica* and *F. gigantica* (Fig. 1 and 2) can be differentiated morphologically by their shape, size, and the shape of cephalic cone (Keiser and Utzinger 2009). Adult *F. hepatica* is large, leaf-shaped measuring 3cm in length by 1.5 cm in width; the color is brown to pale grey. It possesses two unequal suckers; the smaller oral sucker which is powerful and located at the anterior end that extends into a conical projection, the cephalic cone, and the ventral sucker which is larger and located at the base of cephalic cone. The posterior end of the body is more rounded than anterior end. *F. gigantica* is the largest liver fluke which is longer and narrower than F. hepatica, measuring 7.5 cm in length and 1.2 cm. in width. It also possesses two unequal suckers; a shorter cephalic cone and the fluke is more oblong with a larger rounded posterior end (Jeandron et al. 2010; Abdisa and Jilo 2017; CDC 2019). Fasciola worm is hermaphroditic; it possesses two dendritic highly branched testes and one ovary that produce eggs excreted with animal feces. Even though both Fasciola spp. are separate, "hybrid forms" with intermediate morphological characters and genetic components of both species were recorded in some Asian and African countries, where both species were found (CDC 2019).

Classification of Fasciola Species

Fasciola belongs to Phylum Platyhelminthes, Class Rhabditophora, Order Plagiorchiida, Family Fasciolidae, Genus Fasciola and species *Fasciola hepatica*, Linnaeus 1758 and *Fasciola gigantica*, Cobblod 1855.

Life Cycle

Each adult fluke produces more than 20,000 eggs daily, which are deposited in the biliary passages of the host (Stein 2003). These eggs are oval, operculated, yellowish brown in color, large in size, the average length ranges from 130±150/63±90 µm for *F. hepatica* and 150±196/90±100 µm for *F. gigantica* (Marcilla et al. 2002; Stein 2003; Keiser and Utzinger 2009). The deposited eggs pass with the bile to the intestine then are excreted in animal feces. The life cycle (Fig. 3) begins when the eggs embryonate under favorable environmental conditions of temperature, oxygen tension, pH and humidity (at least a film of water).

The embryonation takes more than two weeks depending on the above-mentioned factors, then hatch into a free-swimming miracidium (Stein 2003). The miracidium penetrates the body of a suitable snail, the intermediate host, which is responsible

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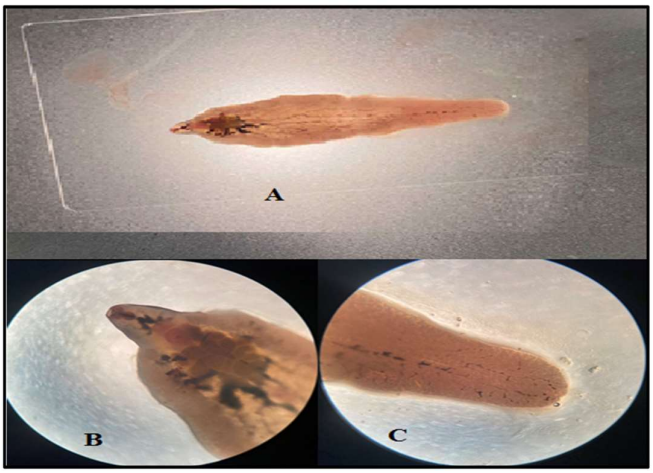

Fig. 1: *Fasciola hepatica* isolated from bile duct of sheep; A: Adult; B: Anterior end of Fasciola showing oral and ventral suckers, pharynx, genital pore, ovary and uterus; C: posterior end of the fluke; B and C under magnification power 20x.

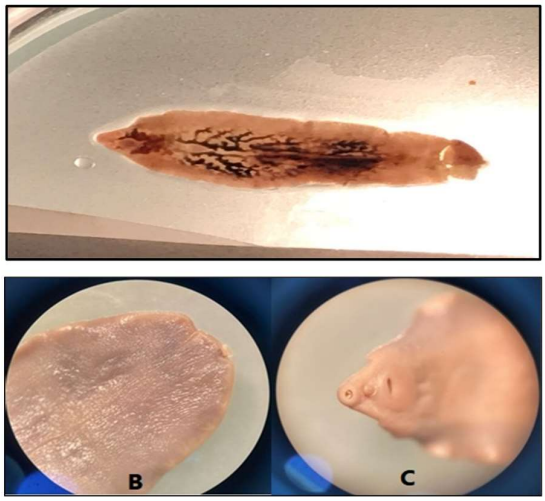

Fig. 2: *Fasciola gigantica* isolated from bile duct of Cattle; A: Adult; B: Posterior end of the fluke; C: Anterior end showing oral and ventral suckers, and genital pore, B and C under magnification power 20x

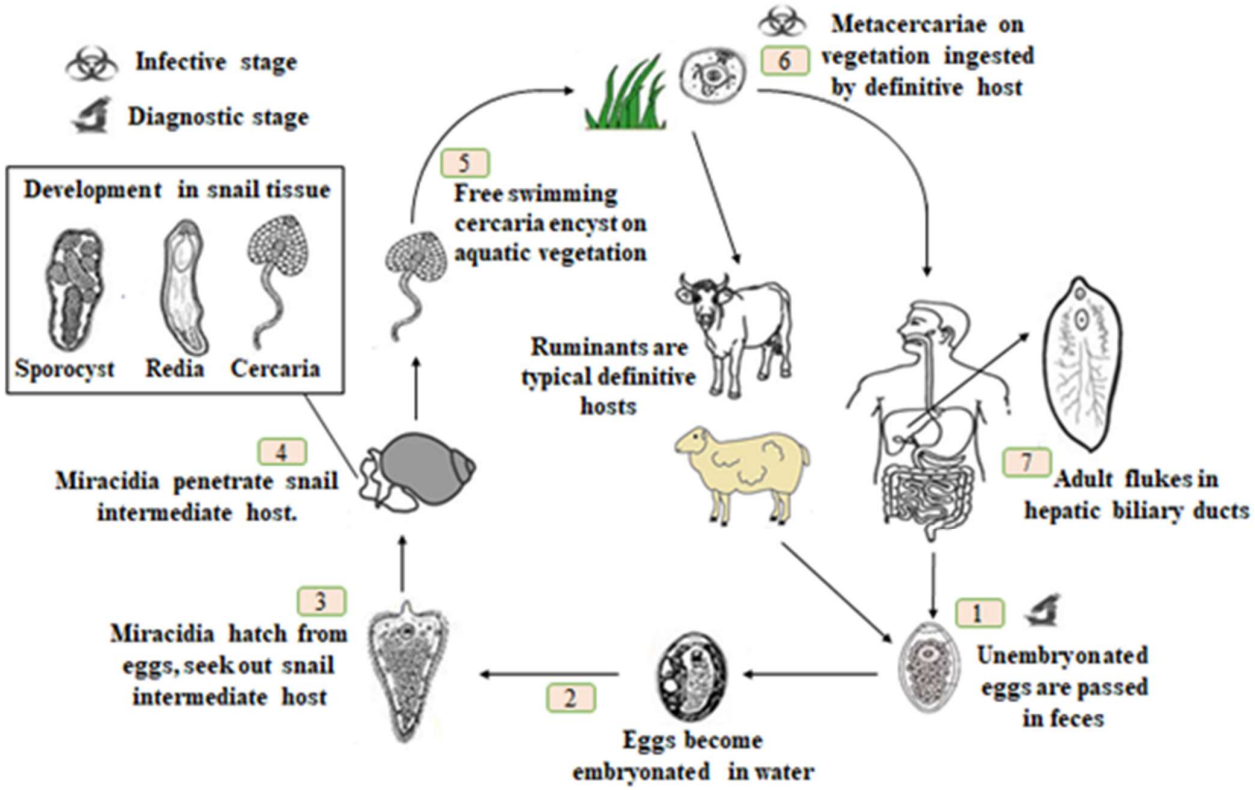

Fig. 3: The Lifecycle of Fasciola spp.

for the completion of the developmental stages and the transmission of the disease. The snails acting as intermediate hosts for Fasciola spp. inhabit a wide range of habitats such as rivers, lakes, springs, irrigation ducts, etc., (Thanh 2012). The snail Lymnaea truncatula acts as intermediate host for *F. hepatica* in Iraq and also, is prevalent in many temperate zones of America, Australia and Europe (Mas-coma et al. 1997, and 2009; Kock et al. 2003). While Lymnaea columella is very common in South Africa (Kock et al. 2003). In Egypt, the snail, L. cailliaudi acts as intermediate host for *F. gigantica*, while L. columella can transmit both *F. gigantica* and F. hepatica. In tropical and subtropical areas of Africa, *F. gigantica* is transmitted by Lymnae natalensis (Thanh 2012). In the intermediate host the miracidia develop into sporocysts, redia and cercaria.

The cercariae leave the snail and swim in water, for a short period of time, then encyst as metacercaria on aquatic vegetation (e.g., watercress) or other objects. Each snail infected with one miracidium can produces up to 4,000 free-swimming cercariae (Rinaldi et al. 2007). When the

metacercaria is taken by the susceptible definitive host, it will excyst by the effect of gastric and intestinal juices in the anterior part of the small intestine and penetrate its wall reaching peritoneal cavity, then to the liver.

The juvenile flukes migrate through the liver tissue and enter the biliary passages, where they become mature and produce eggs (CDC 2019). The incubation period ranges from 2-3 months depending on the number of the flukes and adult flukes may remain alive in the bile ducts of sheep for years, while in cattle most of them start shedding eggs within 5–6 months (Ballweber 2022).

Both Fasciola species can adapt to new animal hosts, thus they infect a wide range of domestic and wild animals and human can also be infected and regarded as incidental host. The commonest definitive hosts for Fasciola spp. are sheep, goats, cattle etc. Besides, other wild animals (deer, rabbits, llamas, rats, kangaroos, and beavers) can be infected with Fasciola spp. (Keiser et al. 2008; Keiser and Utzinger 2009).

Pathology and Symptoms of Fascioliasis

The pathological effects of the disease depend on the parasitic load. Therefore, the severity of the disease varies from asymptomatic to highly symptomatic (Pullan et al. 2008). These effects might be severe in mammalian hosts. This could be attributed to simultaneous infection by the parasite at different disease stages. Fascioliasis clinically can be divided into two phases:

Acute Phase

This phase of the disease starts when the immature Fasciola penetrate the liver tissue of the host (Pullan et al. 2008) causing tissue damage, hemorrhage, inflammation, and fibrosis of the liver. These effects are produced due to digestion of invaded liver tissue by the parasite. The burrows produced by flukes can be observed in the histological examination of liver sections.

The symptoms of the acute stage can be summarized as abdominal pain, gastrointestinal disorders and anemia (Pullan et al. 2008; Becker et al. 2011). Other symptoms of hepatic damage include jaundice, hepatosplenomegaly and ascites (CDC 2019). The infected definitive hosts of the livestock may die suddenly due to intense blood loss, liver dysfunction in addition to secondary bacterial infection with Clostridium novyi. Furthermore, in case of heavy infections, the parasite can penetrate the diaphragm tissue entering to the lung tissue causing pneumonia, infection and fibrosis of the plural membrane and producing lesions in the respiratory system (Pullan et al. 2008; Speich et al. 2010; Becker et al. 2011).

Chronic Phase

This phase of the disease begins when the parasite reaches the bile ducts and lay eggs. The main conspicuous symptoms of this phase include: dilatation and hyperplasia of biliary ducts, their calcification, thickness of gallbladder. In some cases of heavy infections, the bile ducts may be blocked causing severe complications such as, cholangiolitic, acute pancreatitis, cholecystitis and bacterial superinfections (Pullan et al. 2008; Becker et al. 2011).

Other pathological effects of the disease in livestock are intense anemia, since a single Fasciola fluke cane led to losing 0.2-0.5 ml of blood daily (Autissier 2008). Chronic fascioliasis of sheep and cattle may lead to bottle jaw disease (oedema under the jaw) in the infected animals (Duthaler 2012).

Epidemiology of Fascioliasis

Fascioliasis has a global distribution in all continents where sheep and cattle are reared. Due to vast literature on the prevalence and distribution of the infection, in this section only the studies performed in Iraq and some of the neighboring countries during the last decades are reviewed.

The Epidemiology of Fascioliasis and its Relationship to some Factors in Iraq

Some studies have been performed in Iraq reporting the prevalence of fascioliasis in different parts of the country. Rasheed and Kadir (2008) in Kirkuk, reported rates of 2.63%, 0.50%, 0.43% and 4.00% in cattle, sheep goats and buffaloes, respectively. Furthermore, they observed seasonal variation in the rate of infection, since the prevalence was higher (5.0%) in cattle in Autumn, followed by Winter (2.08%). While the highest rates in sheep and goats was in winter, which were 0.88% and 0.68%, respectively. Because the larval stages of Fasciola spp. develop in freshwater snails and their presence depends on environmental conditions like humidity, rain fall, temperature etc. Furthermore, both definitive and intermediate hosts are involved with the external environment (Mas-Coma 2005).

In Erbil, Koyee et al. (2011) inspected slaughtered 53,868 sheep, 17632, goats and 14435, cattle and reported, infection rates of 4.11%, 3.62% and 3.44%, respectively, of fascioliasis. The highest prevalence in sheep was recorded in summer and autumn seasons which was 4.8% for each season, while in winter and summer lower rates (3.86% and 3.64% respectively) were reported. In goats, the highest prevalence (4.61%) was observed in autumn and the lowest (2.84%) in winter. In cattle, the highest prevalence was in spring (4.08%) and the lowest in winter (2.66%).

Al-Kassar (2012) reported the presence of Fasciola spp. stages at a rate of 16.04% in vegetables in Al-Nasiriyah, southern of Iraq. In Babylon, Abdalnabi (2012) performed serological survey for the prevalence of fascioliasis and reported rates of 14.30%, 35.00%, and 68.40% in cattle, sheep and goats, respectively. Hussain and Zghair (2017) reported a rate of 3.61% among 500 examined cows in Karbala, Iraq. Oleiwi et al. (2017) investigated the presence of fascioliasis in 220 blood samples collected from local breed sheep in Abu Gharib district, Baghdad governorate using ELISA test and recorded a seropositivity rate of 12.73% for *F. hepatica* among them.

Abass et al. (2018) investigated the prevalence of fascioliasis in slaughtered cattle, goats and sheep of Kirkuk from 2017 to 2018 and reported rates of 1.35%, 0.63% and 0.28%, respectively. With the highest prevalence (80%) in April and the lowest (6.0%) in November in cattle. On the other hand, the highest prevalence for sheep and goats was recorded in March which were 72.00% and 11.00%, respectively.

Gatie et al. (2018) investigated the prevalence of fascioliasis in cattle and buffalo in Thi-Qar and found 54.7% and 23.71% of them infected. In Koya city, Hassan (2018) investigated the prevalence of cattle and sheep on monthly basis and reported rates of 1.8% in cattle and 0.14% in sheep, with the highest rate (2.30%) among cattle in June and 0.98% among sheep in March while infection was not reported among goats.

In Kirkuk, Abass et al. (2019) investigated the rate of fascioliasis in slaughtered livestock and reported rates of 0.17%, 0.7%, 1.23% and 12.98% in sheep, goats, cattle and buffalo, respectively. Also, they further added that, the rates were higher in spring and winter and lower in summer and autumn.

Al-Mahmood and Al-Sabaawy (2019) in Mosul, reported a natural infection rate of 4% in slaughtered cattle. Al-Alo et al. (2019) investigated the prevalence of fascioliasis in sheep and cattle based on abattoir data of Al-Najaf, southern Iraq, and reported rates of 0.67% in sheep, and 0.66% in cattle. Furthermore, the highest rates in sheep and cattle were during April which were 0.69% and 0.80%, respectively and the lowest rate was 0.38% for both species in October.

Rasheed et al. (2019) investigated the prevalence of fascioliasis among slaughtered sheep in Tikrit and Balad cities, and reported rates of 17.54% and 8.79%, respectively. They further added that in Tikrit, the highest rate (30.58%) was in January and the lowest (10%) was in June, while in Balad city, they reported the highest rate (13.33%) in December and the lowest (3.86%) in June.

In Duhok governorate, Nerway et al. (2021) reported prevalence of 2.0% of fascioliasis among slaughtered livestock, with the highest prevalence of 3.27% in cattle, followed by sheep (1.76%) while the lowest (0.87%) was in goats. The local breed of livestock showed slightly higher rate as compared to imported breed (2.10% versus 1.88%). With regard to gender, the prevalence was higher (2.08%) in males than females (1.08%). The distribution of Fasciola species among livestock differs in sheep and cattle. *F. hepatica* was more common in sheep and goats; while most of the cattle were infected with *F. gigantica*.

Regarding the intensity of infection in livestock, heavy infections were reported in 38.46% of infected animals, with the highest rate (55.74%) being in sheep, followed by light infection which were reported in 35.90% of animals, with the highest rate (62.12%) in cattle, while 28.21% of the animals were infected with a moderate number of flukes with the highest rate (71.43%) being in goats. Most of the liver flukes were recovered from the bile ducts (67.69 %), followed by gall bladder (21.54%) and the least (10.77%) from both the bile ducts and gall bladder.

The Epidemiology of Fascioliasis in Countries Surrounding Iraq

A vast number of studies on fascioliasis, among countries neighboring Iraq were carried out in Iran. Only the studies performed during the period from 2005 to 2019 on sheep, goats and buffalos are listed in Table 1, which showed fluctuation in infection rates.

Regarding seasonal fluctuation of prevalence in Iran, cattle, sheep and goats showed the highest prevalence in spring and summer as reported by Ali et al. (2011), while, Khanjari et al. (2010) reported high prevalence among cattle in spring and among sheep in winter. On the other hand, some studies recorded higher prevalence in cattle, sheep and goats in winter, and low prevalence in summer (Kordshooli et al. 2017; Arbabi et al. 2018; Aminzare et al. 2018).

With respect to gender, some studies recorded significantly higher prevalence in females than males (Ali et al. 2011; Khanjari et al. 2014).

In Saudi Arabia few studies were carried out on the prevalence of fascioliasis in livestock. Sanad and Al-Megrin (2005) investigated the prevalence of fascioliasis in imported and local sheep in Riyadh city, and recorded a rate of 21.9%, with a higher rate among imported sheep as compared to local one, since only 4.96% of them were infected. Degheidy and Al-Malki (2012) investigated the prevalence of Fasciola infection and liver abscess among slaughtered imported cattle, in Al-Taif city and reported rates of 8.6% and 1.1% respectively, they further, added that fascioliasis caused 52.06% loss of liver and meat estimated to be around 75000 SR yearly. In another study in Al-Taif, Degheidy et al. (2013) recorded a prevalence of 3.1% in sheep.

In Jordan only the study performed by Maraqa et al. (2005) reported the prevalence of helminthic infection in imported and local sheep. *F. hepatica* was reported in 3.2% of sheep imported from Australia, while none of the local breed was found infected.

Yildirim et al. (2007) recorded prevalence of *Fasciola hepatica* in 65.2% cattle of Turkey using ELISA test, with significantly higher rate among females than males (70.7% versus 47.8%). Significantly different rates were observed in different age categories while the association among animal was found nonsignificant. In the black sea area, Acici et al. (2017) investigated the rate of F. hepatic in sheep in three areas using ELISA, and reported rates of 32.40%, 25.40% and 34.90% in Samsun, Sinop and Tokat, respectively. Furthermore, Celik and Celik (2018) performed another study on fascioliasis and recorded rates of 7.5% and 14.14% for *F. hepatica* among sheep and goats, respectively. With regard to Syria and Kuwait, fascioliasis was not investigated in these countries.

The Genome of Fasciola spp.

Fasciola gigantica complete nuclear genome is 1.04 Gb, having a total of 20858 genes (Pandey et al. 2020). While, F. hepatic possess the largest parasitic genome accounting of 1.3Gb (Cwiklinski et al. 2015). It possesses 10 pairs of chromosomes, 32% of this genome is composed from repetitive DNA. This genome contains 12 gene-coding proteins, two rRNA genes and 22 transfer RNA genes, with the absence of mitochondrial genes that encode ATP synthase membrane subunit 8 (atp 8 gene) (Huang et al. 2013; Liu et al. 2014). The targeted genes in each genome must be long enough to show reasonable variable differential and similar features allowing differentiation between the genes of the targeted species (Huang et al. 2004). Because some nucleotides remain constant and others are divers, this will lead to the occurrence of small numbers of mutations in the active constraint regions of the genome. Coding regions possess more functional constraints as compared with non-coding regions; therefore, their information is used in phylogenetic studies (Shaw et al. 2005). Phylogenetic and genetic studies of Fasciola spp. have been performed using r DNA and mitochondrial DNA (mt DNA) markers (Le et al. 2000; Huang et al. 2004).

DNA Markers

The DNA markers are modern tools used for the differentiation between Fasciola species, since morphological differences do not give the precise identification of the species due to the overlapping of the morphological features between both Fasciola species in addition to time consuming (Rokni 2008; Hasanpour et al. 2020). Therefore, recent molecular techniques were used for distinguishing both

species of flukes, as it is necessary to identify them accurately because intermediate forms of both species have been raised creating difficulties for taxonomical identification (Walker et al. 2006). For this reason, molecular techniques are considered as the golden method for discrimination between both species.

So far, the used markers include first Internal Transcribed Spacers (ITS-1) of 5.8S and second Internal Transcribed Spacer (ITS2) of 28S ribosomal ribonucleic acid (rRNA) (Marcilla et al. 2002; Le et al. 2008) and the mitochondrial DNA markers (Walker et al. 2006).

Internal Transcribed Spacers (ITS)

The rDNA markers are valuable markers, because they have a number of variable regions between two conservative regions making them a perfect molecular marker in molecular studies (Hills and Dixon 1991). These markers are non-coding regions situated between18S, 5.8S and 28S ribosomal RNA genes as illustrated in Fig. (4). The ITS1 and ITS2 are used in phylogenetic studies for differentiation between Fasciola spp. isolated from various hosts and different locations. This will help in identifying the intermediate forms of Fasciola and in the precise identification of the known Fasciola spp. (Itagaki and Tsutsumi 1998; Itagaki et al. 2005; Prasad et al. 2008).

Mitochondrial DNA Markers

These markers are used in phylogenetic studies of Fasciola spp., they included the cox1, and the Mitochondrial Nicotinamide Dinucleotide Dehydrogenase Subunit-1 (nd1). Both these markers have been used in many molecular studies for determining the relatedness among Fasciola spp. (Chaichanasak et al. 2012). Such studies have been carried out in Bulgaria, China, Turkmenistan, Turkey and Russia using one or both of these markers (nd1 and cox1) for determining the lineages of F. hepatica, and they reported 13 haplotypes using nd1 and 10 using cox1 (Semyenova et al. 2005).

Molecular Studies on Fascioliasis

Molecular Studies of Fascioliasis in Iraq

In Iraq some molecular investigations were performed using different DNA markers for identifying Fasciola spp. Mohammed et al. (2016) used ITS-1 and ITS-2 ribosomal DNA markers to identify *F. hepatica* isolated from cattle in Duhok governorate. Abdulwahed and Al-Amery (2019) used ITS2 and RFLP to identify *F. hepatica* and *F. gigantica* isolated from infected sheep in Al-Kut city, central region of Iraq. In addition, they also, reported a new intermediate form of Fasciola. In Baghdad, Hassone and Salah (2019) performed a phylogenetic study of *F. hepatica* isolated from cattle using cox1 gene and recorded 12 *F. hepatica* closely related forms which were similar to sequences from Spain.

Hamoo et al. (2019) used ITS-1 rDNA as a marker for identifying Fasciola spp. isolated from cattle liver in Kirkuk. They reported *F. gigantica* which showed 100% similarity with globally isolates sequences. The same researchers conducted another study in Aqrah (Hamoo et al. 2020) using the 18S rRNA gene for the identification of *F. gigantica* removed from the liver of infected cattle.

Table 1: The prevalence of Fasciola spp. among livestock in Iran

Hosts	Province in Iran	Prevalence (%)	References
Sheep	Fars	38.30	Sayari et al. 2008
Goats	Fars	5.00	
Cattle	East Azerbaijan	32.10	Eslami et al. 2009
Sheep	East Azerbaijan	32.00	
Calves	East Azerbaijan	0.00	
Buffaloes	East Azerbaijan	17.00	
Horses	East Azerbaijan	50.00	
Cattle	Khuzestan	49.50	Ahmadi and Meshkehkar 2010
Sheep	Khuzestan	28.70	
Goats	Khuzestan	35.90	
Cattle	Isfahan	2.40	Ali et al. 2011
Sheep	Isfahan	6.90	
Goats	Isfahan	4.10	
Cattle	Ilam	53.00	Abdi et al. 2013
Sheep	Ilam	36.50	
Goats	Ilam	10.50	
Cattle	Lorestan	7.60	Ezatpour et al. 2014
Sheep	Lorestan	7.10	Ezatpour et al. 2015
Goats	Lorestan	3.90	
Cattle	Fars	1.65	Mohamadzadeh et al. 2016
Sheep	Fars	0.33	
Goats	Fars	0.24	
Cattle	Jahrom	11.15	
Sheep	Jahrom	5.22	Kordshooli et al. 2017
Goats	Jahrom	2.15	
Cattle	Arak	0.76	
Sheep	Arak	0.75	Arbabi et al. 2018
Goats	Arak	0.42	
Cattle	Guilan	9.00	Khademvatan et al. 2019
Sheep	Guilan	4.20	
Goats	Guilan	3.10	

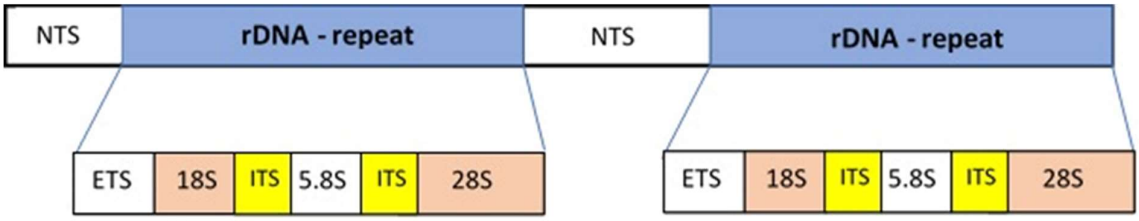

Fig. 4: Diagram of ITS-1 and ITS-2 markers.

Raoof et al. (2020) used the mitochondrial 28S rRNA gene in a molecular study and phylogenetic analysis for Fasciola species isolated from sheep and goats of Sulaymaniyah governorate. They obtained two sequences of *F. gigantica* that showed high similarity with sequences of Indian strains, while four *F. hepatica* sequences were analogous to sequences from Iran and Saudi Arabia.

Mohammed et al. (2021) carried out another molecular study and phylogenetic analysis of Fasciola spp. isolated from cattle, sheep and goats in Duhok using ITS1 and ITS2 as DNA marker. They sequenced 13 products from the study; 7 amplicons were recognized as *F. hepatica* and 5 as *F. gigantica* except one sequence of *F. gigantica* was clustered as monophyletic, these sequences were deposited in GenBank.

Molecular Studies on Fascioliasis in Countries Surrounding Iraq

Simsek et al. (2011) used PCR-RFLP method for targeting mitochondrial cox1 with the help of the restriction enzymes AluI and RsaI for identification of Fasciola spp. isolated from domestic livestock in Turkey. Both fluke species were reported.

In Turkey also, Canakoglu et al. (2019) used molecular tools for identifying Fasciola spp. isolated from cattle and sheep using PCR-RFLP on β-tubulin isotype 3 gene and the enzymes MboII, HphI and HindII. Polymorphism in β-tubulin isotype, 3 genes were reported among *F. hepatica* isolated from sheep and cattle of the two studied parts of Turkey.

Shalaby and Gherbawy (2013) performed a molecular study of Fasciola species isolated from imported sheep in Saudi Arabia, using Random Amplified Polymorphic DNA (RAPD) PCR in amplifying ITS1. Both species of Fasciola were recorded in their study in addition to a hybrid form with different genetic constituents of both species.

Amor et al. (2011) used morphological and molecular techniques for the identification of Fasciola species isolated from buffaloes and goats in Iran. They used the sequences of both ITS-1 and ITS-2 rDNA. The performed tree of these sequences showing 48.1% identity to the haplotypes that most frequently identified for F. hepatic and 38.45% identity to *F. gigantica*. But these sequences differ from each other in the position of some nucleotides of the ITS region. Also, a new intermediate form with 13.45% similarity was reported which showed overlapping of nucleotides in all positions between the two Fasciola species.

Also, Shahbazi et al. (2011) used ITS1 and RAPD-PCR techniques for molecular identification of Fasciola removed from sheep and cattle. They reported intra-specific variation within both species. Shafiei et al. (2014) investigated the morphological and molecular analysis of *F. hepatica* and *F. gigantica* using the ITS1, ITS2, and mitochondrial genes (nd1 and cox) in the southwest of Iran. They isolated *F. hepatica* from sheep and goats, while both species (*F. hepatica* or *F. gigantica*) were isolated from cattle.

In west region of Iran, Kobra et al. (2018) used morphological characters and molecular tools like PCR-RLFP, to identify both Fasciola spp. and their intermediate forms isolated from sheep, goats and cattle. Another study in west of Iran, Shokouhi et al. (2019) carried out a genotyping investigation on Fasciola spp. isolated from sheep and cattle targeting ITS1 by PCR and PCR-RFLP techniques with Rsa1 restriction enzyme and they reported *F. hepatica* in their study.

Javanmarda et al. (2020) identified Fasciola spp. taken from sheep, goats, cattle and humans using multiplex PCR and RLFP-PCR for targeting the genes phosphoenolpyruvate carboxykinase (pepck) and DNA polymerase delta (pold). They found that all isolates were *F. hepatica* without the detection of any hybrid form. But the sequences isolated from cattle were with higher diversity in the three genes than those of sheep and goats. In addition, phylogenetic analyses showed a close relation between *F. hepatica* isolates of human and sheep.

Diagnosis of Fascioliasis in Livestock

The control and management of fascioliasis depend on the early identification and treatment of the disease in infected hosts. Different diagnostic methods have been used for the diagnosis of the disease in livestock such as coprological, morphometric, serological, imaging techniques and molecular techniques (Thanh 2012).

Coprological and Morphometrical Methods

In coprological diagnosis, the stool samples are examined using microscopy which is effective for detecting Fasciola eggs in the stool samples and in bile and duodenal fluid samples. Stool samples can be examined using sedimentation and Kato-Katz method (Hanpithakpong et al. 2008; Bhamidipati et al. 2009). The copromicroscopic techniques are cheap, and can easily be performed. Using this method, the intensity of infection can be estimated that help in determining the effect of anthelminthic used in treatment of livestock (Cesar et al. 2011).

Fasciola eggs are quite big and characteristic, as they are oval, operculated and unembryonated. So morphological characters can be helpful in diagnosis, except during migration of immature flukes in the liver tissue and in such cases, it will be difficult to detect the eggs in stool samples (Hillyer 1999; Marcilla et al. 2002). The copromicroscopy can be good for diagnosing chronic infections, since mature flukes only can lay eggs, but in the cases of acute and ectopic infections this method is useless (WHO 2006).

The morphometric methods are used in many countries for the differentiation of adult Fasciola flukes depending on measurements of body length and other morphological

characters which are differential such as, body length, width, cone length and width, size of suckers, and other measurable characters (Periago et al. 2006; Ashrafi et al. 2006). For precise diagnosis, it is useful to combine morphometric method with Computer Image Analysis System (CIAS). This technique has a wide application in many countries where fascioliasis is prevalent (Periago et al. 2008; Itagaki et al. 2009; Thanh 2012). Morphological features are not used for identification of intermediate forms of Fasciola species, because these forms are genetically different and such diagnostic method do not give accurate results (Knowlton 1993). Furthermore, morphological identification requires a lot of experience, so using molecular techniques can give a more confirmative identification of a species (Hebert et al. 2003).

Immunodiagnosis Methods

The most widely used immunological methods include Enzyme-Linked Immunosorbent Assays (ELISA), which is indirect serological test used for detecting antibodies produced by the parasite (Rokni et al. 2002; Espinoza et al. 2005; WHO 2006). ELISA helps to diagnose even infections with low parasite load in addition to ectopic infections (Mas-Coma et al. 2005; WHO 2006). Although, immunodiagnostic techniques are highly sensitive, but they have some difficulties in recommending the precise treatment, since the circulating antibodies remain for long time after treatment. Furthermore, cross reacting antibodies with other trematodes may develop (Boulard et al. 1995; Hillyer et al.1999).

Molecular Methods

Molecular Methods are the most confirmative means for diagnosis as compared with other methods. In this method polymerase chain reaction (PCR) are used and they gave an accurate diagnosis of both parasite species and even strains (Mas-Coma et al. 2005; Thanh 2012).

For precise identification of species and the measurement of their boundaries in addition to studying their phylogenetic relationships, many molecular methods are used including mitochondrial and nuclear DNA markers, fingerprinting, hybridization methods, sequencing of selected genes, DNA barcoding and microsatellites (Maddison 1997; Baker and Bradley 2006; Thanh 2012). All these methods can be used for differentiation of Fasciola species eggs and from eggs of other trematodes and even can be used in identifying the parasite in infected snail host (Ai et al. 2011).

Treatment of Animal Fascioliasis

For treatment of animal fascioliasis, numerous anthelminthic drugs have been used. Some of these drugs are effective in treatment for adult parasite stages (Spithill 1999). The drug Triclabendazole is effective for mature and immature stages of *F. hepatica* and *F. gigantica*. Therefore, it is used in the treatment of acute and chronic stages of fascioliasis (Waruiru et al. 1994). This drug is given as one dose of 10 mg/ Kg body weight for treatment of adult flukes inhabiting the biliary passages and the migratory juvenile flukes within liver tissue (Mahmoud 2008). Three doses per year are recommended for treatment starting from winter season for treating chronic infection. The second dose is recommended in spring to minimize the pasture contamination by parasite eggs and the third dose in autumn, at the end of dry season (Pfukenyi and Mukaratirwa 2004).

Prevention and Control of Fascioliasis

It is necessary to control the snail hosts which harbor the larval stages of the parasite and contaminate the pasture preventing disease spreading among definitive hosts (Pfukenyi and Mukaratirwa 2004). In this step, molluscicides and pesticides are used (Mzembe and Chaudhry 1981). Molluscicides are used for reducing the snail populations in rivers, springs dams or for the amphibious snails living on the surface of plants (Spithill 1999). The other step is to keep away the animal herds from sites that contain heavy growth of aquatic plants used for animal feeding that might be contaminated with parasite infective stages.

Conclusions

This review indicates that fascioliasis is endemic in Iraq, since both *F. hepatica* and *F. gigantica* in addition to their intermediate forms were reported in the livestock besides the presence of the snail hosts required for the completion of the parasite life cycle. Thus, the application of preventive and control measures are necessary,

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CHAPTER 19

CRYPTOSPORIDIOSIS IN DOMESTIC ANIMALS: AS NEGLECTED ZOONOTIC DISEASE

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INTRODUCTION

Cryptosporidiosis is considered as a major zoonotic infection caused by protozoan parasite, with public health and economic impacts on humans and animal health, since it infects humans and a wide variety of animal species. Cryptosporidiosis is caused by an intracellular apicomplexan protozoan parasite belonging to different species of Cryptosporidium. About 23 species of Cryptosporidium have been identified from human and other vertebrates including domestic animals with different degree of pathogenicity ranging from asymptomatic to severe gastrointestinal disorder (Ryan and Hijjawi 2015). This parasite has a direct monoxenous life cycle (sexual and asexual stages), which is completed within a single host, usually an herbivore animal (Leitch and He 2012).

Humans are most commonly infected with Cryptosporidium hominis by the consumption of contaminated food or water with oocysts of the parasite. The parasite infects the gastrointestinal tract and causes injury to small intestine resulting in severe diarrhea (Feng et al. 2018). Furthermore, Cryptosporidium parvum, which most frequently infects animals, can cause infection in humans as well and is also considered as a zoonotic protozoan (Hatam-Nahavandi et al. 2019).

Several species of Cryptosporidium cause infection in young ruminants especially cattle leading to varying degree of symptoms such as loss of appetite, lethargy, dehydration, retardation of the animal's growth, reduction in milk yield, and in some cases can cause death of the infected animal (Thompson et al. 2008; Thomson et al. 2017; Santin 2013). Till now, the importance of human and animal cryptosporidiosis failed to gain necessary attention. Such attention could be illustrated by the economic losses in domestic animals. Cryptosporidiosis is common in many domestic and wild animals and these animals can serve as sources for the infected stage, the sporulated oocyte that infects humans and contaminate the environment (Robertson et al. 2014).

Domestic animals are susceptible to sporulated oocyst (Hatam-Nahavandi et al. 2019; Gerace et al. 2019). Sporulated oocyst in the feces is considered as the infected stage for wide host range due to the easy way for transmission and the highly resistance nature of the oocysts to disinfectants and environmental condition (Caccio` and Putignani 2014). Several species of Cryptosporidium infect domesticated animals. Among these, *C. bovis* and *C. parvum* are found in cattle, *C. canis* in dogs, *C. baileyi* in birds, *C. cuniculus* in rabbits, *C. felis* in cats, *C. meleagridis* in Turkeys, *C. suis* and *C. scrofarum* in pigs (Petersen 2015).

Historical Perspective of Cryptosporidium

In 1907, cryptosporidiosis was first recognized as a commensal protozoan by Edward Tyzzer, who isolated *Cryptosporidium muris* from asymptomatic laboratory mice. Following the initial description of Cryptosporidium, the discovery was elapsed over 50 years, as the parasite was misdiagnosed with other coccidian members particularly Sarcocystis, because the oocyst of Sarcocystis species have thin walls, which rupture and release sporocysts containing sporozoites like sporulated oocyst of Cryptosporidium (Xiao et al. 2004).

In early 1960s, ultrastructural studies recognized endogenous stages having an attachment organelle of the parasite, which is used as the main character for differentiation between Cryptosporidium and Sarcocystis and the wrong perception of strict host specificity was used for Cryptosporidium. Such recognition led to the new discoveries of various species: *C. garnhami* (humans), *C. bovis* (calves), *C. agni* (sheep), *C. rhesi* (monkeys), *C. anserinum* (geese), *C. cuniculus* (rabbits) (Upton 2000; Pedraza-Diaz et al. 2001; Xiao et al. 2004).

The veterinary importance was highlighted with high mortality and morbidity in turkeys caused by *C. meleagridis* in 1950s and bovine diarrhea caused by *C. parvum* within the early 1970s (Current and Garcia 1991), and the diagnosis of Cryptosporidium species in the respiratory and gastrointestinal tract of mammals, reptiles, birds and fish (Leitch and He 2012). Although, broad range of domestic animals were infected with Cryptosporidium, but the impact remained ignored until 1980s when cryptosporidiosis was recognized as one of the common primary causes of neonatal diarrhea in calves and lambs (Tzipori et al. 1982).

In the twentieth century, several species of Cryptosporidium were named based on the host origin, due to cross-transmission of the Cryptosporidium from one host species to

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another such as *C. wrairi* in guinea pigs, *C. meleagridis* in turkeys, *C. felis* in cats, *C. baileyi* in poultry and game birds (Goodwin 1989; O'Donoghue 1995; Xiao et al. 2004). Recently, several Cryptosporidium spp. are named by using molecular techniques such as *C. andersoni* from cattle, *C. canis* from dogs, *C. hominis* from humans, and C. molnari from fish (Fayer et al. 2001; Alvarez-Pellitero and Sitja-Bobadilla 2002).

Classification of Cryptosporidium spp

According to the classification system proposed by Tyzzer (1907), Cryptosporidium species is classified as:
Phylum: Apicomplexa
Class: Conoidasida
Subclass: Coccidia
Order: Eucoccidiorida
Suborder: Eimeriorina
Family: Cryptosporidiidae
Genus: Cryptosporidium

Life Cycle and Ultrastructure of Cryptosporidium Parvum

Cryptosporidium has complex monoxenous life cycle undergoing both sexual and asexual replications (Bouzid et al. 2013). The parasite undergoes several morphological changes for completing its life cycle. The life cycle starts with the infected stage, the oocyst which is excreted in human or animal feces leading to the contamination of water and environments as presented in Figure 1. The oocyst has obscure internal structures with average size around 4-6 μm. Two forms of oocysts exist: Thin wall oocyst consists of protein lipid carbohydrate matrix which causes re-infection in the wall of the digestive tract and thick wall oocyst consists of inner and outer walls which is released to the environment with feces (Pumipuntu and Piratae 2018). The thick wall of sporulated oocyst enable oocyst to withstand in the environmental conditions (sunlight and drying). Animals are infected by consuming contaminated food (grazing) or contaminated drinking water. The excystation of oocysts occurs in the gastrointestinal tract under favorable condition such as the presence of carbon dioxide, bile salt, pancreatic enzymes, acidic pH and optimum temperature (37°C) which induces excystation of oocysts and release of four sporozoites that invade the outer small intestine enterocytes of the gastrointestinal tract (O'Donoghue 1995; Silva and Sabogal-Paz 2020; Siddique et al. 2021).
The sporozoite of Cryptosporidium is nucleated, spindle shape stage with average size of 5.0 ×0.5 μm. The apical complex of sporozoites is the defining feature of Cryptosporidium and help in the invasion and adherence to gastric epithelial cells in stomach in case of infection with *C. muris* and *C. andersoni* and the intestinal epithelium cells in case of infection with *C. parvum* and *C. hominis* (Fayer 2010).
The sporozoites are engulfed by the host cells and form parasitophorous vacuole which changes the cytoskeleton of the infected cells (Guérin and Striepen 2020). Asexual cycle begins with the conversion of sporozoites into trophozoite inside the parasitophorous vacuole. Mitotic division occurs and results in the production of type I meront, which converts into 6-8 type I merozoites. Merozoites (type-I) can re-attack the cell and replicate asexually and produce two forms, either meronts which holds eight merozoites or meronts (type-II) which holds only four merozoites. The merozoites are less active and larger than merozoites which are reproduced from type I meronts. Merozoites (type-II) infect neighbor enterocytes and produce macrogamont (female) and microgamont (male) which are sexual stages reproduced by fertilization to form diploid zygote which in turn convert to oocysts. Four sporozoites result from meiosis division in oocyst (Tandel et al. 2019).

The Genome of Cryptosporidium Species

The genomes of Cryptosporidium are containing eight chromosomes, with average size of 0.945-2.2 Mb and a total haploid genome size of about 9.2 Mb (Hays et al. 1995; Blunt et al. 1997). Moreover, Cryptosporidium parvum contain two small extrachromosomal cytoplasmic double-stranded RNAs (Khramtsov et al. 1997). The RNAs have an open reading frame (ORF), each of them encodes RNA-dependent RNA polymerase and encodes protein that has restricted homology to protein kinases of the mammalian (Clark 1999). The rRNA gene of Cryptosporidium parvum is composed of small rRNA subunit with a size of 1.7 kb and a large rRNA subunit with size of 3.6 kb, as well as 5.8S rRNA subunit with a size of 151-bp. The analysis of the genome revealed the presence of metabolic pathway and the lack of cellular structures found in other apicomplexans (Wanyiri and Ward 2006). Although, the main energy source for parasite is glycolysis metabolism, but both aerobic and anaerobic metabolisms are presented, hence reflecting the flexibility of the environment (Barta and Thompson 2006). The limitation of biosynthesis and metabolism suggesting the major requirement on nutrient acquisition from the host (Rider and Zhu 2010.). The analysis of genome encoded many transporters which are necessary for the importance of the critical nutrients from the host (Xu et al. 2004, Pain et al. 2005). Genomic analysis presented that Cryptosporidium spp. and Plasmodium spp. shared over 150 ancestral "apicomplexan" proteins, involved mainly in interactions with host cells of the eukaryotic and the biogenesis of the apical complex (Templeton et al. 2004).

Epidemiology of Cryptosporidiosis in Ruminant

The infection of farmed animals with cryptosporidiosis is of veterinary significance, consequentially leading to clinical morbidity, mortality besides major production losses. The zoonotic nature of various species of Cryptosporidium indicated that the public health may be affected by animals' infection (Robertson et al. 2014). The transmission of Cryptosporidium oocysts from host to another occurs via contaminated feces, droppings, food, water and utensils (Zahedi et al. 2020).
Cryptosporidiosis is detected in small and large ruminants worldwide, especially calves and lambs (Thomson et al. 2017). Four types of Cryptosporidium were identified in large ruminants especially cattle and buffaloes are *C. parvum, C. andersoni, C. rayanae* and *C. bovis* (Haghi et al. 2020). The main causative agent of profuse diarrhea and possibly death in neonatal calves is Cryptosporidium parvum, then the disease is asymptomatic in animals older than six weeks (Siddique et al. 2021).
In Iraq, several research studies on cryptosporidiosis illustrated that among domestic animals; cattle are more commonly infected with oocysts of *C. parvum* compared to other domestic animals with prevalence of 54.1%, due to shedding of heavy

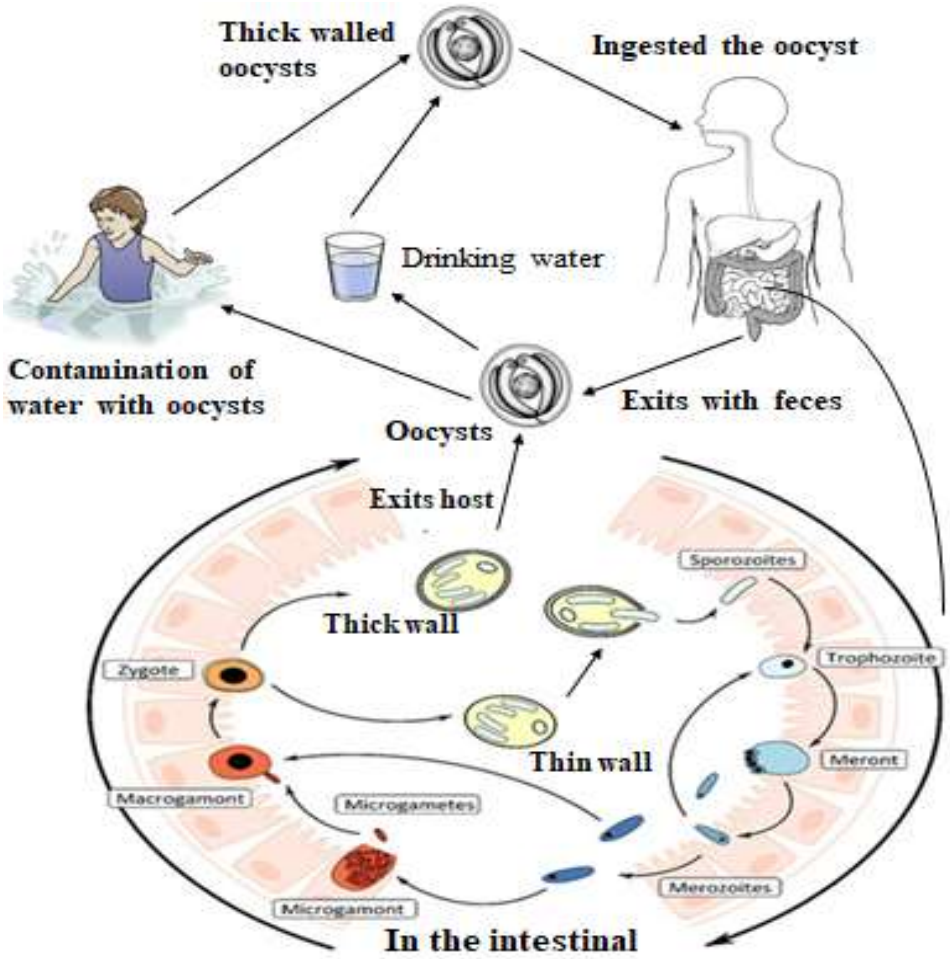

Fig. 1: Life cycle of *Cryptosporidium parvum.*

burden of oocysts (1.1× 108) per gram of feces (Silverlås and Blanco-Penedo 2013; Robertson et al. 2014; Hatam-Nahavandi et al. 2019; Alali et al. 2021). Other species of Cryptosporidium have been reported; Cryptosporidium andersoni diagnosed among young and adult cattle, while *C. bovis* and C. ryanae were detected among post-weaned calves and less commonly among pre-weaned calves (Yang et al. 2020; Alali et al. 2021). The reasons for detection of Cryptosporidium species in many animals' species at different ages are due to changes in the intestinal microflora as well as changes in diet, which may reduce the capacity of the pathogen to infect adult animal enterocytes (Siddique et al. 2021).

Cryptosporidium anderson infects adult cattle more commonly than young cattle and leads to a decrease in milk production in adult cows. While, infection with *C. parvum* may lead to tiredness, weariness, fatigue, profuse watery diarrhea, dehydration, inappetence and death in severe cases among neonatal calves. In case of watery diarrhea, the signs of diarrhea appear after 3-4 days of ingestion of oocysts. Then the infected animals may shed 1×1010 oocysts per day (Mohteshamuddin et al. 2020; Wu et al. 2020; Shaw et al. 2021).

Among small ruminant, sheep and lambs are infected with cryptosporidiosis caused by C. ubiquitum and C. parvum. Cryptosporidium ubiquitum the oocysts can be detected in all age groups of these animals, while C. xiaoi and *C. parvum* mostly infect lambs and goat kids (Dessì et al. 2020). The infection is more severe among small ruminants compared to large ruminants with 100% morbidity and 50-70% mortality. Most common clinical signs among infected small ruminant are diarrhea, anorexia, abdominal pain and depression (Mammeri et al. 2019; Kabir et al. 2020; Santin 2020). Among livestock, few studies have been performed on cryptosporidiosis in sheep and goats than in cattle due to the reason that sheep and goats are grazing in open outdoor area as compared to cattle which is being kept indoors (Robertson et al. 2014). In general, younger small ruminants are more vulnerable to cryptosporidiosis as compared to older ruminants, due to high rate of diarrhoeal prevalence and shedding, whereas, in old animals, subclinical infection occurs which is reflected in low shedding rates (Vieira et al. 1997; Robertson et al. 2014). In Iraq, few and inadequate studies have been conducted on Cryptosporidium in animals and these studies covered few cites with variable infection rates. In Mosul among calves the rate of infection was 29% (Al-Robaiee and Al-Farwachi 2014), in Wasit the rate of infection was 51.7% among calves (Al-Zubaidi 2015), in Baghdad, the rate of infection was 35.6% among cattle (Merdaw et al. 2018), also another study was conducted in Baghdad and recorded a high rate of infection which was 38% among cattle (Alseady and Kawan 2019).

Generally, one study was conducted among horses in Baghdad city with a high rate (64%) of infection (Altaee et al. 2014). Another two studied on Cryptosporidiosis in camels were performed, which reported varying rates of infection about 14% -61% and *C. parvum* was the only species detected (Hussin et al. 2015; Ahmed et al. 2016). However, in case of sheep and goats,

the rate of infection was 13% -74% in sheep and 12% -32.5% in goats and six species of Cryptosporidium were reported namely; *C. parvum, C. hominis, C. suis, C. andersoni, C. ubiquitum* and *C. xiaoi* among sheep and goats (Alali et al. 2021).

Diagnosis of Cryptosporidiosis

Cryptosporidiosis does not have a pathognomonic clinical sign, which means that laboratory verification is mandatory for confirmation of diagnosis. Hence microscopic examination is required for detecting the oocysts in the feces after staining the samples with one of the following stains; modified acid-fast stain, Auramin O (fluorescent stain) or immunofluorescent stain (Jex et al. 2008). The acid-fast method used for detection is a modified method of Ziehl Neelsen and dimethyl sulfoxide, it contains safranin-methylene blue, and modified Koster.

Ziehl-Neelsen stained oocyst appears as red crescent-shaped sporozoites with size around 4-6 µm (Chalmers and Katzer 2013). Concentration of stool samples is necessary for the detection of stained smear, which enhance the detection of fresh, frozen or preserved stool by concentrating the quantities of oocysts (Pacheco et al. 2013). Two common methods are used for the concentration of stool samples; Formalin Ethyl Acetate sedimentation technique and modified zinc sulfate floatation technique (Pumipuntu and Piratae 2018).

Although microscopic examination remains the gold standard for detection, but it is time-consuming, besides that it is lacking both specificity and sensitivity which depends mainly on the expertise of the microscopist to distinguish oocysts from other cysts found in the sample (Jex et al. 2008). Therefore, Immunological techniques has been developed for the detections of the oocyst antigen including Enzyme Linked Immunosorbent Assay (ELISA) and immunochromatographic lateral flow (ICLF) (Fayer et al. 2000; Jex et al. 2008). Although such techniques reduce labor, cost and time consumption and enhance the sensitivity and specificity, but many antigens of infected oocyst are preserved within Cryptosporidium species. Hence molecular technique based on DNA detection have been developed and used to identify species of Cryptosporidium using; polymerase chain reaction (PCR) assay (real time PCR, nested PCR and multiplex PCR), DNA sequence and PCR Restriction Fragment Length Polymorphism, (Simonato et al. 2017; Abdelsalam et al. 2017; Mero et al. 2017).

Molecular Characterization of Cryptosporidium Species

Identification of new species of Cryptosporidium is no longer depend on the morphological description studies because most infected animals harbor multiple Cryptosporidium spp., which are usually difficult to be differentiated from each other on the basis of morphological characters. Hence, molecular diagnostic techniques are developed to identify species based on the distinct genetic differences. In most molecular methods, several genetic studies use DNA markers such as small subunit ribosomal ribonucleic acid (SSU rRNA), gene family of heat shock protein 70 (HSP70), Acetyl-CoA gene, Cp15 and Cp 11 gene, 60 kDa glycoprotein (gp60) and dihydrofolate reductase inhibitors (dhfr) and for the detection of different species of Cryptosporidium isolated from animal sources (Fayer et al. 2000; Khan et al. 2017). Such DNA markers are beneficial due to their universal distribution and had specific and generic primers (Xiao et al. 2004). DNA markers are useful for distinguishing between Cryptosporidium andersoni and C. muris and also, used to distinguish *C. parvum* from *C. canis*and C. hominis (Fayer et al. 2001; Morgan-Ryan et al. 2002). Other DNA markers have been used for differentiating some species of Cryptosporidium such as gene coding proteins; Cryptosporidium oocyst wall protein (COWP) gene which is used for differentiating between Cryptosporidium wrairi and C. parvum, and also, between *C. parvum* isolated from animal and human origin (Xiao et al. 2000). Thrombospondin Related Adhesive Protein 1 (TRAP-C1) (Spano et al. 1998) and b-tubulin gene for identification *C. parvum* (Widmer et al. 1998). Besides noncoding DNA sequences used such as Internal Transcribed Spacer 1 (ITS1) (Morgan-Ryan et al. 2001) and microsatellites (Feng et al. 2000).

Treatment of Cryptosporidiosis

Although, infected animals with cryptosporidiosis have strong immune response and can recover spontaneously without any treatments, some supportive treatments are required for managing heavy infections and to reduce oocyst shedding such as; paromomycin which is anti-Cryptosporidial aminoglycoside used in the treatment of Cryptosporidiosis in cattle, calves, lambs and goats with appropriate dose 100 mg/kg body weight up to 3 weeks after birth (Brainard et al. 2020). Halofuginone lactate is used as prophylactic treatment to treat milder clinical signs and reduce the output of oocyst (Silverlas et al. 2009; De Waele et al. 2010). Another drug such as nitazoxanide, notwithstanding and decoquinate drugs are also used in animals for treatment (Viel et al. 2007; Gargala 2008; Ollivett et al. 2009; Siddique et al. 2021).

Prevention of Cryptosporidiosis

The only method to control cryptosporidiosis and to reduce the high levels of environmental contamination with infected stages is good hygienic farm management practices by using clean feeding and watering cans. The infection could be controlled among neonatal calves by controlling the number of infected stages (oocysts) consumed and ingesting an adequate amount of colostrum within 24 hours of birth which allow the immunity to develop and reducing the cryptosporidiosis severity and malnutrition should be avoided.

Infected cattle should be isolated in dry and clean house from other infected animals to control the spreading of the parasite to other healthy cattle. The infected cattle should be treated with fluid and electrolytes, besides enhancing the digestion of neonatal claves by giving milk in small amounts many times daily to minimize weight loss (Robertson et al. 2014). Other preventive measures should be applied to control the transmission of Cryptosporidium spp. such as reducing the number of ruminants in the animal stocks which lowers the chances of contact between calves and their owners, and separation of young animals which are more susceptible to infection from adult one (Hoar et al. 2001). Batch cultivation, population reduction, and proper disinfection processes would help to keep infection levels under control (Cunha et al. 2019).

Conclusions

From this review it can be concluded that cryptosporidiosis is a zoonotic endemic disease with worldwide distribution

including Iraq. The life cycle of the parasite is direct and the disease occurred when a sufficient number of oocysts were ingested by humans and domesticated mammals such as sheep, cattle, pigs, goats, camels. Therefore, preventive measures and health education programs are required to reduce cryptosporidiosis among domestic animals and humans.

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CHAPTER 20

MAGGOT DEBRIDEMENT TREATMENT AND ITS APPLICATIONS IN VETERINARY MEDICINE

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INTRODUCTION

Maggot debridement treatment (MDT) is known by various names, such as larval therapy, larval debridement therapy, maggot therapy and maggot debridement therapy, around the world. It is the use of sterile, live first and second instar larvae of the fly *Lucilia sericata* to treat chronic wounds that do not heal with conventional medical methods (Fig. 1) (Sherman et al. 2000; Rueda et al. 2010; Gasz and Harvey 2017; Aydın and Uslu et al. 2021; Uslu and Küçükyağlıoğlu 2021). MDT has been described as a safe, effective, and controlled method (Choudhary et al. 2016).

In MDT, the larvae must be free of microorganisms and disinfected before they are placed into the wound. Infection complications can be observed in patients as a result of insufficient disinfection in the laboratory. The sterility of the larvae is microbiologically checked before use to confirm whether they carry infectious agents (Wolff and Hansson 2005).

The majority of fly larvae associated with myiasis are unsuitable for larval therapy because they can damage living tissue. However, *L. sericata* larvae feed only on dead tissue and do not harm living tissue (Table 1) (Hall and Wall 1995). Annually, 15 million US dollars are spent in the United States to treat chronic wounds (Sood et al. 2014).

History of Maggot Debridement Treatment

Maggot debridement treatment uses the sterile first and second instar larvae of the common green bottle fly i.e., *Lucilia sericata* (syn. *Phaenicia sericata;* order Diptera; suborder Brachycera; family Calliphoridae), which feed only on dead tissue, to clean chronic and infected wounds (Baer 1931). The cleaning and healing effects of these larvae on wounds have been known for many years. Military surgeons observed that the wounds of soldiers injured on the battlefield healed faster when infected with larvae (Pechte and Sherman 1983). Indians rubbed bloody meat left under the sun on wounds, which were then infested with maggots and eventually healed. Indigenous tribes from Australia cleaned and healed wounds with fly larvae (Sherman et al. 1996; 2000).

The beneficial effects of fly larvae on wounds were first recorded in writing by Ambroise Paré (1510–1590). Paré, who was a surgeon, reported in 1559 that larval treatment destroyed dead tissue in non-healing wounds of soldiers injured in war without damaging healthy tissue (Nigam et al. 2006; Whitaker et al. 2007; Gupta 2008). Baron Dominique-Jean Larrey (1766–1842), the chief physician of Napoleon's army, declared in 1829 that larvae cleaned necrotic wounds without damaging living tissue, removed dead tissue, and helped in the development of new tissue (Church and Courtenay 2002; Whitaker et al. 2007; Gupta 2008).

Surgeon John Forney Zacharias (1837–1901) was the first to document the use of myiasis larvae for the treatment of open infected wounds. For the first time, Dr. Joseph Jones and Zacharias clinically applied larvae to wounds of soldiers and reported that they remained in the wounds for days and only ate the dead tissue while not damaging living tissue (Nigam et al. 2006; Chan et al. 2007). According to Zacharias, larvae reduced sepsis and accelerated wound healing (Whitaker et al. 2007).

The first deliberate use of fly larvae for wound treatment was by orthopedic surgeon William Baer in 1928. During World War I, Baer had observed the healing effects of maggots on infected wounds of soldiers. He later developed a method to sterilize larvae before their use and observed wound debridement and disinfection and stimulation of tissue growth by the larvae (Baer 1931). This sterile larval application reduced infection and was an effective treatment. MDT grew in popularity between 1920 and 1940. Robinson (1935) surveyed American and Canadian physicians who used Baer's method, and more than 90% reported that they were satisfied with the treatment.

The Lederle laboratory in New York was the first commercial maggot producer; it produced "Surgical Maggots" until the 1940s. However, the widespread use of sulfanamide in the 1930s, the mass production of penicillin in 1944 and its widespread use, and the development of surgical techniques decreased the interest in MDT. In addition, MDT also decreased considerably in these years due to difficulties in larval production and application (Teich and Myers 1986; Nigam et al. 2006; Whitaker et al. 2007). In the 1950s–1980s, MDT was infrequently used for skin and soft tissue wounds that did not respond to medical and surgical treatment (Teich and Myers 1986).

In the late 1980s, antimicrobial resistance became more common with the increasing incidence of diabetic foot ulcers and pressure ulcers. Conventional medical wound care proved insufficient to treat these persistent wounds (Teich and Myers 1986). Due to bacterial resistance against and Myers 1986). Due to bacterial resistance against antibiotics, larval therapy has begun to be used again for treating chronic wounds (Nigam et al. 2006; Whitaker et al. 2007). The work of Dr. Ronald Sherman and colleagues in the

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Table 1: Types of flies used in maggot debridement treatment (Sherman et al. 2013)

Family	Species	Literature
Calliphoridae	*Lucilia sericata*	Baer (1931)
	Lucilia cuprina	Fine and Alexander (1934)
	Lucilia caesar	Baer (1931); McLellan (1932)
	Lucilia illustris	Lerlercq (1990)
	Calliphora vicina	Teich and Myers (1986)
	Phormia regina	Baer (1931); Robinson (1933)
		Reames et al. (1988)
	Protophormia terraenovae	Lerlercq (1990)
		Sherman et al. (2000)
	Chrysomya rufifacies	Sherman et al. (2000)
Sarcophagidae	*Wohlfahrtia nuba*	Grantham-Hill (1933)

Fig. 1: Adult *Lucilia sericata* (from U. Uslu).

1990s increased the interest in MDT. It began to be used once again in the USA from 1989 and in England, Germany, 1990s increased the interest in MDT. It began to be used once again in the USA from 1989 and in England, Germany, Ukraine, Sweden, Switzerland, Israel, and Thailand from 1990 (Mumcuoglu et al. 1998; 1999). The International Society for Biotherapy was established in 1996 to research and develop the use of live larvae and their products in tissue repair (Sherman 2000; Mumcuoğlu et al. 2001).

The US Food and Drug Administration (FDA) authorized the supply of medical larvae as a prescription treatment only in 2004 (FDA 2007). In the same year, larvae were approved for prescription in MDT in England (Geary et al. 2009). The European Medicines Agency (EMA) has allowed MDT to be used in the treatment of a variety of wounds. MDT was approved by the Ministry of Health in Turkey on October 27, 2014 in the Traditional and Complementary Medicine Practices Regulation. By 2011, sterile larvae were being produced by 24 laboratories and exported to more than 30 countries and an estimated 50,000 patients had been treated worldwide (Gilead and Mumcuoğlu 2012).

It is estimated that 629 million people globally will have diabetes by 2045, e.g., 82 million in North Africa, 41 million in sub-Saharan Africa, and 183 million in Southeast Asia, China, Australia and the Pacific region (International Diabetes Federation (IDF) 2017). The top five European countries with the highest number of diabetic patients aged 20–79 in 2019 were Germany (9.5 million), the Russian Federation (8.3 million), Turkey (6.6 million), Italy (3.7 million), and Spain (3.6 million). A foot amputation due to diabetes occurs every 30 seconds in the world.

In countries such as the USA, England, Germany, Israel, Malaysia, Japan, and Thailand, commercial companies produce disinfected larvae, which are used in hospitals for treatment. Sterile larvae used for MDT in Turkey were developed by Prof. Dr. Uğur Uslu in the larva production unit. Due to rapid technological developments in the last 100 years, one-piece, cage-like dressings have been created with materials used in treatment with larvae. "Maggot containment dressings" have been produced, allowing larvae full and free access to the wound and preventing them from escaping from the wound. These developments have expanded the applications of MDT, which is now used worldwide (Sherman 2009).

It has been known for a long time that MDT is a successful method for clearing soft tissue infections. It is a simple, effective, safe, and cost-effective option for the treatment of wounds and ulcers that do not respond to conventional treatments and surgical interventions. Larval therapy is expected to be applied in more countries and patients in the future.

Lucilia sericata Morphology

Classification
Class: Insecta
Order: Diptera
Suborder: Brachycera
Family: Calliphoridae
Genus: *Lucilia*
Species: *Lucilia sericata* (Meigen 1826)

Adult *L. sericata* flies are metallic bluish-green in color and are 5–10 mm in size (Fig. 2) (Apperson et al. 2011; Mun 2013; Sherman et al. 2013). Adult flies have three body parts: head; thorax; and abdomen. The head consists of a pair of antennae, two large compound eyes, and mouthparts. The compound eyes make up most of the head and are red in color. In females, the compound eyes are separated on the forehead (dichoptic state), while in males they are very close together (holoptic state). The ocelli (simple eyes), which are points that allow light perception, are located in a narrow area between the compound eyes. The antennae bear olfactory receptors, mechanoreceptors, and auditory organs.

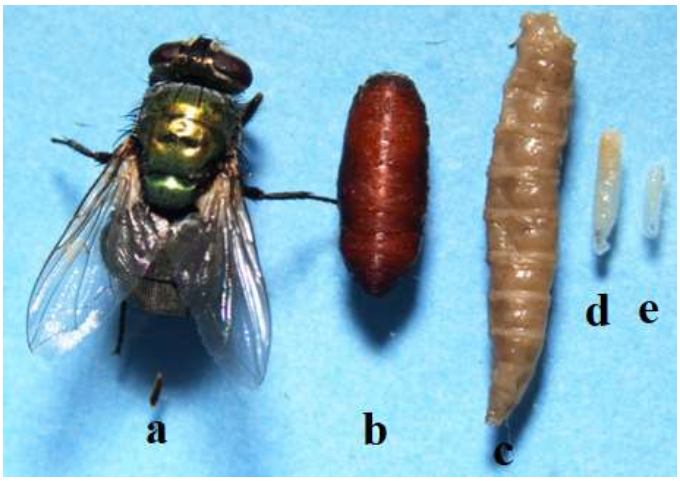

Fig. 2: *Lucilia sericata*; (a) Adult fly, (b) Pupa, (c) Larva 3, (d) Larva 2, (e) Larva 1 (from U. Uslu).

The antenna has three main parts: the basal segment, which connects the antenna to the head; the pedicel; and the flagellum, which bears a plumose arista with long sensory hairs (Ward and Shearer 1997).

The thorax consists of three parts: the prothorax; mesothorax; and the metathorax (Uslu and Küçükyağlıoğlu 2021). The wide mesothorax is connected to the narrow anterior and posterior thorax. The wing muscles of the mesothorax are overdeveloped. The thorax area has acrostichal bristles; the presence of these bristles in the mesothorax is an important diagnostic feature of adult *L. sericata* (Carhan and Yesilırmak 2015).

Adults have a pair of light-brown wings attached to the mesothorax. The wings have a scaly structure. The shape of the veins on the wing and the presence of hairs on the veins are important diagnostic features for differentiation of species. The second pair of wings, called the halteres, is atrophied and knob-shaped. When a haltere is broken, the fly loses its balance and cannot fly (Ward and Shearer 1997; Wall and Shearer 2001; Carhan and Yesilırmak 2015).

Three pairs of legs emerge from the ventral thorax. Each leg has five parts: the coxa; trochanter; femur; tibia; and tarsus. The tarsus consists of five subsegments called the tarsomeres, with one pair of claws on the last segment. Between each pair of tarsal claws there are two pulvilli, which provide adhesion to surfaces. Between the two pulvilli, there is a hair-shaped empodium whose presence or absence is important in fly species identification (Wall and Shearer 2001).

The abdomen contains important organs for respiration, digestion and reproduction. The reproductive organs of males and females are located at the tip of the abdomen. Females lay their eggs singly or in groups with an ovipositor (Wall and Shearer 2001; Carhan and Yesilırmak 2015).

Adult female *L. sericata* lay eggs in clusters. The eggs are white or pale yellow, approximately 1.5 mm long, and slightly conical at one end (Fig. 3) (Wall and Shearer 2001; Sherman 2002; Fleischmann et al. 2004; Yaghoobi et al. 2005). The larvae are cream-white in color, conical in shape with a thin and pointed front and thick tip at the rear, and have no feet. The first instar is 3 mm, the second instar is 4–7 mm, and the third instar is 9–18 mm long and has 12 segments (Fig. 4) (Carhan and Yesilırmak 2015; Nair et al. 2018).

The larva has a stomach and intestine, and anterior, middle, and posterior parts . It breathes through two posterior

Fig. 3: *Lucilia sericata;* Egg clusters (from U. Uslu).

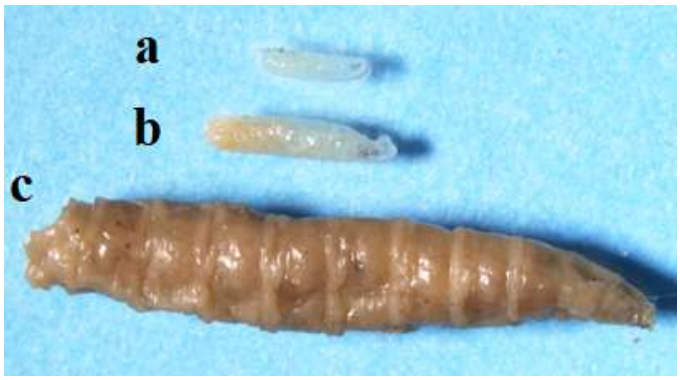

Fig. 4: *Lucilia sericata*; (a) 1st stage larva (b) 2nd stage larva (c) 3rd stage larva (from U. Uslu).

spiracles, and feeds by immersion in liquid media with its anterior sides and secretes digestive enzymes (Fig. 5).

The cephaloskeleton of the second instar larva has a pair of well-developed mouthhooks. The third instar larva is 10–16 mm long and has 12 segments. The anterior spiracle has 7–9 arms, while the posterior spiracle has two clefts.

The peritreme in the posterior stigmata is narrow and closed. The pupa is light brown and 9–10 mm long and 3–4 mm wide. The pupal sheath is white at first, then darkens rapidly (Fig. 6) (Apperson et al. 2011; Nair et al. 2018).

Lucilia sericata Biology

Lucilia sericata is common in temperate and tropical regions of the world (Mun 2013; Sherman et al. 2013). Females lay egg clusters (an average of 2000–3000 eggs) on corpses and carrion, necrotic and decaying tissue, feces, and open wounds. The adult fly population increases in spring and autumn in Asia region/Turkey (Abdolmaleki et al., 2015; Mumcuoğlu and Özkan 2015; Carbonaro 2020). It undergoes complete metamorphosis. The life phases of *L. sericata* are

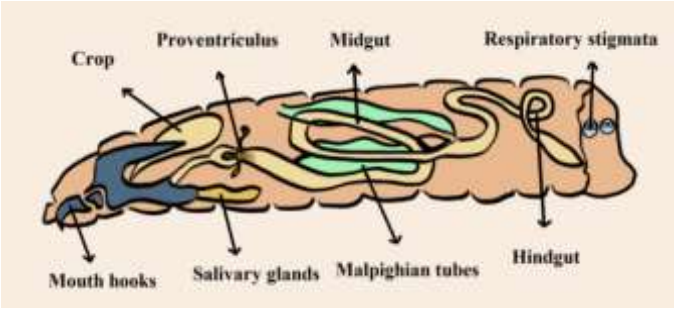

Fig. 5: *Lucilia sericata;* Morphological structure of the 2nd stage larva (Illustrated by U. Uslu).

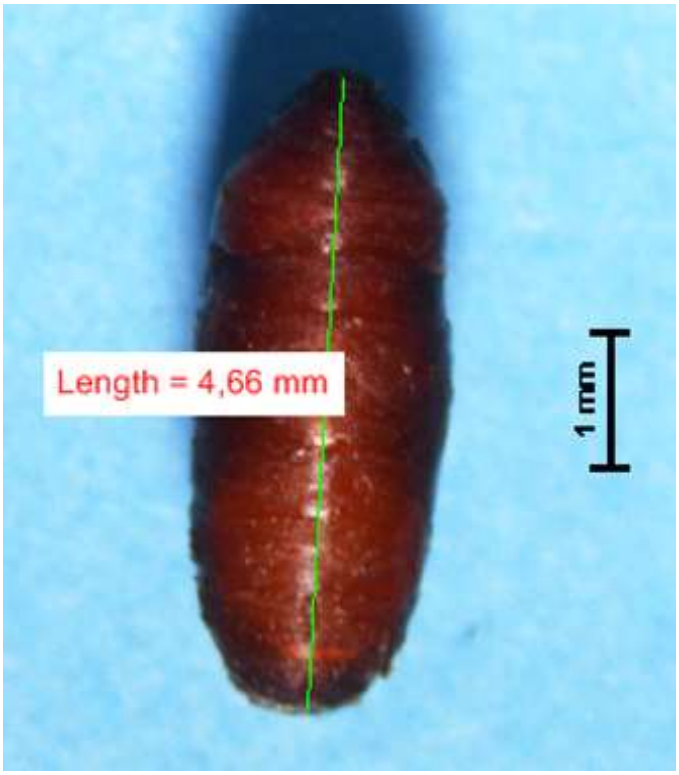

Fig. 6: *Lucilia sericata*; Pupa (from U. Uslu)

the egg, first instar, second instar, third instar, pre-pupa/pupa, and adult (Fig 7-8-9) (Anderson 2000; Carbonaro 2020; Aydın and Uslu 2021).

Egg

The rate of development of eggs, larvae, and pupae is greatly affected by temperature. The minimum time for the first instar larva to emerge from the egg is 12–24 hours at 28 °C and approximately 21 hours at 21°C (Anderson 2000). Larval development takes approximately four days at 20°C and three days at 27°C (Anderson and Kaufman 2020). The period to develop from egg to adult is 6–8 days at 35°C. Development stops at 12–13°C and eggs, larvae, and pupae die at 45°C. *L. sericata* completes its life cycle in 8–10 days (Nair et al. 2018).

Larva

The life cycle of *L. sericata* completes in 16 days at 25 °C (Mellenthin et al. 2006). Larvae hatch from eggs at an average humidity of 50% at 25°C. Development time of larvae and

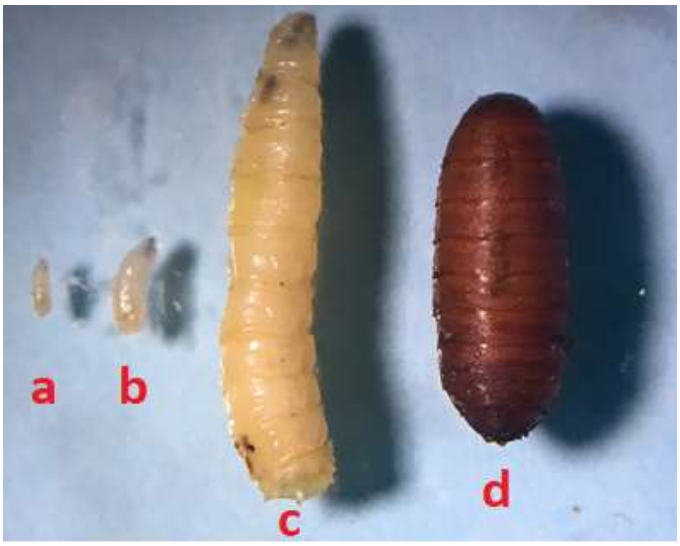

Fig. 7: *Lucilia sericata*; (a) I. Instar larva, (b) II. Stage larvae, (c) III. Stage larva, (d) Pupa (from U. Uslu).

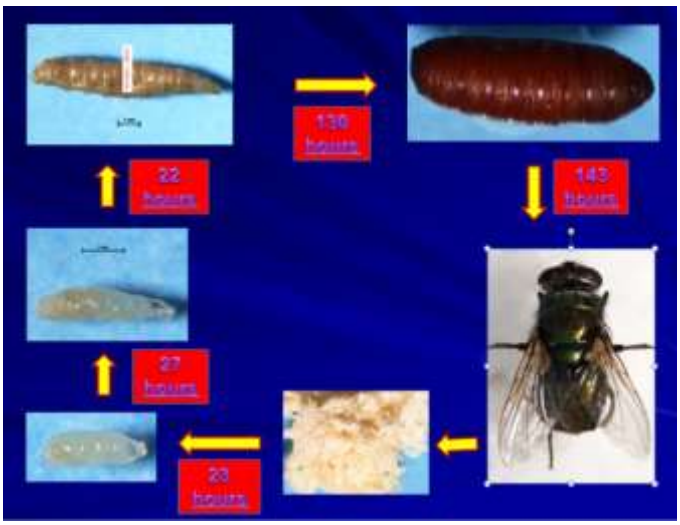

Fig. 8: General life circle of *Lucilia sericata* in the environment (from U. Uslu).

Fig. 9: *Lucilia sericata*; Egg clusters (from U. Uslu).

pupae; The first instar larvae pass in 22 hours, the second instar larva in 15 hours, the third instar larva in 43 hours, the prepupal stage in 100 hours and the pupal stage in 192 hours.

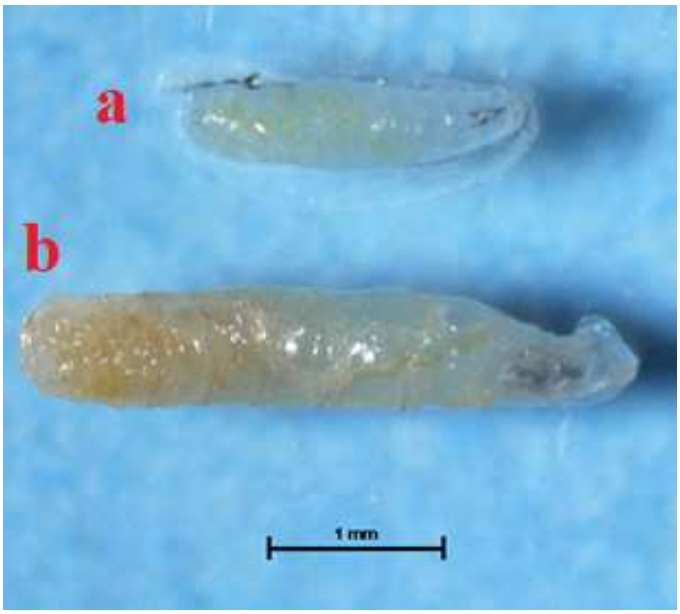

Fig. 10: *Lucilia sericata*; (a) I. Instar larvae (b) II. stage larva (from U. Uslu).

Fig. 11: *Lucilia sericata* (a) Cephalo-pharyngeal skeleton (b) Anterior stigma (c) Posterior stigma (from U. Uslu).

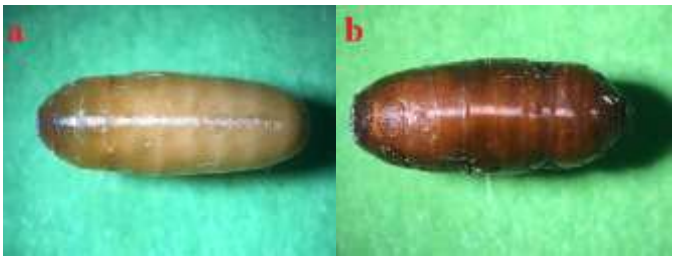

Fig. 12: *Lucilia sericata*; (a) Pre-Pupa (b) Pupa (from U. Uslu).

Pupa

After entering the pupal stage, the larva stops feeding. The adult emerges from the pupa in 7–10 days (Fig. 12) (Anderson 2000). Pupation can begin at 8–11 °C, and the pupal stage is completed in 18–24 days at 12–13 °C (Nair et al. 2008), 10 days at 21 °C, 6–7 days at 27 °C, and 4–7 days at 32 °C (Anderson 2000).

Adult

The new adult fly colony begins to lay eggs after 8–10 days. The number of eggs increases over time, reaching a maximum within a month, and then gradually decreases. Although the *L. sericata* colony has a lifespan of 2–2.5 months, egg production occurs within period of 1.5-month. *L. sericata* flies produce pheromones that attract the opposite sex (Fig. 13) (Nair et al. 2018).

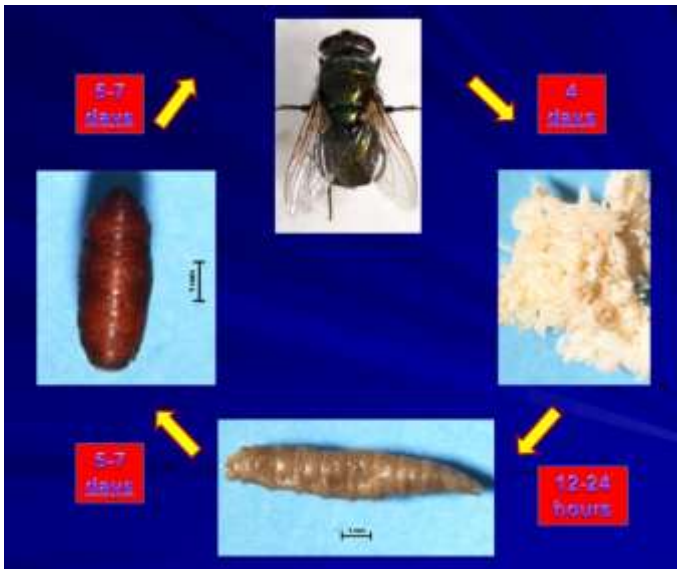

Fig. 13: Life cycle of *Lucilia sericata* at 25.0 ± 2°C (from U. Uslu).

After mating, females lay a maximum of 225–250 eggs at a time and a total of 2000–3000 egg clusters on carrion, on open and foul-smelling wounds on the host, and on dirty and wet wool (Sherman 2002; Fleischmann et al. 2004; Strikewise 2020). The adult of *L. sericata* is metallic green (Apperson et al. 2011) and are soft immediately after emergence and lack the ability to fly (Nair et al. 2018).

Lucilia sericata is known for causing sheep wound myiasis worldwide. *Lucilia sericata* and a similar species, *Lucilia cuprina* (Wiedemann), are known in Britain and Australia for causing sheep strike. Sheep strike is a type of myiasis (invasion of living tissue by fly larvae) and usually is observed near the rear of the sheep. This condition is quite serious and untreated sheep die. The female flies lay their eggs on sheep in areas of the body contaminated with sweat and urine, such as under the tail and the abdomen (Strikewise 2020).

Under laboratory conditions, flies of both sexes remain viable for about two months. If two flies start to lay eggs in April and all their offspring survive, the fly number could reach 191,010,000,000,000,000,000 in August. The number of generations is about 30 under tropical conditions but may be ≤ 10 in temperate climates (Nair et al. 2018). *L. sericata*'s activity is seasonal. It can produce eight generations during the warmer period of the year from May to October (Fleischmann et al. 2004). However, according to some authors, they produce three to four generations per year in temperate climates (Wall and Shearer 2001; Yaghoobi et al. 2005).

The number of *L. sericata* adult flies increases in spring and autumn. Adult flies emerge during the day and are most active in sunny locations. They require protein before laying eggs, and need a sufficient energy intake during the larval stages to progress to the pre-pupal, pupal, and adult stages and for the adults to lay eggs. Adults mainly feed on sugary liquids and choose protein foods to lay their eggs and develop larvae. When adult females find enough protein, they start to lay eggs at three-day intervals after 5–9 days.

Laboratory Conditions for Maintaining Colonies

In larval production units in the laboratory, adult *L. sericata* colonies are kept at 25 °C, 50% humidity, and 12 hours of

light, and provided with granulated sugar and water in the cages. Under laboratory conditions, females lay eggs for at least 1½ months and produce about 225–250 eggs at each laying (Wall 1993) and a total of about 2000–3000 eggs; they live for 22–32 days.

Lucilia sericata Larva Production and Sterilization in the Laboratory

In order for the larval debridement treatment to be successful, the larvae must be purified of microorganisms and disinfected before being placed on the wound. For medicinal purposes, disinfected live first and second instar larvae are used (Sherman et al. 2000; Bandırma 2017; Gasz and Harvey 2017).

Lucilia sericata fly larvae feed on contaminated food in their environment soon after hatching and cannot be sterilized afterwards. However, the embryo inside the egg is sterile. Since the membrane (chorion) surrounding the fly egg is extremely resistant, the surface of eggs is disinfected. The disinfected eggs are then incubated in a Petri dish in sterile culture medium and are allowed to hatch into the larval stage. The optimal disinfectant should have high antibacterial potential but low egg toxicity. After the disinfected larvae hatch, they can only survive for 3–7 days, depending primarily on humidity, temperature, and the amount of food. Survival times may be shortened by transportation, extreme temperatures, pressure, and insufficient oxygen or nutrients (Sherman et al. 2013).

Not all fly larvae are suitable for larval debridement treatment. The most commonly used flies for this purpose are *L. sericata*, which feeds only on necrotic tissue (Uslu 2016; Uslu et al. 2019).

A climate room and sterilization room are required for the production and sterilization of larvae in the laboratory. An average of 12 m^2 of space is required in the climate room to continuously obtain and maintain adult colonies of *L. sericata* in fly cages. In the climate room, a temperature of 24–27 °C, humidity of 40–60%, and a light system are sufficient. Next to the climate chamber, a 20 m^2 functional laboratory is required for larval extraction, sterilization, preparation, development of sterile packages, and independent microbiological analysis (Fig. 14) (Uslu 2016; Uslu et al. 2019).

In the laboratory environment, a plastic cage covered with gauze is used for the fly colony. Water, a 20% sugar solution, pieces of meat or liver are placed in the cage for adult flies to feed on and lay eggs (Fig. 15) (Uslu 2016; Uslu et al. 2019).

L. sericata flies need protein foods before they lay their eggs. In order to obtain eggs, an average of 10 g of chicken liver is placed in fly cages with colonies of *L. sericata* and removed after 3–4 hours. Adult female flies lay an average of 225–250 eggs at three-day intervals (Fig. 16) (Uslu 2016).

Live sterile *L. sericata* L1 and L2 larvae are used for treatment to remove dead tissue, reduce the risk of infection, and stimulate wound healing without damaging healthy tissue and the epithelium of the wound (Mumcuoğlu et al. 1999; Mumcuoğlu et al. 2001). To obtain larvae, the eggs are transferred to a specially prepared liver agar medium in a 90 mm Petri dish and incubated overnight at 25–30 °C in an oven (Fig. 17). The L1 larvae hatched in the oven are placed in a net-covered cage in the climate chamber and provided with a medium made from chicken liver for feeding the larvae. The larvae that reach the second and third instar stages in the

Fig. 14: Green meat fly, *Lucilia sericata*: Air-conditioning room; Fly colony cages; Temperature and humidity digital boards; Fly killer and camera (from U. Uslu).

Fig. 15: Cages in the climate chamber; Water; Sugar; Pupa; Adult fly *Lucilia sericata* (from U. Uslu).

Fig. 16: *Lucilia sericata* eggs on chicken liver (from U. Uslu).

tumper pass into the wandering larval stage after completing the feeding stages. The tumper container is lined with paper napkins, under which the wandering larvae move to escape from the light environment; the humidity is maintained at 45–50% and temperature at 25 °C (Uslu 2016; Uslu et al. 2019).

The wandering larvae enter the pupal stage after 5–6 days. The pupa is light brown and barrel shaped (Fig. 18). After pupa formation is completed, 1000–1500 pupae are placed in plastic cages covered with gauze. Adult flies emerge from pupae in 7–10 days. The newly formed adult fly colony begins

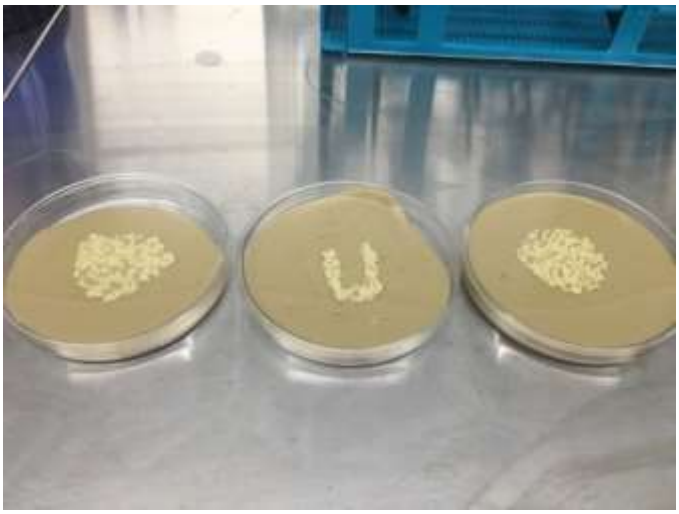

Fig. 17: *Lucilia sericata* eggs on liver agar media (from U. Uslu)

Fig. 18: *Lucilia sericata* III. stage larva and pupa (from U. Uslu).

to lay eggs after 8–10 days. The number of eggs increases over time and reaches a maximum in a month and gradually decreases. Under laboratory conditions, flies of both sexes remain viable for about two months. After mating, *L. sericata* females lay a cluster of a maximum of 225–250 eggs at a time on chicken livers. A new cage is prepared for each new colony (Uslu 2016; Uslu et al. 2019).

Collection and Sterilization of Eggs

Approximately 10 g of chicken, turkey, or beef liver is placed in fly cages with *L. sericata* colonies and kept in the cage for 3–4 hours. Eggs deposited on the liver in the biological safety cabinet-Class II are collected with the help of a loop and then placed in a sterile 50 ml vial (Fig. 19) (Uslu 2016; Uslu et al. 2019).
In order to separate the eggs from each other, 0.05% sodium hypochlorite is added to the vial and the eggs are separated by shaking the vial for 15 minutes in a magnetic stirrer. After discarding the sodium hypochlorite solution, the eggs are sterilized by shaking them in 0.5% formaldehyde for 15 minutes. After filtering the sterile eggs in a Buchner funnel, they are washed 3-4 times with a sterile isotonic saline solution to remove formaldehyde; the eggs are then transferred to sterile liver agar medium in Petri dishes and incubated overnight at 25-30 °C (Fig. 20) (Uslu 2016; Uslu et al. 2019).

Fig. 19: *Lucilia sericata*: Agitation of eggs in the falcon; Appearance of eggs in the falcon (from U. Uslu).

Fig. 20: *Lucilia sericata* eggs in the oven (from U. Uslu).

Larvae are microbiologically tested to confirm whether they carry any infectious agents before use (Wolff and Hansson 2005). The hatched larvae are inoculated into thioglycolate broth, blood agar, and chocolate agar media one day later to check whether they are sterile. Sterile larvae are used for treatment as soon as possible. If the larvae are not sterile, they are discarded after being kept at -20 °C overnight. If necessary, sterile larvae can be stored at 5-8 °C for 2-5 days without losing viability (Martin 1996; Mumcuoğlu et al. 1998; Mumcuoğlu et al. 2001; Blake et al. 2007; Uslu et al. 2019). Sterile larvae to be used for treatment are placed in vials with perforated caps and transported in a Styrofoam box containing ice (at 10 °C) (Jaklič et al. 2008; Nair et al. 2018; Uslu 2016; Uslu et al. 2019).

Table 2: MDT Applications in Some Animals in Veterinary Medicine

Animal Type	Number	Etiology	Number of Successful Treatments	References
Cat	1	Post operative infected wound	1	Uslu et al. (2021)
Dog	1	Gangrenous foot	1	Uslu et al. (2022) In Press
Dog	1 (LDT and HBOT combined)	Severe skin degloving, fracture of left thoracic limb (LTL)	1	Reinstein et al. (2022)
Dog (American Bulldog)	1 (LDT+ fish skin grafts and autologous skin cell suspension)	Serious burn wound	1	Dawson et al. (2022)
Horse	4	Sarcoid (Skin Tumor)	2	Ahmadnejad et al. (2022)
Rabbit	6	Experimentally created infected wound in the lumbar region	6	Barros et al. (2021)
Rabbit	2	Experimental scalding burn to the back area	2	Amiri et al. (2021)
Wistar Rats	24 (12 antibiotics + larval treatment) (12 of them only larval treatment)	Diabetes wound created with intraperitoneal STZ	24	Borkataki et al. (2021)
Sheep	50	Skin wound	50	Durán et al. (2020)
Wistar Rats	12 (6 antibiotics + larval treatment) (6 of them only larval treatment)	Experimental infected wound	12	Borkataki et al. (2018)
New Zealand White Rabbit	6 (3 of them MDT with Lucilia sericata) (3 of them MDT with Sarconesiopsis magellanica)	Experimental dorsal wound infected with Pseudomonas aeruginosa and Staphylococcus aureus	6	Díaz-Roa et al. (2016)
Equide (Horse, Donkey, Poni)	41 (35, 4, 2)	Foot pathology (9), laceration of the limbs (15), wounds (6), fistulous withers (5), other musculoskeletal infection (2) and dehiscence of the linea alba (4).	38	Lepage et al. (2012)
2 Cattle, 1 Horse, 1 Dog	4	Infected wound	4	Castañeda et al. (2010)
Horse	20 (MDT as adjunctive therapy)	Contaminated and septic navicular bursitis	18	Bras and Morrison (2009)
Horse	13	Laminitis, ingrown nails, foot abscess, chronic ulcer on sole of foot, vasculitis, barbed wire wound	12	Sherman et al. (2007b)
Pet Animals (Dog, Cat, Rabbit)	7 (2, 4, 1)	Pressure sore, gunshot wound, fibrosarcoma, multiple bite wound, claw wound	5	Sherman et al. (2007a)
Equide	43	Foot diseases (distal phalanx osteomyelitis, septic navicular bursitis, chronic laminitis, collateral cartilage necrosis, nonhealing foot ulcers)	41	Morrison (2005)
Sheep	6	Dermatitis interdigitalis acuta, pododermatitis purulenta	6	Kočišovă et al. (2006)
Donkey	1	Abscess due to injection (Gluteal Region)	1	Thieman (2003)
Rabbit	3	Decubitus Wound	3	Kočišovă et al. 2003
Donkey	1	Pannikülitis	1	Bell and Thomas (2000)
Guernsey bull	1	Actinomycosis	1	Dicke (1953)

Maggot Debridement Therapy in Veterinary Medicine

Maggot debridement therapy is the treatment of suppurative skin infections with the larvae of *L. sericata*. The larval stages of *L. sericata* cause myiasis in tissues and organs of both humans and animals. *L. sericata* has an important place among myiasis-causing flies in medical entomology. Larval treatment is applied to wounds, burns, and abscesses that are difficult to heal and resistant to antibiotics in humans and animals. It is used for treatment by taking advantage of the antiphlogistic, antiseptic, antibiotic, and granulation tissue accelerating effects of larval secretions.

Chronic wounds are generally classified as diabetic wounds, decubital wounds, burn wounds, venous-ischemic ulcers and arterial insufficiency wounds. The treatment of chronic wounds is generally performed surgically. Plenty of oxygen, a moist environment, and low contamination of the wound have a positive effect on treatment. Wound healing is not possible without removing necrotic tissue and providing blood supply to the wound area. For this purpose, larval treatment, which will perform all these procedures, is widely used (Kurtoğlu and Karataş 2009). Although MDT has been used in humans for the treatment of chronic wounds for centuries (Kočišovă et al. 2006; Michelle et al. 2011; Lepage et al. 2012; Kenawy and Abdel-hamid 2020), it has rarely been used in animal infections (Table 2) (Bell and Thomas 2000; Jones and Wall 2008).

The success rate of MDT has been reported as 70–80%. While larval treatment is progressing positively in human medicine, clinical studies on animal treatments are very few. However, in recent years, there has been a significant increase in the treatment of chronic wounds in veterinary medicine (Choudhary et al. 2016; Kenawy and Abdel-hamid 2020; Uslu et al. 2021; Uslu et al. 2022). MDT is most commonly used to treat different types of wounds in horses and small animals, especially dogs and cats, and as an alternative to amputation (Sherman et al. 2007a; Dar et al. 2013); however, it is used less frequently in farm animals.

Larval therapy is a simple, effective, economical, and safe option for the treatment of necrotic, pustular, or gangrenous wounds and ulcers, which do not respond to conventional treatments and surgical interventions and deep wounds with

insufficient blood circulation and limited penetration of antibacterial drugs and for the control of orthopedic, dermatological and geriatric infections. In animals, the benefits to disadvantaged patients, e.g., those of old age, with severe diabetes, or at risk of amputation and chronic sepsis, increases the value of this treatment.

In addition, concerns about antibiotic resistance and the demand for organically raised livestock and residue-free meat and milk are increasing the value of larval treatment and has led to a growing interest in it in veterinary medicine (Dar et al. 2013; Jones and Wall 2007). Larval treatment also prevents weight loss in animals, as well as eliminates milk destruction that occurs with the use of antibiotics. Larval therapy can also be used to treat abscesses and some tumors in animals. It can be provided at more affordable prices than surgical and chemotherapy treatments to pets and service animals (Hall and Wall 1995; Hinshaw 2000).

In veterinary medicine, MDT has been used to treat actinomycosis in a Guernsey bull (Dicke 1953); panniculitis and abscesses in donkeys (Bell and Thomas 2000); chronic decubitus wounds in rabbits (Kočišovă et al. 2003); foot rot and acute and chronic interdigital dermatitis in sheep (Kočišovă et al. 2006); pressure ulcers and gunshot wounds in dogs and necrotic tumors and multiple bites in cats (Sherman et al. 2007a).

It has been used to treat a variety of conditions in horses: chronic laminitis and necrosis in complicated laminitis cases (Morrison 2005, 2010; Sherman et al. 2007b); ingrown nails, foot abscess, chronic and non-healing foot ulcers, foot infection, chronic distal interphalangeal joint sepsis, collateral cartilage necrosis, and foot and leg wounds (Morrison 2005; Sherman et al. 2007b); nail infections and diseases (Jurga and Morrison 2004; Morrison 2005); septic navicular bursitis (Morrison 2005); osteomyelitis (Jurga and Morrison 2004; Morrison 2005); vasculitis, deep cuts, barbed wire wounds, soft tissue abscesses and wounds, abdominal wounds, and lacerations of the limbs (Lepage et al. 2012; Sherman et al. 2007b) and cancer (Morrison 2005). MDT also prevents the deterioration of the gastrointestinal flora and colitis in horses (Hall and Wall 1995).

Maggot therapy has been increasingly used in recent years to prevent amputation and euthanasia of pet animals (Choudhary et al. 2016). The application of larvae to a wound resulting from the amputation of a dog's gangrenous foot (Uslu et al. 2022) and a post-operative infected wound covering the abdominal and inguinal region of cats (Fig. 21) (Uslu et al. 2021) healed successfully following this treatment.

Effect Mechanism of Maggot Debridement Treatment

Larvae secrete enzymes and substances to dissolve dead tissue on the wound and consume it. Substances identified in larval secretions include allantoin, urea and calcium carbonate, and enzymes such as trypsin, chymotrypsin-like enzymes (LCTa and LCTb), leucine aminopeptidase, carboxypeptidase A and B, serine protease, and collagenase. It has been reported that these larvae cannot survive on clean granulation tissue and die of starvation.

Clinical studies have shown that the mechanism of healing of necrotic wounds with larval treatment has four main stages: debridement, disinfection, initiation of granulation (Lepage et al. 2012; Yaman et al. 2021), and blood flow (Chan et al. 2007).

Fig. 21: Wound appearance before and after treatment with sterile *Lucilia sericata* larvae in a cat (from U. Uslu).

Debridement

Debridement is the removal of tissue that has lost vitality on or around the wounds or contaminated materials from the environment. Maggots feed on dead tissue, cell debris and serous exudates of the necrotic wound. They do not harm living tissue. It is difficult to remove necrotic tissue without damaging healthy tissue, whereas it is very easy for larvae to consume microorganisms in the necrotic tissue together with the tissue itself. Larvae penetrate into the deep parts of the wound and remove the necrotic tissue from the intact tissue with the help of their mouthhooks in a manner resembling microsurgery. They can penetrate into any area of the wound where there is necrotic tissue and clean even very small areas. Mumcuoğlu et al. (2001) reported that each maggot can eat 25 mg of necrotic tissue within one day. Other studies have found that one *L. sericata* larva can consume 0.3 g of tissue per day. Two hundred maggots can consume 15 g of necrotic tissue or tissue fluid in one day (Wollina et al. 2000).

Maggots clean the wound with the help of their pair of mouthhooks. The enzymes they secrete help the larvae to break down necrotic tissue and digest it in their digestive system. Necrotic tissue is liquefied with collagenase, trypsin and chymotrypsin-like proteolytic enzymes secreted from the stomach of the larvae, and the environment is disinfected as the larvae feed on this liquid. The larvae leave behind clean healthy granulation tissue (Mumcuoğlu et al. 1999; Sherman et al. 2000; Mumcuoğlu et al. 2001).

Disinfection

Larvae have antibacterial effects due to eating bacteria and a bactericidal effect due to excretions/secretions. For example, ammonia and ammonium bicarbonate excreted by maggots are useful for wound healing and disinfection. Calcium and calcium carbonate, also excreted by the maggots, directly kill bacteria, stimulate phagocytosis, and accelerate the development of granulation tissue (Sherman et al. 2000; Mumcuoğlu et al. 2001). By producing ammonia and calcium carbonate, the larvae cause the pH of the wound environment to change from acidic to alkaline. Alkalization of the wound site both prevents bacterial colonization and accelerates wound healing.

The secretory and excretory products of the larvae show effects similar to those of broad-spectrum antibiotics against

gram-negative bacteria, such as *Escherichia coli*, *Salmonella*, and *Pseudomonas aeruginosa*, and gram-positive bacteria, such as *Staphylococcus aureus*, *S. epidermidis*, and *Listeria*, which are pathogenic and resistant to antibiotics (Mumcuoğlu et al. 1999). This antimicrobial activity is largely due to phenylacetic acid and phenylacetaldehyde produced by the bacterium *Proteus mirabilis* living commensally in the stomach of the larvae. Microorganisms are destroyed by these antibacterial substances in the larval digestive tract. Mumcuoğlu et al. (2001, 2009) examined green fluorescent proteins produced by *E. coli* migrating in the digestive tract of *L. sericata* larvae and found that 66.7% of live bacteria were in the crop, 55.6% in the midgut, and 17.8% in the hindgut.
In addition, the irritating effect of maggots crawling on the wound and the serous exudate they secrete help mechanically wash the wound.

Initiation of Granulation

Larvae stimulate living tissue with the mechanical effect they create with their movements on the wound, leading to the development of granulation and the healing of the wound. Products such as ammonium, urea, and allantoin secreted by maggots increase the effects of the epidermal growth factor (EGF) and interleukin (IL) 6 and stimulate granulation tissue and the immune system (Hinshaw 2000; Wollina et al. 2002). Calcium carbonate has a stimulating effect on granulation by changing the pH of the wound from acid to neutral or slightly alkaline (pH 8–9).
The continuous movement of maggots on the wound mechanically stimulates the living tissue and helps to shape the granulation tissue. Increased granulation tissue formation and rapid wound closure have been detected with clinical larval treatment (Sherman 2002).

Regulation of Blood Circulation

While the larvae are feeding on dead tissue, free radicals are released during the destruction of cells. These radicals initiate the inflammatory response in the wound area, which starts after 48–72 hours, and is also the cause of pain. As a result, edema of the wounds is reduced and oxygen in the tissue is increased (Mumcuoglu 2001).
Optimal body temperature and oxygen and a moist wound environment are required for larval therapy to be effective.

Advantages of Maggot Debridement Treatment

1. Antibiotic use is not necessary because sterile maggots clean the wounds contaminated with gram-positive and gram-negative and aerobic and anaerobic bacteria.
2. MDT has an important role in reducing pain due to infected wounds, eliminating bad odors, and reducing treatment costs.
3. Larvae can remove most surface dead tissue.
4. Maggots selectively debride necrotic tissue without damaging healthy tissue, including blood vessels and tendons.
5. Maggots can clean the most hidden parts of the wound (sinuses, pockets under the skin, etc.).
6. Maggots are effective in quickly debriding the wound and significantly reducing the bacterial load (they reduce the risk of sepsis).
7. MDT prevents the spread of necrosis to the surrounding tissue.
8. It increases granulation and stimulates wound healing.
9. It is a feasible method to apply.
10. Its anti-infective feature provides superior debridement.
11. There is no need for hospitalization. MDT has no significant side effects.
12. MDT can prevent limb amputation and euthanasia in pet animals.
13. It does not require nursing services and is cheaper than the classical treatment method.
14. The treatment method is completely natural and the wound heals quickly.
15. There is an increase in tissue oxygenation and a decrease in edema with MDT.
16. Maggot therapy may be beneficial to small animals and prevent possible amputation and euthanasia in small animal surgery.
17. Unlike systemic antibiotics, MDT does not destroy the normal gastrointestinal flora (Sherman et al. 2000; Mumcuoğlu et al. 2001; Kenawy and Abdel-Hamid 2020; Uslu et al. 2021; Uslu et al. 2022).

Disadvantages of Maggot Debridement Treatment

1. Larvae can escape from poorly sealed dressings.
2. Application of larvae in cats may cause discomfort, which may be due to irritation or itching. It may also mean that the patient is experiencing pain.
3. MDT may cause itching and discomfort in staff as well as the patient.
4. Psychological and emotional problems may occur.
5. It may cause fever in some patients.
6. It should not be used in patients who are allergic to fly larvae, or any substance produced by the larvae (Courtenay et al. 2000; Hinshaw 2000; Sherman et al. 2000; Mumcuoglu 2001).
7. It may incur a relatively high treatment cost in veterinary medicine and increased shipping time (usually 24–48 hours after order) (Lepage et al. 2012).
8. Effectiveness is limited by the wound environment (pH, fluid, and oxygen).
9. Surgery is slower than MDT (Kenawy and Abdel-Hamid 2020).
10. It is not sold in pharmacies.

Indications for Maggot Debridement Therapy

Indications for MDT include diabetic foot ulcers, venous stasis ulcers, postoperative wounds, osteomyelitis, neuropathic foot ulcers, traumatic non-healing wounds, abscesses, boils, cellulitis, mastoiditis, necrotized tumor masses, neuropathy, paraplegia, hemiplegia, thalassemia, polycythemia, severe burns, and chronic or acute infected wounds. Maggot treatment is the most suitable option for necrotic, pustular, discharging, gangrenous wounds, wounds with insufficient blood circulation, and deep wounds where penetration of antibacterial drugs is limited.

Contraindications for Maggot Debridement Therapy

Contraindications are described as patients' allergic reactions to larvae, hemorrhagic abscesses, and coagulopathies. Fistulas in the respiratory system, internal organs, and endocrine glands and very rapidly progressive necrosis and wounds have

been described (Sherman et al. 2013). Larval therapy should not be used for wounds close to major blood vessels, wounds that may bleed, wounds close to internal organs, and open wounds of body cavities and sinuses (Church 2001; Jones and Wall 2007; Dar et al. 2013).

Duration of Application of Larval Treatment and the Number of Larvae

Sterile *L. sericata* larvae can be administered to patients daily, 1–2 times a week, or weekly. Larval treatment is usually applied twice a week. The larvae should be left on the wound for 24 hours, and the dressing should be removed with the larvae at the end of the period. If the wound is not closed in the controls, the larval treatment is continued. As a basic rule, if the wound has been cleaned, larvae should not be left on the wound.

The number of maggots to be applied to the wound depends on the stage and size of the larvae, the amount of necrotic tissue (Jones and Wall 2007), and the size of the wound. 1-3 mm into the wound. Long larvae are recommended. The size of the wound is important in MDT in veterinary medicine. In general, 7–8 sterile maggots per cm^2 are recommended for necrotic areas. If the wounds are small and superficial, 5–20 larvae can be placed per cm^2, while 400–800 larvae can be applied if the wound is deep and there is too much necrotic tissue. In veterinary medicine, 5–10 (Sherman et al. 2007a) and 8–12 maggots/cm^2 (Kočišovă et al. 2003) have been reported. A dose of 100 larvae during one treatment cycle can debride 50 g of necrotic tissue (Blake et al. 2007).

Larvae should be used as soon as possible, but maturation can be delayed, if necessary, by storing in the refrigerator at 4–8 °C for two (Blake et al. 2007; Dar et al. 2013) to five days (Wolff and Hansson 2005).

Application Techniques: Free-range Larvae or Biobags

There are two methods of larval application (Steenvoorde and Jukema 2004; Jones and Wall 2007). In the direct contact method, the larvae are freely placed directly on the wound surface (Steenvoorde and Jukema 2004). Cage dressings are generally preferred in MDT. Free application is recommended in patients with deep necrotic tissue.

The other technique packages sterile larvae in "tea bags" or "biobags." The larvae are placed between two 0.5 mm thick pieces of gauze (polyvinylalcohol-hydro-sponge) glued on three sides, and then the mouth of the bag is glued. Due to the permeability of the bags, the larvae can feed easily and their secretions can penetrate the wound. Since the larvae cannot navigate directly on the wound, mechanical irritation at the wound edges does not occur.

The biobag method has been successfully used in veterinary medicine (Table 3) (Mumcuoğlu et al. 2001; Mumcuoğlu and Özkan 2009; Sherman et al. 2013). Its implementation is less complex and requires less experience. It saves time in dressing changes (Blake et al. 2007). In wound care, if the owner of the animal does not accept the direct application of larvae, a biobag may be preferred (Jones and Wall 2007). The disadvantage of biobag application is that although larval debridement is effective in nearby wounds, it is inactive in irregularly shaped wounds (Blake et al. 2007). A statistical analysis showed that after 3–4 days of treatment, there was no difference in the amount of larval tissue debridement between the direct contact technique and the biobag.

Maggot dressings are individually adapted in veterinary medicine (Sherman et al. 2007b; Lepage et al. 2012). Wounds on the upper extremities or bodies of animals can be treated using a biobag (Sherman et al. 2007a). After the first day of larval treatment, the larvae can be moistened to prevent them from drying out (Wolff and Hansson 2005; Dar et al. 2013).

Lucilia sericata larvae are removed from the wound when they are fully grown (Wolff and Hansson 2005; Dar et al. 2013). The larvae are 1–3 mm long when applied to the wound (Jones and Wall, 2007) and 10 mm long when removed from the wound (Wolff and Hansson, 2005). Free larvae can be removed using forceps (Dar et al. 2013; Uslu et al. 2021), while larvae penetrating deep into wounds can be carefully removed by irrigating the wound with sterile water (Jones and Wall 2007; Dar et al. 2013). Larvae and dressings are disposed of as clinical waste (Lepage et al. 2012; Uslu et al. 2022).

The duration of application of larvae to wounds varies in different clinical studies. After 48 hours in the wound, they are less useful because of being less active. Therefore, it is recommended that all larvae should be removed after two days (Paul et al. 2009). After the sterile *L. sericata* larvae are removed from the wound, the wound is reassessed to determine whether to continue larval treatment (Dar et al. 2013; Uslu et al. 2021; Uslu et al. 2022). The treatment is continued until the end of the treatment period and the wounds are completely debrided. After the treatment process is completed, wound management with moist wound healing is required (Ewma 2006; Uslu et al. 2021; Uslu et al. 2022), and the wound should be treated with classical methods until it heals. Maggot therapy can be combined with systemic administration of antibiotics to prevent secondary bacterial infections. Wound photographs can be taken to show changes in the wound and the progress of its healing (Chivers 2010; Uslu et al. 2021; Uslu et al. 2022).

Wound treatment in animals can be painful and long-lasting, and it can cause animals to interfere with dressings, leading to a negative effect on the wound. Therefore, when there is a behavioral change in animals, larval treatment is terminated and painkillers may be administered (Jones and Wall 2007; Chivers 2010). Dressing changes require additional analgesia (Chivers 2010).

Sterile larvae are commercially produced and offered for use by many private companies in Malaysia, Japan, Germany, England, Israel, and the USA.

Larval therapy has recently emerged as a powerful medical aid for animals, although it is still limited in veterinary practice and clinical studies are scarce.

MDT, which is accepted because of positive results from laboratory and clinical applications, is applied as an alternative treatment for wound healing. Larval treatment is rare in veterinary wound care. Application of larval therapy in veterinary medicine may involve problems, including treatment costs and supply and transportation of larvae. In addition, the time and effort spent on preventing the removal of dressings by animals is extremely important.

Today, MDT is an FDA-approved therapy for non-healing skin and soft tissue wounds. It is a safe and effective clinical application for debridement and disinfection of some serious wounds in veterinary medicine.

It is extremely important to determine the number of maggots needed in the treatment and whether patients can

Tablo 3: Comparison of free and biobag larvae applications (Sherman et al. 2013)

Features	Free larva	Bio-bag
Larval activity	Larvae have easy access to deep wounds.	Larvae are suitable for sensitive areas such as eyes, ears and mouth.
Debridement power	+++	++
Maggot escape	Larvae rarely escape	Larvae do not escape
Larval application time	24-72 hours	72-96 hours
Larvae Cost	Treatment cost is low	Treatment cost is high
Pain sensation	More painful	Less painful

benefit from the treatment. Veterinary nursing may encounter difficulties in dressing wounds, identifying and preventing pain, and overcoming issues with owner acceptance of the treatment. In veterinary wound care, variability in wound healing may be observed in different species. Wounds in cats heal more slowly than those in dogs. Epithelialization and granulation tissue production are also slow in cats. However, in wounds that do not respond to surgical procedures and conventional medicine, MDT has been reported to be successful and provide complete healing in small animals and horses (Sherman et al. 2007b; Morrison 2010).

Chronic wounds that do not respond to antibiotics in pet animals are an important problem for veterinarians. Larval therapy is an important alternative to amputation in cats and dogs. In veterinary medicine, larval therapy may even be the first treatment option due to increasing antibiotic resistance and chronic wound infections. The benefits to disadvantaged patients, such as older animals and those with severe diabetes, risk of amputation, and chronic sepsis, increase the value of MDT. Small animal veterinarians today are increasingly turning to larval therapy to treat wounds.

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CHAPTER 21

MULTIPLE OVULATION AND EMBRYO TRANSFER IN CATTLE

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INTRODUCTION

Multiple Ovulation and Embryo Transfer (MOET) includes a series of procedures involving the transfer of embryos from superovulated donors to suitable recipients. A broader definition of MOET is the transfer of embryos from cows and bulls with high genetic capacity and yield levels to recipients with lower yield levels but without reproductive problems. The steps of MOET can be listed as selection of donors, selection of recipients, superovulation of donors, synchronization of donors and recipients, insemination of donors, embryo recovery, morphological evaluation of embryos, and fresh or frozen/thrawed embryo transfer and storage of embryos.

The first successful embryo transfer in mammals was carried out by Walter Heape in 1890, with the transfer of embryos from Angora rabbits to Belgian doe. Later on, successful results were obtained in embryo transfer in sheep and goats in the 1930s and 1940s by Warwick and colleagues (Betteridge 2003). However, the first successful result in cattle was reported, but the pregnancies were terminated before the gestation period was completed (Mapletoft 2018). Then, it was reported that the first embryo transfer calf was born with the transfer of the embryo produced from the slaughterhouse material in 1951 in Wisconsin (Betteridge 1981). In the later years, it is stated that especially in the 1970s, Rowson and colleagues developed embryo transfer in farm animals and started to be used commercially (Mapletoft 2018).

In Multiple Ovulation and Embryo Transfer, it is possible to increase herd productivity by transferring embryos collected from high-yielding donors to lower-yielding recipients. Thus, genetic improvement and selection of elite female and male animals can be achieved within the herd. In addition, the generation interval can be shortened and therefore the breeding plans can be accelerated (Alaçam 2015). Diseases that are transmitted by mating can be prevented by embryo transfer (Hasler 2003). The transfer of a dairy cattle from one region to a distant region requires a serious cost. However, transferring embryos in liquid nitrogen to distant regions is both easier and less costly (Kidie 2019). With the transfer of embryos to recipients in distant regions, passive immunity can be provided to calves that will be born from recipients adapted to that region and by this way, calf losses can be reduced (Alaçam 2015).

This chapter will provide comprehensive review of literature about selection of donors and recipients, superovulation of donors, synchronization of donors and recipients, insemination of donors, embryo recovery, morphological evaluation of embryos and fresh transfer or long-term storage of embryos are evaluated in general terms in MOET.

Selection of Donors and Recipients

One of the aims of embryo transfer is to obtain the maximum number of healthy offspring during their lifetime from animals with high yielding genetic capacity. In order to achieve this aim, the selection of especially high-quality donors is of great importance. The desired aim can be achieved by transferring quality embryos collected from donors to suitable recipients. Therefore, the animals to be selected as donors should be selected among the animals with the following characteristics (Erdem et al. 2020b; Karasahin et al. 2021b).

Selection of Donors

High Genotypic and Phenotypic Character

Selection of genetically and phenotypically high yielding animals for embryo transfer is the first step in donor selection. The main purpose of livestock is to increase milk or meat production. Therefore, the use of animals that stand out in terms of yield characteristics in the herd in embryo transfer can be selected to increase the high-yielding animal population and hence production in the herd. In addition, parameters such as fertility, resistance to diseases, ease of calving, and temperament should be considered in the selection of donor animals (Miglior et al. 2017).

Hereditary Transmitted Diseases

There should be no history of hereditary transmitted diseases in animals to be used as donors, especially Bovine Leucocyte Adhesion Deficiency (BLAD) and Complex Vertebral Malformation (CVM) (Kaymaz 2019).

Sexually Transmitted Disease

According to the International Embryo Transfer Association (IETS) donor animals should be free from diseases which are transmitted by mating and affect fertility such as Bovine Spongiform Encephalopathy (BSE), Infectious Bovine Rhinotracheitis- Infectious Pustular Vulvovaginitis (IBR-IPV), Foot-and-Mount Disease (FMD), Bovine Viral Diarrhea Virus

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(BVDV), Bovine Leukemia Virus (BLV), Bovine Tuberculosis (TB), Brucellosis, etc.. It is stated that the zona pellucida (ZP) is an important barrier for infectious agents to infect the oocyte and embryo (Escobar 2018). However, ZP may permit the transmission of some diseases. Pathogens can penetrate the ZP and infect oocyte and embryo. They can also attach to the ZP and infect the recipient and subsequently the hatched embryo. For embryos produced in vivo, pathogens can be effectively removed by following the protocol set by the International Embryo Technology Society (IETS) (Stringfellow and Givens 2000). Most of the transport of pathogens occurs by penetrating ZP. Therefore, embryos should be washed in Dulbecco's phosphate buffered saline (dPBS) at least 10 times before transfer or freezing procedures (Escobar 2018). Studies have reported that BVDV that has penetrated the ZP can be removed by washing (Gard et al. 2009). In the Office International Des Epizooties (OIE) protocol, it is recommended to use 100-fold diluted solution for each washing step of the embryos and to use disposable sterile pipettes during the transfer of the embryos (Escobar 2018; Alkan 2021).

Age and Body Condition Score (BCS)

Some researchers have reported that donor age affects the embryo production. Oocyte/embryo ratio increases after superovulation in older animals. While this rate is about 50% in old cows (10-13 years old), it is around 25% in young heifers (3-6 years old) (Malhi et al. 2005). It is reported that this difference is due to the lower fertilization and division rates in older cows compared to younger heifers (Nak 2021). At the same time, obtaining fewer embryos in older cows may be related to changes in follicular and endocrine mechanisms with advancing age (Malhi et al. 2005). For this reason, it has been reported that less number and poor-quality embryos can be obtained from older donors (10 years and older). However, the quality and number of embryos decrease in very young animals. In the studies performed, fewer embryos were obtained in the heifers aged 7.8-9.9 months, compared to the heifers aged 10 months. Reason for fewer embryos is the lower oocyte development capacity in the prepubertal period and this capacity increases with advancing age (Mikkola et al. 2020). That's why it is recommended not to use animals under 1 year age and over 10 years age as donors in order to obtain quality and maximum number of embryos together.

Importance of AMH in Donor Selection

Detection of ovarian reserves in donor selection is of great importance in predicting superovulation response and embryo yield. The number of primordial follicles on the ovary is called ovarian reserve (Visser and Themmen 2005). Oocyte quality decreases in animals with low ovarian reserve, and thus embryonic development capacity and superovulation response decrease, so embryo quality and yield cannot be at the desired level (Ireland et al. 2008). There is a statistically significant positive correlation between the total number of primordial follicles on the ovary and the number of antral follicles (AFC) (Modina et al. 2014). In addition, there is a positive correlation between Anti Mullerian Hormone (AMH) and AFC levels in cattle (Satılmış 2021). For this reason, AMH measurement can be used in donor animal selection (Hirayama et al. 2017; Nabenishi et al. 2017; Sevgi et al. 2019; Karakaş Alkan et al. 2020).

Selection of Recipients

Although the identification of recipients is not as important as donor selection, it can affect the success of embryo transfer. For this reason, recipients should be selected according to certain criteria, and these are listed below:

- Recipients must be healthy animals with no congenital defects.
- Must have normal and regular estrus cycles, have no problems with their reproductive history.
- Recipients should not be too old.
- Recipients should not have nutritional and metabolic problems.
- Body conditions should be normal.
- Recipients must not carry diseases that may affect fertility (Kaymaz 2019).

Superovulation of Donors

Multiple Ovulation and Embryo Transfer is an assisted reproductive technique involving the transfer of embryos collected from donors having superior yield characteristics with hormonal stimulation and superovulation to the recipients. There are many factors that affect the success of embryo transfer. Among these factors, the superovulation response of the donor animal has an important place. Individual variation of the superovulation response has a direct effect on the yield and quality of collected embryos (Polat et al. 2021). Follicle Stimulating Hormone (FSH) and Pregnant Mare Serum Gonadotropin (PMSG) are widely used for superovulation in cattle (Alaçam 2015). These hormones can also be used together with other hormones such as Gonadotropin Releasing Hormone (GnRH), Human Chorionic Gonadotropin (hCG), progesterone, prostaglandin F2α (PGF2α) and anti-PMSG.

Follicle Stimulating Hormone and PMSG, which are widely used for superovulation, have advantages and also disadvantages. The most important advantage of the purified FSH preparations available in the market is the high embryo yield and quality due to their low LH contents. In addition, its short-chain glyco-protein structure does not cause antibody formation in donors, but its half-life is quite short (average 3-4 hours). Therefore, in order to achieve the desired superovulation response, it requires two injections per day at 12 hours intervals. Furthermore, its cost is higher and is more difficult to obtain than PMSG (Bader et al. 2005; Ağaoğlu and Kocamüftüoğlu 2015). PMSG, on the other hand, is a glycoprotein hormone with FSH and LH like effects in cows. PMSG can be used for superovulation because it is easily available in the market and its cost is lower than FSH. PMSG has a long half-life (approximately 40 hours) and may have a circulating effect for more than 10 days. Due to its long half-life, a single injection is sufficient for the superovulation protocol (Kaymaz 2019). However, due to its long chain glycoprotein structure, it causes antibody formation in donors. For this reason, superovulation protocols using PMSG require the use of anti-PMSG during the first insemination (Dieleman et al. 1993). In addition, due to its long half-life; it has important disadvantages such as anovulatory follicle development, abnormal endocrine changes, decrease in embryo quality and number.

Factors Affecting the Superovulation Response

There are many factors that affect the superovulation response in cattle and these factors can be classified as animal related, gonadotropin preparations and environmental factors.

Animal Related Factors

Factors such as breed, age, nutritional status (BCS), progesterone level, lactation and milk yield of donor animal directly or indirectly affect the superovulation response.
Among the donor animal related factors, the most important factor is the number of follicular waves and the day of the cycle when the superovulation protocol is started. Studies have reported that the presence of the dominant follicle on the ovary at the start of superovultaion protocol negatively affects the superovulation response (Guilbault et al. 1991; Wehrman et al. 1996; Kim et al. 2001). It is reported that in the dominant follicle stage, smaller follicles tend to regress and there is less response to gonadotropin stimulation (Mikkola et al. 2020). The second follicular wave occurs approximately between the 8th and 12th days of the cycle, depending on the number of follicular waves of the sexual cycle (Ginther et al. 1989). Since there will be no functional dominance in this period, it is stated that it will be the most appropriate time to start the superovulation protocol between days 8-12 of the cycle, which coincides with the beginning of the second follicular wave (Mapletoft and Bo 2004). On the other hand, some studies reported that exogenous FSH administration affects the dominant follicle independently of its inhibitory effect on the pituitary and ovary (Diaz et al. 2001; Bó and Mapletoft 2014).
Superovulation response differs according to age and breed in cattle. Superovulation response is higher in Hereford and Brahman breeds compared to other breeds. In a study, significantly higher number of transferable embryos were obtained in Brown Swiss cattle than in Holstein-Friesian cattle (Mollo et al. 2007). Superovulation response is higher in young cows than in older cows and heifers. Especially in old cows (>10 years) and heifers (<1 year), the superovulation response decreases significantly. However, the reduce in selection of older animals as donors is due to the prevalence of genetic selection today (Mikkola et al. 2020). Animals should be in optimum body condition (BCS 2.75-3.50) prior to superovulation.

Gonadotropins Related Factors

Gonadotropins should be administered in appropriate doses. Low or high dose administration negatively affects the superovulation response. As PMSG preparations have a long half-life, an adequate superovulation response is obtained in a single injection of PMSG, while FSH preparations should be administered in two doses, 12 hours apart, due to their short half-life (Baruselli et al. 2006). The ratio of FSH:LH varies in the FSH preparations available in the market. Optimal superovulation response can be achieved with the use of purified FSH preparations with a low LH ratio. However, a rest period of at least 25-30 days should be left between the two protocols in order to perform repeated superovulation in the donor animal (Mapletoft et al. 2002). In addition, the preparation, storage and lot number of the drug also affect the superovulary response (Polat et al. 2021).

Environmental Factors

Environmental factors that cause stress on donors, may adversely affect the superovulation response (Al-Katanani et al. 2002). These factors include poor housing conditions, long-term openness, extreme cold or hot ambient temperature (heat stress).

Synchronization of Donors and Recipients

The synchronization of donor and recipient animals is important for the success of embryo transfer. After recovery of transferable embryos from donors, the success of embryo transfer depends on the selection of the recipients and the timely transfer. Presence of a functional corpus luteum (CL) in recipients during transfer increases the rate of conception after transfer. A functional CL depends on factors such as the management, feeding and estrus monitoring of the recipients (Alkan et al. 2020).
In embryo transfer, embryos at the age of 6.5-7 days from donors are recovered and transferred to appropriate recipients. The embryo tolerates a 12hour synchronization interval between donors and recipients. Therefore, if embryos will be transfered freshly, there should be a maximum of 12 hours between the estrus of the donors and recipients (Alaçam 2015). While superovulation protocol is applied to donors, estrus synchronization protocols are applied to recipients based on GnRH, PGF2α and progesterone or with the combined use of these hormones. Estrous synchronization can be achieved by using PGF2α alone or in combination with progesterone. However, in these protocols, estrus can spread over a period of 2-4 days in recipient animals. Therefore, if a fresh transfer is to be made, donor and recipient animals can be asynchronous. Estrous of donors and recipients can be synchronized with fixed-time insemination protocols (such as Ovsynch, Cosynch) in which GnRH and PGF2α are used sequentially (Kaymaz 2019). However, regardless of the protocol chosen, monitoring and recording estrus in recipients minimizes asynchrony between donors and recipients.

Insemination of Donors

It is recommended to use live, linearly moving, high motility semen, to eliminate the negatives related to fertilization and because it is a costly procedure. Therefore, it is important to detect donors' estrus at the right time. In a study, it was determined that insemination with a single straw at 0, 12 and 24 hours of estrus in donor animals had no effect on embryo quality, but the fertilization rate was higher in those inseminated at 0 and 12 hours (Dalton et al. 2000). The ovulation period (the time between the first and last ovulation) in superovulated donors is 12 hours (Adams 1994). In the traditional method, donors are inseminated twice with double straws 12 hours after the onset of estrus. In the insemination procedure, the catheter should be released into the corpus uteri as soon as it passes the cervix while advancing the catheter towards the horns reduces the quality and number of embryos (Kidie 2019). Sex sorted semen has been widely used in artificial insemination protocols. However, the pregnancy rate in insemination is low due to the fact that it undergoes many processes during production compared to unsorted semen. In MOET programs, sex sorted semen can be used to obtain female embryos. However, studies have shown that the use of sex sorted semen decreases the fertilization rate and increases the rate of degenerated embryos (Hayakawa et al. 2009; Sartori et al. 2018).

Embryo Recovery

In in vivo method, embryos are recovered by various techniques. After fertilization, sequential mitotic divisions take

place in the embryo, during which the embryo moves from the oviduct to the uterine horn. Although there are individual differences, the embryo is found in the oviduct until the 4th day, at the uterotubal junction on the 5th day and in the apex of the uterine horn on the 6th/7th days (Kaymaz 2019). In the MOET programs, embryos are usually collected from the apex region of the uterine horn on the 7th day after insemination. Embryos are collected by two flushing methods, surgical and non-surgical (transcervical) method. However, with the development of the transcervical method in the early 1970s, the surgical method is no longer used now (Phillips and Jahnke 2016). In the transcervical method, a 2 or 3-way (preferably 3-way) Foley catheter is inserted through the vagina and cervix into the uterus at the starting point of the curvatures. The cuff of the Foley catheter is filled with 5-10 ml of air so the catheter is fixed. With the inserted Foley catheter, the uterus should be flushed at least 10 times with an average of 200 ml of flushing medium each time (depending on operator's experience). In uterine flusing, 800-1000 ml of D'PBS (Dulbecco's Phosphate Buffured Saline) or lactated Ringer's solution (with antibiotic added) is used as flusing medium (Kaymaz 2019). One of the important facts in MOET programs is the development of embryo filters. Before the advent of filters, the uterine effluent was collected in a glass bottle or graduated cylinder and the embryos were expected to settle to the bottom for 30-60 minutes. Then, the supernant remaining in the upper part of the collected liquid was discarded and the embryos were scanned in a little liquid remaining at the bottom. However, nowadays, during flusing, the uterine effluent is passed through 50-70 mm stainless steel or plastic filters, allowing embryos with a diameter of 150-200 mm to remain on the filter and to be collected by scanning easily (Phillips and Jahnke 2016). Embryo recovery is highly associated with uterine flushing; operator's experience, flusing time and method, the type and position of the catheter used, the number of media and the number of flusing (Phillips and Jahnke 2016; Kaymaz2019; Alkan et al. 2021).

Morphological Evaluation and Classification of Embryos

Embryos are evaluated morphologically before being transferred or frozen after flushing. Using a magnification of at least 50 under a stereomicroscope, the quality of the embryos is examined for developmental stages and abnormalities on the embryos. The diameter of bovine embryos is between 150-190 mm according to the developmental stages (Phillips and Jahnke 2016). Embryos recoveried from donors are evaluated using the IETS classification system according to their developmental stages and quality grades. This classification system consists of a 2-digit code according to development stage and quality, respectively (Dursun and Karaşahin 2021).

Embryos are classified according to their developmental stage from grade 1, which is an unfertilized oocyte or a 1-cell embryo to grade 9, which is an expanding hatched blastocyst.

Grade 1 embryo is one cell or unfertilized oocyte. Single celled or unfertilized oocyte evaluated after flushing is called UFO. UFOs usually feature a spherical oolemma or vitelline membrane. It contains the part called perivitelline space between the zona and the cell (Phillips and Jahnke 2016).

Grade 2 embryos are the embryos that are at the 2-12 cell developmental stage and are considered to be delayed in stages. In embryos at this stage, fertilization is done but stops due to any degeneration of sequential mitotic divisions. Embryos at this stage are considered dead or degenerated embryos (Phillips and Jahnke 2016).

Grade 3 embryo is an embryo at 16 or more cell stage as a result of sequential mitotic divisions in the embryo and is called morula or early morula. The cellular mass of the embryo appears to occupy the perivitelline space (Bó and Mapletoft 2018).

Grade 4 embryos orcompact morula are individual blastomeres coalesce to form a compact mass in the compact morula stage. The cellular mass of the embryo appears to occupy 60-70% of the perivitelline space, but the blastomeres are undifferentiated (Bó and Mapletoft 2018).

A fluid-filled cavity called a blastocele is present in embryos of Grade 5 or early blastocysts. It has a signet ring appearance with embryo blastocele formation. At this stage, the cell mass appears to occupy 70-80% of the perivitelline space. In the early blastocyst stage, it can be noticed that the inner cell mast (ICM) and trophoblast cells begin to differentiate (Phillips and Jahnke 2016; Bó and Mapletoft 2018).

Grade 6 embryos are called blastocyst. At this embryonic devolopment stage, the arc of trophoblast cell, ICM and blastocele space are clearly distinquished. The embryo occupies almost all of the perivitelline cavity. However, the zono pellucida retains its thickness (Phillips and Jahnke 2016; Bó and Mapletoft 2018).

Grade 7 embryos are expanded blastocysts. At this stage, the enlargement of the diameter of the embryo is clearly noticeable. It is observed that the thickness of the zona pelucida becomes thinner due to the pressure on the zona pelucida imposed by expansion of the embryo (Phillips and Jahnke 2016; Bó and Mapletoft 2018).

Grade 8 embryos are hatched blastocyst. The embryo hatches out through a crack in the zona pellucida due to the pressure created by the expansion of the embryo. Empty zonas and spherical or collapsed blastocysts can be seen separately in areas scanned under the microscope (Phillips and Jahnke 2016; Bó and Mapletoft 2018).

Grade 9 embryos are hatched expanded blastocysts. At this stage, the hatched blastocyst appears to expand again (Phillips and Jahnke 2016).

According to the developmental stages, embryo can be obtained from the same donors at different developmental stages, from unfertilized oocyte to hatched expanded blastocyst during the same flushing (Bó and Mapletoft 2018). The best predictor of viability is that the embryo reach the desired developmental stage on the 7th day after estrus, when they are usually recovered (Bó and Mapletoft 2018). Also, the highest pregnancy rate is achieved from excellent and good quality embryos in developmental stages from compact morula to expanded blastocyst (Hasler et al. 1987).

The quality of the embryos is evaluated according to their morphological appearance, with codes ranging from 1-4.

Code 1 embryo is excellent or good. The blastomeres of the embryo appear uniform in size, color and density. The embryo has a symmetrical and spherical cellular mass. At least 85% of the blastomeres are alive and there is almost no irregularity between them. The zona pelucida maintains its smooth and spherical structure (Bó and Mapletoft 2018).

Code 2 embryo is categorized as fair. There is a mismatch in size, color and density between the blastomeres of the embryo. At least 50% of the blastomeres are viable and moderately irregular between them. The zona pelucida partially preserves

its robust and spherical structure (Phillips and Jahnke 2016; Bó and Mapletoft 2018).

Code 3 is embryo categorized as poor. There are serious irregularities in size, color and density between the cellular mass and blastomeres. At least 25% of the cell mass of the embryo is alive. The zona pelucida is solid but has concave or flat structures on which its spherical structure is disrupted (Phillips and Jahnke 2016; Bó and Mapletoft 2018).

Dead or degenerated embryos are categorized as Code 4. UFO, dead or degenerated embryos are considered in this grade. These embryos cannot be used (Bó and Mapletoft 2018).

The grade of embryo quality is evaluated visually according to its morphological appearance. Code 1 embryo tolerates freezing protocols without reducing pregnancy rate. According to IETS, only Code 1 embryos can be exported. When a Code 2 embryo is transferred fresh, the conception rate is close to code 1 embryos. Although, Code 1 embryos can be stored frozen, transfer of these embryos freshly is highly recommended. Code 3 embryos cannot tolerate freezing/thawing protocols. For this reason, only fresh transfer of Code 3 embryos is recommended, but the rate of conception is lower than Code 2 embryos. Code 4 embryos cannot be used (Phillips and Jahnke 2016; Bó and Mapletoft 2018; Erdem et al. 2020a).

Long-term Storage or Fresh Transfer of Embryo

Although the first freezing procers of cell stated in the 1800s and many studies have been done on this subject. Today, spermatozoa, embryo, oocyte and ovarian tissues are commonly frozen and preserved by using various cryoprotectant materials and cryopreservation techniques (Arav 2014).

The meaning of the word cryopreservation is defined as "cell whose metabolism is preserved by freezing outside the body" (Parnpai et al 2016). Intracellular (permeable) and extracellular (nonpermeable) cryoprotectants are used for cryopreservation. Frozen embryos using various cryoprotectants can be stored for a long time by stopping their metabolism at low temperatures (-196oC). The purpose of freezing the embryo is to preserve the current state of the embryo and to continue its development when thawed (Kaymaz 2019). However, sometimes significant morphological and functional damage may occur in embryos during cryopreservation. Therefore, some principles have been developed in order to minimize the damage to embryos. The basis of these principles are freeze-thaw rates and the characteristics of the cryoprotectants used. Today, there are three different cryopreservation methods, which have been developed and widely used by considering these criteria, as slow freezing, rapid freezing and vitrification.

Slow Freezing

Slow freezing, which is a slow and controlled freezing method, is also called standard, balanced (equilibrium) or traditional freezing method (Kaymaz 2019). In the traditional slow freezing technique, the embryo is frozen with a lower concentration of cryoprotectants by means of a programmable freezing device. However, disadvantage of the technique is that a rather expensive device is needed during freezing and the process takes quite a long time. For a successful cryopreservation procedure, it is desirable to pass this range between +15 and -5oC quickly (Gajda and Smorang 2009). However, in this technique, the decrease from +15 to -5oC, which is the critical temperature for the embryo, occurs more slowly than other cryopreservation techniques. For this reason, the embryo is exposed to the toxic effect of cryoprotectants for a long time, especially in the specified temperature range, and this creates disadvantages such as breakage in zona pellucida and disruption of cell membranes. On the other hand, compared to other cryopreservation techniques, a higher rate of pregnancy is obtained from embryos frozen and thawed by slow freezing technique (Kaymaz 2019).

Rapid Freezing

The rapid freezing method is defined as the freezing method in which the cells are partially dehydrated before the embryo is exposed to the application of high freezing rates (Kaymaz 2019). In the rapid freezing method, mixtures of cryoprotectants (1.5 M glycerol + 1.0 M sucrose solution) that can or cannot penetrate into the cell are used as cryoprotectants (Fahning and Garcia 1992). However, in this method, significant damage may occur in embryos due to the difference in osmolarity during the freezing-thawing process (Vajta and Kuwayama 2006). Therefore, this technique is not often preferred for cryopreservation.

Vitrification

Vitrification is known as glass-like solidification, it is the transformation of cells, tissues and organs into a completely vitreous state inside the cell at low temperatures (Fahning and Garcia 1992). Vitrification is an embryo freezing technique based on the principle of rapid cooling and preventing ice crystal formation by using high concentration cryoprotectant substances. During vitrification, highly concentrated cryoprotectant substances increase the viscosity of the intracellular water at a sufficient cooling rate, solidifying the cell contents and cryopreservation occurs. Advantages of the vitrification technique can be listed as being short, simple, not requiring any special equipment, and being routinely usable. However, there are disadvantages such as the need for solutions in different dilutions for the removal of cryopretectans, the need for a stereomicroscope during thawing, the need for experienced personnel to perform the procedure, and the toxic effect of high concentrations of cryoprotectants. Due to these disadvantages, in order to minimize the toxic effects of cryoprotectant substances, shortening the equilibration time or two-stage equilibration is applied (Massip 2001, Vajta and Kuwayama 2006). Generally, glycerol, ethylene glycol and glycerol/propanediol combinations can be used for vitrification (Prentice and Anzar 2011).

Average pregnancy rates obtained after freezing-thawing the embryos from farm animals using the vitrification method are between 60-75%. In addition, the most suitable embryo development stage for the vitrification technique is the blastocyst stage (Youngs 2011).

Fresh Transfer

Cryopreservation process of embryos, the contact of embryos with different cryoprotectants and exposure to the external environment during this process negatively affect the survival

of embryos. Therefore, a higher conception rate is achieved with fresh transfer of embryos (Karasahin et al. 2021a). Rate of conception is 68-77% with the fresh transfer of embryos, while this rate is between 56-68% in the transfer of frozen-thawed embryos (Karasahin et al. 2021a). Surgical and non-surgical transfer process did not have an effect on the pregnancy rate, but the non-surgical method is more practical and less costly (Hasler 2001). Therefore, embryo quality, transfer site, CL type and diameter have important effects on pregnancy rate during embryo transfer in cattle heifers (Alkan et al. 2020). In a different study, the rate of conception was 64% in fresh transfer, 53% in vitrified frozen, and 70% in ethylene glycol treated (Soler et al 2007).

As a result, conception rate after fresh transfer of embryos to recipients is higher than the frozen-thawed method. However, since there is not always a suitable recipient for fresh transfer, it is important to freeze the obtained embryos with appropriate methods (Tekeli and Yesilkaya 2021).

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CHAPTER 22

IN VIVO EMBRYO PRODUCTION IN SHEEP AND GOATS

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INTRODUCTION

Continuity of animal production and increase in yield in animal husbandry is possible by providing a fast and sustainable reproductive performance. In the sheep and goat industry, it is necessary to improve the genetic material and spread the male and female genotypes in order to increase the yield per animal and to obtain high-yielding offsprings. For this purpose, many continuously developing new reproductive biotechnological methods/assisted reproduction techniques are used in small ruminant breeding that play an important role in the production and management of sheep and goats. By means of these techniques, it is possible to accelerate animal breeding, to increase the productivity levels of animals, and to obtain new gene sources reliably. Today, embryo production and transfer is one of the most important assisted reproductive techniques that increases the rate of genetic progression in sheep and goat breeding (Ishwar and Memon 1996; Karakas Alkan 2021; Tekeli et al. 2021). Within this scope, information about *in vivo* embryo production in sheep and goat breeding will be presented in this chapter.

Embryo Transfer

Embryo transfer is an assisted reproductive technique that involves the transfer of numerous embryos from genetically superior parents to recipients (Gibbons and Cueto 2011, Widayati 2012). Embryo transfer can be performed after fresh or frozen/thawed embryos collected from the genital tract of the donor animals (*in vivo*) or obtained under laboratory conditions (*in vitro*) (Cognie et al. 2003; Amiridis and Cseh 2012; Tekeli et al. 2021).

The Importance of Embryo Transfer

Embryo transfer technology provides solutions for many issues such as rapid spread of genotypes or high yielding new breeds, shortening of the generation interval, reducing the risk of spreading diseases, enable generations to easily adapt to different production and administrative systems, obtaining sex-determined offspring and advanced biotechnological method such as *in vitro* fertilization, cloning, transgenesis and micromanipulation of the embryos. Also, it is possible to collect 4-6 times higher the number of embryos from a superior donor in a year through this method (Gibbons and Cueto 2011; Amiridis and Cseh 2012).

The first embryo transfer in sheep and goats was carried out about 90 years ago (Warwick et al. 1934). Later, 19 embryos with 2-16 cells were transferred to 18 recipient sheep for the first time by Hunter et al. (1955) and 8 lambs were born alive. These applications founded the basis of surgical embryo transfer in sheep and goats (Ishwar and Memon 1996). Sugie and his team made important developments in the field of biotechnology in sheep and goats in their studies in the 1960s but they could not share their findings with other researchers due to language problems (Betteridge 2003; Grazul-Bilska et al. 2006). During last 30 years, embryo transfer technology has been widely applied in many countries around the world in order to increase the number of animals with high genetic potential regarding milk, meat, wool and mohair production (Gibbons and Cueto 2011).
Embryo transfer applications are controlled by International Embryo Technology Society (IETS) and all information is collected by this center. This center records fresh and frozen/thawed embryo transfers in farm animals. IETS reports that 32,000 embryos were produced *in vivo* in sheep and goats in 2019 (Viana 2020).

In Vivo Embryo Production

In recent years, multiple ovulation and embryo transfer (MOET) technique has played an important role in the breeding and reproduction of farm animals in the world, and beneficial results have been obtained through this application. This method also allowed the creation of genetically superior herds and the development of appropriate freezing procedures in order to protect these gene resources (Lehloenya 2008; Gibbons and Cueto 2011; Karakas Alkan 2021).
In vivo embryo production in sheep and goats includes stages such as donor selection, synchronization, superovulation and fertilization (natural mating or artificial insemination), collection and evaluation of embryos (Baldassarre and Karatzas 2004; Gibbons and Cueto 2011; Karakas Alkan et al. 2017). Although many advances have been made in *in vivo* embryo production in small ruminants, there are still differences in superovulation response in sheep and fertilization rates in goats. In addition, premature corpus luteum regression in sheep poses an important problem in *in vivo* embryo production. Therefore, there is still a need for new research on embryo production in small ruminants (Cognie et al. 2003; Lehloenya 2008; Karakas Alkan 2021).

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Selection of Donors

Donor animals should be highly productive, have high genetic potential, have no fertility problem, have normal and regular estrus cycles, do not carry a hereditary or infectious disease, have no metabolic and nutritional problem, and have a high market value. However, the most important selection criterion is the donor's yield in terms of meat, milk, wool, mohair production (Tekeli et al. 2021).

There are many factors that affect a successful embryo transfer in sheep and goats. These are the management of donor and recipient animals, estrus synchronization of donor and recipient, donors' superovulation response, fertilization rate, embryo collection and evaluation, embryo transfer technique, and other factors affecting the survival of transferred embryos (Ishwar and Memon 1996). However, the most important factor affecting the success of embryo transfer, especially in sheep and goats, is the superovulation response that varies individual to individual (Cognie et al. 2003; Lehloenya 2008; Karakas Alkan et al. 2020; Karakas Alkan 2021).

Superovulation response and number of transferable embryos considerably limit *in vivo* embryo production in cattle, sheep and goats (Cognie et al. 2003; Sevgi et al. 2019). Individual differences are observed even in animals housed in the same group and undergoing the same superovulation protocol in *in vivo* embryo production. For this reason, it is very important to select animals that can respond better to superovulation treatments in recent years (Rico et al. 2009; Monniaux et al. 2010; Soquilla and Mingala 2017; Karakas Alkan et al. 2020).

Studies during the past decade have focused on the role of anti-Müllerian hormone (AMH) in predicting animal responses to gonadotropin treatments (La Marca and Volpe 2006; Monniaux et al. 2010; Monniaux et al. 2013; Sevgi et al. 2019; Karakas Alkan et al. 2020). In different animal species, higher AMH concentrations were associated with a better performance in terms of superovulation response and a higher potential for embryo production (Monniaux et al. 2011). AMH is known to be the best endocrine biomarker to predict follicle number and superovulation response in assisted reproductive technologies (La Marca and Volpe 2006). Since this hormone is expressed in granulosa cells of developing follicles and the highest AMH concentrations are found in antral follicles, AMH concentration measurement can be used to determine the follicle pool of donor animals (Monniaux et al. 2013; Karakas Alkan et al. 2020). Some researchers have reported a positive correlation between the number of small antral follicles and serum AMH concentrations (Gruijters et al. 2003; Monniaux et al. 2013; Abdel Aziz et al. 2017). In addition, a significant correlation was found between AMH concentrations and total corpus luteum (CL) and transferable embryo numbers in cattle (Rico et al. 2009). Karakas Alkan et al. (2020) reported a strong positive correlation between AMH concentrations measured in donor goats on the day of initiation of the synchronization protocol and the number of total corpus luteum (CL), total count of oocyte/embryo and transferable Code 1 quality embryos. In addition, superovulation response was measured to be higher in donors whose AMH concentration was high on the day of synchronization. Cut-off value in ROC curve analysis performed for the selection of donors with highest superovulation was determined to be 4.77 ng/ml (Karakas Alkan et al. 2020). Lahoz et al. (2014) reported that the determination of the plasma AMH concentration in a single blood sample before FSH treatment is a suitable method for selecting the best oocyte donors for embryo biotechnologies in prepubertal sheep. Based on this information, the measurement of AMH concentration stands out as a good criterion in order to determine donors who will respond better to superovulation treatment.

Synchronization and Superovulation Treatments

Synchronization is the process of planning estrus and ovulation according to the intended time (Alaçam 2015). Synchronization is very important for the application and success of the artificial insemination and embryo production procedure (Abecia et al. 2012). Since sheep and goats are seasonally polyestrous animals, estrus synchronization protocols should be applied to donors before superovulation. In addition, estrus synchronization protocol is required to use the same donors for recurrent superovulation practices throughout the year (Thibier and Guerin 2000).

In sheep and goats, mostly progestogens are used for estrus synchronization before superovulation. For this purpose, intravaginal sponge and controlled internal drug releasing devices are often preferred because of their ease of application and cheaper cost. Medroxyprogesterone acetate (MPA, 50-60 mg) and flurogestone acetate (FGA, 30-40 mg) are used as progestogens. Hormonal applications can be made both during the breeding season and during seasonal anestrus (Amiridis and Cseh 2012; Karakas Alkan et al. 2017). When progestogens are applied as long as the corpus luteum is active in the estrous cycle, they suppress cyclic activity and therefore ovulation with negative feedback on GnRH and gonadotropins. With the termination of the progestogen application, the estrus is synchronized (Amiridis and Cseh 2012).

The most commonly used estrus synchronization protocol in sheep and goats includes administration of progestogen for 9-14 days and administration of luteolytically effective prostaglandin 48 hours before the removal of progesterone source (Baldassarre and Karatzas 2004; Rahman et al. 2008; Amiridis and Cseh 2012).

Superovulation applications provide ovulation and maturation of oocytes by stimulating the development of more than one oocyte from the ovary (Tekeli 2015; Kose et al. 2012). Unpredictable differences in superovulation response are the most critical step in embryo production programs in sheep. There are endogenous (genetic, age, follicular wave, breeding season, etc.) and exogenous (gonadotropins, route of administration, dose, nutrition, etc.) factors that affect the superovulation response (Gibbons and Cueto 2011; Amiridis and Cseh 2012).

Pregnant mare serum gonadotropin (PMSG) and follicle stimulating hormone (FSH) are the two most commonly used gonadotropin preparations for superovulation. Apart from these hormones, horse pituitary extract (HAP) and human menopausal gonadotropin (HMG) are also used in superovulation protocols (Gonzalez et al. 2001). These gonadotropins have some advantages and disadvantages (Loi et al. 1998).

PMSG hormone can stimulate follicle development, estrogen production, ovulation, luteinization and progesterone synthesis. PMSG has great efficacy due to its longer biological half-life in comparison of FSH activity, and a single dose of 750-2000 IU can be administered subcutaneously or intramuscularly 1-2 days before the end of synchronization protocols. High superovulation responses have been observed in protocols

with PMSG. However, the quality embryo numbers obtained in this protocol are well below the acceptable limits in commercial embryo transfer applications. The reason for poor embryo quality can be explained in several ways (Gonzalez et al. 2001; Amiridis and Cseh 2012). PMSG has a half-life of 21 hours in sheep due to its high molecular weight. Short half-life causes deterioration of follicle development, formation of anovulatory follicle stimulation, premature follicle luteinization and reduced embryo quality (Gibbons and Cueto 2011). In addition, PMSG causes excessive follicular steroid secretion. This impairs sperm and gamete transport, oocyte maturation and embryo development. At the same time, ovarian stimulation decreases as anti-PMSG antibody develops in repeated applications of PMSG (Gonzalez et al. 2001).

FSH is the most commonly used gonadotropin with the best results in superovulation protocols of small ruminants (Gibbons and Cueto 2011). FSH is a member of the glycoprotein hormone family. Natural or recombinant FSH preparations are used in superovulation protocols (Ben-Menahem 2018). FSH induced superovulation treatments in sheep and goats culminate in a higher number of quality embryos and ovulation rate per donor than PMSG (Gonzalez et al. 2001; Gibbons and Cueto 2011). However, due to the short half-life of FSH preparations, repeated doses are required (Amiridis and Cseh 2012). The most widely accepted superovulation protocol for the stimulation of multiple ovulations in sheep and goats is the administration of FSH in decreasing doses towards the end of the progestogen administration. Unlike PMSG, the biological half-life of FSH is 3-4 hours. For this reason, 6-8 applications should be made at 12-hour intervals. A total of 200 mg of FSH (e.g. 50, 50, 30, 30, 20, 20 mg) per animal is administered to sheep and goats for superovulation. Multiple ovulations usually occur 60 hours after removal of the intravaginal progesterone source (Gibbons and Cueto 2011).

A long-term administration of progesterone (for 11 days) is included in the traditional superovulation protocol and FSH administration is started 48 hours before the removal of the progestogen source from the vagina (9 days after the intravaginal insertion of the progesterone source). FSH is administered in six decreasing doses (total of 200 mg) at 12 hours interval. A single dose of prostaglandin is administered with the first FSH injection. The progesterone source is removed from the vagina on the 11th day. GnRH injection is administered 24 hours after removal of progesterone source from the vagina and donors are allowed to mate with fertile males at 12 hours after GnRH administration (Thibier and Guerin 2000; Menchaca et al. 2007; Gibbons and Cueto 2011; Agaoglu et al. 2014; Karakas 2015).

In fact, traditional superovulation protocols were first designed in 1980s. However, in these years, there was not much information about the ovarian follicular dynamics of ruminants. With new developments, it has been suggested that follicular development must be considered when designing the control of ovarian activity in goats. From this point onward, the presence of the dominant follicle is very important when starting superovulation (when a new follicular wave is started). For this reason, the day 0 (day 0= day of ovulation) protocol comes to the fore in studies. It is thought that it is easier to initiate a new follicular wave when starting FSH treatment through this protocol (Menchaca et al. 2007).

In the Day 0 protocol, a short-term (5 days) progesterone administration is performed together with the prostaglandin injection on the first day. During the removal of progesterone, eCG is applied and ovulation is stimulated with a GnRH analogue after 36 hours. Superovulation treatment begins at 72-84 hours after removal of the intravaginal device and two prostaglandin injections are made during the last two FSH administrations. For the synchronization of ovulations, GnRH is injected at 12 hours after the last FSH administration. When the results gained after the traditional and Day 0 protocol are compared, it is observed that the Day 0 protocol is better method in providing the superovulation response in goats (Menchaca et al. 2007).

Tasdemir et al. (2011) investigated the effect of Day 0 protocol on ovarian response and embryo fertility in Ankara and Kilis goats during anestrus. Estrus synchronization rates and CL numbers were similar in both goat breeds whereas, the rate of transferable embryos was higher in Angora goats than Kilis goats. However, it has been concluded that the Day 0 protocol can induce a superovulation response in goats out of the breeding season, but the breed may have an effect on the superovulation response (Tasdemir et al. 2011).

Natural Mating and Artificial Insemination

After synchronization and superovulation protocols in sheep and goats, donors are usually mated by natural mating method (Karakas Alkan 2021). In natural mating, it is generally recommended to mate donors every 12 hours from the beginning to the end of estrus (Gibbons and Cueto 2011). However, artificial insemination (fresh or frozen/thawed) can also be preferred some times. The use of cervical insemination in embryo production in sheep often does not yield successful results. Therefore, if artificial insemination is to be performed, the laparoscopic method would be more appropriate. In this method, semen is released into the horn of the uterus close to the fertilization site. Thus, a decrease in the required semen dose and an increase in the fertilization rate can be achieved (Ishwar and Memon 1996; Gibbons and Cueto 2011; Karakas Alkan et al. 2017). The optimal time for laparoscopic insemination with fresh semen in sheep is 32 hours after the onset of estrus (Brebion et al. 1992). When frozen/thawed semen is used, laparoscopic insemination can be performed at 40-55 hours after the removal of progesterone source (Evans et al. 1986). In goats, if laparoscopic insemination is to be performed with fresh semen, insemination is recommended at 20-24 hours after the onset of estrus (Vallet and Baril 1990), and if frozen/thawed semen is to be used, insemination is recommended at 46 hours after the removal of progesterone source (Fieni et al. 1990).

If cervical insemination is performed with fresh semen, it should be $800x10^6$ sperm/ml for sheep and $400\text{-}600x10^6$ sperm/ml for goats. This rate is $80x10^6$ sperm/ml for sheep and $100x10^6$ sperm/ml for goat for laparoscopic insemination. In cases where frozen semen will be used, the required dose for insemination in sheep and goats should be $100x10^6$ sperm/ml (Baril et al. 1989; Vallet and Baril 1990; Gibbons and Cueto 2011).

Regardless of the type of superovulation application, fertilization losses are frequently encountered, especially in sheep with a high ovulatory response. Fertilization losses occur at equal rates in sheep after natural breeding or artificial insemination (cervical artificial insemination). This is thought to be due to problems during the passage of spermatozoa through the cervix and this problem can be solved with the intrauterine laparoscopic insemination procedure. Intrauterine

insemination is an effective method to eliminate fertilization losses, especially in donors with a high ovulation rate. However, if rams or goats with known fertility are suitable for breeding, natural breeding should be preferred in superovulation applications (Ishwar and Memon 1996; Cognie et al. 2003; Gibbons and Cueto 2011).

Obtaining the Embryo

In vivo obtained embryos are collected by flushing the uterine horns with a medium at 6-8 days after mating. Flushing of the uterus can be done in 3 different ways; laparotomic, laparoscopic and cervical. Regardless of the method the obtaining of embryo, the corpus luteum numbers must be determined before flushing. It should be compared with the embryo numbers obtained later (Paramio 2010; Amiridis and Cseh 2012; Paramio and Izquerdo 2014).

Laparotomic Method

As animals are anesthetized in this method, feed and water should be withheld from the donors for 12 h before the operation (Lehloenya 2008). At day 6/7 following the mating, midventral laparotomy is performed to the donors under general anesthesia and a Foley catheter is placed in the uterine horns and flushed with appropriate solutions (Modified Dulbecco's Phosphate Buffered Solution with glucose and sodium pyruvate supplementation). In this method, the embryo retrieval rate is quite high, as the oviduct and uterus are visualized through the incision and flushed. This procedure can be applied 2-3 times for each individual. Since abdominal adhesions are formed following the application, it limits the possibility of repeated application (Ishwar and Memon 1996; Holtz 2005; Amiridis and Cseh 2012; Karakas Alkan et al. 2017). In order to prevent postoperative adhesions, 50-100 ml of 6% dextran solution can be left in the peritoneal cavity (Ishwar and Memon 1996).

Laparoscopic Method

Animals should be fasted for 24 hours before the surgical procedure. The animals under general anesthesia are laid upside down at an angle of 45 degrees and a pneumoperitoneum is created by injecting approximately 4-5 liters of CO_2 gas into the peritoneum. One of the uterine horns is grasped through a small incision and removed with the help of a forceps. Flushing is performed by placing a Foley catheter at the apex of the uterine horn close to the utero-tubal junction. While embryo collection with the laparotomic method can be applied 2-3 times in an animal, the embryo collection process with the laparoscopic method can be repeated approximately 7 times (Ishwar and Memon 1996; Karakas Alkan et al. 2017). Although the laparoscopic embryo collection method is less invasive, this method also requires the animal to be placed under general anesthesia and special equipment and expert technicians are required for the surgical procedure (Holtz 2005). In addition, the rate of obtaining embryos with this method is lower than the laparotomy method (Gibbons and Cueto 2011).

Cervical Method

Adhesions may sometimes occur in genital organs and tissues following laparotomic and laparoscopic methods. The repeated use of donor animals in both methods is limited due to abdominal adhesions. Cervix is relaxed with PGE_2, estradiol and oxytocin in the cervical method and embryos can be collected through the cervix (Ishwar and Memon 1996; Fonseca et al. 2013). The procedure can be performed under general or epidural anesthesia and the cervix is carefully pulled caudally by placing the vaginal speculum first. Then, the cervix is widened with a dilator and the catheter is directed into the uterus and the uterus is flushed several times with 20 ml of washing medium. In this method, embryo recovery rates were determined as 60-80%. In addition, it has been reported that estradiol and PGE_2 do not have a negative effect on the viability of embryos. However, it is less preferred as use of estradiol is prohibited in many countries (Ishwar and Memon 1996; Suyadi and Holtz 2000; Lima-Verde et al. 2003; Karakas Alkan 2021).

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CHAPTER 23

OVUM PICK UP (OPU) BY TRANSVAGINAL ULTRASONOGRAPHY IN CATTLE

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INTRODUCTION

In recent years, ovarian follicular aspiration has been used to obtain oocytes from cattle for in vitro embryo production (IVP). In order to increase the success of IVP and embryo transfer in cattle, it is necessary to obtain good quality oocytes. The oocyte retrieval method significantly affects in vitro developmental capacity and morphology of cumulus-oocyte complex (COC) (Konishi et al. 1996; Hasler 1998; Merton et al. 2003). Bovine oocytes can be collected from the ovaries of living animals or slaughterhouse. Follicle aspiration with transvaginal ultrasound is a non-invasive technique developed for the collection of oocytes from antral follicles of living animals (Dellenbach et al. 1984). Bovine oocytes are obtained from antral follicles by OPU technology from living animals with or without hormonal stimulation. By these method, immature oocytes are collected (Wrenzycki 2018). In vitro fertilization of oocytes obtained with OPU is recognized as a reproducible and the most flexible technique used to generate embryos from living donors. Since OPU does not affect the normal estrous cycle of the animals, it can be applied to many suitable donor animals from 6 months old calves to 3 months pregnant cows (Hasler et al. 1995). That's why, it becomes a good alternative to multiple ovulation embryo transfer (MOET) and its use is increasing day by day (Bousquet et al. 1999; Faber et al. 2003; Pontes et al. 2010). Repeated oocyte retrieval in cattle was first performed through endoscopy from the right paralumbar fossa by Canadian researchers (Lambert et al 1983). It was first reported by a Danish team that follicular oocytes in cattle can be aspirated with the aid of ultrasound (Callesen et al. 1987). However, follicle aspiration from bovine ovaries by transvaginal ultrasound was first performed by a Dutch team (Pieterse et al. 1988). Aspiration of follicles and collection of oocyte from the ovaries of living donors brought a new perspective to assisted reproductive programs and enabled the use of the donor animal with superior genetic potential for IVP for many years (Bols and Stout 2018).

This section will provide information about transvaginal ultrasound-guided follicle aspiration in cattle, also known as OPU. The definition of OPU technique, necessary equipment and application procedure will be discussed. Afterwards, biological and technical factors affecting the success of OPU will be evaluated.

OPU Procedure and Equipment

Equipment required for OPU comprises three main components. These are ultrasonography device with a suitable probe (linear, convex, micro convex), a needle guide system connected to the oocyte collection tube and an aspiration pump. In addition, a probe holder is needed to manipulate the probe and needle accurately. The puncture needle is mounted next to the probe by means of the probe holder. Thus, it is displayed on the ultrasound monitor as the puncture needle advances into the sonographic field. The needle is usually connected to the aspiration vacuum pump with a teflon or silicone tube. Thus, follicular contents are aspirated as pressure is applied to the vacuum pump. Oocytes and follicular fluid are aspirated into a collection tube (Bols and Stout 2018).

Epidural anesthesia is performed using a local anesthetic to prevent excessive stretching during transrectal manipulation. Although not mandatory, before OPU, cows may be sedated with some sedatives such as detomidine hyoscine-Nbutylbromide and hydrochloride to relax the intestines. After anesthesia, the rectum is emptied, the perineum and vulva are disinfected, and tail is fixed on the right side. The probe holder is inserted through the vulva and placed into the vagina. The operator inserts his hand through the rectum and brings the ovary to the tip of the probe. Thus, the ovaries and follicles are displayed on the ultrasound screen. Next, the operator slowly pushes the needle until the vaginal wall is pierced and the needle can be viewed on the ultrasound screen. With rectal palpation, following the position of the needle, the needle is directed into the follicle. Vacuum pump is activated by the foot pedal as the needle enters the follicle. In this way, COC can be collected into tubes containing oocyte collection medium (Bols and Stout 2018).

Factors Affecting OPU Results

Technical Factors Affecting OPU

OPU success rate is evaluated by factors such as the oocyte recovery rate, aspiration vacuum pressure, operator experience, and needle diameter. According to many operators, the oocyte recovery rate varies between 7% to 70%. Different aspiration vacuum pressures and needle diameters have been used in commercial and experimental cattle OPU applications over the years. Therefore, it is very difficult to directly compare the oocyte recovery rate (Bols et al. 1997).

Ultrasound Probe

During the follicle aspiration process from the ovary, the follicles must be adequately visualized with ultrasound.

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Fig. 1: OPU Equipment.

Fig. 2: OPU Application.

Accurate visualization of the follicles on the ovary ensures that the cumulus cells are aspirated without damage and less trauma to the donor. Some technical factors for example ultrasound probe, needle diameter, and aspiration vacuum pressure can affect the number and quality of COC. Although sector probes are mostly preferred by operators in OPU systems, follicle aspiration can be performed using a linear probe. However, it is reported that oocyte number and quality not statistically different between the sector and linear probe (Bols et al. 1996; 1997; 2004).

Needle Type and Diameter

The advancement in ultrasound technology has eliminated the differences between the image resolutions of ultrasound devices. Therefore, the most important technical factor for successful follicle aspiration is the needles used (Scott et al. 1994; Seneda et al. 2001). Traditionally, in the beginning, most operators used needles approximately 60 cm long, 1.5 mm in diameter, which is easy to use and make (Looney et al. 1994). However, these needles are not disposable, they become dull very quickly and cannot regain their former sharpness even if they are regularly maintained. For this reason, alternative OPU systems using disposable 18 gauge (1.02mm) epidural and hypodermic injection needles have been developed. These needles have some advantages such as being sterile, easy to manipulate and change, and having different diameters and lengths (Rath 1993; Bols et al. 1995). The amount of aspirated follicular fluid increases as the diameter of needles increases. In addition, the use of large diameter needles increases the rate of oocytes with compact cumulus cells and the number of collected oocytes (Bols et al. 1995). On the other hand, the bevel of the needle tip affects the rate of oocyte with compact cumulus cells. Two different types of needle tips, short and long bevel, are available in market. Some researchers revealed that the use of long bevel needles increases oocyte yield (Bols et al. 1997).

Aspiration Vacuum Pressure

In OPU systems, the aspiration vacuum pressure at the needle tip varies according to the aspiration device, the length of the tube used and the needle diameter. The fluid aspiration rate can be increased up to three times by changing the needle diameter even if the pressure applied through the vacuum device is the same. Therefore, the needle diameter should not be ruled out while evaluating the aspiration vacuum pressure. The increase in aspiration vacuum pressure increases the number of collected oocytes. However, it has been reported that high aspiration pressure (70-130 mm/Hg) decreases the rate of compact cumulus cell oocyte. Accordingly, it is stated

that low aspiration vacuum pressure increases the rate of reaching the blastocyst by increasing the rate of compact cumulus oocyte (Bols et al. 1996).

Biological Factors Affecting OPU Results

OPU Application Frequency

The quality and number of oocytes collected through OPU technique has a great importance on increasing the number of blastocyst produced in vitro. The highest rate of reaching blastocyst are gained with good quality COCs (Bols et al. 2018). Repeated application of the OPU technique to the same donor animal allows obtaining more oocytes from donor animals with superior genetic characteristics (Pieterse et al. 1991). Furthermore, the frequency of OPU application to the same donor animal affects the quality and count of oocytes collected (Merton et al. 2003). Although it has been reported by some researchers that OPU frequency doesn't affect the count of COCs collected or the number of follicles aspirated per application, the majority of researchers reported that twice-weekly OPU application enhanced follicle count and blastocyst rates (Bols et al. 1997; Garcia and Salaheddine 1998).

Nutritional Status of the Donor Animal

Nutritional status is one of the important factors affecting fertility in cattle (Robinson 1990; 1999). Malnutrition causes problems such as abnormal estrous cycles, low pregnancy rates, delayed puberty, and low birth weight. Malnutrition had specific effects on follicular growth. Negative energy balance (NEB) in cattle negatively affects pre-ovulation follicle diameters and follicular growth (Armstrong et al. 2001). Nutritional status affects follicular development and ovulation with its role in hypothalamus, hypophysis and ovarian activity (Armstrong et al. 2003). Furthermore, malnutrition has negative effects on the body condition score (BCS), in vitro developmental adequacy of the oocytes collected from the donor animal, and blastocyst rate (Dominguez 1995; Ruiz et al. 1996).

Breed of the Donor Animal

In the OPU technique, oocyte yield depends on the antral follicle pool available for aspiration (Bols et al. 1998). In cattle ovaries, the follicles develop in a wave-like pattern and the antral follicle population in the ovary varies according to the periods of the estrous cycle (Adams et al. 1992). Follicle growth rate, follicular wave number and maximum size of the dominant follicle differ significantly between Bos indicus and Bos taurus cattle breeds (Figueiredo et al. 1997; Bó et al. 2003). Therefore, in repeated OPU applications in cattle, the number and development of antral follicles in the ovary vary according to the frequency of aspiration and animal breed (Goodhand et al. 1999; Viana et al. 2010). Follicular wave numbers and antral follicles per wave is higher in Bos indicus cattle breeds than in Bos taurus breeds. This situation causes collection of a higher amount of oocytes from Bos indicus cattle breeds (Segerson et al. 1984; Viana et al. 2000; Seneda et al. 2001).

Age of the Donor Animal

Oocytes can be obtained for IVP from calves as early as 2 months of age (Lohuis 1995). Like other mammalian species, antral follicles are observed in cattle during fetal development and folliculogenesis happens in the fetal ovary in the last trimester of pregnancy (Erickson 1966). However, laparotomy under general anesthesia is required to obtain oocytes from young calves. With laparotomy, the ovaries are exposed and the follicles are aspirated. Collecting oocytes from prepubertal calves is important for cattle breeding, as it allows shortening of the intergenerational time and acceleration in genetic progress (Lohuis 1995; Yang et al. 1998). Also, it has been reported by Duby et al. (1996) that the development adequacy of oocytes collected from calves less than 6 months old is weaker. In calves, the use of the OPU technique depends on the pelvis size for insertion of vaginal probe. In Holstein and Friesian heifers, oocyte can be collected with OPU from 6 to 9 months of age, based on the size of the vaginal probe used (Rick et al. 1996; Bols 1999; Bols and Stout 2018). The OPU technique can be applied to many suitable donor animals, ranging from 6-month-old calves to 3-month-old pregnant cows (Hasler et al. 1995). However, rate of reaching blastocyst of oocytes collected from donors aged 1 to 3 years is higher than older donors (Moreno et al. 1992; Ali et al. 2021).

Heat Stress and Seasons

Heat stress significantly affects fertility in cattle and nearly 60% of the cattle population in the world is exposed to heat stress. In tropical, subtropical and temperate regions, a significant decrease in fertility is observed in summer due to heat stress. It is stated that pregnancy rate, which is 40-60% in the winter months, decrease to 10-20% in the summer months (Gwazdauskas et al. 1975; Badinga et al. 1985; Cavestany et al. 1985). High temperature affects follicular dynamics and cellular functions in various female reproductive system tissues (Badinga et al. 1993). Effect of heat stress on the vitality and quality of oocytes in the ovaries is negative. (Sartori et al. 2004). Embryo development is adversely affected, the quality of the bovine oocyte decreases, and the lipid composition of oocyte membranes changes, in summer season. In addition, studies in Holstein cows are reported that after in vitro fertilization, the ability of collected oocytes to reach the blastocyst is lower in summer than in winter (Al-Katanani et al. 2002).

Synchronization of Follicular Waves

Many studies have been conducted to monitor different sized follicle populations or individually identified follicles using ultrasonographic imaging. In the bovine ovary, it is stated that follicular growth occurs in a wave-like manner and an estrous cycle usually comprises of two or three follicular waves. The formation of the follicular wave in cattle is started by the growth of approximately 8-41 small follicles with a diameter of 3-4 mm (Adams and Pierson 1995; Adams 1999). The dominant follicle of one wave causes regression of other follicles and suppresses the emergence of the next follicular wave. In most OPU/IVEP programs, follicular aspiration is performed on random days of the estrus cycle and follicles of at least 2 mm in diameter are aspirated (Pontes et al. 2011; Dos Santos et al. 2016). The period of the estrous cycle during follicle aspiration affects oocyte quality, oocyte recovery rate and IVP (Hendriksen et al. 2004). Approximately 85% of follicles aspirated on a random day of the estrous cycle have varying degrees of atresia based upon the apoptosis process

(Hendriksen et al. 2000). Atresia in these follicles is related to the presence of a dominant follicle during aspiration. The presence of the dominant follicle negatively affects the development of other follicles by secreting inhibin and estradiol (Wolfsdorf et al. 1997). It is stated that higher oocyte recovery rates are achieved and better-quality oocytes are collected due to the absence of atresia events in aspirations performed in the follicular growth phase where there is no dominant follicle (Bacelar et al. 2010; Gimenes et al. 2015). Also, it has been reported in some studies that oocytes collected at this stage have a higher rate of reaching blastocyst (Machatkova et al. 2004). Follicular wave synchronization in donor animals makes the follicles more homogeneous according to their size and developmental stages, and ensures the collection of more quality and competent oocytes. Bovine estrous cycle and physiology of follicular wave allow pre-OPU follicular waves to be synchronized. Hence, before OPU, synchronization of follicular wave can be used to improve IVEP results (Seneda et al. 2020).

Ovarian Superstimulation

Follicular dynamics can be manipulated by exogenous gonadotropin administrations during the estrous cycle in cattle (Aerts and Bols 2010). The count of oocytes collected in OPU depends on the number of antral follicles larger than 3 mm which are suitable for puncture. The purpose of pre-OPU ovarian superstimulation is to increase the count of oocytes available for puncture per session. With exogenous gonadotropin application in cattle, the count of antral follicles per session is increased. Thus, the number of oocytes collected from animals with superior genetic characteristics increases (Chaubal et al. 2006). The proportion of large healthy follicles can be increased with the gonadotropin treatments before OPU (Rouillier et al. 1996). The diameter of the follicles in the ovary during OPU is one of important criteria to improve the effectiveness of IVEP systems (Vassena et al. 2003). There is a heterogeneous follicle population of different diameters in the ovary on any day of the estrous cycle (Carolan et al. 1996). In OPU/IVEP systems, most of oocytes are harvested from follicles of 3 to 8 mm in diameter before the preovulatory LH surge (Dieleman et al. 2002). In order to increase the developmental competence of the oocyte in these follicles, many superstimulation protocols including different gonadotropin hormones and doses are being practiced (Blondin et al. 1997). Use of gonadotropin hormones before OPU, can increase the ratio of follicles with intermediate diameters (>6 and <10 mm) in the ovary (Goodhand et al. 1999). Therefore, gonadotropin applications during follicular development can be used to achieve ideal follicles for IVEP (Oliveira et al. 2016).

Three different gonadotropins are used for ovarian superstimulation in cattle. These are equine chorionic gonadotropin (eCG), hypophysis extracts of animals such as pigs and sheep and human menopausal gonadotropin (hMG) (Mapletoft et al. 2002; Aerts and Bols 2010). Due to the differences in properties such as half-life and FSH / LH release of gonadotropins, many different protocols have been developed to superovulation or superstimulation with these hormones. Since the ultimate goal of pre-OPU superstimulation is to generate additional follicles rather than inducing multiple ovulations, changes in the dose and timing of gonadotropins used are required (Bols and Stout 2018). For this purpose, many methods have been tried in pre-OPU superstimulation protocols, such as using a single dose of FSH, different administration routes, reducing the amount of FSH and dissolving it in different polymers (Bó et al. 1994; Chaubal et al. 2007; Ongaratto et al. 2010; Vieira et al. 2016).

Dominant Follicle Ablation Before Superstimulation

The ovarian response after superstimulation and superovulation varies considerably between animals (Looney 1986). Ablation of the dominant follicle (DF) or hormonal applications can be performed to maximize the superstimulation response and increase the count of antral follicles. As the dominant follicle in the ovary triggers the secretion of inhibin and estradiol, resulting in suppression of follicular growth, it adversely affects the superstimulation response (Aerts and Bols 2010). When the large follicle developing in the ovary reaches an average diameter of 8-9 mm, a difference occurs between other follicles and future DF follicles. This situation is termed deviation (Ginther et al. 1997). All follicles that develop before deviation have the capacity to become DF. Following the deviation, the largest follicle becomes DF, while the others follicles regress. In a study conducted in heifers, it was determined that any follicle with 5 mm diameter reaches DF when all other follicles are removed. Thus, it has been understood that any antral follicle has the capacity to become DF (Gibbons et al. 1997). For this reason, ablation of DF involves aspiration of all follicles greater than 5 mm. In superstimulation applications, ultrasound-guided follicle ablation is to eliminate the suppressive effect of DF (Berfelt et al. 1994; Baracaldo et al. 2000). Ablation of DF caused by the emergence of gonadotropin-sensitive a new antral follicles wave in an average of 1-2 days. Follicle ablation can be used in embryo production centers with the necessary equipment and trained personnel. In addition, it is stated that dominant follicle ablation is as effective as protocols involving the combined use of progesterone and estradiol in follicular wave synchronization for superstimulation in cattle (Bungartz and Niemann 1994; Bó and Mapletoft 2014).

Health Status of Donor Animals During and Following the Opu Process

The OPU is a technique that can be applied at different frequencies in animals with or without gonadotropin administration. It allows multiple pick-up oocytes from donor animals. The multiple use of donor animals is important in terms of the number of oocytes to be obtained in subsequent applications and the changes that repetitive applications will create in animals (McEvoy et al. 2006). OPU causes short and long-term changes in ovaries of donors. These changes are related to the function and the shape of ovaries, and differ in their severity and nature. The most natural change is related to the follicular dynamics in the ovary and the endocrine status of the donor animals (Petyim et al. 2000; 2001; 2003). General differences in follicular dynamics are not a problem for OPU unless they persist over a long period of time (Carlin et al. 1999; Galli et al. 2001). Nevertheless, it is stated that luteal tissue or CL-like structures may form after follicular aspiration and this may affect the quality of the collected oocytes (Stubbings and Walton 1995; Petyim et al. 2003). Apart from this, another factor that negatively affects the follicular and luteal activity in the ovary following the follicular aspiration

procedure is the scarring of the ovarian tissues with recurrent follicle puncture. Repeated OPU applications, results in development of an excessive amount of connective tissue in tunica albuginea and in the ovarian stroma (Kruip et al. 1994; Alberio et al. 2002; Chastant-Maillard et al. 2003). The flexibility of ovarian tissue against sclerosis has been reported by many researchers. It is stated that cattle can tolerate the follicular puncture process, which breaks down the ovarian surfaces and significantly change the tissue dynamics (McEvoy et al. 2002; Bogh et al. 2003). Besides, needle penetration applies to the vaginal and the risk of rupture or pathogen contamination during the procedure should not be forgotten (Younis et al. 1997; Cho et al. 2004).

In OPU procedures, one of the issues that require utmost care in order to perform the follicular aspiration process in a healthy way and to prevent the entry of pathogens is the application of epidural anesthesia to the donor animals. Epidural anesthesia is a very important part of the transvaginal follicle aspiration technique. Epidural anesthesia minimizes uncomfortable movement and abdominal strain of the donor animal (McEvoy et al. 2006). Sterile needle should be inserted into the epidural space between the coccygeal vertebrae to administer epidural anaesthesia (McEvoy et al. 2002). With the sudden movements of the donor during anaesthesia administration, the needle tip may pass through the epidural space, contact the underlying intercoccygeal disc. These accidents can cause long-term or permanent problems for the donor. Nonetheless, if an infection occurs at the epidural injection site, it can cause serious complications and suffering (Hall and Clark 1991).

Conclusion

The OPU technique has been accepted as a suitable method for collecting oocyte, in consequence of intensive studies to collect oocytes from cattle. It is widely used in commercial applications and research on bovine oocyte. OPU has become an alternative to MOET as it allows the production of more embryos in a short time. Producing more embryos in a short time ensures that the generation gap is reduced and genetic progress is accelerated. In addition, it forms the basis for the use of advanced technologies such as transgenics and cloning.

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CHAPTER 24

BOVINE IN VITRO EMBRYO PRODUCTION (IVEP)

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INTRODUCTION

Efforts to increase animal based foods have been going on for centuries. *In vitro* fertilization (IVF) is known as the general definition of obtaining an embryo *in vitro* condition. For this purpose, breeding studies are carried out and biotechnological techniques are frequently used in these studies. Embryo production with *in vitro* fertilization in bovine is one of the biotechnological methods (Alkan 2021; Satılmıs and Guler 2021). The first live offspring from embryo transfer was obtained from an Angora rabbit by Walter Heape in 1890 (Gordon 2004). However, success with the IVF technique in bovine and other mammal animals could not be accomplished for a long time. The first successful application of IVF for cattle was reported in 1977. Iritani and Niwa (1977) proved that calves can be produced with the IVF technique as a result of their long term studies. The first live calf was obtained by the IVF method by Brackett et al. (1982). Then, the first twin calf was obtained by culturing zygote in the mouse oviduct by Hanada et al. (1986). In Ireland, the first calf was obtained by performing all of the steps in the laboratory (*in vitro* maturation, *in vitro* fertilization, and *in vitro* culture) in 1987. In 1980s, IVF took place only as a research technology among embryo transfer technologies. *In vitro* fertilization studies in bovine have gradually increased. *In vitro* fertilization studies were supported by OPU in the 2000s, and recently *in vitro* fertilization studies are being used for genetic selection (Hasler et al. 2017).

Embryo transfer studies at the cellular and molecular level in bovine are not common. Because the applicability of embryo transfer studies is limited and difficult. However, it is known that the required experimental material can be obtained at a low cost by *in vitro* fertilization method. Superovulation in embryo transfer studies is very expensive and causes excessive manipulation on animals. (Erdem et al. 2020; Alkan 2021). This situation provides an occasion for *in vitro* fertilization studies both financially and ethically. The demand to maintain the genetic existence of high-yielding animals that have been slaughtered in the last 10-15 years has contributed to the increase in IVF studies (Galli et al. 2003; Gordon 2004).

The State of Bovine *In Vitro* Fertilization Today

According to the data collected from International Embryo Technology Society (IETS), it has been reported that *in vitro* fertilization studies have increased gradually in the last 15 years, especially in the last 4 years. According to IETS 2019, data of *in vitro* production of embryos from abattoir-derived oocytes by region are given in Table 1 and transfer of *in vitro* produced bovine embryos by region are given in Table 2 (Viana 2020).

In Vitro Embryo Production in Bovines

Embryo production by *in vitro* fertilization is an assisted reproductive technique, all stages of which are performed in the laboratory (Satılmıs 2019). Stages of embryo production by *in vitro* fertilization are listed below.

- Obtaining oocytes from the ovary;
  - From abattoir-derived ovary: Obtaining oocytes from abattoir-derived ovaries with Aspiration, slicing, and dissection technique.
  - By ovum pick up (OPU) technique: Obtaining oocytes from OPU from live animals.
- Maturation of oocytes,
- Fertilization,
- Embryo culture (Gordon 2003; Saleh 2017; Ferré et al. 2020).

Obtaining Oocytes from Ovary

Immature oocytes from ovaries is collected from abattoir derived ovary or from live animals by the OPU technique (Karaşahin 2021). To collect oocyte from abattoir derived ovaries; aspiration technique, slicing technique and dissection technique are used. However, aspiration technique is the the most commonly used method for this purpose (Gordon 2003).

Aspiration Technique

One of the most commonly used techniques in bovine is follicle aspiration. Antral follicles on the ovary can be aspirated with the help of pipette, vacuum aspiration needle or injector. The aspiration technique is advantageous as it is faster than other methods. Fast oocyte collection is a very important criterion for embryo production units. With this method, antral follicles of 2-8 mm in size can be aspirated and 10-20 mL injectors with 18–22-gauge needle without gasket are recommended for aspiration (Gordon 2003).

Slicing Technique

Slicing is another method of obtaining immature oocytes from abattoir derived ovaries. During slicing, the ovary is held with the help of forceps and the follicles are cut with the help of a scalpel. This process is carried out in an already prepared 90 mm petri dish containing the washing solution. The oocytes collected from the follicles are poured into the washing

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Table 1: *In vitro* production of embryos with abattoir-derived oocytes by region (Vianna 2020).

Region/	Donors			Oocytes			Transferrable embryos		
Country	Dairy	Beef	Total	Dairy	Beef	Total	Dairy	Beef	Total
Africa	0	143	143	0	2,343	2,343	0	373	373
Asia	0	0	0	0	0	0	0	0	0
Europe	662	204	866	7,499	2,017	9,516	1,449	649	2,098
N America	0	60	60	14,316	5,650	19,966	3,013	1,085	4,098
Oceania	0	0	0	0	0	0	0	0	0
S America	0	2,860	2,860	0	71,590	71,590	0	14,318	14,318
Total	662	3,267	3,929	21,815	81,600	103,415	4,462	16,425	20,887

Table 2: Transfer of *in vitro* produced (IVP) bovine embryos by region (Vianna 2020).

Region/	Embryos transferred							
Country	OPU				Abattoir			
	Fresh	Frozen		Total	Fresh	Frozen		Total
		Domestic	Foreign			Domestic	Foreign	
Africa	1,257	1,976	0	3,233	0	0	0	0
Asia	0	0	0	0	0	0	0	0
Europe	24,554	22,103	322	46,979	2	29	0	31
N America	183,229	150,412	53	333,694	84	1,980	0	2,064
Oceania	7,134	2,897	0	10,031	0	0	0	0
S America	222,302	169,900	0	392,202	8,751	205	0	8,956
Total	438,476	347,288	375	786,139	8,837	2,214	0	11,051

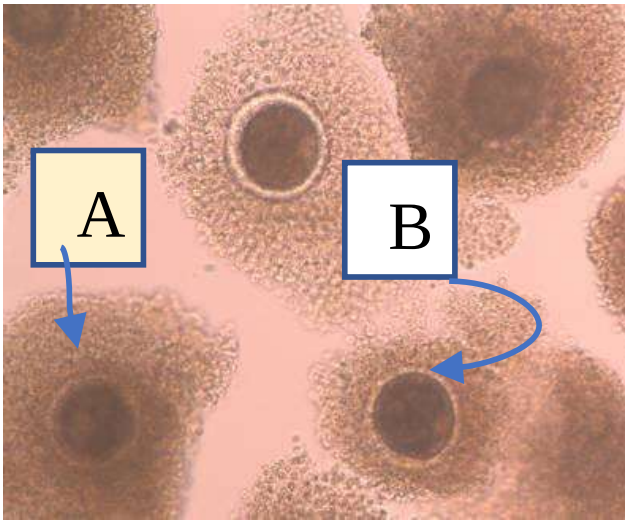

Figure 1. A and B quality oocytes (Satılmıs 2019)

solution and examined under a microscope (Saleh 2017). The slicing technique is faster than aspiration and more oocytes are collected, but the collected oocytes are of poorer quality (Wang et al. 2007). Sometimes, slicing method is used in combination with the aspiration technique. In this method the follicles on the ovary are aspirated first and then sliced. It is reported that the number of collected oocytes by this method increases significantly (Hoque et al. 2011).

Dissection Technique

In the dissection method, the follicles on the ovary are dissected and separated. The dissected follicles are poured into previously prepared petri dishes that contain collection solutions. In this way, the cumulus cells of the collected oocytes are minimally damaged. It is reported that the number and quality of collected oocytes by the dissection method is quite high. With the dissection technique, an average of 32-44 oocytes per ovary can be collected. The most important advantage of this technique is that atretic and non-atretic follicles can be more easily identified. However, this method requires a lot of experience (Carolan et al. 1994; Gordon 2004).

Ovum Pick Up (OPU) Technique

In the ovum pick-up method, oocyte is collected by aspirating the follicles on the ovaries of live animals. OPU is a systematic non-invasive technique that can be performed with help of an ultrasound probe, aspiration pump, and aspiration needle. The use of OPU in humans has also predicted its usability in bovine. It has been determined that OPU is a flexible and repeatable method after bovine applications (Pieterse et al. 1991). Unlike OPU technique is advantageous over Multiple Ovulation Embryo Transfer (MOET) because donors can be used more frequently (Faber et al. 2003; Pontes et al. 2010). OPU is a conventional and practical technique because it can be used in the postpartum $2^{nd}/3^{rd}$ week, pregnancy, and calves from 6 months of age (Galli et al. 2001; Besenfelder et al. 2012; Qi et al. 2013). In addition, the OPU technique has proven that the oocyte is not only collected from abattoir derived ovary. The OPU technique has also increased the success of *in vitro* fertilization studies and the number of collected embryos (Galli et al. 2004).

Quality Evaluation of Oocytes

The selection of bovine oocytes for the *in vitro* maturation process is based on some basic morphological evaluations (Satılmıs and Guler 2021). Classification schemes of oocytes were made with the help of a microscope. Cytoplasm density of oocytes, the density of cumulus cells and some visible morphological features are included in this scheme. Bovine oocytes are classified according to some morphological features from 1 to 4. Grade 1 oocytes have compact and multi layered cumulus, homogeneous ooplasm, cumulus-oocyte complex (COC) clear and transparent. In Grade 2 oocytes, the cumulus structure is multi layered, the ooplasm is homogeneous, there is darkening of the zona, the POPs are darker and less transparent. Grade 3 oocytes are those in which the cumulus structure is less layered, the ooplasm is irregular and has dark clusters, rather dark compared to COC

grades 1 and 2. In Grade 4 oocytes, the cumulus cells are enlarged and scattered, ooplasm is irregular, COC is dark and irregular (Gordon 2004). In addition, immature oocytes can be classified as A, B, C, and D according to the distribution of cumulus cells (Cetica et al. 1999) where cumulus cells appear as 5 or more layers around the zona are categorised as A Quality, cumulus cells cover more than 1/3 of the circumference of the zona in 2 or fewer layers or a small part of them may not be cumulus are categorised as B Quality, oocytes are usually completely bare or have much fewer cumulus cells than B grade oocytes are categorised as C Quality and the cumulus cells look like a spider or swollen are categorised as D Quality.

Some researchers reported that most of the grade A oocytes (87.7%) are in the germinal vesicle (GV) stage. In addition, it was determined that the meiotic maturation of grade A oocyte was higher (76.5%) compared to other grades (Cetica et al. 1999). For this reason, A and B quality oocytes are matured in IVF studies. It is known that C and D quality oocytes are not used for maturation. The maturation capability of oocytes is affected by factors such as oocyte size, presence of cumulus cells and characteristics of the animal. Another factor affecting the maturation quality of oocytes is the size of the aspirated follicle. Therefore, aspiration of follicles with a size of 2-8 mm on the ovary is recommended (Gordon 2003). It has been reported that low-quality embryos are collected from cultured oocytes as a result of aspiration of follicles smaller than 2 mm or larger than 8 mm (Katska and Simorak 1984; Carolan et al. 1994).

In Vitro Maturation (IVM)

The oocyte is a special cell of the reproductive system as it is a pool where the genetic information of two organisms is combined. Oocytes undergo a series of molecular and conformational changes for development. Oocytes are formed as a result of mitosis of embryonic germ cells in the ovarian cortex during the embryonic process. Mitotic proliferation is completed by 7.5 months of fetal life. This event indicates that germ cell mitosis is completed before birth. Afterward, mitotic activity ceases. After this stage, germ cells enter the prophase of meiosis and transform into oogonia. During subsequent growth, oogonia develop into mature oocytes (Alexander 2012).

Oocytes mature both cytoplasmically and cellularly. For germ cell formation, the diploid somatic cell transforms into a haploid cell. During fertilization, as a result of the fusion of the sperm to the oocyte, two haploid cells combine to form a mixed-genome diploid cell (zygote) obtained from both parent cells. During the meiotic split in the transformation of somatic cells into germ cells, a decrease in the number of chromosomes is observed. In this process, changes occur in the cytoplasm depending on the accumulation of substances. Due to the unequal divisions seen in females, they have more cytoplasm than males (Alexander 2012).

It has been reported that the rate of oocyte maturation *in vitro* is lower than *in vivo*. The reason for the slow maturation of the oocyte in *in vitro* conditions is that the follicular fluid content is not known exactly. In addition, maturation success is greatly affected by the quality of the used oocyte. For this purpose, it has been reported that metabolic analysis of follicular fluid and characterization of oocyte quality may be beneficial to better perform oocyte maturation *in vitro* (Wrenzycki 2018).

Nuclear Maturation of the Oocyte

Nuclear maturation takes place during meiosis of the oocyte and includes the process from the germinal vesicle stage to metaphase-II. This phase takes place similarly to the mitotic division of somatic cells and consists of some phases. These include synthesis phase (S), Mitotic or meiotic phase and Developmental or GAP phase (G). DNA replication takes place in synthesis phase (S). The maternal cells are required for the synthesis of molecules, and genetic distribution takes place between the two female cells. In mitotic or meiotic phase (M) chromosomes and cytoplasm divides into two and disperse into female cells. Developmental or GAP phase (G) is formed between M and S phases. The difference of this process from mitosis is that it involves two successive S phases and as a result, diploid cells are formed. As a result of the miotic cell cycle, cells become diploid and then meiosis takes place. Meiosis consists of two parts, meiosis-I and meiosis-II, and these parts are separated from each other by a gap called interkinesis. The stages of meiosis are named as those of mitosis. Names of stages are prophase, metaphase, anaphase, and telophase. Diplotene is the first division phase of meiosis, the chromatins are scattered and despiralized. Germinal vesicle is available. Diakinesis is the end of the prophase of meiosis-I. Chromosomes were divided into four uniform copies and fragmentation of the germinal vesicle (GVBD) occurs. The GVBD event occurs as the nuclear membrane begins to fold during diakinesis, the nuclear pores disappear, and then the nuclear membrane fragments are separated from the small double-walled sacs and disappear rapidly. In bovine, GVBD occurs after follicular development or ovulatory LH release. At the metaphase-I stage, chromosomes are localized with maximum density at the equator. As a result of GVBD, the cumulus cells and their connections around the oocyte are destroyed. In anaphase-I, condensed chromosomes are pulled to the poles. In the telophase-I stage, a group of chromosomes degenerates to form polar body-I between the oocyte and the perivitelline space. In metaphase-II, the second meiosis begins and the chromosomes travel and reach at the equator of the oocyte. And finally in anaphase-II/telophase-II, spermatozoa enter the oocyte cytoplasm (Monniaux et al. 2009). In the first meiosis, the chromosomes match and exchange by recombination. The number of cells is doubled, but the number of chromosomes does not decrease to half of the number of chromosomes per cell. In meiosis-II, mitosis-like division takes place to reduce the number of cells (Monniaux et al. 2009; Tripathi et al. 2010; Brunet and Verlhac 2011; Alexander 2012). As a result of the meiosis of the spermatozoon, four haploid cells are produced from the diploid cell at the beginning, while only one oocyte is obtained from a diploid cell in the meiosis of the oocyte. It is reported that in each meiosis, one of the cell matches completes its development in the body of the other cell in the form of a primary polar body. The meiotic distribution of cytoplasm among female germ cells is not equal. After completing its maturation, the cell receives most of the cytoplasm to form the polar body. Degenerated chromosomes of the primary polar body are dysfunctional and secretly expelled from the surface. It is known that during and after all these events, the chromosomes are despiralized in the pronucleus. The haploid female germ cell formed during fertilization infuses with the male haploid germ cell to form a diploid cell and forms the zygote, which is a diploid organism (van den Hurk and Zhao 2005; Kimura et al. 2007).

Cytoplasmic Maturation of the Oocyte

Cytoplasmic maturation refers to oocyte growth, changes in the structure and distribution of oocyte organelles and the accumulation of biologically active substances necessary for further development of the oocyte. Complex changes occur in the position and structure of all organelles, especially mitochondria, until metaphase-II. In the cytoskeletal stage, the cytoplasm containing microfilaments and microtubules greatly assists nuclear maturation during the separation of chromosomes. Ribosomes in the cytoplasm produce peptides necessary for oocyte maturation and subsequent steps. Thus, cytoplasmic maturation involves the storage of ATP, mRNAs, proteins, and transcription factors (Brevini et al. 2007; Miyano and Manabe 2007; Ferreira et al. 2009). After the resumption of nuclear maturation, protein synthesis is rapidly activated and a significant part of the mRNA is degraded and dies (Aerts and Bols 2008). Storage of metabolic substances in the cytoplasm induces oocyte growth. Mammalian oocytes grow in two different characteristic structures. In the first phase, growth is temporarily associated with the follicle from which it develops while in the second phase, the oocyte maintains its current size, although the follicle continues to grow until ovulation. Organelles stored in oocytes during the first growth phase are responsible for the progression of meiosis. In addition, these macromolecules are responsible for condensation of penetrating spermatozoa, formation of a male pronucleus, shaping of fertilization, formation of zygote, and reaching the 6-8 cell stage (van den Hurk and Zhao 2005). The growth and development of the oocyte in the follicle also depends on the somatic cells around the cumulus and granulosa layers. Connection is established between these cells and the oocyte by gap junctions. Energy and substrates (nucleosides, amino acids, phospholipids, and ions) required for oocyte development are transported through this relationship (Feng et al. 2007; Mermillod et al. 2008; Sirard 2016). Defects in somatic cells or junctional errors between gap junctions prevent oocytes from reaching optimal size, maturation, and fertilization (Hutt and Albertini 2007). Synchronization of cytoplasmic and nuclear maturation is very important for the development of oocytes and also in *in vitro* embryo production (Eppig et al. 2004).

In Vitro Maturation Environment and Affecting Factors

As a result of successful *in vitro* maturation, the oocyte should be able to fertilize and provide the development of the embryo. However, the maturation success of the oocyte is affected by some factors such as the diameter of the follicle and the oocyte, the estrous cycle, the medium used for the maturation of the oocyte and its content, maturation time, temperature, humidity and environmental gas components, osmotic value of maturation media, the technique of collection of oocyte and the characteristics of the animal. Bovine oocytes are generally incubated for 20-24 hours at 38.5°C in an environment containing 5% CO_2 for *in vitro* maturation. The appearance of cumulus expansion or any of the primary polar body formations after incubation is considered maturation (Alexander 2012). For *in vitro* maturation, there are many different preferred media such as Tissue Culture Medium (TCM-199), Synthetic Oviductal Fluid (SOF), North Carolina State University (NCSU-37), and Ham's F10a. Hormones, bovine serum albumin, follicular fluid, growth factors, vitamins, serums and different antioxidant substances can be added to these mediums. Supplement to the maturation medium is intended to improve the medium (Grøndahl 2008; Strejček and Petrovičová 2012).

In Vitro Fertilization (IVF)

In vitro fertilization consists of a complex mechanism involving oocyte maturation, semen separation and capacitation. IVF is the process of bringing the capacitated sperm and mature oocyte together in the laboratory and fusing the nuclei of these two cells. One of the most important steps in IVF technique is the selection of semen to be used. The selected sperm must go through a maturation process to fertilize the oocyte. Spermatozoon shows capacitation, hyperactivation and acrosome reaction during maturation. In *in vivo* conditions, hyperactivation and capacitation of spermatozoa take place in the female genital tract before encountering the oocyte. The acrosome reaction begins during the penetration of the oocyte into the cumulus cells and zona pellucida. Some biochemical changes in spermatozoa inside the female genital tract are called capacitation. In *in vitro* fertilization studies, capacitation is provided with ingredients that mimicking the female genital tract environment. Hyperactivation is a necessary step for spermatozoa to enter the zona pellucida (ZP) and cumulus cells. At this stage, Ca^{+}, ATP and cAMP are used. Acrosomal Reaction (AR) involves the distribution between the acrosome membrane and the surrounding plasma membrane so that hydrolytic acrosomal enzymes can be released (Gordon 2003). IVF is considered successful if one of the criteria listed below is identified like presence of the tail of the sperm in the ooplasm or the head of the spermatozoon in the ooplasm, the presence of male and female pronuclei in the ooplasm, the detection of first and second polar bodies, the presence of sperm in the zona pellucida or perivitelline space, and the detection of the telophase stage of the second meiosis (Donnay et al. 2002).

In Vitro Fertilization Media and Used Medium

It is reported that the optimal condition for fertilization is 38.5-39°C, environments containing 5% CO_2 and maximum humidity (Hammam et al. 2010). The most commonly used media for IVF are Tirode's Albumin Lactate Pyruvate Solution (TALP), Synthetic Oviduct Fluid (SOF), Brackett and Oliphant Medium (BO) and Potassium Simplex Optimization Medium (KSOM) (Nedambale et al. 2006). Also, SOF (synthetic oviductal fluid) is used instead of TALP medium. In addition, substances such as heparin, caffeine, epinephrine, penicillamine, taurine, hypotaurine, BSA, glycine and hyaluronic acid can be added to assist fertilization media (Gordon 2003).

Preparation of Semen for *In Vitro* Fertilization

In IVF, "Separation" is applied to the sperm to increase the fertilization ability. With this process, the ones with the highest motility are selected among the available spermatozoa (Ohnami et al. 2012; Wrenzycki 2018). Some techniques have been developed to remove substances (bacteria, enzymes, abnormal spermatozoa, chemicals used during freezing) that adversely affect IVF success in straws. These are migration-sedimentation, a density gradient centrifugation and filtration technique (Kaymaz 2012). Swim-up and percoll gradient

techniques are mostly used for the separation of sperms. Percoll gradient is among the centrifugal technique. It has been reported that the acrosome integrity of spermatozoa collected by the separation method with the percoll gradient technique is higher than the swim-up technique (Somfai et al. 2002). Important tips during the application of the separation technique should be kept in mind. It should be fast and easy, obtain a large number of motile spermatozoa, be economical, separate toxic substances, leukocytes and bacteria, allow to work in high volume semen, and most importantly, it should not cause damage to spermatozoa (Kaymaz 2012).

In Vitro Culture (IVC)

It is the stage of washing the zygote with a culture medium after *in vitro* fertilization and culturing it for 6-9 days until it completes the developmental period. It is very important for embryo development that the zygote is cleared of denatured cells, spermatozoa residues and cells that may contaminate the culture medium before being placed in the culture medium. For embryo culture, simple media were used in the first days of culture. Following considerations regarding culture media should be kept in mind to enhance success of In vitro culture: 1. Preparation of the medium considering the needs of the embryo at the developmental stage, 2. Additions to the culture medium should be made as early as possible in the early developmental period of the embryo, 3. Adding components such as growth factors to the culture to obtain a high rate of the blastocyst, 4. If BSA is to be added to the culture medium, support should be obtained from non-acidic oil preparations, 5. Examination of multi-step culture systems for embryos, 6. Frequent use of embryo culture medium to control the standard (Gordon 2003).

Embryo Culture System Medium

It is desired that the oxygen in the *in vitro* culture medium should be below 10%. The use of 5% O_2, 5% CO_2 and 90% N_2 gases is generally preferred in culture systems. CO_2 stabilization is an important factor in culture systems. It is desired that the pH of the environment should be between 7.2-7.6. It is reported that the ideal temperature in the culture environment is between 38-39 °C, and the most ideal light is filtered yellow light (Garcia et al. 2006).

At the start, Bovine Oviduct Epithelial Cells (BOEC) were used as embryo culture medium, and then mediums such as Tissue Culture Medium (TCM-199), Synthetic Oviduct Fluid (SOF), Hamster Embryo Culture Medium-6 (HECM-6), Chatot Ziomek Bavister Medium (CZB), CR1aa (Charles Rosenkrans), KSOM and Complete Defined Medium (CDM) were introduced. Complex embryo culture media such as TCM-199 are designed for *in vitro* development of somatic cells rather than early embryonic development of mammals. Bovine embryos and somatic cells develop a large number of embryotrophins in the serum, which play different roles in embryo development (Gordon 2003; Feugang et al. 2009). Today, SOFs have become the most widely used mediums as embryo culture medium. SOFs were designed as a result of biochemical analyses of oviduct fluid (Gordon 2003). In recent years, successive culture medium systems have been developed for embryo development. In this system, the embryo culture medium mimics the physiological changes of the oviduct environment *in vivo*. It is reported that the application of these culture media systems is more complicated, but the results obtained are more successful (Wrenzycki 2018). Donnay et al. (2002) reported that excess glucose can have a toxic effect in the early embryonal period, but more glucose is needed after the morula stage. This indicates that it may be advantageous to use a sequential culture medium system.

In the use of the sequential embryo culture medium system, the development of embryos is decelerated as a result of the freezing-thawing process (Garcia et al. 2006). To prepare a successful embryo culture medium, oviduct biochemistry should be analyzed and simulated well and optimum conditions should be provided for some parameters. It has been reported that the oviductal fluid has an osmolarity of approximately 245-290 mOsm / kg and a pH of 7.2-7.6. At the same time, concentrations of Na^+, $Ca^{2+,}$ Mg^{2+}, and Cl^- are low in the oviductal fluid, while potassium (K) and bicarbonate ($HCO3^-$) levels are high. In the *in vitro* environment, lactate, pyruvate and glutamine are preferred as energy sources in the early embryonal development period, while glucose is preferred after the morula stage. Amino acids and macromolecules are used as protein sources in culture systems (Gilchrist et al. 2015). To increase the success in embryo culture medium systems, somatic cells have been used. This situation forms the basis of co-culture systems. Mostly granulosa cells, oviduct epithelial cells, rat liver cells and uterine cells are used for co-culture systems. Co-culture systems have a positive effect on embryo development by eliminating certain toxic components in the environment and reducing the O_2 concentration (Lonergan et al. 2001; Lonergan and Fair 2016).

Evaluation of Embryo Quality

Interest in studies on morphology, ultrastructure, cryo resistance and gene structure of embryo have been increased recently. There are many factors that affect embryo quality such as the color of the blastocyst, its compactness, and the size of the hatched blastocyte. It is not possible to detect abnormal chromosome distribution or chromosomal disorders in bovine embryos with current morphological methods. However, genetic analysis can be performed by ultracentrifugation or multiphoton laser microscopy while in the zygote stage. Another option is the biopsy method. During the pre-implantation period, the viability of the embryo is mostly affected by a normal morphology, rapid division rate and early pregnancy factors (EPF, cytokines and luteotrophic factors) released from the embryo. These factors have a direct effect on the transport and activity of the ovum (Makarevich 2012). To evaluate the quality of embryos in bovine, examinations from different perspectives are made under the microscope with 50X or 100X magnifications. The size of the bovine embryo varies between 150 and 190 µm. Structural features of an ideal embryo are the compact and spherical zona pellucida, the distinguished perivitelline cavity, any granules and vesicles containing cytoplasm, distribution of the color, and the uniform zona pellucida (Bo and Mapletoft 2013). Following points should be considered while evaluating the morphology of embryos:

1. Presence or absence of cellular fragments is easy to assess during early embryonic development. Evaluation becomes more difficult in later stages. The cells are based on the zona pellucida and the perivitelline gap cannot be seen. For these reasons, evaluation becomes very difficult.

2. The degree of compaction; Generally, the roundness and sharpness of the outer periphery of the morula are evaluated.
3. Color and texture of the blastomere varies from light to dark. Many factors affect this change. Embryos collected from *in vitro* fertilization are darker in color than embryos collected *in vivo*.
4. Irregular division occurs due to time-synchronous divisions (Makarevich 2012).

Different developmental stages of embryos are:

1-cell: unfertilized oocyte (UFO)

2-12 cells: contains 2-12 cells

Early morula: These contain 16-32 cells, the large non-compact cluster of blastomeres,

Morula: These contain 32-64 cells, compact cluster of small blastomeres

Early blastocyst: blastoceles < 50%

Blastocyst: blastoceles > 50%, expanded blastocyst

Embryo increases in size and the zona pellucida wall becomes thinner and in result, zona pellucida rupture. The blastocyst is then hatched, the embryo is discarded and expanded hatched blastocyst is completely discarded and increase in size resulting in empty zona pellucida (Makarevich 2012).

Embryo evaluation is one of the most critical stages of the embryo transfer procedure. The quality and developmental stages of the embryos are evaluated with a standard scale determined by IETS. Embryos are categorised into 9 according to their degree of development and into 4 according to their quality (Erdem et al. 2020; Dursun and Karasahin 2021). Quality-I embryos are excellent or good consisting of more than 85% living cells. The degree of development of embryos is compatible with their development period. Cell distributions are diffuse and zona pellucida is present. Irregularities within the cell are minimal. A high rate of pregnancy is obtained from perfect-quality embryos. Embryos at this stage are suitable for freezing. Quality-II embryos are evaluated as a medium, consisting of 50% living cells. The color is slightly lighter and there is a partially irregular cell mass. They are not suitable for freezing as they lose their viability during freezing and thawing process. For this reason, 2nd quality embryos are used for fresh transfer if suitable recipients are found. Quality-III embryos are weak or of poor quality consisting of 25% living cells and have a more irregular structure. These are not suitable for freezing, and the chances of survival in fresh transfers are very low. Quality-IV embryos are known as degenerated oocyte or 1-cell zygotes (Bo and Mapletoft 2013).

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CHAPTER 25

CURRENT STATUS AND FUTURE PROSPECTS OF STEM CELL THERAPY IN ANIMAL HEALTH

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INTRODUCTION

Regenerative medicine is a relatively new field in biology that deals with the repair and regeneration of diseased and damaged tissue, as well as to rectify the congenital anomalies. Over time, regenerative medicine has gained much importance because of its promising results and wide-ranging applications. Among the various options for regenerative medicine, stem cells are at the forefront and are comprehensively studied. These cells can repair injured tissue, that the body would otherwise be unable to regenerate. Although regenerative medicine techniques and stem cells have been previously used, however, the concept of stem cell therapy was first coined by Caplan (1991). In his series of studies on stem cells, he suggested isolation and culturing of the stem cells in vivo and in vitro setup. Research has shown that there are many types of stem cells, including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) (Gazit et al. 2019). Amongst, MSCs have been the most studied and used in preclinical and clinical trials because of their invaluable properties such as easy availability, simple culturing techniques and less or no ethical concerns. In addition to their regenerative potential, MSCs can also modulate the recipient's immune system and thus are gaining much recognition and scope in veterinary medicine (Kolios and Moodley 2013). The poor response or unavailability of conventional treatment regimens has shifted the focus of researchers on the use of MSCs in veterinary medicine. MSCs offer potential treatments for many animal diseases including orthopedic, reproductive, dermal, hepato-renal, digestive, cardiovascular, neuromuscular, dental, and respiratory systems. Although, since long, the scientists have been understanding the nature and behavior of MSCs, but still there is a lot to comprehend. This chapter aims to highlight the current status of stem cell applications in regenerative biology in preclinical and clinical trials.

Stem Cells Origin and Types

Stem cells have been defined and redefined many times, however, scientists agree on their undifferentiated nature, self-renewal ability, and plasticity to differentiate into various types of mature cells (Morrison et al. 1997). Depending on the source of stem cells, they can be broadly classified as embryonic stem cells (ESCs), adult stem cells (ASCs) and induced pluripotent stem cells (iPSCs) (Evans and Kaufman 1981; Takahashi and Yamanaka 2006). The ESCs are obtained from early blastomeres before they lose their totipotency. From adult individuals, ASCs are traditionally obtained from bone marrow and adipose tissue. From fetal adnexa, stem cells are routinely retrieved from the amniotic fluid, however, amniotic membrane, Wharton's jelly, cord blood, placenta and other tissues are also rich sources of ASCs (Sarfraz et al. 2021). The discovery of iPSCs is relatively a new addition in stem cell class, obtained by dedifferentiation of adult cells (Takahashi and Yamanaka 2006).

Depending upon their degree of differentiation potential, stem cells are classified as totipotent, pluripotent and multipotent stem cells (Wagers and Weissman 2004). Totipotent stem cells exist only in very early embryonic stages, just before gastrulation, and are able to give rise to a variety of adult body tissues. Furthermore, they are also capable of developing extra-embryonic structures (Evans and Kaufman 1981; Martin 1981). After gastrulation, these cells yield pluripotent stem cells which are proposed to form all types of adult cells except extra-embryonic tissues. The cells produced by successive cell divisions of embryo, having even less differentiation potential than that of pluripotent stem cells are termed as multipotent stem cells. They can differentiate into number of cell types, but their differentiation ability is limited. Other types of stem cells are oligopotent and unipotent stem cells, which can deliver cells of their own lineage only (Thomson and Marshall 1998).

Human ESCs were first reported in 1998, which opened new horizons for gene-expression studies and their functions in early embryonic development and differentiation (Thomson et al. 1998). The studies also focused on development of drugs by identifying and targeting the genes and tissues of interest. However, with the passage of time, the use of ESCs for trials raised huge moral and ethical concerns and cultural predicament which limited their use. Due to these hurdles, the focus of research was shifted to other sources of stem cells for

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stem cell-based treatments. Lately, iPSCs were developed by two Japanese scientists by reprogramming the adult mouse fibroblasts into pluripotent stem cells in 2006 (Takahashi and Yamanaka 2006). This was a huge discovery in the field of regenerative medicine because these cells appeared similar to ESCs in their genotypic, phenotypic and growth kinetics behavior. However, the dedifferentiation of adult cell can create chromosomal changes which may lead to teratological disorders and hence raised a massive concern about their safety and use in the regenerative medicine.

Another source of the stem cells is adult animals, which contain both the hematopoietic (HSCs) and non-hematopoietic stem cells (Non-HSCs). The bone marrow contains both HSCs and non-HSCs or mesenchymal stem cells (MSC). The MSCs are multipotent in nature as they can give rise to diversified cell types including osteo, chondro, adipo, myo and many other cell types. This differentiation is endogenously activated to regenerate the dead, diseased, and injured cells in a tissue (Caplan 1991). The history of MSCs appeared before 1968, when a population of osteoid cells with fibroblastic morphology was extracted from bone marrow (Friedenstein et al. 1968). Studies in the late twenties showed that these cells could differentiate into bone, cartilage and fat-like cells (Dennis et al. 1999). This provided the basis for determining that MSCs exert their healing abilities by differentiating into other tissue types (Miyahara et al. 2006; Quinn and Flake 2008). In many studies, the immune modulation activity of stem cells was probed, and it is hypothesised that MSCs primarily modulate the immune system and are involved in the tissue repair, therefore, they exhibit regenerative ability. Now, it is stated that perivascular MSCs population in the tissues is involved in aiding these cells to sense local or remote tissue injury and riposte to it by focused relocation to the site of damage and involvement in the therapeutic process (Niess et al. 2016). On the basis of this, MSCs should be termed as "medicinal signalling cell" instead of "mesenchymal stem cells" as stated by Caplan (2017).

The MSCs are relatively easy to collect in large number, have good kinetic potential, and their use in not restricted by ethical concerns, therefore, they are considered promising stem cells for therapeutic procedures. With the increased focus of the scientific community of MSC, their exact definition needed to be well elaborated to set a common ground, therefore, The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) formulated a set of canons to define human MSC, which was also comprehended in a position paper (Dominici et al. 2006). The core concepts articulate that; MSCs must adhere to the plastic in standard culture conditions; they must express mesenchymal markers including CD73, CD90, CD105 (Sandhu et al. 2017; Jurek et al. 2020) and not more than 2% of them must have the expression of HLA class II, CD14 or CD11b, CD34, CD79a or CD19, and CD45; and they must yield cells of osteogenic, adipogenic and chondrogenic lineage on appropriate induction in an in vitro setup.

Sources for Isolation of Mesenchymal Stem Cell

There is a long list of tissues and organs used to isolate and characterize MSCs. The results indicate that the cells from each tissue possess a unique set of features besides the basic features of stem cells and these features should be kept in consideration while choosing an appropriate source MSCs for stem cell based therapeutic purposes. In domestic and companion animals, MSCs have been isolated from their adult individuals as well as from their respective fetal annexes including bone marrow (Sasaki et al. 2018; Zhang et al. 2018; Arévalo-Turrubiarte et al. 2019), fat tissue (Sasaki et al. 2018; Arévalo-Turrubiarte et al. 2019; Rashid et al. 2021b), synovial fluid (Bearden et al. 2017; Arévalo-Turrubiarte et al. 2019), patellar fat (Rashid et al. 2021a), umbilical cord (Zhang et al. 2018; Denys et al. 2020), Wharton's Jelly (Sarfraz et al. 2021), cord blood (Kang et al. 2012), periosteum and muscle (Kisiel et al. 2012; Arévalo-Turrubiarte et al. 2019), peripheral blood (Sato et al. 2016; Longhini et al. 2019), periodontal ligament and gingiva (Mensing et al. 2011), placenta (Carrade et al. 2011), amniotic fluid (Sarfraz et al. 2021) and endometrium (Rink et al. 2017). In lab animals, MSCs have also been isolated from soft tissues including the brain, liver, pancreas, kidney, spleen, thymus, lung and muscle (Meirelles et al. 2006). For a long time, the most frequently chosen sources of stem cells have been adipose tissue and bone marrow, because they yield higher numbers of cells as compared to other tissues. Of the two, the former has gained attention due to its minimal invasiveness to the donor, but has similar properties to stem cells isolated from bone marrow, including trilineage differentiation and immunophenotyping (Bearden et al. 2017; Sasaki et al. 2018). They differ in terms of growth kinetics, plasticity and secretory activity (Arévalo-Turrubiarte et al. 2019; Fideles et al. 2019; Villatoro et al. 2019). Even within same type of tissue, for example adipose tissue, the anatomical location for cell isolation matters (Yaneselli et al. 2018; Rashid et al. 2021b).

The established fact is that the phenotypic and genotypic characteristics of the stem cells are greatly influenced by the type and location of the tissue, therefore, it is of utmost importance to consider these properties while choosing the cells for regenerative and therapeutic purposes. Furthermore, there is no general rule of thumb to prefer one type of tissue over the other for stem cell isolation. Apart from the in vitro culture conditions, the cellular properties are partly influenced by the donor's conditions like species, age, health etc. Because of different variations, there is no single yard stick for comprehensively comparing the results of one study with another (Dominici et al. 2006). Though all the MSCs isolated from different species and sources show plastic adhesion and differentiation, yet their extent of surface antigen expression is highly variable and is no way to compare these results with the criteria described specially for CD73, CD90, CD105 and other hematopoietic lineage markers (Boxall and Jones 2012).

Donor-recipient Relationship for Therapeutic Applications

Since stem cells are aimed to treat injured, diseased, and degenerated tissues, it is important to understand the relationship between donor and recipient. This relationship can be one of three types, xenogeneic, allogeneic, or autologous (Rashid et al. 2021a). Xenogeneic stem cells are cells used across species, the term allogenic means to use stem cells from the same species, while the term autologous is used to use cells from the same individual. An autologous use of stem cell seems the most convenient and efficient than allogeneic, however, both have some advantages over the other. The limiting factors for the use of autologous stem cells include the time for in vitro cell expansion, health status and age of patient (Zajic et al. 2017; Fideles et al. 2019; Taguchi et al. 2019). Contrarily, the

allogeneic stem cells are ready to use, obtained from a young and healthy individual as well as passed a number of safety and scrutiny tests.

The limitation in the use of allogeneic stem cell therapy is the likelihood that major histocompatibility complex class-I on a donor's MSCs is identified by the recipient's T cells, leading to immediate cytotoxicity of donor cells. Moreover, major histocompatibility complex class-II molecules can be identified by recipient's T cells, leading to humoral or cytotoxic immune reactions. Major histocompatibility complex molecules may also be indirectly recognised by antigen presenting cells (APCs) to produce antibodies in B cells (Wieczorek et al. 2017). Numerous studies using allogeneic cells in preclinical and clinical trials show immune responses in in vivo and in vitro settings (Joswig et al. 2017; Oliveira et al. 2017; Cabon et al. 2019; Ursini et al. 2019). These immune reactions have raised questions about the immune-privileged status of MSCs in allogeneic use. Even with the repeated exposure of allogeneic stem cells, the immune reaction was not diminished, contrarily, severe local side effects have been reported in the host's immune response (Joswig et al. 2017; Bertoni et al. 2019; Cabon et al. 2019). Still, some studies suggest non-statistical difference in host's response on repeated exposures (Magri et al. 2019). Not only the immunogenic properties of clinically tested MSCs vary, their MSC expression also vary which seems largely depended on the species and breed of origin, tissue, culture environment and even on the individuality of the donor (Ménard et al. 2020). Although many studies have demonstrated the immunomodulatory properties of allogeneic mesenchymal stem cells, the issue of immunogenic response has plagued the use of these cells, making autologous cells still the best choice for regenerative biology in contemporary settings.

Therapeutic Potentials of Mesenchymal Stem Cells

More recently, stem cells have been thought to heal diseased and damaged tissues by differentiating and replacing these cells. MSCs are now found to be involved in complex immune regulatory mechanisms, including paracrine, vesicle release, immune regulation, and cell-to-cell transfer of organelles.

Paracrine Secretions

The immune cells including natural killer cells (Spaggiari et al. 2006), dendritic cells (Gao et al. 2017), macrophages, as well as B and T cells to be affected by MSC paracrine signalling. Likewise, many factors and cytokines are thought to have immunomodulatory effects, among them are tumor necrosis factor (TNF) stimulated gene-6 (TSG-6), interleukin 10 (IL-10), transforming growth factor-beta (TGF-β), prostaglandin E2 (PGE2) and indolamine-2,3-dioxygenase (IDO).

TSG-6 is an inflammation-related protein that is also involved in anti-inflammatory and protective functions (Day and Milner 2019). TSG-6 is released by MSC and is involved in cellular structure, vesicle size, growth kinetics, plasticity and survival, hence is vital for MSC stemness (Romano et al. 2019). TSG-6 triggers the switch from M1 to M2 phase, which alleviates the signs of inflammation in many diseases (Wang et al. 2015b; Um et al. 2017; Song et al. 2018; An et al. 2020).

IL-10 is a known anti-inflammatory factor that limits Th1 and Th2 responses and the axillary roles of dendritic cells and macrophage; as well as inhibits T-cell proliferation (De Vries 1995). IL-10 is secreted by the contact of T-cells and inflammatory milieu (Najar et al. 2015; Um et al. 2017).

TGF-β is an important growth factor that contributes to cell propagation, plasticity, angiogenesis, wound healing and embryonic development (Gordon and Blobe 2008). The homing and migration of MSCs are also affected by TGF-β (Deng et al. 2017a; Dubon et al. 2018). TGF-β, like TSG-6, triggers the transition of macrophages from an inflammatory (M1) to an anti-inflammatory/regulatory (M2) state, thus helping to regulate T cells (Schmidt et al. 2016; Gazdic et al. 2018; Liu et al. 2019; Wu et al. 2020).

PGE2 is a major prostaglandin that blocks pro-inflammatory cell migration, modulates chemokine production, and promotes regulated cell differentiation (Kalinski 2012). It is important in NK-cell inhibition (Spaggiari et al. 2008) and in M2 conversion of macrophage polarization (Jin et al. 2019). Recently, it was reported to aid in the clearance of apoptotic cells by MSC (Zhang et al. 2019).

IDO is involved in multiple roles, including lymphocyte arrest (Spaggiari et al. 2008; Franquesa et al. 2015) and M2 transformation of macrophage, (François et al. 2012). This enzyme is secreted in an inflammatory milieu by MSCs (Luk et al. 2017).

The above discussion suggests that MSCs can alter the progression of events through paracrine responses, thereby altering and regulating local niches.

Release of MSC-derived Extracellular Vesicles (MSC-EVs)

The role of MSCs in regulating inflammatory cells is not limited to affecting cells through a paracrine mode but can also modulate niche by secreting vesicles in the extracellular environment. These vesicles are enveloped and protected by components of the plasma membrane, so they can be transported over long distances in the body (Jung et al. 2013; Mäkelä et al. 2015).

The vesicles are involved in M2 conversion of macrophages (Hyvärinen et al. 2018), T-cell suppression (Crain et al. 2019) and upregulation of IL-10 (Park et al. 2019). Vesicles have shown therapeutic potential in respiratory (Khatri et al. 2018), renal (Eirin et al. 2017), neurological disorders (Deng et al. 2017b; Ruppert et al. 2018) hepatic (Haga et al. 2017) and cardiac cell damage (Liu et al. 2017). They help to promote healing through formation of new blood vessels and the production of extracellular connective tissue matrix (El-Tookhy et al. 2017).

Studies have shown that vesicles can function in a cell-free environment, thereby avoiding the possible side effects of MSC immune response elicitation (Mäkelä et al. 2015). Nonetheless, cell-to-cell interactions are still required to confer immunomodulatory properties (Luk et al. 2016 & 2017; Gao et al. 2017).

Major hurdle, so far, is the lack of a gold standard technique for the isolation and standardization of MSC-EVs. The most commonly used techniques to isolate MSC-EVs include ultracentrifugation, isolation kits, ultrafiltration, and chromatography. Different techniques yield vesicles of different sizes, characteristics and degree of purity. Therefore, it is believed that each type of MSC-EV has its own unique function. Furthermore, there are many discrepancies and ambiguities in the available literature on MSC-EVs (Reiner et al. 2017; Toh et al. 2018) leading the International Society for Extracellular

Vesicles to require the use of generic terms for such vesicles, unless they are fully defined. Moreover, the society urged to explain the methods of isolation and characterization of MSC-EVs in detail so that the similar results could be reproduced.

Apoptosis-Derived Immunosuppression

Phagocytosis is not only involved in the clearance of dead and dying cells, but also contributes to the immune response, therefore, have immunomodulatory functions. This effect was recoded in an experiment in which heat inactivated MSCs increased IL-10 and decreased interferon, suggesting that the elicitation of immune function is independent of MSCs (Luk et al. 2016).

Contemporary studies suggest that cells of the innate immune system mediate MSC immunomodulatory effects. MSCs have been shown to undergo phagocytosis when physically interact with T-cytotoxic cells and monocytes/ macrophages. The macrophages/monocytes that engulf MSC subsequently exhibit indolamine-2,3-dioxygenase activity (Galleu et al. 2017) to inhibit T-cell proliferation (Cheung et al. 2019).

Transfer of Organelles

In addition to the mechanisms described above, MSCs were also observed to transfer their mitochondria and other organelles through tunnels (Spees et al. 2006). The transfer facilitates in respiration in the recipient cell. When MSCs transferred their mitochondrial contents to immune cells, they showed better phagocytic and antimicrobial activity (Jackson et al. 2016). Other similar studies have shown that organelle transfer mechanisms can be used to pave the way for the treatment of physiological disorders and pathological conditions.

Homing of the Damaged Tissue by MSCs

In addition to their potential to modulate the immune system through direct interaction and release of extracellular vesicles, MSCs home the damaged tissue and release the growth factors, chemokines and cytokines. The chemokines upon activation, are involved in the cell migration (Wynn et al. 2004; Chamberlain et al. 2008; Zou et al. 2011). MSCs homing also originates from different growth factors, including TGF-β1 (Gao et al. 2014), vascular endothelial growth factor (Ball et al. 2007), insulin-like growth factor-1 (Xinaris et al. 2013), fibroblast growth factor (Wang et al. 2015c) and hepatocyte growth factor (Forte et al. 2006). MSCs homing is also influenced by physical stimuli like stress and strain (Xiaorong et al. 2019). It is preferred that the stem cells should be administered in the parenchyma of the desired tissue, however, it is not always possible (Nowakowski et al. 2016), therefore, general administration is performed.

In general, IV administrations, MSCs face many challenges, including migration from the systemic circulation to desired tissues (Nowakowski et al. 2016) mainly due to entrapment in the lung (Gao et al. 2001; Eggenhofer et al. 2012; Jasmin et al. 2014). In the lung tissue, integrins are over activated resulting into cellular interactions (Wang et al. 2015a). Another obstacle to systemic infusion of MSCs is their short lifespan, because they disappear 24 hours after infusion (Eggenhofer et al. 2012; de Witte et al. 2018), therefore no long-term benefits can be obtained, yet some effects can be attained with their apoptosis-linked immunomodulation (de Witte et al. 2018).

Alternatives to IV, intra-arterial (IA) and intraperitoneal routes are experimented because they bypass filtering organs and tissue entrapment, thus providing better tissue distribution. In the IA injection of MSCs, the organs showed better cellular uptake, especially in the liver (Mäkelä et al. 2015). Contrarily, IA administration is complicated by challenging procedures and possible intravascular occlusion and thrombosis (Sole et al. 2013). It was later shown that the problem of thrombosis could be avoided by injecting cells without a tourniquet (Trela et al. 2014). Similar to IA, intraperitoneal administration of cells showed favourable results because the cells housed the desired tissues and did not induce immune reactions (Gooch et al. 2019).

In the recent years, stem cells have been widely studied and used in clinical and preclinical trials which lead to a better understanding of their mode of action, therapeutic activity and healing power. Modern understanding redefines their nature and role in regenerative medicine, thus opening up new horizons and perspectives in the field of regenerative medicine. Many questions about MSC implantation have already been answered that allow us to use these cells effectively, but at the same time, new problems have arisen that need to be addressed. However, with current knowledge about the role of stem cells in veterinary regenerative medicine, we can better address animal diseases and pathologies.

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CHAPTER 26

PATHOGENESIS AND PREVENTION OF AVIAN GOUT

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INTRODUCTION

Avian gout is a disease of urate deposition in poultry caused primarily by Sodium urate crystals (Dalbeth et al. 2019). Due to excessive uric acid production and uric acid metabolism disorder, a large amount of uric acid is excreted through the kidney, causing kidney damage and dysfunction, further blocking uric acid excretion, and resulting in uric acid poisoning and uric acid crystal deposition, leading to gout. Gout can occur in chickens, turkeys, pigeons, geese and pheasants, and mainly in caged chickens. The main features of avian gout are loss of appetite, light crown color, depressed, white semi-mucus-like feces containing large amounts of uric acid and reduced or zero egg production in adult hens. There are two general types of avian gout: one is arthritic gout and the other is visceral gout (Guo et al. 2005; Hong et al. 2020). The joint type of gout is the deposition of uric acid in and around the joint cavity. At first, the joint is swollen. The swelling is soft and the boundary is not obvious. After that, the area gradually becomes hard and forms nodules. The nodules are large, similar to the size of a bean, and later rupture. There is cheese flow out, forming ulcers. The sick bird is often in a squatting or one-legged standing position, with slow movement and lameness. Visceral gout is the deposition of uric acid in the viscera, which is not easy to diagnose clinically, and can be seen in the pleura, peritoneum, lungs, pericardium, liver, spleen, kidneys, intestines and intestinal lining with lime-like, flocculent, crumbly white uric acid crystals on the surface. The most distinctive phenomenon is the "tinea kidney", in which the kidney is covered with snowflake patterns formed by urate deposits. Gout is one of the most common mammalian and avian metabolic diseases, and is an arthritic form of hyperuricemia (Yang et al. 2020). Due to the lack of uric acid oxidase and glutamine synthetase in poultry, it is difficult for ammonia to be converted into urea through the guanine cycle and it is difficult to be excreted. Therefore, poultry are prone to hyperuricemia and gout (Zhang et al. 2018). In order to improve egg production and growth rate of broilers as soon as possible, farmers usually give broilers high-fat and high-protein feeds, which are rich in nucleoproteins and purine bases, easily inducing avian gout. Poor feeding conditions and improper feed ratios have made poultry gout a common disease in poultry, causing great economic losses to farmers. Gout involves a variety of mechanisms, such as increased synthesis and decreased metabolism of uric acid caused by abnormal purine metabolism and astrovirus infection (Shao et al. 2017; Zhang et al. 2018). When the level of uric acid in the blood exceeds the saturation of the blood, the excess uric acid forms crystals deposited in the joints and tissues of the body, causing recurrent inflammation in the surrounding area. This chapter reviews the causes, mechanisms and prevention of avian gout with the purpose that it can provide reference for the prevention and treatment of avian gout.

Causes of the Disease

There are many causes of gout. The cause of gout is usually complicated and difficult to be determined. Existing studies have shown that there are more than 20 causes of avian gout, which can be divided into two categories. One category is the excessive production of uric acid in the body, and the other is the disturbance of urate excretion.

Hereditary Factors

There is a genetic susceptibility to gout for certain strains of chickens. Certain breeds of chickens have defective renal tubular secretion of uric acid, which can cause gout even when fed on the diets with normal protein levels. Some researchers have also bred some hereditary hyperuricemia chickens (HUA chickens) from chickens with high incidence of joint gout, and hereditary factors are often the main factors of joint gout. Studies have shown that long-term feeding of high-calcium diets can cause kidney damage and induce gout in chickens.

Nutrient Metabolic Factors

Excessive protein content in feed, especially too much animal protein, can easily produce excessive uric acid. Common ones include animal offal, meat and bone meal, fish meal, meat scraps, etc. In addition, it also contains plant-based feeds such as soybeans and mushrooms. It has been reported that gout can be caused when the addition of fat-free horsemeat and 5% urea to the Turkey diet increases the protein content of the diet to 40%. All hens developed gout after 3 to 5 months of continuous feeding with fat-free horsemeat. Studies have shown that, under the condition of normal kidney function, feeding diets with slightly higher protein level will temporarily increase plasma uric acid level, but will not cause gout. Other studies have found that high-protein diet can promote the occurrence of articular gout in HUA chickens.

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Infectious Factors

All pathogenic microorganisms that are nephrophilic and can cause renal function damage may block the excretion of urate, thereby causing chicken gout, such as nephropathogenic infectious bronchitis (IB), infectious bursal disease (IBD), inclusion body hepatitis in chickens, avian egg drop syndrome, avian influenza, Marek's disease (MD), avian mycoplasmosis, pullorum disease, staphylococcus, aspergillus, pneumocystosis, Eimeria, and histo-trichomoniasis (Pegram et al. 1981; Slemons et al. 1990; Trampel et al. 2000). At present, nephropathogenic infectious bronchitis is the most studied and reported (Xu et al. 2019). Guo et al. (2005) found that the pathogenesis of nephropathogenic infectious bronchitis virus induced avian gout, which indicated that nephropathogenic infectious bronchitis virus infection induced the change of global transcriptomic and metabolomic profiles of kidney and Fabricius (Xu et al. 2019; Kuang et al. 2020). In addition, NIBV mediated endoplasmic reticulum stress and apoptosis in kidney, which were closely related to the occurrence of chicken gout (Fung et al. 2014; Chen et al. 2021).

In addition, gosling astrovirus (GoAstV) is also believed to be the main causal pathogen of gout, but the full-blown disease of gout cannot be well reproduced by infecting the goslings with GoAstV. Therefore, Liu et al. (2020) studied the changes in GoAstV and GPV infection in goslings by PCR and DNA sequencing. The HE staining demonstrated that the kidneys were congested with renal tubular necrosis, abscission of renal tubular epithelial cells, and eosinophilic protein-like substance in the renal tubules. The ureteral lumen was enlarged with necrotic debris and basophilic staining of urate deposits. The liver, spleen and lung were markedly congested and edematous. The hyperemia and hemorrhage of the cerebrum, cerebellum, and trachea were present. The necrosis and dissolution of cells and hemorrhage in blood capillaries in the heart and leg skeletal muscle were also observed. Basophilic intranuclear inclusion bodies were found in kidney, liver, harderian gland, bursa of fabricius, lung, and spleen (Liu et al. 2020). Studies have shown that different varieties of geese can be infected with GoAstV. New type of goose stellate virus infection leading to gout in goslings mainly occurs in 5 to 20 days old goslings, goslings infected at about 5 days old onset, incidence of about 80-90% and mortality of about 20-30%, and the highest can reach 50% (Monroe et al. 1993; Niu et al. 2018). Goslings in the early onset of infection appear slow action, close eyes, shrink neck spirit, drooping double wings, drooping feathers, fluffy messy, and other clinical symptoms. As the condition gets worse, sick young appetite decreases gradually. Excretion of stone ash cinder looks white and thin dung appears claddy. Serious conditions can cause paralysis until the death. Young goose growth is retarded, and body resistance is reduced. The typical clinical symptom of gout occurrence of young goose is obvious joint swelling. In the late stage of the disease, white urate crystals are precipitated at the eyelid (Zhang et al. 2018).

Coccidiosis of Eimeria tender occurs mostly in chickens aged from 3 to 6 weeks. The parasite parasitized in the cecum and rectum mucosa epithelial cells, and had an incubation period of 4 days after infection. At the beginning of the disease, the patient was listless, with feathers inverted, head curled up, eyes closed, loss of appetite, and feces sparse. From the end of the 4th to 5th day, the chick suddenly excreted a large amount of blood stool, showing obvious anemia. Studies have shown that the serum uric acid content in tender chickens with Eimeria coccidiosis is significantly increased, which may be related to excessive ammonia and nucleic acid removal, especially the intensified tissue destruction and accelerated nucleic acid decomposition, but whether it is related to local destruction of cecum or other systemic toxin effects remains to be studied. However, according to Ruff et al. (1981), chest RNA was significantly reduced on day 4 of *E. tenella* infection. In addition, the study found changes in kidney structure and uric acid clearance in young chickens with Eimeria coccidia. Padmavathi and Witlock (1981) proved that the cause of death in tender chickens caused by Eimeria infection was renal tubular dysfunction caused by elevated uric acid levels.

Toxic Factors

Toxic factors mainly damage the kidneys and reduce uric acid excretion, thereby causing visceral renal gout. Some nephrotoxic drugs combine with plasma proteins to produce antigenicity, which can cause allergic reactions in the body and diffuse kidney damage. For example, improper medication and excessive use of nephrotoxic drugs such as antibiotics, sulfonamides, furans, and chlorocyclic hydrocarbon pesticides in the process of prevention and control of poultry diseases can easily reduce renal function, weaken the ability to excrete uric acid, and cause a large accumulation of uric acid which causes gout. In addition, the excretion of sulfonamides in an acidic environment can easily cause crystals to be precipitated from the urinary tract, while the renal excretion capacity of crystalline sulfonamides is extremely low, resulting in obstruction of the renal tubular lumen. The mycotoxin poisoning factors are more harmful, which can seriously damage the kidneys and cause hyperuremia and visceral gout. Nephrophilic chemical poisons such as Potassium dichromate, cadmium, thallium, zinc and lead can also cause gout. Visceral gout in birds can be caused by intoxication with allopurinol drugs used to treat hyperuricemia in humans.

Unbalanced Vitamin Content

Long-term lack of vitamin A in the diet can lead to keratinization of renal tubular and ureteral epithelium, and impaired urate excretion after kidney injury, leading to gout. Lack of vitamin D can make the body mineral especially calcium phosphorus metabolism disorder and cause gout. Lack of pantothenic acid, biotin, choline can directly or indirectly lead to kidney diseases and cause gout. For example, high levels of vitamin D can enhance the absorption of calcium in the gut, which can cause hypercalcemia.

Other Factors

Cold and wet environments function in the development of gout. The lowering of the ambient temperature can promote the crystallization of urate. When uric acid is at a high level in the body of avian animals, cold temperatures and humidity will cause local microvascular contraction, thus slowing down the blood flow and promoting the deposition of large amounts of urate in the joint cavity of avian joints, resulting in gouty arthritis.

Lack of drinking water can also promote the deposition of urate. The rotation and absorption of nutrients in the body and the excretion of wastes in the body require water as a medium. When the lack of water is too long, the concentration of uric

acid and other minerals in the blood and renal tubules will increase. Then urine is concentrated and urate is continuously deposited in the ureter, which finally causes gout.

Pathogenesis

Avian gout spreads all over the world, with an incidence of 85% and a mortality of 30%. Urate overproduction and underexcretion of urate can be considered to lead to hyperuricemia in the body of birds and monosodium urate crystal deposition to form avian gout.

Urate Overproduction

There is no arginase in the livers of avian animals, so that protein cannot be excreted through the ornithine cycle into urea, and only through the purine nucleotide cycle to form purines (Lee et al. 2013). Purine is a nitrogen-containing ring structure substance, which is widely present in the nucleic acid of plant tissues. Purine can be produced by the decomposition of nucleoprotein in food, or it can be formed by the decomposition of the core protein. Urate is the end-product of purine nucleotide degradation, so diet therapy occupies an important position in this disease. Purines can be produced by the decomposition of nuclear proteins in food (exogenous), and in the body (endogenous) (Li et al. 2021). Hence, with excessive intake of protein, purine substances increase serum urate and the risk of incident gout. High uric acid combines with calcium and sodium ions to form uric acid salts. The stability of urate is easily affected by the environment. It is protected from deposition in the blood by massive plasma proteins that maintain stability. However, high urate levels in the blood form ultrafiltration urate colloidal particles, which are filtered into tissues with low protein content, destabilizing them and causing deposition. Urate deposited in the joint cavity can act as a damage-related molecule to stimulate innate immune pathways, and activate the nuclear transcription factor NF-κB through TLR4 and TLR2, promoting the synthesis of pro-IL-1β and inflammasome components, and eventually leading to gouty arthritis. Xi et al. (2019) studied the expression levels of inflammatory factors and inflammatory signaling molecules in the kidneys of goslings with gout and found that TLR2/TLR4, MyD88, NF-κB, IL-1β, IL-8 and TNF-α in the kidneys of goslings with gout are significantly increased, resulting in severe renal inflammatory damage, which further exacerbates renal excretory dysfunction (Xi et al. 2019).

Uric Acid Excretion Disorder

The only organ for excretion of uric acid in poultry is the kidney, so that the normal excretion of uric acid in poultry depends on the structure and function of kidney (Shideman et al. 1981). Under normal circumstances, uric acid is excreted through renal tubule secretion and reabsorption, both of which directly affect uric acid levels in the body. If the secretion of uric acid by the renal tubules is reduced, it will cause the obstruction of uric acid excretion and induce hyperuricemia. Previous studies have shown that New Hampshire chickens exhibit an innate genetic susceptibility to articular ventilation due to an impaired uric acid transport mechanism and a defective gene for uric acid secretion in renal tubules (Cole et al. 1980). In addition, some researchers selected inherited hyperuricemia chickens from the chickens with high-incidence of jointed gout (Austic et al. 1976). If the serum uric acid concentration in poultry keeps increasing, it will cause persistent excessive excretion in the kidney, which in turn induces up-regulation of URAT1 (uric acid anion exchanger 1 encoded by SLC22A12) and GLUT9 (glucose transporter 9 encoded by SLC2A9), leading to kidney damage (Liu et al. 2015; Qin et al. 2018). At the same time, uric acid deposits in the way of urate in the blood on the joints, cartilage, soft tissue, and visceral surface. URAT1 and GLUT9 also mediate the reabsorption of uric acid in the proximal tubules, which in turn seriously hinder the excretion of uric acid (Le et al. 2008). On the contrary, OAT1 (organic anion transporter 1 encoded by SLC22A6) participates in basolateral urate excretion, helping the kidney to regulate excess excretion of uric acid (Habu et al. 2005). Therefore, changes in the functions of different urate transporters can directly cause changes in uric acid excretion.

Another major cause of uric acid excretion disorder in poultry is the accumulation of hyperuricemia and sodium urate crystals, which leads to the formation of gout (Hong et al. 2020). Uric acid is slightly soluble in water and can form urate deposition with cations such as Na^+, K^+ and Ca^{2+} under certain pH conditions (Kanbara et al. 2012). In other words, the stability of urate colloid is closely related to electrolyte balance and acid-base balance of body fluid. Many studies have reported that a high calcium diet and long-term vitamin A or vitamin D deficiency are important causes of gout in poultry (Guo et al. 2008). High dietary calcium levels can cause hypercalcemia, which leads to metabolic alkalosis and increased parathyroid secretion (Konstantinov 1970). Then it can increase calcium ion concentration and deposition in renal tubular epithelial cells, leading to the formation of kidney stones and chronic renal insufficiency. By this time the nephron is continuously destroyed, so that it is not enough to compensate for all renal function, resulting in blocked excretion of uric acid, and deposition on the serous membrane surface of the kidney, heart, liver, mesentery, air sac and peritoneum, eventually causing gout and renal failure (Marcocci et al. 2011; Ding et al. 2019). In addition, when the poultry body is in a state of high calcium, the ratio of calcium to phosphorus will be unbalanced, and low phosphorus can promote the occurrence of gout. Relevant studies showed that when the calcium content in gosling's diet reaches 3.1% (the normal requirement is 0.8~1%), the ratio of calcium to phosphorus reaches 7.8:1.0 (the normal ratio is 1.1:0.7), and gout occurs in gosling (Alagawany et al. 2021). It is worth mentioning that vitamin A can maintain mucosal integrity and protect mucosal barrier by regulating the proliferation and differentiation of epithelial cells (Miyaishi et al. 1992). But long-term vitamin A deficiency can lead to keratinization of renal tubules and ureteral epithelium, which is not conducive to the excretion of uric acid and phosphorus, which eventually could lead to urate deposition and renal failure (Chandra et al. 1984). Another cause of metabolic disorder of avian fluid electrolyte is insufficient intake of vitamin D. Lack of vitamin D can lead to the mineral metabolism disorder, proportion imbalance, and excretion obstacles, especially calcium and phosphorus. However, when dietary vitamin D supplementation is excessive, it will cause intestinal absorption of more calcium, which is easy to cause hypercalcemia and indirectly lead to the occurrence of gout.

Similarly, any nephrophilic protomicroorganism can cause renal function injury and urate excretion obstruction, such as new astrovirus, avian nephritis virus (ANV), aspergillus nephritis,

and nephropathogenic infectious bronchitis virus (NIBV) (Jin et al. 2018). These pathogens act on renal tissue directly or indirectly, causing functional lesions at the early stage and organic lesions at the later stage. Wu et al. (2020) found that GoAstV infection in goslings caused an increase in uric acid produced by purine metabolism, and a decrease in uric acid excreted by the kidneys, resulting in the accumulation of uric acid in goslings, and eventually leading to hyperuricemia and the occurrence of gout (Wu et al. 2020). Trampel et al. (2000) reported the first case of urethral cryptosporidiosis in adult hens. Under the influence of cryptosporidium parasitism, ureteral epithelial cells of infected birds prolifically shed, resulting in partial ureteral obstruction and visceral gout. Some nephrotoxic drugs will produce antigenicity after combining with plasma proteins, which can cause allergic reactions in the body and diffuse damage to the kidney - degeneration, necrosis, shedding, agglutination of renal tubular epithelial cells, blocked excretion of uric acid, etc. In addition, the excretion of sulfonamides in acidic environment makes it easy for crystals to precipitate from the urinary duct, while the excretion ability of kidney to crystalline sulfonamides is very low, which leads to obstruction of renal tubule lumenage and reduces the ability of kidney to excrete uric acid.

Other Factors

Cold and wet environments play a role in the development of gout. The lowering of the ambient temperature can promote the crystallization of urate. When uric acid is at a high level in the body of avian animals, cold temperature and humidity will cause local microvascular contraction, thus slowing down the blood flow and promoting the deposition of large amounts of urate in the joint cavity of avian joints, resulting in gouty arthritis.

Lack of drinking water can also promote the deposition of urate. The rotation and absorption of nutrients in the body and the excretion of wastes in the body require water as a medium. Once the lack of water is too long, the concentration of uric acid and other minerals in the blood and renal tubules will increase. Then urine is concentrated and urate is continuously deposited in the ureter, which finally causes gout.

Prevention

Due to the complex causes of avian gout, there is no specific drug to treat it at present. The most effective way to prevent avian gout is to take different measures for different causes.

Pay Close Attention to Feed Management

Poultry have different nutritional needs at different feeding stages. High-calcium and high-protein feed is one of the main causes of gout in poultry, and regulating the content of calcium and protein in feed is the most effective method. After decades of development, the formula and technology in the feed industry have been very mature. The feed required by poultry at different growth and development stages is subdivided to make the feeding of farms more targeted. In the process of storing feed, we should pay attention to the warehouse environment to ensure that it is cool, ventilated and dry. For warehouses with relative humidity greater than 50%, quicklime can be sprinkled on the corner ground to absorb moisture in the air, reduce humidity and prevent mildew of feed. The technicians of many manufacturers can also come on site for disease diagnosis and treatment services, which is conducive to the control of the disease from the feed source.

Strengthen Disease Prevention and Control

Most gout is caused by diseases, most of which are nephropathogenic, infectious bronchitis virus, infectious bursal disease, Salmonella infection and other diseases (Choi et al. 2009; Lin et al. 2015). It is suggested that the vaccine immunization of infectious bronchitis and infectious bursal disease should be well done in the chicken stage, so that the body can produce effective titers of antibodies to resist wild virus infection. At present, there is no good vaccine for salmonellosis. It is recommended to use antibiotics, but pay attention to the rest period for broilers and eliminated chickens. Commercial chickens entering the laying period are prohibited from using antibiotics, and non-antibiotics materials such as traditional Chinese medicine, probiotics, phage feed additives, biochemical products and plant essential oils can be used for prevention and control of the disease.

Scientific Use of Drugs

Drug abuse can lead to liver and kidney damage, resulting in urate metabolism and excretion disorders (Jospe-Kaufman et al. 2020). Here we do not forbid the use of veterinary drugs, but use them in a proper way. Aminoglycosides have strong toxicity to the kidney, but they are basically not absorbed orally. Only when injected, the drugs can quickly reach the kidney through blood circulation and cause damage to the kidney. The drug administration method is very important. It is easy for sulfonamides to form crystalline urine when passing through the kidney, resulting in damage to the renal tubular wall and affecting the excretion of uric acid. When used, sulfonamides can be used together with baking soda to alkalize the generated urine, which can reduce the crystallization of sulfonamides. It is suggested that farms with frequent occurrence of this disease should consulted professional licensed veterinary doctors for clinical medication guidance, so as to reduce the incidence of the disease.

Treatment

For poultry gout with complex pathogenic mechanism, there is currently no particularly effective drug. Different types of gout need to be targeted differently.

High-calcium and high-protein feed is one of the main causes of gout in poultry, and reducing the content of calcium and protein in feed is the most effective method. Dexamethasone is currently the most effective drug for the treatment of avian gout. Dexamethasone belongs to the hormone class, increasing enzyme activity in the body to increase the rate of metabolism to achieve the purpose of reducing uric acid and urate deposition in the body to relieve gout. Relevant literature shows that the cure rate of dexamethasone for gout is as high as 91.8%. Allopurinol, as a competitive inhibitor of xanthine oxidase, is also useful in the prevention and treatment of avian gout. Allopurinol can effectively reduce the content of uric acid in the body, but attention should be paid to its side effects on gastrointestinal tract. In recent years, rapid progress has been made in the process of treating gout with Chinese herbal medicine, such as avian gout powder, and kidney kangning.

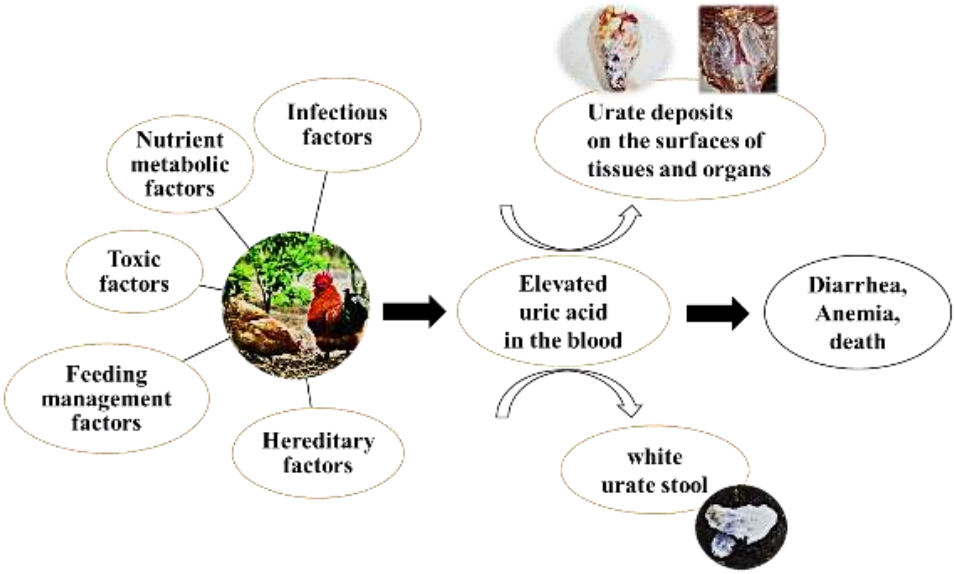

Fig. 1: The general process of avian gout

Relevant experiments show that, compared with allopurinol, Piper-betle-L showed significant efficacy in the treatment of avian gout (Chakravarthi et al. 2021). Probenecid is of great significance in the treatment of chronic avian gout. Probenecid reduces urate concentration by inhibiting urate reabsorption and promoting urate excretion, which accelerates the formation of urate dissolution. But benzosulfonate has no obvious effect on acute gout. Gout can also be treated by adding Sodium bicarbonate and Potassium chloride to drinking water to regulate the acid-base balance in avian. It works by alkalinizing urine, making uric acid less likely to accumulate and crystallize in urine. It has the advantages of quick effect and can greatly reduce the mortality of poultry. There are many other drugs to treat avian gout, such as colchicine, non-steroidal anti-inflammatory drugs, and utlopine. A recent study showed that pepper extract alleviates gout symptoms by increasing antioxidant capacity in broilers (Vikrama et al. 2022). Generally speaking, the cure rate of traditional Chinese medicine is higher than that of western medicine. Specific use of drugs should be selected according to the actual situation.

Thoughts on the Study of Avian Gout

Avian gout is highly similar to human gout. In terms of clinical manifestations, avian joint gout is very similar to human gout, mainly in joints. The manifestations of the disease are joint swelling, being soft and painful at the beginning, gradually hardening of swelling parts, aggravation of pains, the formation of not mobile or slightly mobile nodules, and presence of pain swelling occurs in distal joints, such as toe joints, and anterior toe joints, but it can also infringe on wrist joints, anterior wrist joints and other places. In terms of pathogenesis, both avian gout and human gout are caused by urate deposition due to increased uric acid production or decreased uric acid excretion. All are affected by factors such as heredity, diet, and environment. Excessive intake of purines and proteins can lead to an increase in the production of uric acid, which is easily deposited in the form of sodium salts and calcium salts, resulting in gout. The pathological basis is similar. The liver of poultry does not contain arginase, so the protein consumed can only generate purine, which forms uric acid insoluble in water under the action of xanthine oxidase, and easily forms urate with sodium or calcium, which leads to gout on the surface of renal tubules and joint cavities. However, humans lack in uric acid oxidase, which cannot metabolize purine into soluble urea and expel it from the body like other mammals (Hong et al. 2020). Uric acid is also the final metabolic product. At present, the commonly used animal models of gouty arthritis only simulate the pathological phenomenon of urate crystals precipitated in the joints, which is inconsistent with the pathogenesis of human gout. In addition, uric acid oxidase exists in rodents, which has a different metabolic pathway compared with purine in humans. Uricase-deficient mice obtained by gene knockout are expensive for basic research. However, there are many similarities between avian gout and human gout, and the avian gout has a high incidence and can be seen in many kinds of birds. Therefore, it is of great significance to establish an animal model of avian gout for the study of the pathological mechanism of human gout and drug screening.

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CHAPTER 27

MECHANISM RESEARCH AND TREATMENT OF REPEAT BREEDING SYNDROME IN COWS

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INTRODUCTION

Repeat breeding syndrome (RBS) is a critical reproductive disorder in repeated breeder cows, with pregnancy losses after three or more artificial inseminations but no anatomical or infectious abnormalities (Gunther 1981). The incidence of RBS in cow was reported to be 25% in Spain and Australia, 22% in USA and United Kingdom and 10% in Sweden (Bartlett et al. 1986; Eddy 1994; Gustafsson et al. 2002; Garcı´a-Ispierto et al. 2007; Canu et al. 2010). RBS is one of the most important factors affecting economic success in the dairy and beef industries, such as increasing intervals to conception, wasteful semen and insemination costs, increased veterinary examination and treatment costs, and additional maintenance charges (Casida 1961; Bartlett et al. 1986; Lafi et al. 1992; Perez-Marin et al. 2012). RBS contributes to declining fertility worldwide, which poses a severe threat to the breeding of cows. Unfortunately, RBS remains unmanageable mainly due to the lack in obvious factors. This chapter is focused on the mechanism research and clinical treatment of repeat breeding syndrome.

Mechanism Research

The physiological cause of repeat breeding syndrome is multifactorial. Nevertheless, many researchers prefer to focus on the following mechanisms: fertilization failure and early embryo death.

Failure of Fertilization

Failure of fertilization may result from inferior oocyte quality of female or unqualified artificial insemination (AI), including inopportune insemination. Poor oocyte quality can be caused by the following factors: chromosomal abnormalities, heat stress, and hormone disorders (Wiltbank et al. 2006; Sartori et al. 2010; Roberto et al. 2013; Sood et al. 2017). Plenty of research shows that repeat breeding cows have poorer oocyte quality and less quantity, which delays cytoplasmic maturation and obstructs fertilization (Kurykin et al. 2011; Kafi et al. 2017). During the research study in heifers directed by two scientists, seventy-four percent of the embryos generated by superovulation on day 7 after insemination were morphologically normal, and merely 28% were generated by the superovulated repeat breeding group (Gustafsson et al. 1983). A comparison of the amount in gene expression of repeat breeding cow and healthy cumulus-oocyte complex showed 178 genes were distinctly expressed. There were lower expression levels of ANXA1, Lactoferrin, HEM45, LOX-1, and GSTA4 gene in repeat breeding Holstein Friesian heifers, which provides reliable data for seeking the underlying candidate marker genes for RBS (Roberto et al. 2013). Heat stress, one of the major problems for cow fertility, has a harmful impact on the maturation of oocytes and the sustainable development of predatory embryos (Ferreira et al. 2011; Walsh et al. 2011; Silva et al. 2013). For instance, at the embryo development towards the blastocysts period, heat stress can increase the apoptotic index and disturb the expression of some genes in both *in vitro* fertilized and parthenogenetic embryos (Roberta et al. 2016). Hormone disorders is another main factor contributing to the failure of fertilization (Ariane et al. 2011). During the estrous cycle, hormonal asynchrony was found in repeat breeding heifers, with progesterone (P_4) concentration of approximately 0.5-1.0 nmol/L and lower plasma P_4 concentrations after AI (Albihn et al. 1991; Wiltbank et al. 2006). In addition, delayed LH peak, prolonged duration of estrus, and delayed ovulation were considered the evident clinical symptoms in some reports (Bage et al. 2002; Sood et al. 2017). Finally, fertilization failure can also be caused by unsuccessful AI, including in-accurate oestrus detection, incorrect time and unbefitting skill of AI, obstruction of fallopian tubes in females and inferior semen quality in males (Ayalon et al. 1984; Sartori et al. 2010; Yusuf et al. 2010).

Death of Early Embryo

Early embryo death, a major source of repeat breeding syndrome, is mainly generated by inferior oocyte quality and an inadequate uterine environment. The embryonic mortality of ruminants ranges between 20 and 50% (Humblot 2001; Diskin et al. 2008). The highest early embryo death rate is in the first week of a pregnant lactating cow (Walsh et al. 2011). Firstly, nutrition plays a vital role in the cytoplasmic maturation of

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oocytes in cows. Accumulation of cytoplasmic lipid deposits and their misdistribution have deleterious effects on oocyte maturation and quality (Adamiak et al. 2006; Leroy et al. 2008). A mass of oocytes with lipid droplets was an inescapable threat to their quality, mature capacities, and cryo-tolerances (Abe et al. 2002). It was reported that the reproductive performance of animals has a connection with their weight. As the body score surpasses 3.5, the cow has delayed initial oestrus, late conception and additional services to be pregnant (Crow et al. 2008). The body condition is inextricably linked to the daily feeding management, and several studies show deleterious influence on oocyte quality and blastocyst rate by immoderate intake (Humblot et al. 1998; Boland et al. 2001; Webb et al. 2004). Secondly, an inadequate uterine environment resulting from endometritis or other diseases can reduce embryo death by growth factors, bacterial toxins, and inflammatory mediators, including endometrial epidermal growth factor, prostaglandins, and reactive oxygen species, and cytokines (Sheldon et al. 2006; Wagener et al. 2017). Cytological endometritis has been reported to have deleterious effects on repeat breeder cows (Salasel et al. 2010; Janowski et al. 2013; Pothmann et al. 2015; Pascottini et al. 2016).

Treatment

Hormonal Treatments

Hormonal treatments, such as progesterone (P_4), gonadotropin-releasing hormone (GnRH), human chorionic gonadotropin (HCG), or insulin, have been widely used to improve the rate of conception for repeat breeding cows (Young et al. 1988; Thuemmel et al. 1992; Selvaraju et al. 2002; Kharche et al. 2007). During early pregnancy in the repeat breeding cows and heifers, the administration of P_4 (75 mg/d for cows, 40 mg/d for heifers, intramuscular injection on daily days 6-10 after AI) may increase plasma P_4 enough to reduce peripheral circulation demand for P_4 from the ovarian circulation, thus decreasing embryonic mortality due to insufficient P_4. However, it did not affect the conception rate (Thuemmel et al. 1992). Supplementation with PRID (1.55g of P_4) between days 5 and 19 after AI-enhanced the likelihood of pregnancy in 1-2 parity repeat breeding cows. It is worth mentioning that the proportion of abortions tended to be lower in PRID cows. However, there are some contradictory results regarding the advantage of GnRH therapeutic effects (Perry et al. 2009).

On the one hand, some reports indicated that the GnRH or synthetic analogues could improve the pregnancy rate in crossbred cows. There are several preparations of GnRH, such as gonadorelin, buserelin, cystorellin, and fertirelin, and the dose used for them ranges from 10 to 500μg in clinical treatments for several decades (Chenault et al. 1990; Stevenson et al. 1990; Ahuja et al. 2005). A study showed that there is a dose-effect relationship in RBS crossbred cow bred, which was previously exceeded 5 times reproductive procedure, particularly in the times of first service and conception rate (Kharche et al. 2007). On the other hand, it was shown that a dose of GnRH at the time of AI did not influence conception rates in beef cattle with obvious behavior of estrus (Heuwieser et al. 2011). Insulin and IGF-I can function as significant accommodators of follicular growth, reproductive hormone fluctuation, oocyte maturation, and embryonic development. Cows in the insulin treatment group were administrated subcutaneously with a long-acting purified form of bovine insulin (0.2 IU/kg/day on days 8, 9 and 10 of the estrous cycle). The results showed that P_4 concentration increased after insulin injection. However, the pregnancy rate had no first service pregnancy rate, and the overall pregnancy rate did not differ between the insulin injection group and the control group (Selvaraju et al. 2002).

Regulation of Fatty Acids by Diet Therapy

Fatty acids (FA) were not only closely related to the formation and metabolism of many important chemical compounds (hormone, ketosteroid and cholesterin) in reproductive health but also were essential factors for the structure and function of some organelle in germ cells (Abayasekara et al. 1999; Jump et al. 1999; Cheng et al. 2004; Leroy et al. 2005). Dietotherapy of FA can improve milking and reproductive performance, speculated by the possibility of harm reduction in the negative energy balance of high-producing dairy cows. Studies were conducted on diets with some individual fatty acids or FA groups. The results showed that ω-3 PUFAs could positively influence reproductive status, for instance, follicular development, persistent corpus luteum, and high fertility (Staples et al. 1998; Mattos et al. 2004). Results from dietary studies showed that ω-3 added to the ration of ruminants can prolong the presence of corpus luteum by reducing PGF_{2a} and increasing embryonic survival (Diskin et al. 2008; Gulliver et al. 2012). Ambrose and his colleagues verified that dietary supplementation of ALA (alpha-linolenic acid, a type of ω-3) was provided for Holstein cows for twenty-eight successive days before artificial inseminations caused larger preovulatory follicles and fewer embryonic losses (Ambrose et al. 2006). During the non-breeding season, a more significant proportion of saturated FA in cow oocytes could account for a smaller conception rate (Zeron et al. 2001). Feeding fish oil is rich in ω-3 (a blend of eicosapentaenoic acid-EPA and docosahexaenoic acid-DHA, two types of ω-3), from −2 to +2 weeks of breeding, and the experimental results indicated that the group of repeat breeding cows who achieved pregnancies fed by fish oil in the synchronization of estrus and artificial insemination, possessed a greater diameter of the preovulatory follicle and higher expression of mRNA of interferon-stimulated gene transcripts (Aamir et al. 2019).

Aquapuncture Therapy

Acupuncture, a simple, safe and effective technique, has been used successfully to treat reproductive disorders in cows, pigs, and women (Fung 1984; Lin et al. 1988; West 2000; Lin et al. 2001). As an alternative treatment, it is recognized gradually by the Oriental. Eighteen repeat breeding cows and heifers were treated by aquapuncture, in which 5 and 10 ml of 50% glucose solution were injected at Shenpeng and Baihui acupoints, respectively. The pregnancy rates after the treatment were 77.7 and 66.6% based on P_4 concentration and rectal palpation, respectively. However, the final rate obtained from the actual delivery of the fetus was 44.4%, which had a similar consequence to GnRH treatments (Lin et al. 2002).

Embryo Transfer Protocol

Embryo Transfer (ET) is diffusely applied to enhance the amount of excellent production performance animals and build

superiority gene population. Many studies have investigated the function of embryo transfer for increasing breeding efficiency in repeat breeding cows (Tanabe et al. 1985; Son et al. 2007; Block et al. 2010; Yaginuma et al. 2019). Rodrigues and his colleagues used the conception rate to compare RBS Holstein cows by AI with those by ET. The conception rate was higher in the group by ET (41.7%) than that by AI (17.9%) (Rodrigues et al. 2007). However, there was no remarkable distinction in the pregnancy rates on two months between health and RBS (82% vs. 70%) (Tanabe et al. 1985). Timed artificial insemination superovulated donors and timed embryo transfer in embryo recipients is a choice to avoid oestrus detection. In a study, conception rates were enhanced subsequent CIDR TAI or TET, compared with the rates subsequent AI after a single injection of $PGF_{2\alpha}$ in the synchronization in RBS cows. There was a higher conception rate in the ET than in the appropriate AI group or the TAI (53.8% vs. 18.5% vs. 7.7%) group (Son et al. 2007). In a word, the embryo transfer protocol provides a capacity for mitigating the threat of inferior quality of oocyte and embryo and remedying the imperfect functionality of the uterine in repeat breeding cows.

Vaginal Treatment

Seminal plasma (SP) and milk osteopontin (mOPN) in the vagina are new approaches in recent years. The normalized endometrial Epidermal growth factor (EFG) profile and preserved fertility in RBS cows were studied (Dagvajiamts et al. 2020; Hay et al. 2022). A series of research have manifested there is a likelihood that SP enhances female reproductive performance by regulating some factors of uterine function and ameliorating prenidatory environment in mice, sows, horses, cows, and humans (WS et al. 1989; Gooneratne et al. 1989; Tremellen et al. 2000; Alghamdi et al. 2004; Robertson 2007; Dagvajiamts et al. 2020). EFG is a critical factor in controlling uterine function and embryo development, which is regulated by estrogen in mice and rats. The healthy cows have two peaks at days 2-4 and 13-14 of the estrus cycle with EGF concentrations of their uterine endometrium, and the RBS cows are deficient in these peaks relatively (Katagiri et al. 2004; Katagiri et al. 2013; Katagiri et al. 2016; Kafi et al. 2017). In a recent study, 5% SP with PBS and only PBS were infused into the vagina of two RBS cows' groups at day 3 of their estrous cycle, respectively (31 vs. 36). The results showed that about 60% of RBS cows had normalized two maximum values of endometrial EGF concentrations again and produced more pregnancies than the controls (44.4 % vs 19.4%) (Dagvajiamts et al. 2020). However, the mechanism of EGF concentrations in the uterus altered by SP infusion into the vagina remains unknown. Nowadays, studies have shown that SP improved females' fertility, which is likely associated with sperm motility and function or the direct mode of action on uteri found in other varieties (Katila et al. 2012; Bromfield 2016). OPN, a glycosylated phosphoprotein, was firstly separated from the bovine diaphyseal bone and widely detected in a variety of epithelial cells and seminal plasma, blood, milk, urine, oviduct, uterus and placenta (Franzen et al. 1985; Cancel et al. 1997; Sodek et al. 2000; Johnson et al. 2003; Tsuji et al. 2007; Goncalves et al. 2008; Schack et al. 2009; Dudemaine et al. 2014). Cow OPN has 262 amino acids and displays a molecular weight of 16- 60 kDa (Kumura et al. 2004; Christensen et al. 2016; Dagvajiamts et al. 2020). mOPN, a bountiful origin of OPN in cows, could promote sperm capacitation, cleavage, blastulation and embryonic development *in vitro* experiments (Killian et al. 1993; Christensen et al. 2016). Hay and his team separated mOPN from cow milk, identified 3 major protein bands of 31, 37 and 61 kDa and compared three vaginal treatments (mOPN vs. SP vs. PBS only) to normalize EFG profiles and conception rate of RBS cows. The result shows that mOPN regained the normal endometrial EGF profile (56.1% vs 58.1% vs 23.8%) and improved the conception rate (43.5% vs 40% vs 23.8%) in RBS cows. Besides, there are approximate levels between seminal plasma OPN and milk OPN (Hay et al. 2022).

Conclusion

In summary, researchers on mechanism research and treatments of repeat breeding syndrome generally pay more attention to endocrinology and genomics nowadays. We can utilize multi-omics in all kinds of fluids of RBS and healthy cows in the future. In addition, a high-throughput mass spectrometric discovery approach can be used to unravel new information and potential candidate proteins for repeat breeding syndrome. Finally, the complexity of exosome biology of RBS deserves more investigation, which is beneficial for discovering new treatments.

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CHAPTER 28

PATHOGENESIS AND NUTRITIONAL REGULATION OF FATTY LIVER HEMORRHAGE SYNDROME IN LAYING HENS

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INTRODUCTION

Fatty liver hemorrhagic syndrome (FLHS), a disorder of lipid metabolism in laying hens, was first reported by Couch in 1956 as fatty liver syndrome (FLS) (Wolford and Polin 1972). It was later renamed as FLHS by Wolford, because hens with this disease often have varying degrees of hemorrhage in their livers (Wolford and Polin 1974).

The disease occurs in caged hens at the peak of egg production and is characterized by a marked decrease in egg production, sudden death of well-fattened hens, high body weight of dead hens, and severe liver fat deposition on autopsy with certain hemorrhagic features. The disease has become common in many countries, and it has been reported that 74% of the total mortality of caged hens in Queensland, Australia, is due to FLHS (Rozenboim et al. 2016). In addition, FLHS has been reported as the most common non-infectious cause of mortality in northern California, USA (Mete et al. 2013). With the rapid development in the global poultry industry and the expansion of intensive farming, FLHS occurs in chicken farms and professional farmers and has a tendency to increase year by year. The mortality rate is generally less than 5%, sometimes even up to 30%, which brings huge losses to the poultry industry. The disease is mostly disseminated, slow and not as obvious as other infectious diseases, so it has not attracted much attention from the relevant parties. In view of the serious economic losses brought by FLHS to the farming industry, we will elaborate on the disease from the causes and nutritional control, aiming to provide a more effective way for the poultry industry to prevent and control this disease.

Pathogenesis

FLHS is formed by a combination of many factors; nutritional, genetic, environmental, endocrine and toxicological factors are associated with the occurrence of FLHS. Among them, nutritional factors are considered to be the main factors by most researchers.

Nutritional Factors

High-energy, Low-protein Diets

The proportion of energy feeds in the diet is too large and carbohydrates that are not used up by the animals are easily converted to fat in the body. Zhang et al. (2011) found that dietary carbohydrates significantly increased the transcript levels of fat synthesis-related genes SREBP-1c and ChREBP-mRNA and also significantly increased ACC (acetyl CoA carboxylase) and FAS (fatty acid synthase) activity as a way to induce hepatic lipid deposition.

Low protein diets do not provide enough protein to synthesize apolipoproteins, so the fat in the liver cannot be transported out effectively, which leads to FLHS. Low protein in the diet may be one of the causes of fatty liver in laying hens.

High-protein Low-energy Diets

The proportion of protein is too high whereas the energy is too low in high-protein low-energy diets. The high proportion of protein in the diet reduces the proportion of corresponding energy to meet the requirements of laying hens so that some of the amino acids produced after protein decomposition will generate glucose to provide energy, and in this process, amino acid deamination produces large amounts of nitrogen, which synthesizes uric acid in the body's liver, thus increasing the metabolic burden on the liver, inducing or leading to fatty liver.

Energy and Protein Sources

It was found that the incidence of FLHS was significantly higher in laying hens fed with maize as an energy source than that in those fed with wheat as an energy source. It was found that liver fat content was significantly higher in the maize-soybean type diet group than that in the maize-fish meal type diet group, and that triglyceride content in the liver of laying hens provided with the maize/soybean meal was 30 to 50% higher than in those provided with the barley/soybean meal. In addition, the use of oats as a protein source significantly reduced the liver fat content and the degree of hemorrhage in FLHS laying hens (Cross et al. 1987).

Choline, Methionine, B Vitamins, VC, VE and Calcium Deficiency

The output of fat in the liver of laying hens depends on lipoproteins, and lipoproteins cannot be synthesized without choline. VB12, VC and VE can promote the formation of choline from methionine and betaine. Therefore, when these

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substances are deficient, lipoprotein synthesis is blocked and fat cannot be effectively exported from the liver, which in turn leads to massive accumulation of FLHS. The lack or deficiency of calcium in the feed during the peak egg production period makes laying hens to increase the intake as a way to meet the body's demand for calcium, which often leads to excessive energy intake and promotes the development of fatty liver.

Moldy Feed

Moldy feed can produce a lot of harmful substances, especially aflatoxin, which can cause serious damage to the liver of laying hens and lead to disorders of lipid metabolism in liver cells, thus causing fatty liver. It has been reported that the addition of 1.5 mg/kg of aflatoxin to the feed will cause a significant increase in the fat content of the liver of laying hens. In addition, T2 toxin in *Fusarium oxysporum* is also a cause of FLHS.

Drug Use or Poisoning

Some animal drugs can reduce hepatic lipoprotein synthesis by inhibiting hepatic protein synthesis, resulting in dysregulation of lipid metabolism. The antibacterial drug tetracycline is one of the drugs that can cause fatty liver. Tetracycline, in the process of inhibiting bacterial protein synthesis, also affects hepatic lipoprotein synthesis, causing blockage of hepatic fat output and therefore a large accumulation of fat to form FLHS. In addition, heavy metal poisoning, such as mercury, lead and arsenic, can inhibit protein synthesis and cause fatty liver (Schuman et al. 2000).

Genetic Factors

According to an earlier report, genetic analysis of 39 cases of FLHS laying hens showed that 11 laying hens were the offspring of the same rooster, indicating a strong genetic correlation of FLHS (Zhang et al. 2011). Moreover, the liver fat content of laying hens of different breeds or strains under the same feed and environmental conditions was not the same. In addition, inbred strains of single-crowned white Laihang hens (UCD-003) were more susceptible to FLHS, indicating a genetic susceptibility to the occurrence of FLHS (Schuman et al. 2000).

Environmental Factors

Caging

FLHS generally occurs only in caged hens. The intensive caging of hens results in a lack in exercise or insufficient exercise, leading to a dramatic reduction in energy expenditure and a decrease in fat mobilization, which, combined with excessive feeding and increased lipogenesis, can easily cause excessive fat deposition in the body, especially in the liver. As of 2018, cage systems are the dominant method of egg production in all major egg-producing countries/regions except the EU. Several metabolic disorders, including FLHS, are associated with cage systems.

Stress Response

Many stress factors, such as change of feed and feeders, change of flock, shock, injection of vaccine, hunger and lack of water, will cause the body to produce glucocorticoids, which will stimulate gluconeogenesis and promote the formation of fat, so that fat production in the body is increased.

High Temperature

FLHS in laying hens occurs mostly during the peak laying period in summer and less frequently in winter. Because laying hens have low energy requirements under high temperature, excess energy is easily converted into fat and stored in the body. In addition, high temperature can accelerate the synthesis of fatty acids, resulting in a large accumulation of fatty acids in the liver, which enhances the formation of fat. High temperature also reduces the function of the thyroid gland, resulting in reduced secretion of thyroid hormones and weakened lipolysis, which also contributes to the occurrence of FLHS. High temperature itself is a kind of heat stress, which can also contribute to this disease from the heat stress pathway.

Hormonal Factors

Estrogen, thyroid hormone, cortisol, etc., can affect the development of FLHS by altering lipid metabolism. Most researchers have shown that the estrogen level of FLHS laying hens is significantly higher than that of normal laying hens. On the one hand, estrogen stimulates fat production in the liver of laying hens and estrogen reduces the oxidative capacity of fatty acids in the mitochondria of liver. Choi et al. (2012) showed that exogenous estrogen could induce fat accumulation in liver of male chicks and the average liver weight of estrogen group was significantly higher than that of normal group, and serum TC and TG contents were significantly increased. In addition, serum thyroid hormone levels in FLHS hens are significantly lower than in normal hens. It is generally believed that thyroid hormone reduces body fat deposition by increasing basal metabolic rate and promoting lipolysis. Therefore, the decrease in thyroid hormone levels could also contribute to FLHS (Zhu et al. 2021). Environmental endocrine disrupting chemicals (EDCs) seriously threaten the health of chickens. Bisphenol A (BPA) is classified as an EDC. BPA can act as a selective estrogen receptor modulator (SERM). Exposure to BPA may influence de novo fatty acid synthesis through the increased expression of lipogenic genes, thereby contributing to hepatic steatosis (Marmugi et al. 2012). In addition, BPA could inhibit the insulin signaling and impair the liver insulin sensitivity (Batista et al. 2012). Gao et al. (2021) proved that BPA could aggravate high-energy and low-protein-induced FLHS of laying hens by promoting fatty acid synthesis and inhibiting fatty acid β-oxidation.

Glucocorticoid (GC) excess is another common feature of fatty liver, and clinical studies indicated that the chronically elevated GC level is associated with the occurrence of fatty liver (Targher et al. 2006). But corticosterone (CORT) is the main active form of GC in chickens, and CORT has been regarded as the valid indicator of stress. Researchers found that excessive CORT administration caused liver fatty degeneration, increased abdominal fat, which indicated the classic symptoms of fatty liver bleeding syndrome (Jiang et al. 2008). Further Hu et al. (2017) used exogenous injected CORT and successfully replicated the model of fatty liver hemorrhagic syndrome, which is manifested with mitochondrial dysfunction, lipid peroxidation and inflammation, thereby increasing the susceptibility of liver to more severe damages (Tang et al. 2013; Hu et al. 2017). Glucocorticoid receptor (GR) is the main receptor to accept the signal of corticosterone, and the actions

of GC are primarily mediated by GR (Hollenberg et al. 1985). GR is up-regulated in the liver of FLHS chickens, which indicated that GR is associated with FLHS (Hu et al. 2018; Hu et al. 2020). Corticosterone-induced FLHS could lead to demethylation of N6-methyladenosine (m6A) and post-transcriptional activation of lipogenic genes, which provides new sight in the molecular mechanism of FLHS pathogenesis in the chicken (Feng et al. 2021).

Oxidative Stress Factor

The prolonged accumulation of lipids in the liver results in the formation of lipid peroxides by peroxidation, which generates a large number of free radicals. Free radicals can act on liver membrane and organelle membrane, and also directly oxidize intracellular macromolecules, damaging cell function and integrity. Xing et al. (2020) found that the liver MDA (malondialdehyde) content of laying hens with FLHS was significantly increased and SOD (superoxide dismutase) and GSH-Px (glutathione peroxidase) were significantly decreased, indicating that the occurrence of FLHS was indeed related to oxidative stress. In addition, oxidative stress in the liver can cause endothelial dysfunction and alterations in coagulation and fibrinolysis, leading to hemorrhage in the liver.

Intestinal Flora Factor

The pathogenesis of fatty liver has developed from the previous theory of "double click" to the theory of "multiple strikes" (Tilg and Moschen 2010). The "multiple strikes" hypothesis is that multiple factors act on genetically predisposed individuals together, leading to the occurrence of fatty liver. In "multiple strikes", more and more experiments have shown that the change of the enteric-liver axis is related to the occurrence of fatty liver, and intestinal microbes have been confirmed as a key factor in the enteric-liver circulation, so the intestinal flora has a great relationship with the formation of fatty liver (Federico et al. 2016). Probiotics have been proposed to improve intestinal flora as a way to prevent and treat fatty liver (Guo et al. 2021).

Molecular Mechanisms

Although some research studies have been conducted on the pathogenesis of FLHS from various perspectives, there are still many challenges in understanding the pathogenesis of the disease, which affects the understanding and prevention of the disease. However, non-alcoholic fatty liver disease (NAFLD) in humans, which has similar pathological changes and clinical diagnosis to FLHS, may provide an insight into the pathogenesis of FLHS (García-Fuentes et al. 2002). According to the pathological histological description, NAFLD ranges from non-alcoholic fatty liver (NAFL), which is pure steatosis with fatty infiltration but no signs of hepatocyte damage, to non-alcoholic steato-hepatitis (NASH), which is pure steatosis with fatty infiltration but no signs of hepatocyte. The former is simple steatosis with fatty infiltration but no symptoms of hepatocellular damage, while the latter has symptoms of inflammation and swelling with or without liver fibrosis (Chalasani et al. 2018).

The seminal view of the pathogenesis of NASH is the "second strike" theory, which states that lipid peroxidation and inflammatory responses are caused by oxidative stress following steatosis (Day and James 1998). Later, researchers have found that there are layers subject to complex and "multiple strikes" in this process, including genetic susceptibility, biological environment, behavioral factors, metabolism, and gut microbiota (Buzzetti et al. 2016; Eslam et al. 2018). Current research studies on NAFLD involve the interaction of multiple cell types in the liver, where lipotoxic intermediates, reactive oxygen species, endotoxins and adipokines can drive the aggregation and signaling of immune cells (including Kupffer cells) and the activation of hepatic stellate cells, which in turn form fibroblasts, produce fibrogenic factors and collagen, and drive the development of cirrhosis through apoptosis (Schuppan et al. 2018). Thus, when the ability of the liver to process primary metabolic energy substrates (carbohydrates and fatty acids) is diminished, chronic oxidative metabolism is observed to enhance the production of reactive oxygen species, creating a pro-oxidant state, and this overall increase in the pro-oxidant/pro-inflammatory state leads to intracellular damage, with hepatocellular injury characterized by endoplasmic reticulum stress, apoptotic signaling pathways, and dysfunctional unfolded protein responses, which subsequently predispose to cirrhosis and hepatocellular carcinoma (Neuschwander-Tetri 2010, Friedman et al. 2018).

It is worth noting that the occurrence of fatty liver hemorrhage syndrome in laying hens is also closely related to insulin resistance. Insulin resistance is a defective metabolic response of target cells (such as muscle cells, hepatocytes and adipocytes) or the whole organism to hormonal influences. Insulin signaling begins with the binding of insulin to the insulin receptor (IR). Then, insulin receptor substrates (Insulin 3 receptor substrates, IRSs) undergo phosphorylation, which subsequently triggers the recruitment of phosphatidylinositol 3-kinase (PI3K) and the activation of protein kinase B (AKT) activation. Systemic insulin resistance means that the ability of insulin to lower blood glucose concentrations to appropriate levels is also hampered by disruption of the Glucose transporter 4 (GLUT4) receptor on the surface of the myocyte membrane, resulting in reduced glucose uptake (Wolford and Polin 1974). Among insulin receptors, Insulin receptor substrate 2 (IRS-2), upon activation, acts as a regulator of SREBP-1 and affects DNL (Luedde et al. 2014). In insulin resistance, IRS-2 is downregulated, SREBP-1 is overexpressed, DNL is upregulated, and β-oxidation of fatty acids is inhibited, thus further promoting hepatic lipid accumulation (Wree et al. 2016). Insulin resistance is a major feature of FLHS and is essential for lipotoxicity, oxidative stress and activation of the inflammatory cascade (Del Campo et al. 2018). Excess FFA in the body causes mitochondrial β oxidation, resulting in mitochondrial dysfunction and oxidative stress, which can inhibit insulin signaling and promote the release of inflammatory cytokines by activating NF-κB (Yang et al. 2019).

In addition, the main product of hepatic de novo lipogenesis is triglycerides and the liver is the main site of cholesterol and phospholipid synthesis (Piotrowska et al. 2011). It has been shown that β-estradiol-17-dipropionate can significantly induce hypercholesterolemia and hypertriglyceridemia. Once exogenous and endogenous lipids are secreted from the liver into the blood, they are transported to the ovaries as a component of lipoproteins. Fatty liver is usual for laying hens and occurs when increased lipogenesis exceeds the ability to synthesize and secrete lipoproteins (Shini et al. 2020).

Cidea and Cidec are effector factors inducing cell death. Peng et al. (2019) showed that FLHS significantly increased the

expression levels of Cidea and Cidec mRNA in liver and adipose tissue, and both could be lipid droplet-associated proteins that play an important role in promoting hepatic lipid accumulation and steatosis. In addition, high expression of Cidea and Cidec promoted lipid accumulation in mouse liver, but silencing Cidea and Cidec reduced hepatic lipids. In addition, AMPK pathway-related genes SERBP1a,1c could directly activate Cidea and increase the expression of Cidea mRNA, and Cidea can promote the expression of ACC and FAS, which are related to lipid synthesis.

Gao et al. (2019) study on AMPK signaling pathway of laying hens showed that in liver of FLHS laying hens, the mRNA expression levels of lipid synthesis-related genes ACC, FAS, GPAT and cholesterol synthesis-related genes HMGR and HNF4α were significantly increased, while the mRNA expression levels of fatty acid oxidation-related genes CPT1 were significantly decreased. It can be concluded that the changes in the expression levels of each gene in AMPK signaling pathway are involved in the occurrence of FLHS in laying hens. Therefore, AMPK signaling pathway plays an important role in the formation of FLHS in laying hens.

Diagnostics

Clinical Symptoms

When the chickens are in the peak egg-laying period, they appear depressed, lying down, drowsy and sometimes stand unsteadily. Laying hens with FLHS showed obvious pale and swollen crests and fleshy whiskers, sagging abdomens, and decreased egg production, which could not reach the peak of egg production. Laying hens often die suddenly without obvious symptoms, with high body weights of dead hens (Zhang et al. 2021).

Dissection and Histological Changes

Dead chickens were found to have increased subcutaneous fat. It was found that the entire abdominal cavity was deposited with a large amount of yellowish fat. The mesentery was also covered with more fat. Some chickens also formed thicker fat pad. The liver was grayish yellow and swollen, brittle and fragile, greasy and shiny in appearance. The surface was rich in oil-like droplets and accompanied by varying degrees of bleeder or plaque, and the abdominal cavity was accumulated with light red liquid, sometimes with blood clots next to the liver. When cut with a knife, the knife surface is attached with fatty oil droplets, the cut surface of the liver is raised, the liver lobule is filled with fat, blurred, and the structure disappears. The gall bladder appears enlarged and filled with bile (Tilg and Moschen 2010).

Microscopic observation showed a large number of fat vacuoles and obvious steatosis in the liver tissue. Fat droplets of varying sizes accumulate in the hepatocytes, and the nuclei are often crowded to the side of the cells by the fat droplets. The boundary between cells disappeared. The hepatic cord and sinusoidal structure disappeared, and the liver tissue was completely saturated with fat (Trott et al. 2014).

Nutritional Regulation

Adjustment of Energy-to-protein Ratios

The ratio of energy to protein in the diet of laying hens can be adjusted so that the ratio of protein to energy is 63 to 65:1. Too high or too low energy-to-protein ratios will cause FLHS in laying hens. For laying hens whose body weight exceeds the standard, the objective of preventing FLHS can be achieved by restricting feeding or reducing dietary energy (Wang et al. 2020).

Addition of Choline, Soy Phospholipids, Methionine, Carnitine, Biotin, VB12, Selenium and Calcium

Choline is an important substance in the body of laying hens. It is the precursor for the formation of lecithin. Lecithin is an essential raw material for lipoprotein synthesis, and sufficient choline helps to generate lecithin, which in turn synthesizes lipoproteins to help lipids transportation. Research studies have found that lecithin can significantly up-regulate the expression of the apolipoprotein gene apoB100 in the liver, increase the level of VLDL, improve the capacity of liver fat transport and reduce the occurrence of FLHS. Lecithin can also control appetite and prevent overeating. In addition, supplementation of soy phospholipids in the diet can effectively improve liver function and abnormal metabolism of blood lipids, and prevent the occurrence of FLHS (Yang et al. 2017).

Carnitine plays an important role in regulating lipid metabolism. It can help long-chain fatty acids to enter the mitochondria and promote the oxidation of fatty acids. In addition, the daily average egg production rate of the layers provided with biotin was significantly increased, and the liver fat rate and abdominal fat rate of the layers were significantly reduced. It may be that biotin improves the performance of laying eggs, so that the fat in the liver of laying hens can be continuously transported to the ovaries, reducing the deposition of liver fat.

Selenium has a protective effect on vascular endothelium. Adding a certain amount of selenium to the diet can significantly reduce liver bleeding in laying hens. When the hens are in the peak egg production period, enough calcium should be added to the feed to meet the body' needs of the laying hens and ensure normal egg production.

Change of Energy and Protein Sources

In the supply of layer diets, replacing corn with wheat and barley of equal energy, and replacing soybean meal with fish meal and yeast meal can reduce the occurrence of FLHS to a certain extent. Most researchers believe that when corn is used as an energy source for laying hens, the liver fat content and hemorrhage are much higher than that for laying hens which use wheat or barley as energy sources; when fish meal, yeast powder, etc. are used as protein sources, the occurrence of FLSH in laying hens is significantly lower than that of laying hens which use soybeans as a source of protein (Cross et al. 1987).

Addition of Vegetable Oil

Vegetable oil contains a lot of unsaturated fatty acids (PUFA), and PUFA has the effect of regulating lipid metabolism. Studies have shown that n-3 PUFA can significantly reduce liver fat (Peng et al. 2019). In addition, adding sunflower oil and linseed oil to the diet can significantly reduce the fat content of the liver of laying hens and inhibit the occurrence of FLHS. Davis et al. (2016) also found that adding flaxseed to the diet can inhibit the development of fatty liver to a certain extent and reduce steatosis.

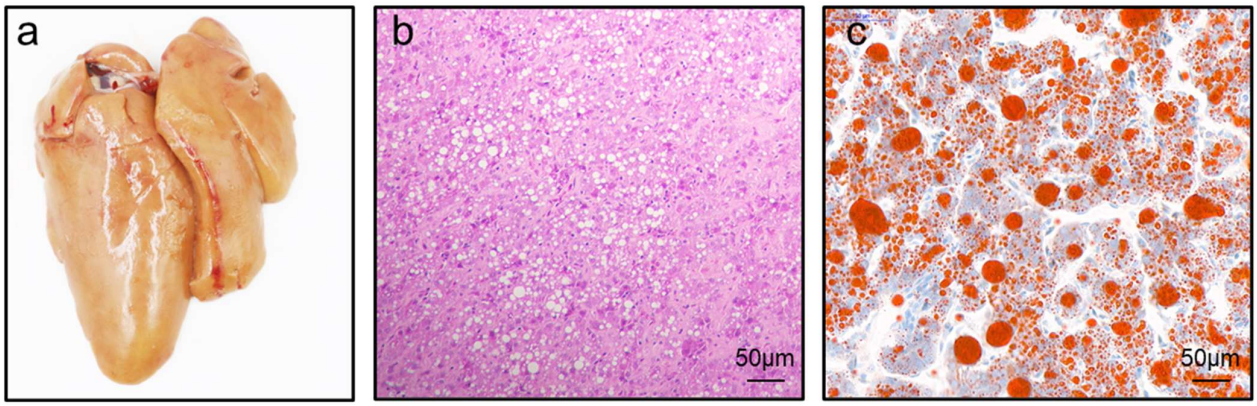

Fig. 1: Observation of liver tissue and sections of fatty liver in laying hens. (a) Morphological observation of liver tissue of fatty liver in laying hens. (b) Representative images of HE staining of fatty liver in laying hens. (c) Representative images of oil red O staining of fatty liver in laying hens

Prevention of Feed Mildew

Moldy feed contains a lot of harmful substances, among which mycotoxins can cause lipid metabolism disorders in the liver by damaging liver cells, leading to FLHS in laying hens. Therefore, during the feeding process, attention should be paid to moisture prevention and feed mildew, and dry and fresh diets for laying hens should be provided.

Addition of Antioxidants

The fat accumulated in the liver for a long time will undergo a peroxidation reaction to produce a large number of free radicals, which can cause damage to liver cells through oxidative stress. Therefore, adding antioxidants such as VC, VE, betaine, pentoxifylline (PTX), selenium, and dihydropyridine to the diet can effectively reduce the oxidative stress of liver cells, thereby reducing the occurrence of fatty liver (Diaz et al. 1994).

Addition of Probiotics, Metformin, Chitosan, Eucommia, Resveratrol, Flaxseed, etc.

Many studies have shown that intestinal microbes play an important role in the formation of fatty liver, and the different stages of fatty liver and fibrosis are closely related to the intestinal microflora (Yang et al. 2017; Zhu et al. 2021). Therefore, probiotics can be added to the diet to improve the intestinal flora and prevent the occurrence of FLHS. In addition, metformin can reduce lipid deposition in the liver. It can increase the activity of liver ATGL (lipase) and activate the AMPK signaling pathway. The activation of the AMPK pathway inhibits the expression of fat synthesis-related genes ACC and FAS (Sun et al. 2021).

Early studies have found that adding chitosan to feed can significantly reduce the triglyceride content of the liver of mice, as well as the fatty degeneration of the liver. Adding 10-20g/L of eucommia leaf decoction to drinking water can significantly reduce the fat content of the liver of laying hens. Xing et al. (2020) found that resveratrol could significantly reduce fatty liver lesions in laying hens, so resveratrol can be tried to control FLHS in laying hens. Schumann et al. (2003) showed that consumption of flaxseed can reduce the content of TC and TG in serum and liver lipid rate, reduce the mortality of laying hens, and have a better therapeutic effect on FLHS of laying hens.

Betaine a methyl donor, as an important component of the methionine cycle, could also effectively prevent FLHS of chickens. Betaine could be used as a methyl donor to relieve FLHS induced by corticosterone or high-energy and low-protein diet, effectively reduce abdominal fat and inhibit liver steatosis, reduce fat synthesis, and promote the β oxidation of fatty acids in liver (Leng et al. 2016; Omer et al. 2020).

In addition, daily management in the feeding process can be strengthened to ensure a peaceful and clean environment in the chicken house. Disinfect regularly and maintain good ventilation to avoid the accumulation of toxic gases in the house. Try to grasp the feeding density to ensure that each chicken has enough space for exercise. When it is hot in summer, pay attention to the cooling treatment in the house to reduce the occurrence of heat stress. At the same time, avoid choosing susceptible breeds when selecting breeds, check the flock regularly and cull those chickens that exceed 15-20% of their normal body mass, and check the diet from time to time to prevent chickens from accidentally eating moldy and toxic feed, which can increase the risk of FLHS. These are also key measures to reduce the risk of FLHS in chickens (Gao et al. 2021; Meng et al. 2021).

In the following Fig. 2, the authors summarize the nutritional regulation for the prevention and control of FLHS, with a view to providing reference for the prevention and control in production practice.

Conclusion

Investigation of the formation mechanism of fatty liver in laying hens can provide a fundamental solution to the problem. Most researchers believe that nutritional factors are the direct cause of FLHS in laying hens. The lack or imbalance in nutrients makes the lipid metabolism of laying hens unbalanced. The production of fat in the liver is more than the output, which continuously accumulates to form fatty liver. Other factors such as genetics and environment have accelerated or weakened the formation of FLHS to a certain extent. In recent years, many domestic and foreign scholars have found that AMPK signaling pathway is closely related to the occurrence of fatty liver. In the laying hens with FLHS, the gene expression in AMPK signaling pathway has changed. For example, carbohydrates can significantly increase the expression of fat synthesis-related genes. The expression of ACC and FAS can induce lipid deposition, so many factors may affect the AMPK signal

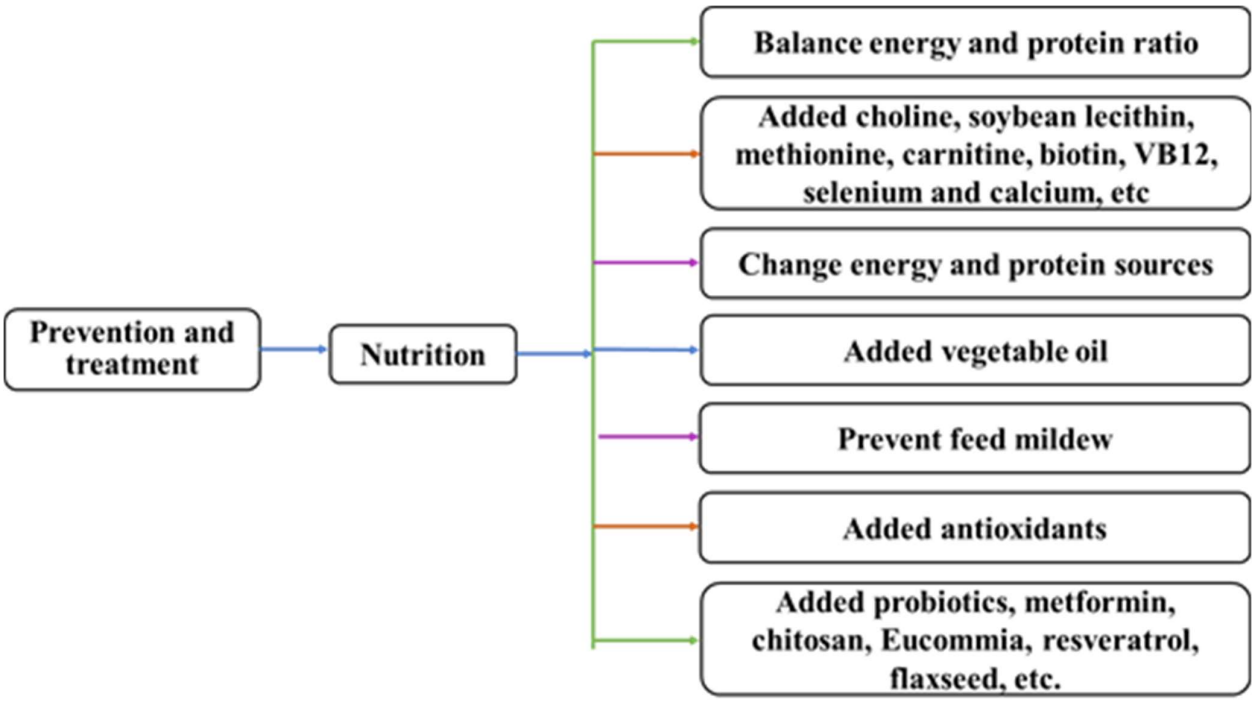

Fig. 2: Nutritional regulations to prevent and treat FLHS

pathway to cause the occurrence of FLHS. To prevent and treat FLHS, we can start with the AMPK signal pathway, and inhibit the occurrence of the disease from the molecular mechanism by activating the AMPK signal pathway. In addition, the relationship between AMPK signaling pathway and the occurrence of fatty liver still needs to be further studied.

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CHAPTER 29

METABOLIC DISORDERS OF POULTRY

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INTRODUCTION

The efficient production of poultry products like meat and eggs is mainly dependent on good health. If poultry birds are in poor condition, then there will be lower volumes of food products for the human population. Therefore, maintaining health and avoiding disease in the worldwide poultry business is a huge concern. Poultry health has a significant impact on the security of a vital food supply, human health, and worldwide economics (Aslam et al. 2020).

Some of the health issues in chicken are indubitably linked to modern society's demand for enormous quantities of poultry meat and eggs for human use. This necessitates intensive production, which entails growing large numbers of birds in limited spaces (Hafez and Attia 2020). Birds are inevitably stressed during their productive time because of big population numbers and high output. Stress is also exacerbated by extensive preventative medicine and vaccination. Stress, which is caused by exposure to external forces and situations, disrupts the body's equilibrium and leads to the onset of a variety of disorders. Metabolic or non-infectious disorders are an important group of stress-related diseases (Asfia et al. 2021).

There are a variety of metabolic illnesses in chicken, including sudden death syndrome, ascites, and fatty liver hemorrhagic syndrome. Metabolic disease is caused by a series of initiating stimuli that cause certain patterns of gene expression to be activated, causing biochemical equilibria to tip into non-homeostatic states. These non-homeostatic states, if left untreated, cause tissue degradation and loss of function in one or more organs, as well as signs and symptoms that lead to clinical diagnosis of disease (Angel 2007). Gene expression studies are the beginning to indicate that various sets of genes are expressed differently in metabolic illness tissues compared to healthy tissue. This altered pattern of gene expression could be caused by a number of factors including the environment, genetics, and nutrition (Mavroudis et al. 2018).

Unfortunately, metabolic problems will always be present in most poultry species to some degree. Since we have probably discovered all of the nutrients required by poultry during the last 50 years or more. Modern metabolic problems are rarely caused by overt shortage or excess of any nutrient or collection of nutrients. Skeletal problems are clearly caused by dietary shortages such as calcium, phosphorus, or vitamin D3, and so cannot be classified as metabolic disorders. Induced deficiencies, which occur when diets appear to be adequate in terms of total nutritional levels, are frequently referred to as a metabolic disorder (Brickley et al. 2005). However, in this chapter, with a few significant exceptions, diseases that are induced by nutrient deprivation are not considered as traditional metabolic disorders.

Fatty Liver Haemorrhagic Syndrome

Couch (1956) first described fatty liver haemorrhagic syndrome (FLHS) as excessive accumulation of fat along with haemorrhages in the liver. FLHS is a metabolic disorder that affects chickens all over the world (Shini et al. 2019).

Etiology and Pathophysiology

FLHS affects caged birds on high-energy rations, and it is most prevalent in the summer. The most typical complaint is sudden mortality of birds in full production. High-energy diets and limited exercise are linked to the illness (Robinson and Kiarie 2019).

In this condition, birds have pale comb. Bird's ovary is generally active but metabolic stress leads to oviposition which could be the reason of fatal haemorrhage. Fatty liver condition only appears in birds with a positive energy balance, hence body weight monitoring is a useful diagnostic tool (Crespo and Shivaprasad 2013).

Furthermore, FLHS is mostly seen in female birds. When the egg production starts, the level of estrogen in the blood increases as does the amount of fat in the liver. Administering estrogen to layers and even male birds could induce FLHS. This reveals that high-producing birds are more prone to FLHS because of more estrogen production from their active ovaries (Shini 2014).

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Clinical Findings

Affected birds from FLHS are generally found dead with no indications of illness. Due to diminished egg production or blood loss, affected birds frequently have pale combs. FLHS-positive layers have higher levels of leptin, oestrogen, and osteocalcin in their blood. In laying hens that already relies on a significant daily calcium flux in and out of the skeleton, there appears to be a comparable rise in bone turnover (Shini et al. 2019).

Lesions

Excessive fat in the liver is a key finding, along with variable degrees of bleeding. The liver is also frequently enlarged, has a "putty colour," and is friable. Large levels of oily fat are commonly seen in the abdominal cavity (Zaefarian et al. 2019).

Sudden death along with fat accumulation and liver bleeding in birds indicates FLHS. This FLHS is recognized during postmortem examination based on lesions like hepatic haemorrhages, enlarged and engorged liver (Yeh 2008). As a result, liver becomes friable. Pale yellow color liver is not a typical postmortem lesion in case of FLHS. Normally, layers fed with a lot of yellow maize or a lot of xanthophyll pigments have a yellow-colored liver with no haemorrhages. There are varieties of diet components that can cause liver bleeding without extra fat formation. Similarly, feeding rancid fat might result in hepatic bleeding without causing fat buildup. In FLHS-affected birds, the liver dry matter is at least 40% fat. Fatty liver disease affects the bird's calcium metabolism, thus compromising skeletal integrity and eggshell quality (Harrison and McDonald 2006).

Diagnosis

Keep an eye on bird's body weight and feed intake on each day. Replace carbs with supplementary fat while maintaining overall energy. Body weight and feed intake of birds should be monitored on daily basis because this condition usually occurs when there is positive energy balance (Crespo and Shivaprasad 2013).

Control and Prevention

When potential issues are identified, curative action such as the adoption of reduced energy diets and/or a modification in feed management should be considered. Furthermore, birds should be protected from high environmental temperature because it is also a predisposing factor in the development of positive energy balance (Wolford and Polin 1972).

FLHS is reduced by using byproduct feeds such as distiller's grains, alfalfa and fish meal. Supplementing with selenium has also been proven to lower FLHS, although the mechanism of action is unknown. When a farm has a history of FLHS, the diet should include at least 0.3 ppm selenium, preferably organic selenium, and up to 100 IU vitamin E/kg food, as well as adequate doses of an antioxidant like ethoxyquin. When layers are fed chelated trace minerals instead of inorganic minerals, there have been reports of a higher prevalence of fatty liver. However, there is no documented link between increased organic minerals in layer diets and the occurrence of FLHS (Rakshit 1981).

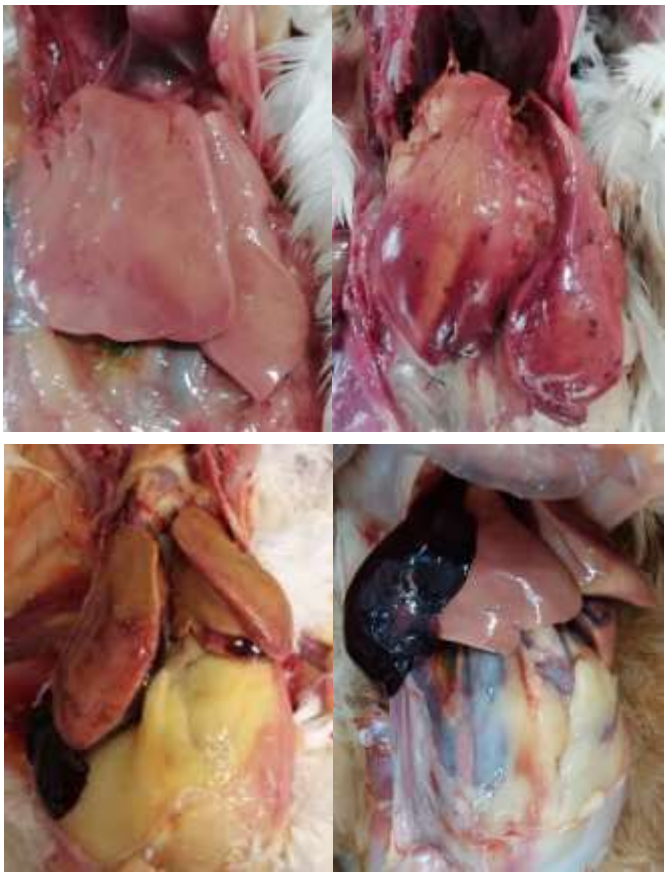


Fig. 1: Various phases of FLHS lesions in livers from varying degree of haemorrhages to hematoma

Gout (Urate deposition)

Visceral or articular gout or urolithiasis is common side effect of kidney failure. Renal failure decreases uric acid clearance from the blood, leading to hyperuricemia and the formation of insoluble products (monosodium urates) within the kidney or on surface of other visceral organs (Bulbule et al. 2013). These urates are semisolid and have white chalky appearance, and they should be distinguished from fibrinoid or purulent inflammatory exudates caused by infectious diseases such synovitis, peritonitis, perihepatitis, and pericarditis. In addition to visceral organs, these urates can also be observed in the synovial fluid and tendon sheaths of different joints, especially the hock joint (Mubarak and Sharkawy 1999).

Visceral gout

Visceral gout develops after rapidly progressing renal failure. In this condition, urates deposition occurs on the peritoneum, hepatic capsule, pericardium and joints. These urates are look like feathery crystals or spherical basophilic masses under the microscope. Infiltration of inflammatory cells is also observed (Pollock 2006). These visceral urates are usually formed in older layers after urolithiasis. Atrophy of the affected kidney is observed due to the obstruction of uroliths in the ureters. The other part of the kidney shows compensatory hypertrophy. Asymmetry in size and weight of both parts of kidney can be used during diagnosis. Meanwhile, the ureters are also distended due to accumulation of staghorn urates or uroliths which are white and brittle in nature (Mudasir et al. 2017).

Predisposing aspects in the development of visceral gout and urolithiasis are infectious and non-infectious causes. Cryptosporidiosis, infectious bronchitis virus, chicken astrovirus and avian nephritis virus are important infectious causes which intensify the visceral gout condition. Non-infectious causes are ingestion of more than 3% calcium, deficiency of vitamin A, and toxicity of mycotoxins such as oosporein, ochratoxin and citrinin. Ascites can also lead to the development of gout because hypoxic condition which arises in ascites induces higher production of uric acid. Aminoglycosides and heavy metals toxicity have also been reported to worsen the condition of visceral gout. Urolithiasis is commonly observed in Leghorn breed of birds, feeding more calcium diet for several weeks before sexual maturity. After 8-10 weeks of age, feeding 3-5 percent calcium, leads to the development of insoluble calcium-sodium-urate uroliths in the renal tubules (Pegram and Wyatt 1981).

Articular gout

Articular gout is less prevalent and develops as a result of persistent increase in the uric acid levels in the blood. Articular gout occurs in birds who are overfed with protein and/or have a K+Cl:Na ratio of less than 1. In articular gout, urates deposition occurs on the wing joints and toe synovial membrane, triggering a granulomatous reaction to urate crystals (tophi). Birds with inherited abnormalities in uric acid metabolism or those fed an excessive amount of protein may develop articular gout (Schlesinger 2004).

Urolithiasis appears to be the most common issue in laying hens fed high calcium levels well before sexual maturity. Although the problem is frequently complicated by IBV infection, it seems self-evident that Leghorn birds should be fed no more than 1% calcium before to maturity. Feeding pre-lay diets containing 2% calcium or layer meals containing 3% calcium for 2-3 weeks before laying the first egg is not an issue. Surprisingly, uroliths rarely occur in adult male breeders fed high calcium diets. High quantities of crude protein raise plasma uric acid levels, presumably creating conditions favourable to urate production (Perez-Ruiz and Lioté 2007).

Control

Numerous mycotoxins have been shown to affect kidney function, thus overall mill quality control and/or the use of feed additives to reduce their adverse effects would likely be advantageous (Imran et al. 2020).

Electrolyte therapy is frequently investigated since most forms of renal disease are linked with an increased loss of water and electrolytes. Potassium and, to a lesser extent, sodium salts, particularly citrates and bicarbonates, are suggested to overcome this issue.

Urinary acidification can be used to prevent or cure urolithiasis, and it can be done without necessarily causing a broad metabolic acidosis. Kidney dysfunction can be reduced nutritionally by avoiding overfeeding of nutrients like calcium, crude protein, and electrolytes (Pak 1991).

Ascites Syndrome

The accumulation of fluid in the abdomen which is non-inflammatory in nature is known as ascites or water belly. This condition is reported in 1970s in the fast-growing broilers. It's defined by an accumulation of transudate in the abdominal cavity, which is the result of a series of events linked to the requirement to supply sufficient oxygen to the tissues (Girmachew et al. 2020). Initially, the illness was particularly common in fast-growing male broilers kept at high altitudes with a degree of cold stress, but it now affects broilers at any altitude. In case of ascites, up to 8% death has been reported in extreme cases, while 1-3 percent mortality is now more common. Yellow protein clots may be present in the fluid which is present in the hepatic area, peritoneal cavity, or pericardial space (Scheele et al. 1991).

Causes of Ascites Syndrome

Increased sodium intake, lung or liver damage, vascular damage, increased vascular hydraulic pressure, increased tissue oncotic pressure, or decreased vascular oncotic (usually colloidal) pressure can all cause ascites, but it's most commonly linked to venous hypertension caused by right heart failure in response to increased pulmonary resistance (Julian et al. 1986).

Increased vascular hydraulic pressure in the venous system is the most common cause of ascites. This increase in pressure is most commonly associated with right ventricular failure (RVF), which is also related to hepatic fibrosis. The majority of cases are due to a hereditary susceptibility to pulmonary hypertension (PH), which causes heart failure and fatal ascites in many cases (Wideman Jr et al. 1999).

PH is common in birds due to hypoxia caused by high altitude, resulting in increased number of RBCs and thickening of the blood. This condition also develops as a result of sodium toxicity-induced RBC stiffness, and less commonly as a result of lung disease. Cold stress, even if just for a few minutes, during the first three weeks of life has been shown to increase the risk of developing ascites syndrome (Julian 1988).

Aflatoxin or toxins from plants like Crotalaria can induce liver damage in chicken. The most common cause of liver injury in broiler hens is obstructive cholangiohepatitis (induced by Clostridium perfringens infection), which leads to ascites. Amyloidosis of the liver can also produce ascites in both meat-type and breeder ducks (Julian 2005).

Pathogenesis

Increased pressure in the pulmonary arteries causes PH as the heart pumps more blood through the lungs to meet the body's oxygen needs. As a result of the volume and pressure overload, the right ventricle dilates and become hypertrophied, resulting in valvular insufficiency and ascites (Aftab and Khan 2005).

The lungs of birds are stiff and immovable within the thoracic cavity. To accommodate increasing blood flow, the capillaries can dilate to a certain limit. As meat-type chicken grow, their lung size will not increase in proportion to their body weight, especially muscle mass. In fast-growing broiler, increased blood flow causes primary pulmonary hypertension and cor pulmonale, as well as occasional occurrences of RVF and ascites (Biswas 2019).

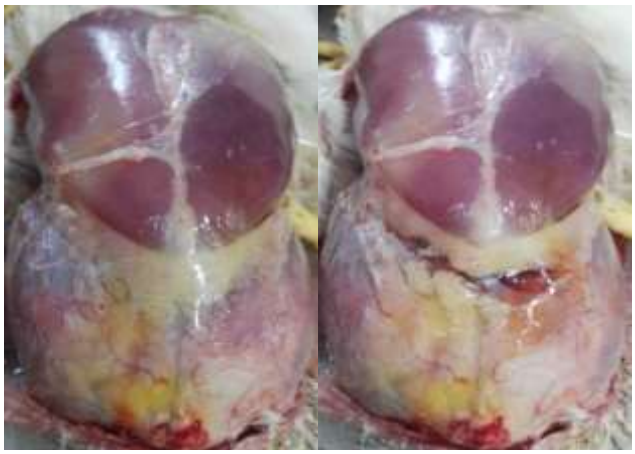
Clinical Findings

In ascites, affected broilers are cyanotic, their abdominal skin may be red, and their peripheral veins may be clogged.

Because growth is slowed as RVF progresses, so affected birds look smaller than their peers. Rapid growth rate, on the other hand, is a known predisposing factor, and the largest broilers are occasionally affected, with males being affected more frequently than females (Nain et al. 2009). Ascites causes an increase in respiratory rate and a decrease in exercise tolerance. Broilers that have been affected commonly die on their backs. Ascites is not present in all broilers who die from pulmonary hypertension syndrome. Death can happen unexpectedly and without showing any clinical signs (Wideman et al. 2013).

Lesions

The majority of lesions are caused by increased venous hydraulic pressure as a result of RVF. In the hepatoperitoneal spaces, clear yellow colored fluid is present along with fibrin clots. Liver may be edematous, congested, swollen and firm in consistency with clotted protein clinging to the surface (Baghbanzadeh and Decuypere 2008). Hydropericardium can range from mild to severe, and pericarditis with adhesions can occur rarely, mainly as a result of secondary illness. Right ventricular dilatation and mild to substantial right ventricular wall hypertrophy may be observed. In most cases, dilatation of vena cava and the right atrium has been observed. The left ventricle can narrow. The lungs are severely swollen and congested (Chapman and Wideman Jr 2001).



Diagnosis

Broilers with ascites as a result of RVF or PH have an enlarged heart, a thicker right side of the ventricle, and varied amount of clear fluid in the abdomen and pericardial sac. Even if there is no accumulation of fluid, the affected broilers have most likely died from PH (Baghbanzadeh and Decuypere 2008).

Control

Ascites produced by pulmonary hypertension syndrome can be avoided by reducing the bird's metabolic oxygen requirements by slowing growth or reducing meal density or availability. Temperature, humidity, and air movement in the environment should be managed to minimize excessive loss of body heat, especially in the early neonatal period. Even a brief exposure to cold stress during the initial weeks of life has been shown to predispose flocks to this problem (Julian et al. 1989). Ascites can be controlled by avoiding lung and liver injury and also by minimizing sodium level in the diet. For meat-type hens, altitudes greater than 3,000 feet (900 meters) are unsuitable, and growth must be controlled to avoid mortality. At higher elevations, extra care is required to avoid cold (Singh et al. 2011).

Round Heart Disease or Spontaneous Cardiomyopathy

Young turkeys with spontaneous cardiomyopathy die suddenly due to cardiac arrest and are unrelated to other poultry cardiomyopathies (Czarnecki 1984; Beaufrère and Brash 2021).

Etiology and Pathophysiology

In turkeys, the actual cause of spontaneous cardiomyopathy is unknown. However, experiments in turkeys employing furazolidone to induce dilated cardiomyopathy found that membrane transport was disrupted, resulting in heart failure. The amount of creatine kinase, glycolysis, glycogen, myofibril, Krebs cycle enzymes, fatty acid oxidation, and soluble proteins is reduced. The sarcoplasmic reticulum's calcium-transport ATPase activity is boosted. Ischemia may play a role in the pathophysiology of spontaneous cardiomyopathy in turkeys, based on this pattern of biochemical alterations (Zhang et al. 2013; Huang et al. 2000).

Although the majority of deaths occur during the brooding stage, the affected birds' heart weight to body weight ratio increases throughout the developing period. Because of the persistent cardiac insufficiency, the affected birds growth rate is slowed, resulting in attacks by their healthy peers. Body weights of affected turkeys are lowered by an average of 1.4 kg that survive to market age (Simpson et al. 2017).

Hypoxia is another important issue during egg incubation or transfer of chicks from hatchery to farm. It's probable that air stratification in poorly ventilated facilities without circulation fans, contributes to cardiac damage leading to later manifestations of the disease (Tintu et al. 2009).

Clinical Findings

The majority of deaths from spontaneous cardiomyopathy happen in the first four weeks of life, with mortality peaking at two to three weeks. Although many poults die immediately, some may have ruffled feathers, drooping wings, and a filthy appearance. Prior to death, they may exhibit laborious, gasping respiration. Mortality is rare after 3 weeks (Julian et al. 1992).

Lesions

The affected poult has a substantially enlarged heart due to dilation of both ventricles, congested lungs, and an enlarged liver in the first 4 weeks of life. There may be ascites, anasarca, pulmonary edema, and hydropericardium. In addition to dilatation, enlarged hearts in older poults are accompanied by substantial hypertrophy of the ventricles (Genao et al. 1996).

Congestion, damage to the myofibrils of the cardiocytes, and localized infiltration of lymphocytes are among the histological abnormalities of dysfunctional hearts.

In most cases, the diagnosis is made mainly on the history and gross abnormalities during necropsy; while an ECG can be used, but it is rarely employed. Similar disorders may be caused by sodium and polychlorinated biphenyls or related substances (Czarnecki 1984).

Treatment

There is no treatment available. Hypoxia has been linked to an increase in incidence during incubation, transportation, and brooding. Improved ventilation during these times appears to be crucial. According to some experts, high quantities of copper in the diet or drinking water have been linked to an increased risk of spontaneous cardiomyopathy. Good brooding methods may help to minimize mortality (Czarnecki 1986).

Sudden Death Syndrome

Sudden death syndrome (SDS), often known as "flipover" or "heart attack," is becoming a more common cause of death in healthy broilers having rapid growth (Crespo and Shivaprasad 2013).

Etiology and Pathophysiology

The etiology of this disease is undefined. However, it can be a metabolic condition which prompts the cardiac arrhythmia in birds. It is assumed to be a metabolic disorder linked with lactic acidosis, carbohydrate metabolism, intracellular electrolyte imbalance and loss of cell membrane integrity. Current investigations linked this disorder to cardiac arrhythmias. It has also been found that occurrence of arrhythmias to be 27% in broiler and 1% in leghorns; however, it is uncertain whether this susceptibility is genetic or dietary (Olkowski 2007). SDS mortality in affected flocks has been observed from 3 to 15.6 percent. It normally affects 1-5 percent of the flock and is the leading cause of death between 21 to 35 days. Affected birds look healthy and have plenty of flesh. SDS is most common in males (Ononiwu et al. 1979; Siddiqui et al. 2009).

Clinical Findings

In SDS there is no definite clinical signs and gross lesion. Infected birds having specific microscopic lesions in subendocardial purkinje cells and cardiomyocytes. These lesions in heart may support in the diagnosis of this disorder (Proudfoot et al. 1982).

In terms of gross pathology, there aren't many alterations. On opening of the heart, atria contain post-mortem blood clots while ventricles are normally empty with slight hypertrophy of left ventricle. The lungs are frequently oedematous, however this usually happens when birds lie down on their backs after death and fluid drains to the lung area due to gravity. Other changes are empty gall bladder, dilated intestine with creamy contents and fresh feed in the digestive tract. In some cases of SDS, enlargement of liver and kidney has also been reported along with varying degree of pinpoint hemorrhages (Chung et al. 1993).

Potential Treatment and Prevention

There is no treatment of SDS. Through preventive measures we can limit the incidence of SDS. SDS can be prevented by varied degrees of nutrient restriction. There are no obvious correlations between the development of SDS and any of the diet components, additives, or climatic influences. When ionophore anticoccidials are utilized (perhaps because of the accompanying faster development rate), or if the food comprises a readily available carbohydrate source such as glucose, the condition appears to be more common. SDS can be developed intentionally by dietary lactate, however the onset time can be regulated by food (Rotter et al. 1985; Imaeda 2000).

Inducing a phase of initial slow growth is the simplest way to avoid or minimize the incidence of this syndrome. Reduced day length, limited feed intake, and/or the adoption of low-nutrient rich diets can all help to overcome this problem. A more feasible strategy is to utilize feed with a 5-7 percent drop in nutritional density, and growth-promoting substances in the beginning. With greater attention in genetic selection, the growth rate and meat yield in broilers have improved in recent years (Newberry et al. 1985, Pass 1983).

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CHAPTER 30

DIABETES MELLITUS IN CATS AND DOGS: CLASSIFICATION AND ETIOLOGIES

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INTRODUCTION

Diabetes Mellitus (DM) is referred as a group of metabolic dysfunctions resulting from impaired synthesis or decreased insulin sensitivity and characterized by hyper-glycemia, hyper-lipedemia and hyper-chloestremia (Khan et al. 2013; Elimam and Baragob 2015). Diabetes is considered as a chronic disease which can affect humans, dogs and cats and other animals such as apes, pigs and horses. In veterinary field, the classification of DM is based on human DM and mechanism. Four types of DM has been described; DM type-1 (insulin deficiency diabetes), DM Type-2 (insulin resistant diabetes), gestational DM and specific type DM (American Diabetes Association 2021). In DM type-1, the pancreas is damaged or incapable to produce enough insulin while DM type-2 occurs when the enough insulin is produced, but body is failed to utilize the insulin due to exhaustion of insulin receptors on cells. Diabetes mellitus is one of the most common disorders among dogs and cats where a prevalence of 0.4-1.2 % has been reported. type-1 DM is particularly more common in dogs as compared to cats and is associated with an absolute insulin deficiency as a result of immune mediated destruction of pancreatic β-cells. Furthermore, gestational DM has also been reported in dogs whereas there are no case reports in cats. However, cats are generally more susceptible to DM type-2 as it accounts for approximately 90% of all the cases in cats (Gottlieb and Rand 2018). The most significant clinical signs associated with DM generally include polyphagia, polydipsia, polyuria and sudden loss of weight. The clinical signs are generally not evident until the blood glucose concentration surpasses the threshold level of glycosuria which is 180-220 mg/dL in dogs whereas 220-270 mg/dL in cats. The likelihood of prediabetic or subclinical disorder is generally rare in dogs and cats unlike humans. It's diagnosis is basically made on basis of typical clinical signs, glycosuria and persistent hyperglycemia (Nelson and Reusch 2014). Most importantly, late diagnosis or delayed therapeutic intervention may result in further complications such as hypercholesterolemia and hypertriglyceridemia followed by ketoacidosis and ketonuria (Gottlieb and Rand 2018). Contrary to humans, there is no elaborated consensus in the veterinary literature on the prevalence and pathobiology of different types of diabetes in dogs and cats. In this chapter, the pathology of diabetes with the prevalence in dogs and cats is discussed along with the associated risk factor.

Diabetes Mellitus in Cats (Feline DM)

The prevalence of feline DM varies from 0.25 to 1% (1 in 400 to 1 in 100) (0.25%) according to the studied population and area. Feline DM shows the similar characteristics of clinical and pathological signs as exhibited by humans. Type-2 DM or Non-insulin dependent diabetes limited to persons having characteristics such as obesity and median to older age following low insulin level or the accumulation of amyloids in pancreas and damaged beta cells (β-cells) that consequently lead to retinal and neuronal complications (Niaz et al. 2018). Feline DM is analogous or like different types of human DM and most commonly, in felines type-2 DM persists. Beta cells in healthy cats are capable of responding to the fluctuating pattern of insulin requirements in the body and stimulate the more insulin secretion in case of increased demand (Ahren and Pacini 2005). Factors related with type-2 DM, hamper the ability of insulin secretion (Ahren and Pacini 2005; Alejandro et al. 2015). Mechanisms that damage the beta cells lead to an overall reduced potential to proliferate to catch up the higher insulin demand of body, faulty or decreased insulin production, insufficient insulin gene expression followed by uncontrolled beta cell death. Long term hyperglycemia generally results in a continuous cycle of progressive loss of insulin production (Poitout and Robertson 2008; Link et al. 2013). Other specific types of DM involve all other causes of DM. In cats, it can be associated with loss of pancreatic cells by neoplasia or pancreatitis (adeno-carcinoma is reported in 8–19% of euthanized animals). More than 60% of the diabetic cats, pancreatitis may be present based on bio-chemical and imaging results (De Cock et al. 2007; Caney 2013; Zini et al. 2015). In literature, It is reported that pancreatitis alone does not only contribute severely to cause DM but also enhances the beta cell destruction and probability of DM remission (Zini et al. 2015). The other specific types of DM may include increased insulin resistance in hyper-somatotrophism (acromegaly) and hyperadrenocorticism (Cushing syndrome)(Niessen 2010; American Diabetes Association 2021) in which the use of prescribed insulin doses are not enough to control the blood glucose level (Niessen 2013).

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Table 1: Major risk factors associated with the development and etio-pathogensis of diabetes mellitus in cats and dogs

Cats	Dogs
Immune mediated insulitis	
Hyperlipidemia	
Islets amyloidosis	
Obesity	
Pancreatitis	
Genetic (breed like Burmese cats)	Genetic
Concurrent hormonal diseases	Concurrent hormonal diseases
1. Acromegaly	1. Hyperthyroidism
2. Hyperthyroidism	2. Diestrus-induced excess growth hormone
	3. Hyperadrenocorticism
Drugs	Drugs
4. Progestogens	6. Progestogens
5. Glucocorticoids	7. Glucocorticoids
Infection	Infection
8. Heart diseases	11. Cardiac and renal diseases
9. Renal diseases	12. Hepatic disorders
10. Concurrent illness	13. Concurrent illness

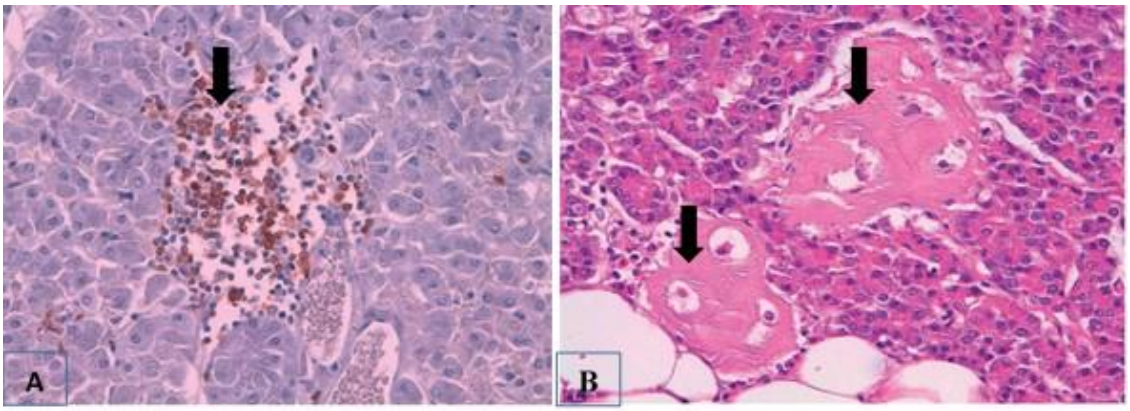

Figure 1: Histomicrograph of diabetic cat: A; Pancreatic beta cells showing infiltration oflymphocytes (black arrow) in 18 year-old hyperglycemic rats (A). Immunohistochemistry for CD3, counterstained with H&E (40 X). B; Islet amyloidosis (black arrow) in a sixteen year old female spayed cat with diabetes mellitus (B) (Hematoxylin & Eosin, 40 X). (Nelson and Reusch 2014)

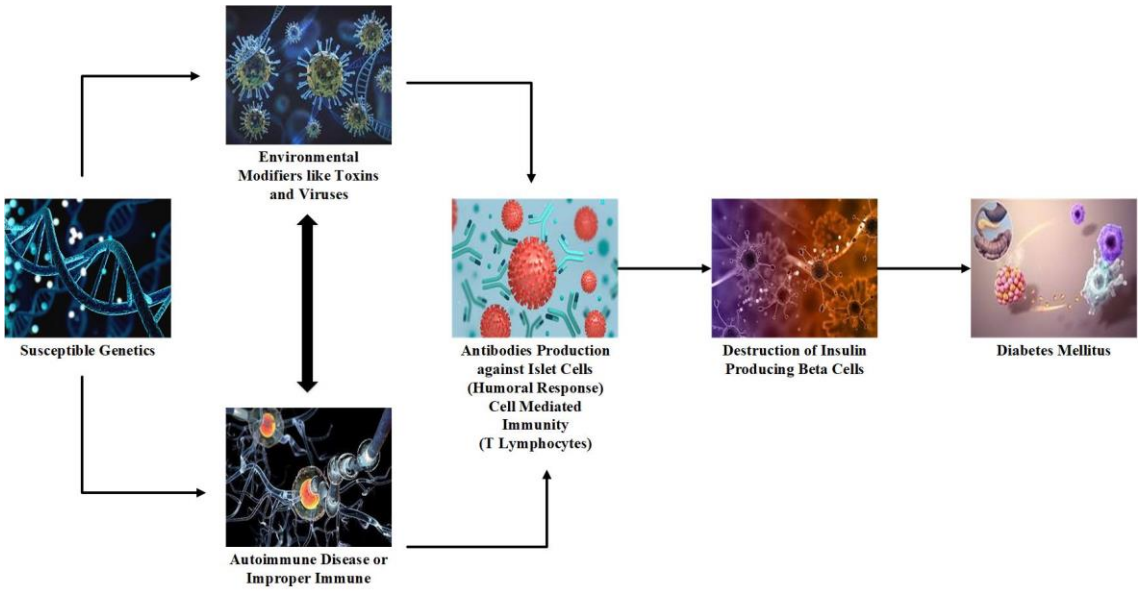

Figure 2: Genetic susceptibility and Development of diabetes Mellitus.

Pathogenesis of DM in Cats

Feline DM can also be classified under human classification system as they develop different types of DM related with acromegaly, hyper-adrenocorticism and pancreatic carcinoma (Rand 2013). Infiltration of inflammatory cells in the beta cells (Codner et al. 2012), commonly seen in human type-1 DM, is rarely seen in cats (Figure 1). Mostly the level of insulin is too low to diagnose that cannot be detected in diabetic cats (Zini et al. 2016). These results showed that fasting hyper-insulinemia is

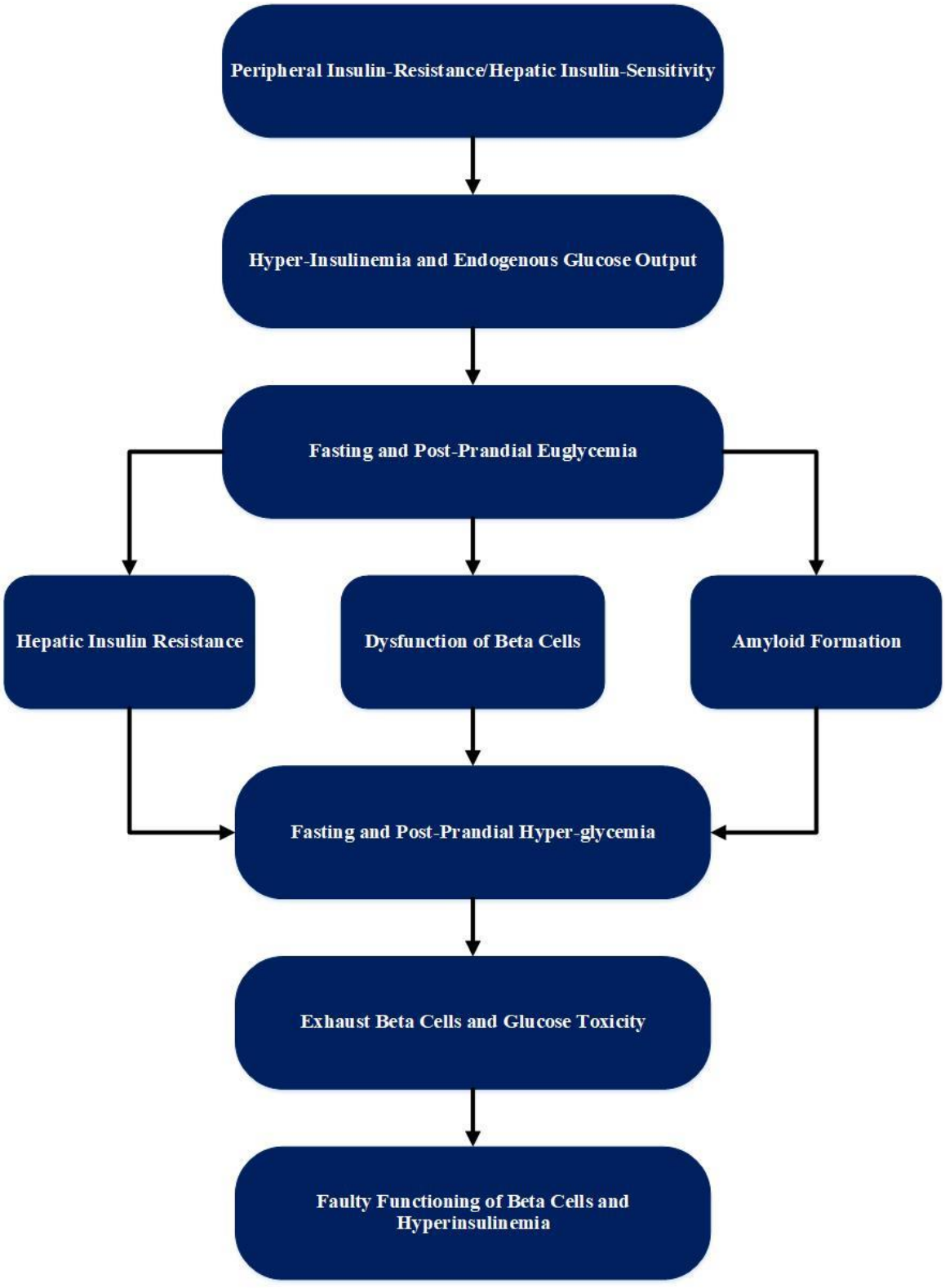

Figure 3: Sequential events in the development of Type 2 diabetes from obesity in cats

not found in case of overt diabetes in cats unlike what is observed in hyper-glycemic patients during early stage of type-2 DM in which the levels of fasting insulin are usually seen elevated in comparison to healthy individuals (Hecking et al. 2013). This fact can be linked with the more rapid beta cells destruction in cats than the humans, however, insulin deficiency is not permanent character in many cats and beta cells are seen to recover to a small degree that enables them to maintain normal glycemic level. The remission rate of diabetic cats has been reported to vary between 25 and >80%, supporting the ability of beta cells to recover (Hoenig 2014; Zini et al. 2016). The exact understanding of insulin resistance or exhaustion of its cellular receptor and their relationship with different factors are not yet clear. In obese cats, glucose transporter (GLUT4) expression on insulin sensitive cells (muscles and fat) were seen significantly lower compared to non-obese cats, whereas the expression of GLUT1, not-insulin sensitive, remained unchanged. Expression of different genes of

insulin signalling in hepatic and skeletal muscles was observed to be lower in obese than in lean cats, identical to humans with insulin-resistance (Nelson and Reusch 2014). The fat cells in cats generally act as complex endocrine glands like humans that secrete adiponectin which decreases in obese condition. Adiponectin improves sensitivity of insulin and also possess strong anti-inflammatory characteristics; and a decline in it, therefore, can contribute to development of insulin resistance and inflammation (Radin et al. 2009). The fat tissue is involved in secretion of adiponectin along with different types of proinflammatory cytokines and obesity is thus regarded as a low grade chronic inflammatory state. It is reported that insulin resistance developed in response to obesity may revert after decrease in the body weight. Though obesity induces development of DM in cats but not all obese cats are susceptible to DM. Beta cells dysfunction leading to impaired glycaemic control is the main pre-requisite for diabetes development in cats. Beta cells dysfunction occurs as a result of amyloid deposition in cats as well as in humans whereas nonhuman possess an amyloidogenic amino acid based structure that potentiates the formation of amyloid aggregates within the pancreatic islets (Hull et al. 2004; Henson et al. 2011). It is generally unclear why some but not all hyperglycemic cats develop amyloid aggregates and whether its accumulation could lead to disease development. Hyperglycemia is known as an additional potential factor, which has a definite negative impact on the physiological performance of beta cells and survival in cats, a mechanism generally known as glucotoxicity (Link et al. 2013). However, the cellular mechanisms involved in insulin sensitivity and its impaired secretion through chronic hyperglycemia are poorly understood.

Diabetes Mellitus in Dogs

Type-1 DM

Clinically type-1 DM is diagnosed more frequently in dogs in which the patient is totally dependent on the exogenous insulin. Specially dogs have adapted to diets like grains and vegetables. Dogs, like cats, don't have salivary amylase however amylase, secreting from the exocrine part of the pancreas, is present in sufficient amounts and are able to digest starch (Murray et al. 2001; Hoenig 2014). Generally, at the time of DM diagnosis, most dogs are found to be insulin dependent. The histological investigation revealed a decline in pancreatic beta cells population and an overall reduced size of pancreatic islets. In addition, the pancreatic beta cells appeared to be degenerated and vacuolated in the DM affected dogs. The juvenile dogs are considered highly susceptible to the lethal form of DM where the pancreas becomes totally deficient of beta cells along with hypoplasia or aplasia of pancreatic islets. Adult dogs with mild changes in pancreatic islets and beta cells are more likely to develop DM after exposure to some environmental factors like insulin antagonistic conditions, pancreatitis and various drugs. Numerous reports have highlighted the role of immune mediated insult in development and occurrence of DM, particularly in dogs. In DM affected dogs, immune mediated insulitis is identified to be based upon cellular infiltration by inflammatory cells in pancreatic islets along with evidence of immunoglobulins against islet cells, insulin, insulinoma antigen and intracellular glutamic acid decarboxylase. Therapeutic control of diabetes and an extensive life-long insulin therapy is crucial for maintenance of a normal diabetic state.

type-2 DM

Insulin resistance in obese dogs has been reported but it seldomly develops type-2 DM in the dogs. Some of the etiopathogenic factors involved in the development of type 2 DM in human beings and cats are generally non-existent in dogs. Some mechanisms such as sensitivity of pancreatic beta cells to variations in glucose concentration and beta cell's derived insulin secretory response have been lost in human beings and cats unlike dogs despite of obesity years leading to induced insulin resistance and compensatory insulinemia (Hoenig 2014; Nelson and Reusch 2014).

Gestational Diabetes in Dogs

This type of special DM is found in human model diagnosed firstly as carbohydrate intolerance with the onset of recognition or during gestation period (American Diabetes Association 2021). A similar condition reported in old female dogs but not in cats. Due to long oestrous cycle, the female dogs ovulate almost seven months apart leading to the elevated level of progesterone after the formation of corpora lutea. Elevated progesterone stimulates the secretion of growth hormone form mammary glands which results in causing the carbohydrate intolerance and insulin resistance in older female dogs. The female dogs diagnosed with gestational diabetes are frequently observed with elevated level of progesterone and growth hormone. Documenting increased baseline blood insulin concentration supports the presence of functional beta cells. These dogs presumably have a suitable mass of intact beta cells to maintain carbohydrate tolerance when insulin-resistance is not present (during the periods of ovarian inactivity when progesterone level remain low (0.5 ng/ml) but they are unable to secrete a satisfactory amount of insulin to maintain euglycemia in the presence of insulin resistance (Fall et al. 2008). Early diagnosis and ovariectomy lead to improve the insulin resistance, while some functional beta cells are present, may restore normal glycemic level without the use of any insulin therapy. Failure in early diagnosis and correction of insulin-resistance may result in loss of beta cells function greater likelihood for long-term dependency of insulin therapy for glycemic control.

Risk Factors

Different factors that are involved in the development of DM includes obesity, lack of exercise, old age and the use of different drugs like glucocorticoids and progestins. It has been reported that males are more prone to DM than females. Furthermore, obese cats are at four times more risk of contracting DM as compared to other cats (Slingerland et al. 2009; Nelson and Reusch 2014). Sensitivity of insulin receptors differs significantly among the individuals which is directly related with the development of DM. Cats and dogs with low insulin sensitivity along with increasing age are at a significantly higher risk of contracting hyperglycemia with weight gain. The male cats having an overall lower insulin sensitivity, when subjected to a feed trial resulted in a comparatively more weight gain as compared to female cats which could be a reason of a higher risk of DM development in male cats. At cellular level, the underlying mechanisms of development of insulin resistance and its interrelations specially with gender are not yet clearly understood. Phenotypes of specific breeds result

from selective-breeding for chosen characteristics like morphology, texture and coat colour along with body size. The desired breed specific characters are frequently accompanied by susceptibility to genetic disorders in comparison with out-bred populations. In cats and dogs, inbreeding coefficients and extensive linkage disequilibrium are reported which are more prone to genetic disorder (Figure 1). Detailed characterization of the genetic and other risk factors involved in the pathogenesis of DM and their interrelations are not yet clear with their underlying mechanism and detailed research work is needed yet to explore this issue (Samaha et al. 2020).

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CHAPTER 31

KETOSIS IN DAIRY ANIMALS

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INTRODUCTION

Ketosis is also known as acetonemia characterized by the presence of ketone (acetone, acetoacetate and 3-hydroxybutyrate) compounds inside the blood and these compounds are eliminated through urine and milk. Generally, ketosis is a metabolic disorder, but in dairy animals it is a lactation syndrome correlated with high milk yielding cows having negative energy balance. Ketosis occurring in the dairy animals is of two types i.e., clinical, and subclinical with signs and without signs respectively.

The transition period (three weeks before and after calving) is important one in high milk producing dairy cattle. During this period (late gestation and early lactation), there is need of more energy. National Research Council also recommended that sufficient nutrients should be included in the diet of animals particularly in the form of bypass nutrients (Marghazani 2012; Das et al. 2014; Gyanendra et al. 2019). To avoid from environment stress, more care should be practiced during last three weeks of pre-partum in dairy animals (Gerloff 2000). It is found that due to intake of energy deficient diet, the huge metabolic burden shifted on the liver due to import of non-esterified fatty acids (NEFA). NEFAs are liberated out from fat stores (triacylglycerol) due to negative energy balance or shortage of glucose. The more concentration of NEFAs into the liver led to increase in oxidation process that finally buoy up liver-connected diseases i.e., ketosis and hepatic lipidosis. Taken together, more acceleration in the gluconeogenesis contributes glucose utilization by the mammary glands (lactose synthesizes) that in turn cause stress in the hepatic tissue of early-lactating animals (Ringseis et al. 2015).

There are two main types of ketosis i.e., clinical with visible signs and subclinical without signs (Youssef et al. 2010). There are many factors involved in the establishment of ketosis during lactating period including body condition score and release of fat during calving (Busato et al. 2002). Ketosis is not considered as life threatening disease (death uncommon) but its negative impact on high yielding dairy cattle have been observed that includes decreased milk production, increased culling rate of early lactating animals, occurrence of displaced abomasum, chances of metritis, less fertility rate, and economical losses (Rajala-Schultz et al. 1999; Oetzel 2013; Steeneveld et al. 2020). More frequency in cases of ketosis, even at normal level imparts overall impact on economic losses to the dairy industry.

Classification of Ketosis

Generally, there are two types of ketosis primary and secondary which differs on the basis of disease source (Herdt 2000). Most of evidence show the occurrence of ketosis in starting days of calving or during early lactating phase (McArt et al. 2012). The terms subclinical and clinical are also commonly used for ketosis classification. Clinical form of ketosis is manifested by increased ketones in biological fluids (blood, urine, milk) along with other symptoms including appetite, decreased weight, and dryness of dung. While the subclinical form of ketosis shows high level of ketones in the blood, milk, and urine, along with absence of major signs. The reason is loosed-type housing system in majority of dairy forms which causes difficulty in observing the signs in specific or individual animal. It was thought that clinicalor subclinical forms of ketosis should be according to the b-hydroxybutyrate (BHB) level inside the blood. Though, it was experienced after careful observation of the animals that increased ketonemia condition did not show clinical signs. Each animal tolerance to clinical ketosis were different and accordingly some animals show clinical signs whilst others may not show (Herdt 2000). It is long-established that term hyperketonemia is best for description of ketosis rather than its clinical or subclinical forms.

Biochemistry and Cycle of Ketones Production

The production source of ketones is associated with fatty acids and amino acids from the fat reservoir tissues in the body and feed (Coelho et al. 2013; Kohlmeier 2015). The process of ketogenesis happens in mitochondria of hepatocytes. Adipokine signal triggering the release of fatty acid from the fat reservoirs results in increasing level of glucagon and epinephrine and decrease in the concentration of insulin. Such happening led to development of hypoglycemic and fasting states (Owen 2005). Coenzyme A is attached with fatty acids and transported to mitochondria. Fatty acids through beta-oxidation splits 2 carbons from the acyl-CoA compound in each cycle for the formation of acetyl-CoA. This acetyl-CoA transported into citric acid cycle for the formation of citric acid, and then citric acid incorporated into the tricarboxylic acid cycle (TCA), finally high energy produced (Stryer 1995). These mechanisms are shown in flow diagram (Figure-2). Every cell of the body can metabolize the acetyl-CoA by TCA cycle,

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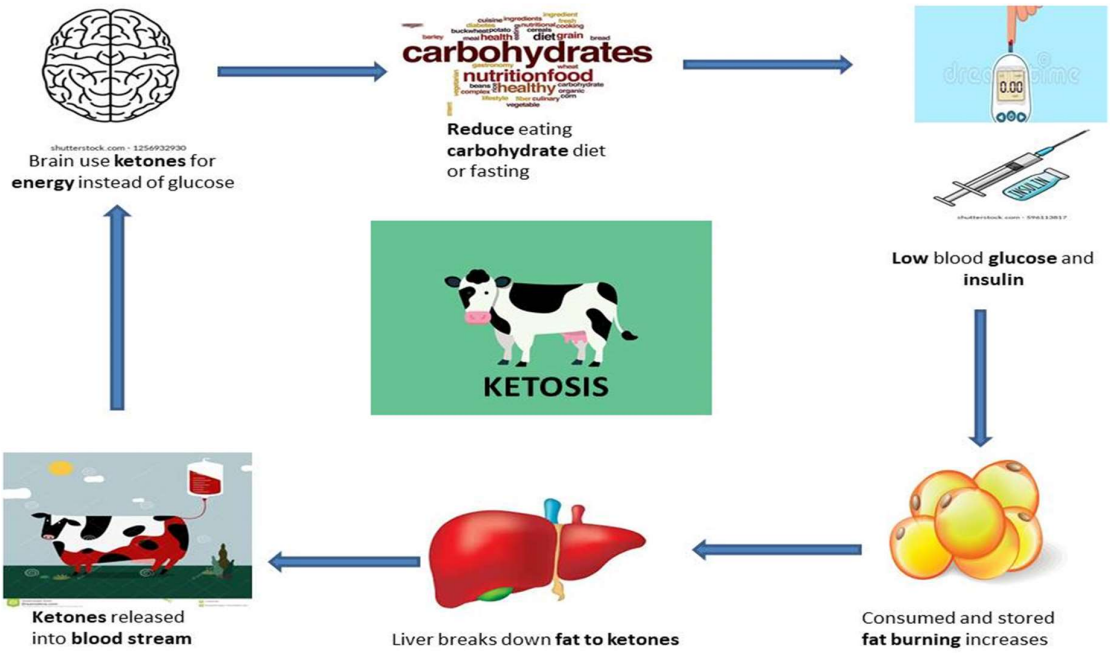

Fig. 1: Factors leading to Ketosis in dairy animals (Zhang and Ametaj, 2020).

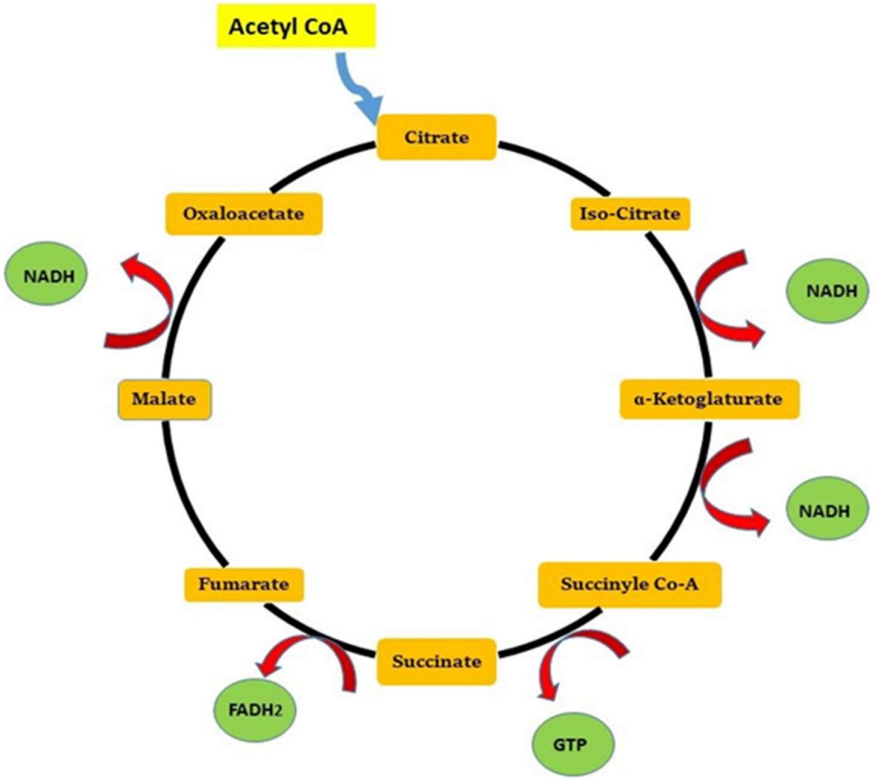

Fig. 2: Kreb's Citric Acid Cycle (Tri-Carboxlic Acid Cycle): (Martínez and Chandel 2020; Protasoni and Zeviani 2021)

but it is also involved in ketogenic process in the hepatocytes (Laffel 1999). During decreased level of glucose inside the blood, oxaloacetate intermediate compound of TCA cycle transported and is utilized in the production of glucose (gluconeogenesis). Oxaloacetate is used in gluconeogenesis prevent its condensation with acetyl-CoA, hence no entrance of acetyl-CoA into the TCA cycle. In nutshell, the energy was generated in the form of ketones from acetyl-CoA. In ketone occurring process, acetyl-CoA 2 molecules converted into acetoacetyl-CoA by condensation with the help of thiolase. In short, acetoacetyl-CoA plus a new molecule of acetyl-CoA produced the hydroxy-β-methylglutaryl (HMG)-CoA with the help of HMG-CoA synthase. HMG-CoA converted into acetoacetate (ketone body) with HMG-CoA lyase enzyme. D-β-hydroxybutyrate type of ketone formed from acetoacetate through D-β-hydroxybutyrate dehydrogenase action. Due to

result of continuous degradation of acetoacetate, acetone (ketone) and CO_2 produced. In the end, ketone compounds not used as fuel of energy by the liver but transported as energy source towards brain and other tissues of body. Primarily ketones not only produced from fatty acids, but also produced from ketogenic amino acids. These amino acids are converted into the intermediate compounds of citric acid cycle through deamination (Kohlmeier 2015).

Causes and Factors Involved in Ketosis

In context of particular biochemical and physiological etiology of ketosis, no proven data is available till date. There is no single cause, while it is well established that misbalancing in nutrient availability, particularly low energy, is a crucial factor. It is proved that glucose deficiency is associated with the occurrence of ketosis during early lactation phase of dairy cattle. Because 60-85% glucose is utilized by the udder for the collecting lactose contents in milk (Reynolds 2005). Deficiency in ACTH or cortisone hormones is also one of the causes of ketosis that cause pathological changes in adrenal gland (Dressler et al. 2019). Another possible cause of ketosis is the deficiency of oxaloacetic acid (OAA) in hepatic tissues (Bach 1978).

Some risk factors are associated directly or indirectly with occurrence of ketosis. Decreased milk yield and production is affected by the presence of ketones inside the body fluids (Dohoo and Martin 1984). Reported reproductive pathological conditions also associated with ketosis (Dohoo and Martin 1984). Ketotic dairy animal were seen with reduction in conception rate during first artificial insemination shot. This may help in culling of dairy animal at early phase of milking (Oetzel 2013). High milk producing ketotic animals had chance of displaced abomasal disease (Koeck et al. 2013). Among feeding management factors, the dairy cows at calving should not be fatty enough, otherwise it may cause reduced feed intake during early lactation. It is found that body condition score of 2.5-3.0 on a 1-5 scale is optimal and any increases in it enhance risk of ketosis (Melendez et al. 2004). Due to occurrence of associated other diseases, ketosis thought to be gateway disease during starting milking phase. Prevention and treatment of ketosis could decrease the possible chance of associated diseases.

Signs of Ketosis

Ketosis is related with the presence of ketones in fluids and characterized by hypoxia along with drop in milk yield. Decreased intake of diet by lactating animal is indication of first clinical sign during ketosis. In some cases, change in rumen motility (active to inactive) was also observed.

Other important clinical signs observed during ketosis were anorexia, decline in milk yield, body weight loss, poor body appearance, dry dung, and sometimes nervous signs. Nervous dysfunction signs are observed in nervous ketosis type which includes not normal licking, pica, absence of normal gait, incoordination, bellowing and aggression. While in cases of subclinical form of ketosis, less signs or no signs are found (Herdt 2019).

Diagnosis

In ketosis, hypoglycemic condition appeared due to less carbohydrate diet intake by the dairy animals. As a result, body produce one chemical named as Beta-hydroxybutyrate (BHB), which facilitate provision of energy when body is in need of sugar. The common approach related with diagnosis of ketosis in dairy animal is blood, milk, and urine testing. In normal circumstances, quantification of BHB level in biological fluid with the help of specialized meter indicates the occurrence of ketosis. The cut off value of BHB considered for non-ketotic cows is <1.2 mmol/L whilst level of BHB >1.2 mmol/L in cows are considered as ketogenic (Itle et al. 2015).

Numerous diagnostic tests (strips, kits, powders, and tablets) are used for the detection of ketosis which give results within seconds and minutes. Acetoacetate from urine (Ketostix strip, Bayer, Leverkusen, Germany) and BHB from milk or blood (Ketolac, Biolab, München, Germany) could be detected by using strips. These tests qualitatively confirm the presence of ketone in body fluids (Geishauser et al. 1998, Geishauser et al. 2000; Carrier et al. 2004; Oetzel 2007). The quantitative confirmatory methods of ketosis detection are using electronic BHB meter, digital devices, and high technological equipment (thin layer chromatography, high resolution gas chromatography and flame ionization detector/mass spectrometer (HRGC-FID/MS). With these techniques, fatty acids profile can be quantified inside the blood and milk and their connection with BHB level (BHB < 1.2 mmol/L). The fatty acids could be valued biomarker that guides in predicting the future occurrence of hyperketonemia (Zhang et al. 2012; Zhang et al. 2013; Li et al. 2014; Zeng and Cao 2018). Diagnosis before occurrence of ketosis may be helpful in the productive and profitable management of lactating animals. In advance, inclusion of various feed additivesor supplements and pharmaceutics could prevent the ketosis.

Prevention and Treatments

Various factors are associated with ketosis including glucose utilization in the formation of lactose (Kronfeld 1972), excess fat on animal body during calving (Smith et al. 1997), less energy diet in post calving period (Dann et al. 2005), not normal hepatic function (Tendler et al. 2007), endocrine glands illness such as ACTH or glucocorticoids, more or less inclusion of proteins diet, mineral and vitamins deficits, and more inclusion of ketogenic diet. Ketosis can be prevented effectively by avoiding these along with few other factors.

One of the approaches for the prevention of ketosis is inclusion of forages (rich in carbohydrates) in the diet of high milk yielding cows during transition period. It will also prevent the occurrence of hypoglycemic cows (Vickers et al. 2013). Second approach, addition of feed additives (supplements, pharmaceutics, herbal) in the diet can also avoid the ketosis in dairy cows (Mammi et al. 2021).

There are shortcomings in well-planned treatment regimens of ketosis and in efficacious ketosis treatment. The most of focused studies were done on treatment of ketosis rather than improvement of production or yield of dairy animals. Variety of treatments had been practiced in dairy cattle industry, with variable outcomes. More treatments had been reported along with other existing diseases but specifically and common one treatment only for ketosis practiced in dairy animals is described hereinafter.

It was proved in 1930s that hypo-glycemic condition occurred in ketosis (McSherry et al. 1960). From that period, glucose/dextrose is used primarily for treating the ketosis. Dextrose treatment play role in correcting the physiology of

the animal as glucose connected with the milk production further corrected the hypoglycemia and fat metabolism (Herdt 2000). It is believed that concentration of glucose is more in 500 ml bottle (50% dextrose). 50% dextrose will boost the glucose level inside the blood eight times more than normal level immediately after injection and normal after 2 hours (Sakai et al. 1996). Due to dextrose administration, the impact on BHB levels is short lived (<24 hours) and dextrose should be repeated for additional impacts (Wagner and Schimek 2010). It had been reported that continuous dextrose administration resulted in hyperglycemia and abomasum dysfunction by decreasing its motility (Holtenius et al. 2000; Zadnik 2003; Sahinduran and Albay 2006; Šamanc et al. 2009). Though, dextrose administration single treatment in connection with onward effects yet not well-known. Dextrose should be selected by the veterinary clinicians as secondary treatment ketonemia. Dextrose could provide reverse effect in animals which were hypoglycemic along with associated signs in nervous system. Later on, animals were administered with specific treatment regime for long period of time (Herdt and Emery 1992; Wagner and Schimek 2010).

Glucocorticoids is one of the corticosteroids that practiced in managing ketosis due to its impact on increasing level of glucose (Herdt and Emery 1992). It is proven that steroids having lessening effect on insulin whist increasing influence on breakdown of fat and protein reservoirs. Dexamethasone injection resulted in increased level of glucose and insulin hormone after 48 hours (Jorritsma et al. 2004). Corticosteroids were not recommended for long term usage in ketosis, due to less efficacious effects along with chances of side effects.

At early stage of lactation, dairy animals are inherently considered as insulin resistant (Bauman 2000). In characteristics, it is in-fact part of the complex mechanism of homeorhesis. During negative energy balance, it allows early lactating animals to produce increased amount of milk. Increased insulin resistance is manifested in animals affected by ketosis than other healthy animals (Sakai et al. 1996). In ketosis affected animals, insulin is used for treatment of ketosis because of its anabolic effect. This hormone lessens breakdown of fat, increases synthesis of fat and use of ketone bodies as energy sources. This interconnected metabolic activity decreases the level and significance of ketonemia. Literature shows limited evidence in favor of insulin as therapy.

It is found that vitamin B_{12} (cyanocobalamin) is involved in the process of gluconeogenesis, that's why it is administered in ketonemia. It is speculated that vitamin B_{12} have ability in stimulation of methylmalonyl-coenzyme (CoA) A mutase, which is main component of krebs cycle and gluconeogenesis (Kennedy et al. 1990, Gordon et al. 2017). The increasing level of this enzyme will produce more efficient energy for the cells. Butaphosphan is one of the compound and precursor of phosphorus production administered in ketosis for its role in gluconeogenesis (Rollin et al. 2010). Phosphorus involved in the phosphorylation of variety of compounds which occurs during gluconeogenesis. Butaphophan-cyanocobalamin pharmaceutical product could be administered in management of ketosis if its impact on milk yield and risk on disease should be validated.

During 1954, it was discovered that ketosis can be treated with propylene glycol (Johnson 1954; Maplesden 1954). Propylene glycol 100% (300 ml) administered orally once a day upto the period of one week (Gordon et al. 2013). In rumen it is transported into the blood, or before converted into the propionate (Nielsen and Ingvartsen 2004). It is involved in the TCA cycle by boosting the oxidation of acetyle CoA, increasing the glucose formation and triggers the insulin (Studer 1993). Insulin level is increased after 15 minutes administration of propylene glycol, and hormone surge maintained into the blood for 2 hours (Studer 1993). Many veterinarians use the propylene glycol by incorporating it into the feed as feed additive (Nielsen and Ingvartsen 2004), so, ruminal environment slowly adjusted for long term till formation of propionate (Nielsen and Ingvartsen 2004). In relation with hepatic oxidation theory, this will result in reduced feed consumption, more fat utilization and prolonging the problem of ketosis, while the clinical significance of this has not been resolved (Allen et al. 2004).

Recently documented review article suggested that monensin is one of the ionotropic antibiotics popularized in developed countries (Europe, USA, and Canada) for the prevention of the ketosis in dairy cows. It produces impact by raising the level of propionate in rumen, by reducing the BHB level inside the blood and by improving the indicators of functional liver (Mammi et al. 2021).

The combined treatment regimens were practiced in resolving the ketosis. Implementation of multiple pharmaceutical products had showed good outcomes in comparison with usage of single product. One of the problems with usage of combined therapy is economically not sound. Taken together, in future research trials should be validated regarding application of each product individually. Additional research is needed for efficacious treatment plan of ketosis which should be economically sound and can be implemented in livestock sector.

Economic Losses Due to Ketosis

The economic losses associated with ketosis depend on individual and herd-level and also associated with other factors. According to one report, the cost for one case of clinical ketosis and subclinical ketosis in average way estimated as €709 and €150. Normal herd level ketosis rates (clinical plus subclinical) were €3,613 for a default farm and €7,371 yearly for a high-risked farm (Steeneveld et al. 2020). In another report, it is recorded that cost of ketosis is €21/cow/year (Van Soest et al. 2019). In Canada, one case of subclinical ketosis treatment cost is $203 (Gohary et al. 2016).

In earlier literature, it is also reported that the losses in dairy animals due to ketosis is about 2.2 to 3.1 lbs of daily milk (4.4 to 6.6%) (Dohoo and Martin 1984; Chapinal et al. 2012), 4.1 lbs daily decline (5.5%) in yield (Duffield et al. 2009), and 865 lbs during 305 days milking period (7%). At herd level, frequency of ketosis cases is about 25-60%. More frequency in cases of ketosis, even at normal level influences overall impact on economic losses to the dairy industry.

Conclusion

Ketosis is an important metabolic disease of early lactating dairy animals affecting production performance and consequent economic losses. This disease can be avoided by proper nutrition and good management of dairy cattle during transition period and at early stage of lactation. The application of quick detection tests can support in early management of problem and preventing more income losses.

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CHAPTER 32

THERAPEUTIC STRATEGIES OF ENDOCRINE AND METABOLIC DISORDERS

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INTRODUCTION

The endocrine system comprises of glands secreting different hormones directly into the blood stream. Basically the hormones reach almost every cell and this communication is slower than the nerve communication, but is more persistent. Other than endocrine glands, additional sources of hormones are also present in the body such as prostaglandins are produced and secreted from plasma membranous phospholipids of many cells. Some prostaglandins are responsible for causing pain and inflammation, especially in case of arthritis, while some others show their actions with pituitary hormone oxytocin to cause uterine contractions during childbirth. Some organs produce specific hormones such as intestine (cholecystokinin, secretin, and gastrin), liver (hepcidin, insulin-like growth factor, and thrombopoetin), kidney (angiotensin, renin, and erythropoietin) and even the heart (atrial natriuretic peptide). The major endocrine glands, including the pituitary, thyroid and parathyroid glands, pancreas, adrenal glands and the reproductive organs (ovaries and testes) are represented in Figure 1. All the endocrine glands are interconnected with one another and secrete different hormones for regulation of homeostasis, growth and development, and metabolism and reproduction in the body by direct transmitting messages to the target organ receptors. To keep the balance in hormonal levels, complex feedback system is present, working in form of positive and negative feedback mechanisms to control the secretion of hormones (Shier et al. 2007).

The pituitary gland is a pea-sized endocrine gland, also referred to as 'master gland' of the body because in addition to secret its hormones, it controls other body glands to secrete hormones. Another name for pituitary is hypophysis (Greek word for 'lying under') that indicates its location just on the underside of the brain. Pituitary gland has two main parts; the anterior (front) and the posterior (back) lobe. Pituitary stalk connects hypothalamus and pituitary via blood vessels and nerves. Hypothalamus communicates with anterior lobe of pituitary through hormones and posterior lobe of pituitary through nerve fibers. The hypothalamus is the region of brain located below the thalamus and above the pituitary and contains a control center for autonomic (heart rate, blood pressure, body temperature, digestion) and endocrine (release of pituitary hormones via hormone releasing factors) functions of the pituitary because of its complex interactions. Thus, hypothalamus is the prime link connecting the nervous system and the endocrine system (Guyton 1981).

The thyroid gland is a butterfly-shaped gland located on either side of the trachea. It is comprised of follicular and less numerous parafollicular cells (C cells), secreting the thyroid hormones including triiodothyronine (T3), thyroxin (T4) and calcitonin. These hormones affect different body organs and tissues like adipose tissue, musculoskeletal system and the heart. From hypothalamus, thyrotropin releasing hormone (TRH) is released and is responsible for the secretion of thyroid stimulating hormone (TSH) from anterior pituitary, which ultimately stimulates the thyroid to release T3 and T4 to affect other tissues. These hormones also inhibit the release of TRH from hypothalamus through feedback mechanism. Other factors can also inhibit TSH secretion including glucocorticoids, stress and warmth. Calcitonin secreting from parafollicular cells regulates body calcium levels depending on serum calcium rather than feedback mechanism. Just posterior to thyroid glands, the parathyroid glands are located. These glands are comprised of three types of cells with different functions. Parathyroid hormone (PTH) is produced and secreted by chief cells of parathyroid glands. This hormone causes active vitamin D production from kidneys, stimulates calcium reabsorption and inhibits phosphate reabsorption from renal tubules. In addition, PTH releases calcium from bones in response to low serum calcium concentration, while an increased calcium concentration prevents the production and release of PTH. Adrenal glands are the triangular shaped glands located at the apex of both kidneys. The outer surface (cortex) of gland secretes mineralocorticoids (aldosterone), glucocorticoids (cortisol) and additional sex hormones. The inner portion (medulla) of gland secretes adrenaline and noradrenaline. Corticotropin releasing factor (CRF) from hypothalamus stimulates pituitary to release adrenocorticotropic hormone (ACTH) and melanocyte stimulating hormone (MSH). The ACTH acts on adrenal glands

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to produce and release aldosterone and cortisol. When adequate concentration of cortisol and aldosterone is achieved, the hypothalamus stops producing ACTH and MSH. The pancreas is situated just behind the stomach in upper left abdomen and surrounded by the liver, spleen and the intestine (Werbel and Ober 1993). Pancreas is a heterocrine gland with 99% exocrine and 1% endocrine function. The endocrine portion of pancreas involves clusters of cells called islets of Langerhans that is comprised of alpha cells secreting glucagon, beta cells secreting insulin and delta cells secreting somatostatin hormones. Low glucose level in the serum stimulates glucagon release from pancreas to initiate glucose production via gluconeogenesis. Other factors can also trigger glucagon release such as trauma, exercise and some infection. Insulin is crucial for efficient metabolism and cellular utilization of glucose. Ovaries are the part of female reproductive system present on each side of the uterus, while testes are the part of male reproductive system present inside the scrotum. From hypothalamus, gonadotropin releasing hormone (GnRH) is released to act on pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH) which ultimately cause the release of estrogen and progesterone from the ovaries in women. In men, LH stimulates testes for testosterone production and release (Rhee 2021).

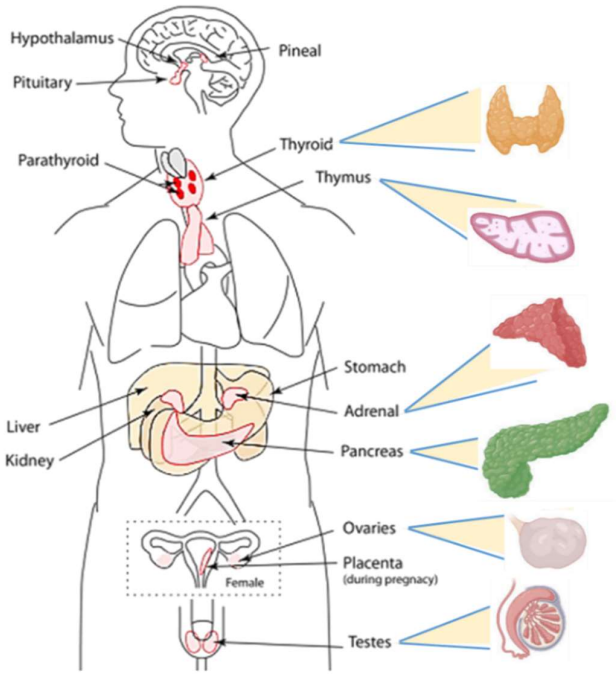

Figure 1: The endocrine system. Major endocrine glands include the hypothalamus, pituitary gland, thyroid and parathyroid gland, pancreas, adrenals, ovary, placenta and testis. Adapted from source: http://hyperphysics.phy-astr.gsu.edu/hbase/Biology/endocr.html

Endocrine and Metabolic Disorders

When body metabolic process fails to work properly in form of having too much or too little hormones or other essential substances for keeping healthy, a number of metabolic disorders arise. Hormonal alterations from endocrine glands result in metabolic syndrome. The major causes of metabolic syndrome are sedentary lifestyle, dietary habits, smoking and excess weight that may result in lethal consequences. Endocrine disorders extent enormous conditions and affect quality as well as quantity of life. Major endocrine disorders include hypopituitarism, acromegaly, diabetes insipidus and diabetes mellitus, hypogonadism, prolactinoma and Cushing's syndrome. Among endocrine disorders, thyroid diseases are more common (Goodman and Synder 2007).

Hypothalamus Deficiencies

The hypothalamus is a small portion of the brain located on the undersurface of brain and lies just below the thalamus and above the pituitary gland. It synthesizes hormones (releasing factors) and produces nerve impulses that regulate the secretion of pituitary gland. Hence, tumors of the hypothalamus or any other deficiency may directly affect pituitary hormones or can interfere with the appetite control center, causing obesity (Guyton 1981).

Pituitary Gland Related Acromegaly

Acromegaly is an uncommon systemic disorder affecting the whole body (Vance 2010) that arises as a result of excessive production and secretion of GH. Meanwhile GH regulates the overall growth of bones, muscles, internal organs and triggers the insulin-like growth factor 1 (IGF-1) secretion. In case of acromegaly, both GH and IGF-1 levels are increased, giving rise to metabolic alterations and tissue enlargement which eventually causes visible body malformations (Fleseriu et al. 2010). This disease generally occurs between the age of 30 and 50 with slow progression, actually delaying the diagnosis at later stages. Generally, GH neutralizes the insulin effects on glucose utilization and controls tissue reaction to insulin; therefore, excessive GH may result in insulin resistance. The patients suffering from acromegaly often experience higher levels of serum triglycerides demonstrating abnormal lipid metabolism associated with acromegaly (Colao et al. 2004).

Galactorrhea or Hyperprolactinemia

Galactorrhea is a lactating condition in non-breastfeeding women or in men, mostly caused by tumors of pituitary (prolactinoma or adenoma) secreting prolactin. In women, mostly the tumors are in form of microadenomas <10 mm in diameter, however in men, the frequency of these microadenomas is comparatively less. Lesions of pituitary gland can raise prolactin levels by constricting the pituitary stalk. This can decrease the actions of dopamine (prolactin inhibitor). Some medications, like antipsychotics (phenothiazine), antihypertensives (alpha-methyldopa) and opioids can also cause galactorrhea or hyperprolactinemia. Increased levels of thyroid releasing hormones increases the TSH and prolactin secretions, so, hyperthyroidism can be an important cause of hyperprolactinemia. It can be linked to hypogonadism due to GnRH deficiency acting on pituitary gonadotropins (Goodman and Synder 2007).

Erectile Dysfunction

Erectile dysfunction (ED) is an inability in men to have an erection hard enough to gain or maintain sexual satisfaction. It is the most common sex problem that almost every man irregularly meets an erection problem. In primary ED, the person is unable to erect or retain an erection, while in

secondary ED, the person was previously able to erect but in late age, develops ED. The latter is more common than former ED. In the USA, about 50% of men by age 40-70 are usually affected, however, ED is not necessarily occur with advanced age and can be treated at any age. For erection, the penis requires to have sufficient blood flow, the normal functioning nerves, enough libido (sexual desire) and the adequate amount of testosterone (male sex hormone) and hence any of the system's problems can be a reason for ED (Guyton 1981).

Central Diabetes Insipidus

Diabetes insipidus occurs as an outcome of deficiency of ADH (vasopressin) due to hypothalamic-pituitary disorder (central diabetes insipidus) or renal resistance to ADH (nephrotic diabetes insipidus), results in polyuria and polydipsia. ADH stimulates water preservation by enhancing the epithelial permeability of the renal tubules to water. At higher levels, this hormone causes vasoconstriction. Like aldosterone, vasopressin preserves cellular and vascular hydration as well as humoral homeostasis. The main stimuli for ADH secretion in the body are the volumetric consumption detected by vascular baroreceptors and an increased osmotic pressure of water detected by hypothalamic osmoreceptors. ADH is produced in the hypothalamus and stored in posterior pituitary. If hypothalamic nucleus and neurohypophysis region remain intact, the hormone is secreted into the circulation. Even 10% of intact neurosecretory neurons can avoid central diabetes insipidus as these neurons cover supraoptic and paraventricular nucleus of hypothalamus and pituitary stalk (Joshi et al. 2011).

Hypopituitarism

It is a rare disorder that prevails as an outcome of decreased secretion of one or more pituitary hormones. A number of factors may involve including inadequate blood supply to the pituitary, some inflammatory diseases, infections, autoimmune disorders, head injury, irradiation, and surgical removal of pituitary tissues or tumors of pituitary gland or hypothalamus (Guyton 1981).

Growth Hormone Deficiencies

Growth hormone (GH) deficiency results in overall short stature, also known as dwarfism especially in children. In adults, it might not usually affect height as the bones are completely developed and grown-up but they may lose energy (Enger et al. 2003).

Prolactin Deficiency

Prolactin deficiency reduces or stops the ability of women for breast milk production after childbirth. A rare birth complication known as Sheehan syndrome is the main cause of low prolactin concentrations and other hormones of pituitary. It usually develops due to shock and excessive loss of blood during delivery, partially damaging the pituitary. Deficiency of prolactin in men has no serious effects.

Gonadotropins (FSH and LH) Deficiency

Deficiencies of FSH and LH in women before menopause can cause cessation of menstrual cycle, infertility, vaginal dryness and loss of feminine sexual characteristics. In men, deficiency of these hormones leads to reduced sperm production, erectile dysfunction, testicular atrophy and loss of masculine sexual characteristics with secondary infertility, while in children this deficiency lead to delayed puberty.

Thyroid Gland Related Hyperthyroidism

Hyperactivity of thyroid gland is hyperthyroidism; also known as hypermetabolic state or thyrotoxicosis. Thyroid storm is uncommon but life-threatening disorder prevailing in 1–2% patients with hyperthyroidism. Thyroid storm occurs during excessive stress owing to adverse drug reactions, diabetic crisis or additional serious contests. The most common type of hyperthyroidism is Graves' disease; an autoimmune disorder followed by active secretion of excessive thyroid hormones due to TSH-mimicking antibodies. It commonly occurs in women during their middle-ages and can also affect men. A number of factors can cause hyperthyroidism such as high iodine-loaded drugs (iodinated IV substances or amiodarone), autoimmune destruction of thyroid gland or acute toxicity of thyroid hormones (exogenous), resulting in hypersecretion of thyroid hormones (Weetman 2000).

Hypothyroidism

Hypothyroidism is a condition of inadequate thyroid hormone secretions resulting in a decrease in overall body metabolism. Primary and secondary hypothyroidism are two major categories of hypothyroidism. Any deficiency in hormonal secretion or thyroid destruction can cause primary hypothyroidism. In the USA, Hashimoto's Thyroiditis is the leading cause of primary hypothyroidism. It is an autoimmune disorder characterized by thyroid cells being attacked by the body's immune system, resulting in destruction of thyroid cells. Secondary hypothyroidism results due to hypothalamic or pituitary disease leading to inadequate secretions of either thyroid hormones or thyroid stimulating hormone. Myxedema coma is a lethal condition of chronic hypothyroidism followed by severe hypotension, hypoglycemia, bradycardia and lower serum sodium levels (hyponatremia). Hypothyroidism is commonly caused by iodine deficiency. As iodine is critical for production and release of thyroid hormones, the disease manifests as congenital hypothyroidism since birth characterized by physical deformities, brain dysplasia and delayed growth. It can be treated by adequate iodine supplementation (Durante et al. 2018).

Parathyroid Gland Related Hypoparathyroidism

Hypoparathyroidism is a condition characterized by hypocalcemia, occurring as a result of insufficient levels of serum PTH or resistance to the effects of PTH. Besides, some congenital, acquired or autoimmune diseases can cause hypoparathyroidism. Resection of the gland during thyroidectomy or some injury lead to acquired hypoparathyroidism (Bilezikian 2020).

Adrenal Gland Related Acute Adrenal Insufficiency

In this condition, body needs more levels of mineralocorticoids and glucocorticoids than the normal capacity of adrenal glands. The major reason of such condition is the abrupt cessation of

steroid drugs following prolonged use. It may occur when the patient is unable to get the proper dose during stress, e.g., after major trauma, surgery or during a disease. Acute disease is categorized into primary, secondary and tertiary class depending on respective endocrine gland dysfunctioning. Primary adrenal insufficiency is associated with adrenal dysfunctioning, secondary adrenal insufficiency is associated with pituitary dysfunctioning, while tertiary adrenal insufficiency is related to the hypothalamic dysfunctioning (Werbel and Ober 1993).

Chronic Adrenal Insufficiency

Failure of adrenal cortex to secrete adequate levels of cortisol leads to chronic adrenal insufficiency. Like acute dysfunctioning, it is also categorized as primary, secondary and tertiary class depending upon either direct or indirect damage of the cortex. Primary adrenal insufficiency is commonly regarded as Addison's disease. It is both endocrine and metabolic disorder arise from direct injury to the adrenal cortex due to some autoimmune diseases, adrenal hemorrhage, tuberculosis, meningococcemia pathophysiology and acquired immunodeficiency syndrome (AIDS) (Arlt and Allolio 2003). It is a chronic illness with long-term attack. As known, adrenal cortex secretes aldosterone and cortisol. Aldosterone maintains a balance of sodium and potassium level in serum. Under stress conditions (heart ischemia, trauma, serious illness or infection), the adrenal glands become unable to secrete adequate level of corticosteroids (cortisol) to encounter body's need, exacerbating Addison's disease. In secondary class, the cortex is intact, but the pituitary becomes unable to secrete ACTH to stimulate the release of cortisol, so it is one step slighter than primary adrenal insufficiency. In the tertiary adrenal sufficiency, ACTH is not released from pituitary due to pituitary-hypothalamic disease (Hahner et al. 2010). Hyperpigmentation owing to excessive MSH production is the key distinctive feature of primary adrenal insufficiency, because ACTH and MSH are secreted from solitary pro-hormone peptide precursor pro-opiomelanocortin. MSH stimulates melanocytes to produce melanin. Both secondary and tertiary adrenal insufficiency are related to lower MSH levels, thus cannot be associated with hyperpigmentation (Arlt and Allolio 2003).

Hyperadrenalism (Cushing's syndrome)

Hyperadrenalism refers to as Cushing's syndrome and may occur due to overproduction or excessive exposure of serum cortisol from adrenal cortex. It commonly occurs among women of age 20-50 years. It may occur due to prolonged use of corticosteroids or pituitary or adrenal tumors. Regardless of the cause, increased levels of cortisol can lead to the interruption in normal metabolism of lipids, proteins and carbohydrates which may result in muscle weakness, fragile bones and hyperglycemia (Thomas et al. 1984).

Pancreas Related Diabetes Mellitus

Diabetes is one of the most prevalent endocrine disorders characterized by a number of combined ailments recognized by high blood sugar levels or hyperglycemia (Joshi et al. 2011). Diabetes is a metabolic syndrome characterized by lack of balance in insulin synthesis and action. Insulin plays crucial role in the absorption and cellular utilization of glucose (Taxitiemuer et al. 2011). Beta cells dysfunctioning leads to deficient production of insulin and amylin eventually causing obesity. Hepatocyte nuclear factor 4-alpha from liver plays role in gene transcription of pancreatic beta cells. In type 2 diabetes, about 2-5% of beta cells are non-functional, gene mutations in liver may progress to maturity-onset diabetes of young people, and still it is noninsulin-dependent diabetes. Diabetic ketoacidosis (DKA) is an acute endocrine disorder characterized by plasma glucose concentration >350 mg/dL (>19.4 mmol/L), serum bicarbonate levels <15 mEq, ketone production and metabolic acidosis which is a clinical symbol of DKA. Its mortality rate is about 14%. In this condition, insulin deficiency and increased levels of glucagon lead to volumetric depletion, hyperglycemia as well as acidosis due to electrolyte imbalance. It is mostly caused by infections, myocardial infarction, trauma or pregnancy. Gestational diabetes occurs during pregnancy and even newborn is at greater risk. Clinically, diabetes is described by hyperglycemia and imbalances in lipid metabolism. For differential diagnosis, the threshold values for fasting blood glucose and random blood glucose are ~140 mg/dL (~7.7 mmol/L) and ~200 mg/dL (~11.1 mmol/L) respectively. Glycosylated hemoglobin's % age (glycated hemoglobin or HbA1c) is an indicator of controlled diabetes in patients within 3 months. Body's inability to control serum glucose levels may give rise to microvascular complications in the eyes, kidneys, nervous system and the heart (Pe et al. 2003; Kousar et al. 2021).

Hypoglycemia

A very common disorder related to diabetes is hypoglycemia in which blood glucose level is <60 mg/dL (3.3 mmol/L) and can vary person to person. At this stage, the body reduces insulin secretion to avoid sudden hypoglycemia. Meanwhile, there is more release of counter-regulatory hormones like adrenaline and noradrenaline and at last, cognitive degeneration occurs. There are substantial mental state alterations as the glucose level falls below 50 mg/dL (2.8 mmol/L). If untreated, morbidity and mortality increases. To avoid this, proper diagnosis and effective therapy are required. Hypoglycemia usually occurs as a result of high dose of insulin, reduced eating or both. Tissues of central nervous system differ from other ones in their way of metabolizing proteins, fats and sugar, especially they depend entirely on glucose as the source of energy. If blood glucose levels drop abruptly, the brain becomes starved. In patients without history of diabetes, hypoglycemia is regarded as postprandial or fasting hypoglycemia and is reflected by hyperinsulinemia, often observed in patients undertaking gastric surgery. Hypoglycemia can be triggered by pancreatic tumors or insulinoma, enzyme deficiency, liver disease, infections and drug overdose (sulfonylureas, insulin) (Pe et al. 2003).

Therapeutic Strategies

Pharmacological Interventions in Combating Endocrine and Metabolic Disorders

Endocrine and metabolic disorders can be caused by a number of risk factors including chronic inflammation, glucose intolerance or oxidative stress causing mitochondrial dysfunctions, shifting natural balance of endocrine and

metabolic system towards disordered state, as shown in Figure 2. The recent guidelines for treatment and management of metabolic and endocrine disorders recommend lifestyle modifications (physical activity, diet and weight loss) as 1st line therapy. Still, these strategies may be inadequate, impracticable or fail to combat metabolic alterations. Conversely, pharmacological interventions like antidiabetic drugs or antihyperlipidemic drugs have been approved to inverse metabolic dysfunctions and weight gain. Besides, their clinical use, a variety of these drugs are used off-label, such as metformin (Kouidrat et al. 2015). The reason for such use is cost effectiveness and well-tolerance to combat weight gain, so can easily be added to dietary plan and lifestyle intervention. Major disorders of endocrine system and their treatments are given in Table 1.

Melatonin is used clinically for treatment of metabolic disorders (to normalize circadian cycle), diabetes and obesity and is investigated so far in human and animal models of oxidative stress and dyslipidemia (Bonnefont-Rousselot 2014; Navarro-Alarcón et al. 2014). Thyroid related disorders like hyperthyroidism can be treated with antithyroid drugs (propylthiouracil and methimazole), radioiodine or surgery. These drugs have fewer side-effects. To achieve euthyroid condition, radioiodine in combination with β-adrenergic antagonist can be administered alongwith T3 and T4 level monitoring. Surgery is recommended if pharmacological treatment fails. Hypothyroidism treatment usually depends on age alongwith TSH level monitoring every 6-8 weeks. The prescribed dose of levothyroxine is 1.6 μg/kg/day in young patients, while in older patients the dose starts 25-50 μg/day followed by 12.5-25-μg increments every 4-6 weeks (Klein and Danzi 2007). A study showed improved cholesterol and fatigue in patients treated with levothyroxine for 12 weeks (Razvi et al. 2007). Hypercalcemia is initially treated with intravenous saline and euvolemic condition is controlled by some diuretics. Subcutaneous calcitonin and bisphosphonates can also be recommended. Parathyroidectomy can be the treatment of choice as it improves symptoms, maintains euvolemic condition and can safely be performed (Marx 2000). Medical treatment (calcimimetics, bisphosphonates, and estrogen modulators) can be considered if surgery is contraindicated. Hypocalcemia is treated with calcium and vitamin D. For acromegaly, surgical resection of pituitary mass to normalize serum IGF-1 and GH, is the 1st line therapy (Ezzat et al. 2006). If serum levels remain increased even after surgery, pharmacological treatment is advised. It includes pegvisomant (recombinant pegylated GH receptor antagonist) either as monotherapy (130 mg) or combined (77 mg) with somatostatin analog (Neggers and Vander-Lely 2009). Radiotherapy may be prescribed alongwith careful follow-up of serum IGF-1 monitoring every 6 months, if medication therapy or surgery fails (Ezzat et al. 2006).

Treatment of Cushing's syndrome involves management of hypertension (Arnaldi et al. 2003), with careful monitoring of pituitary deficiencies in case of surgical resection of pituitary adenoma. This can be treated with chemotherapeutics, including mitotane which inhibits steroid production or hormone replacement therapy. Addison's disease can also be treated with hormone replacement therapy, adequate sodium diet or hydrocortisone (20-30 mg/day), or fludrocortisone (0.05-0.1 mg/day) to mimic circadian cycle.

Medications for the treatment of diabetes include thiazolidinediones, biguanides, meglitinides, sulfonylureas, alpha-glucosidase inhibitors and insulin alongwith novel agents including incretin mimetics and DPP-4 inhibitors (Nissen and Wolski 2007; Kousar et al. 2021). Glitazones are PPAR-γ (peroxisome proliferator-activated receptor gamma) agonists are recommended for glycemic control in patients with multiple complications. Rosiglitazone and pioglitazone were approved for such treatment by FDA in 2002 (Lebovitz 2002). Among lipid lowering medications, HMG-CoA reductase inhibitors or statins, are the most commonly prescribed drugs to prevent cardiovascular and metabolic disorders as these are also well-tolerated and efficacious. For obesity therapy and weight management, orlistat (an intestinal lipase-inhibitor) and the combination of topiramate and phentermine are approved by the FDA (Pucci and Finer 2015).

Table 1: Major endocrine glands, their hormones, disorders due to hormonal fluctuations and treatment options.

Endocrine gland	Hormone	Major disorder	Treatment of choice
Pineal gland	Melatonin	Insomnia	Cognitive behavioral therapy
Hypothalamus	GHRH, TRH, CRH, GnRH, somatostatin, dopamine, vasopressin	Hypopituitarism, hypothyroidism	Hormone replacement therapy, medications, surgery
Pituitary gland	TSH, LH, ACTH, GH, MSH, PRL, vasopressin, oxytocin	Acromegaly, Cushing's disease, diabetes insipidus	Hormone replacement therapy, medications, surgery
Thyroid gland	T3, T4, calcitonin	Goiter, hypothyroidism, hyperthyroidism, autoimmune thyroid disease	Levothyroxine, methimazole, surgery, radioactive iodine treatments
Parathyroid gland	PTH	Hyperparathyroidism, parathyroid cancer, hypoparathyroidism	Vitamin D (calcitriol), calcium supplements, surgery
Liver	IGF (somatomedin), angiotensinogen, thrombopoetin, hepcidin	Metabolic disorders	Enzyme replacement therapy, medications, mineral supplementation
Pancreas	Insulin, glucagon, somatostatin	Diabetes mellitus, obesity, pancreatitis	Medications, surgery
Adrenal glands	Cortisol, aldosterone, DHEA, androgenic steroids, adrenaline noradrenaline	Addison's disease, Cushing's disease, pheochromocytomas	Hormone replacement, steroids, surgery
Ovary and placenta	Estrogen, progesterone, HCG, human placental lactogen	Endometriosis, ovarian cysts, ovarian epithelial cancer, PCOS	Clomiphene, metformin, surgery
Testis	Testosterone, inhibin	Epididymitis, hydrocele, hypogonadism	Antibiotics, anti-inflammatory drugs

Abbreviations. ACTH: Adrenocorticotrophic hormone, ADH: Anti-diuretic hormone or vasopressin, CRH: corticotropin-releasing hormone, DHEA: Dehydroepiandrosterone, FSH: Follicle-stimulating hormone, GH: Growth hormone, GnRH: gonadotropin-releasing hormone, GHRH: growth hormone-releasing hormone, HCG: Human chorionic gonadotropin, IGF: insulin-like growth factor, LH: Luteinizing hormone, PCOS: Polycystic Ovary Syndrome, PRL: Prolactin, TRH: thyrotropin-releasing hormone, TSH: Thyroid-stimulating hormone.

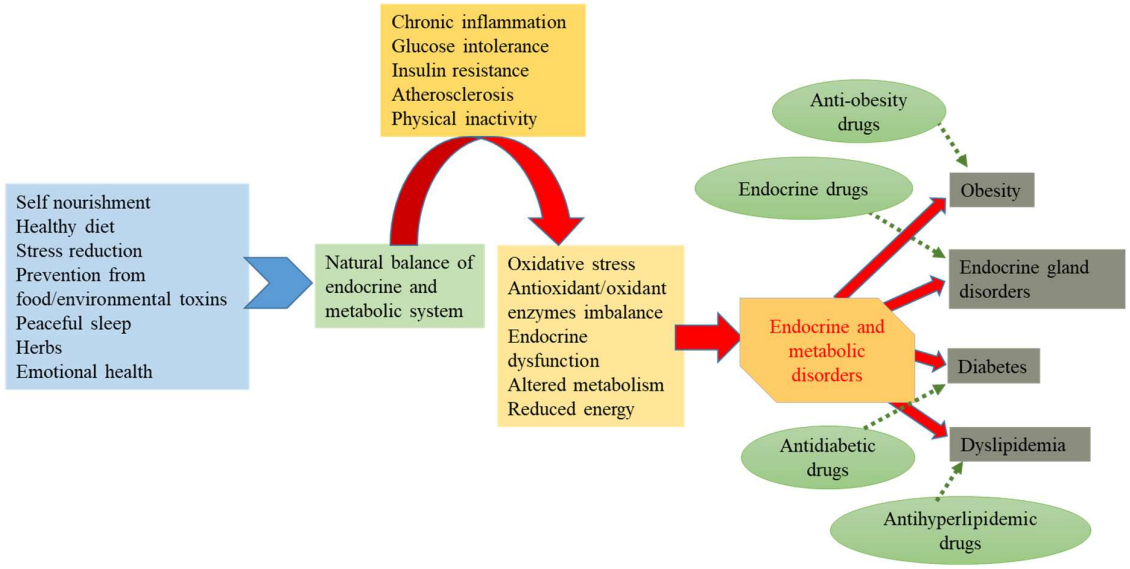

Figure 2: Factors needed to maintain the natural balance of endocrine and metabolic system and major risk factors responsible for mitochondrial dysfunction, preventing it from properly processing fuel, and no cellular energy is produced. This leads to imbalance in antioxidant/oxidant enzymes. As a result of antioxidant/oxidant enzymes imbalance and reduced energy, endocrine dysfunction and metabolic disorders occur. The potential sites of action of different drugs are presented in green ovals.

Nanocarriers in Combating Endocrine and Metabolic Disorders

Blood Triglycerides and HDL

In the recent decades, safe utilization of nanoparticles (NPs) has gained more attention of biomedical scientists and researchers for their therapeutic effects in endocrine and metabolic disorders. Studies show that silver NPs have ability to alter the levels of lactate dehydrogenase enzyme in the body; which converts dietary sugar into usable energy for cells and plays an important role in cellular respiration (Naghsh et al. 2013). But, long term use of these NPs revealed toxicity to lung tissues with abnormally raised serum biochemical and hematological parameters when experimental studies were performed on rats (Alkaladi et al. 2014).

Blood Glucose and Diabetes

Metabolic syndrome is a group of disorders characterized by high blood pressure, high blood glucose and triglyceride level. In studies, silver NPs did not much affect the blood glucose levels. Another study showed that synthetic insulin coated with dextran sulfate-chitosan nanocarrier system possesses 85% association efficacy and better controlled release was detected at pH 6.8 (Kim and Moon 2012). Ceramic nanoparticles composed of calcium phosphate, silica or titanium are more stable and biocompatible and have shown improved spatial features of insulin therapeutic effects. Rosiglitazone in form of polyethylene glycol-NPs shows improved cellular uptake of drug with anti-inflammatory response of macrophages in addition to anti-diabetic effects (Giacalone et al. 2018). Micelles having amphiphilic or surfactant molecules with hydrophobic core also exhibited controlled release of insulin in the treatment of diabetes mellitus. Besides, liposomes are small spherical vesicles with hydrophobic center and hydrophilic groups outside. Liposomes of folic acid with insulin solution showed hypoglycemic effects with 20% more bioavailability than single insulin administration in the treatment of diabetes mellitus (Li et al. 2016).

Abdominal Obesity and Tissue Fats

Obesity is associated with overweight characterized by impaired fat metabolism and can be an outcome of other pertinent diseases including cardiovascular disease, type 2 diabetes, asthma and inflammation of joints. A study has documented that gold NPs have beneficial effects for possible therapy of obesity and pertinent diseases (Chen et al. 2013). Moreover, lipid nanostructures formulation shows beneficial effects in normalizing triglycerides, cholesterol and blood glucose levels in the treatment of obesity. All these efforts combine the captivating properties of NPs with commercial application in controlling endocrine and metabolic disorders.

Bioactive Compounds in Combating Endocrine and Metabolic Disorders

Many bioactive compounds are effective in endocrine and metabolic disorders, including diabetes, hyperlipidemia and obesity. Bioactive compounds are obtained from wholegrain, fruits and vegetables exerting pharmacological effects in humans and have an essential role in the body's defense mechanisms. Such compounds are advantageous to use due to ease of accessibility, safety and fewer side effects (Gothai et al. 2016; McAnany and Martirosyan 2016). Bioactive compounds can also be obtained from microorganisms, making valuable secondary metabolites. Medicinal plants possessing different therapeutic activities like anti-inflammatory, antioxidants, antidiabetic and antihypoglycemic and anti-carcinogenic potential credited to bioactive principles (Bowling et al. 2007).

Resveratrol

Resveratrol is a stilbenoid, a kind of natural phenol and a phytoalexin that possess both anti-inflammatory and

antioxidant properties, maintains fluid homeostasis and improves mitochondrial and cellular functions. The origin of resveratrol is grapes skin, blueberries, red wines and seeds. It regulates glucose metabolism and insulin secretion from pancreatic beta cells and prevents oxidative stress (Khalid et al. 2018).

Quercetin

Quercetin is a flavonoid and documented to be effective for the treatment of DM. The main sources of quercetin are red onions, broccoli, apple and tea, and possesses antioxidant, anti-inflammatory and anti-apoptotic activities. It controls phosphorylation of extracellular signaling regulated kinase (ERK1/2) and enhances β-cell functions and glucose-induced insulin secretion (Peng et al. 2017).

Minerals

Minerals are solid substances present in nature and can be composed of one or more elements combined together. Minerals have a significant role in managing obesity. Iodine supplementation is essential for proper functions of thyroid gland and hormones. Daily intake of calcium (1200 mg) decreases fats in overweight and obese persons, whereas, low calcium intake consequences in decreased lipolysis and increased weight (Sharma et al. 2018).

Probiotics

Probiotics are live bacteria and yeasts valuable for digestive system in maintaining balance of intestinal gut flora and improving nutrient digestibility. Probiotics include *Bifidobacterium* and *Lactobacillus* and are effective for the treatment of obesity. *Bifidobacteria* and *Lactobacilli* synthesize bioactive compounds of conjugated linoleic acid, which have anti-atherosclerotic, antidiabetic, antioxidant and anti-obesity properties (Rashid et al. 2020).

Phytochemicals

Phytochemicals are the compounds produced by plants such as carotenoids, triterpenes and polyphenols (flavones, flavonoid, flavonols, phenolic acids, curcuminoids, stilbenes and anthocyanins) and possess antioxidative, antiadiposity and cardioprotective activities. Certain phytochemicals act as thermogenic compounds including caffeine, salicylic acid, ephedrine and capsaicin, and prevent excessive accumulation of fats in body tissues by burning extra calories (Zheng et al. 2009).

Cinnamon

Cinnamon is a spice made from the inner bark of trees of genera *Cinnamomum*. It is filled with antioxidant properties and a number of other beneficial effects in the treatment of endocrine and metabolic disorders related to insulin sensitivity, glucose and lipid metabolism, oxidative stress, inflammation and body weight (Hussain et al. 2019, 2021).

Curcumin

Curcumin is a polyphenolic compound produced by plant rhizome of *Curcuma longa* specie, possessing anti-inflammatory, antioxidant and antitumorigenic properties. Curcumin decreases oxidative stress by inhibiting aconitase enzyme of citric acid cycle (Sjögren et al. 1996). It also decreases lipogenesis by enabling β-oxidation of fatty acids and is beneficial in endocrine disorders (Noorafshan and Ashkani-Esfahani 2013).

Preventive Strategies of Endocrine and Metabolic Disorders

Restriction of Food Toxins

Physiologically, hormones are present in continuous fluctuations and any imbalance in their levels is primarily due to dietary habits of a person. Different chemicals, preservatives, colorants and refined sugars are routinely used by food industry, additionally, junk food contains higher quantities of salts and trans-fatty acids which can alter hormonal balance in body. Smoking and alcohol abuse should also be avoided. The need is to restrict unhealthy food containing excessive fats, chemicals or extra sodium to maintain body hormonal levels normal (Maqbool et al. 2016).

Sleep Quality

A good quality sleep refers to take 6-8 hour night sleep, which is beneficial in keeping the glands healthy for normal production, metabolism and maintenance of hormones. A medical condition called insomnia or sleeplessness arises as a result of irritability or stress that may cause poor functioning of glands, lessen immunity, weight gain and restricted cognitive ability (Ruge et al. 2019). Melatonin hormone regulates the sleep-wake cycle and has therapeutic effects for sleep disorders. Some food materials containing antioxidants (e.g., chamomile tea) aid in maintaining better quality of sleep and decrease the occurrence of insomnia while some other disturbs sleep patterns include alcohol, smoking and caffeine beverages, so should be avoided at bedtime (Adib-Hajbaghery and Mousavi 2017).

Stress Management

Little stress is essential for survival and protection of human and animal health, however, chronic stress possess devastating effects on body organs and glands that may harm physical and mental status (Hartney 2020). Stress and anxiety are common problems of society and can ruin the balanced life. In females, chronic stress can postpone or stop ovulation while in males, it can result in low testosterone levels. The goal of therapy is to avoid or reduce the negative outcomes of stress on body. Stress management therapy includes calming and relaxing the mind, positively controlling the emotions and handling stress peacefully. Besides, physical activity provides a better solution in stress management as it reduces stress hormonal levels and enhances neuronal production of endorphins to feel good. Routine exercise helps in lowering body's cortisol levels and maintaining endocrine and metabolic balance (Bittar et al. 2016; Krause et al. 2019). Yoga and meditation practice are beneficial approach for controlling stress and emotions, improving cognition, balancing hormones, relaxation, self-discovery and awareness, and keeping spiritual harmony with nature. Stress reducing major food items includes chocolates, coffee, walnuts, banana, oranges, fish, oats, eggs, tea, wholegrain and probiotics (Singh 2016).

Reduce Exposure to Environmental Toxins

A number of environmental toxins are responsible for hormonal imbalance when the body fails to eliminate the toxic chemicals. This leads to toxicity of inner body environment resulting in metabolic and endocrine disorders. So the solution is to avoid bodily exposure to chemicals by adapting preventive measures, including unnecessary usage of drugs, pesticides, household chemicals, deodorants, sunlight exposure and radiations. A healthy food with antioxidants, dietary fibers and probiotics are necessary to regulate and maintain a normal body hormonal level and metabolism (Zheng et al. 2018).

Conclusion and Recommendations

The chapter summarizes the endocrine system physiology comprising of endocrine glands, their respective hormones, disorders of endocrine and metabolic system as well as their therapeutic strategies. The synchronized and balanced hormonal production by endocrine glands is essential for maintenance of good health. Any fluctuation either in production or release of hormones disturbs the body homeostasis and give rise to various metabolic disorders, some of which are life threatening. Pharmacological interventions aim to identify and treat specific metabolic and endocrine disorders. Besides, nanocarriers and bioactive compounds show promising effects to interact with living systems with better health outcomes. Latest research on beneficial use of bioactive compounds in endocrine and metabolic disorders is presenting significant growth and will be upcoming in future. Balanced and nutritious food, stress management and sleep quality together with active life style are essential to retain normal endocrine and metabolic functions and play crucial role for emotional and hormonal health.

Confliction of Interest Statement

All the authors declare no confliction of interest in anyway.

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CHAPTER 33

BOTANICAL CONTROL OF POULTRY COCCIDIOSIS

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INTRODUCTION

The poultry sector is vibrant, fast growing and potential sector playing an important role in food security and economy of developing countries (Abbas et al. 2020; Zhang et al. 2020). Each year, over 50 billion chickens are raised as a source of meat, accounting for over one-third of protein source in food of humans (Quiroz-Castañedaet al. 2015). However, the poultry sector is facing challenges due to outbreak of certain diseases of parasitic, viral and bacterial origin. Among parasitic diseases, Coccidiosis is major parasitic diseases affecting poultry industry all over the world (Blake et al. 2020). Coccidiosis is a parasitic disease caused by different species of genus Eimeria, which is obligate and intracellular protozoa. This is host-specific protozoa and also related to other protozoa like Besnoitia, Babesia, Cystoisospora, Caryptosporidium, Plasmodium, Neospora, Theileria, Toxoplasma and Sarcocystis. There are seven species which are causing coccidiosis in *Gallus gallus domesticus*. These species of Eimeria include *Eimeria brunetti*, *E. acervulina, E. maxima, E. necatrix, E. mitis, E. tenella* and *E. praecox* (Blake et al. 2021; El-Shall et al. 2022).

Coccidiosis is transmitted by ingestion of sporulated oocysts of Eimeria from feces and litter. All Eimeria species show noticeable tropism for definite areas of the gut (Lai et al. 2011). The lifecycle of Eimeria includes variety of asexual reproduction (Walker et al. 2013; Abbas et al.2017a, 2017b), followed by a sexual phase known as gametogony as shown in Fig. 1. Following fertilization, oocysts are excreted and sporulated in the environment. Sporulated oocysts are infectious for avian hosts. The pathology related to every Eimeria species varies, taking place in exclusive sections of the gut and inflicting both malabsorptive or hemorrhagic lesions at intestine (Abbas et al. 2019a, 2019b; Burrell et al. 2020). By infecting the digestive tract, coccidiosis reduces poultry output by compromising the final body weight, intestinal health, and meat quality of broiler chickens. (Swelum et al. 2021; Yaqoob et al. 2021). Coccidiosis is controlled by use of synthetic anticoccidial drugs. Since 1939, a wide range of anticoccidial drugs have been used against poultry coccidiosis (Nogueira et al. 2009). However, due to development of anticoccidial drug resistance (Abbas et al. 2019a, 2020), toxic effects on bird's health this method now be came ineffective. Heavy cost is spent on anticoccidial drugs annually. Fortunately, Eimeria infections create long-lasting and powerful immunity including vaccination as substitute to anticoccidial drugs (Abbas et al. 2011; Chapman, 2014). On the other hand, vaccination may trigger severe hemorrhagic reactions and lack of a "standard" protocol for assessing vaccine efficacy makes the development and validation of vaccine complicated against avian coccidiosis (Shirley et al. 2005).

Due to anticoccidial drug resistance and lower efficacy of Eimeria vaccines, alternative novel compounds are center of recent research now a days. Among alternative anticoccidial agents, use of phytogenic compounds has shown significant results in control of coccidiosis (Abbas et al. 2017a; Abou-Kassem et al. 2021). Recently, there has been a surge of interest around the world in adopting herbal remedies as safe alternatives to treat a variety of ailments with minimum chances of resistance (Abd El-Hack et al. 2020). Because of their growth-promoting and natural immunostimulating properties, different botanicals are extensively researched against poultry coccidiosis (Muthamilselvan et al. 2016; Abbas et al. 2017c, 2019a).

This chapter contains valuable information on potential of different botanicals against Eimeria and positive effects on performance of poultry. Furthermore, herbal medicines, their extracts, bioactive substances, particular anticoccidial characteristics are also summarized for future perspectives of research against coccidiosis. Different herbal blends along with their associated bioactive ingredients having anticoccidial potential with different species are also discussed.

Mechanism of Action of Botanicals Against Coccidiosis

Antioxidants

The antiprotozoal activity of botanicals has also been attributed due to ability to reduce oxidative stress by scavenging oxygen free radicals which induce oxidative stress

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Table 1: Major *Eimeria* species infecting Poultry

Species Name	Infection Site	Pathogenicity Level	Reference
Chicken			
E. acervulina	Upper small intestine	Medium	Tyzzer1929
E. brunetti	Distal small intestine and colon	High	Levine 1942
E. hagani	Upper small intestine	Low	Edgar and Siebold 1964
E. maxima	Middle small intestine	Medium	Tyzzer1929
E.mivati	Upper small intestine	Low	Tyzzer 1929
E. mitis	Upper small intestine	Low	Tyzzer 1929
E. necatrix	Middle small intestine	High	Tyzzer1929
E.praecox	Upper small intestine	Medium	Tyzzer1929
E. tenella	Ceca	High	Raillet and Lucet 1891
Turkey			
E. adenoeides	Small intestine, caeca, colon	High	Moore and Brown 1951
E. gallopavonis	Large intestine, caeca	Moderate	Hawkins 1952
E. meleagrimitis	Small intestine	Moderate	Tyzzer1929
E. dispersa	Small, large intestine	Low	Tyzzer 1929
Pigeons			
E. columbarum	Intestine	Low to Mild	Mitraand Das Gupta 1937
E. labbeana	Intestine	Low to Mild	Pinto 1928
Ducks			
E. anatis	Small intestine	Mild	Scholtyseck 1955
E. danailovi	Small intestine	High	Gräfneret al. 1965
E. saitamae	Small intestine	High	Inoue 1967
Geese			
E. anseris	Small intestine	Moderate	Kotlan 1932
E. nocens	Small intestine	Moderate	Kotlan 1932
E. truncate	Kidney	High	Raillet and Lucet 1891

Table 2: Botanicals reported for Anticoccidial effects against *Eimeria* Species

Botanical Name	Active Compounds	Anticoccidial Activity	Other Beneficial Effects	*Eimeria* Species	Reference
Ageratum conyzoides	Flavonoids, Vernoside and Berberine	Reduced lesion and oocyst score	Improves weight gain, FCR, chicken	Mixed species	Hussain et al. 2021
Allium sativum	Alliin, diallylsulphide, Allicin, Sulphur derivatives	Lowers fecal oocyst count, lower pathology	Increase in performance, organ weight, FCR	Mixed species	Sidiropoulou et al. 2020
Aloe vera	Trepenoidscarbohydtrates Flavonoids	Lesser fecal oocyst counts.	Reduces mortality Immunomodulation	*E.tenella*	Akhtar et al. 2012
Artemisia brevifolia	Artemisinin	Diminishes oocyst and lesion scores	Improves weight gain, FCR	*E.tenella*	Hussain et al. 2021
Artemisia sieberi	Artemisinin	Diminishes oocyst scores in infested chickens.	Improves FCR and promotes weight gain.	Mixed species	Kheirabadiet al. 2014
Azadirachta indica	Azadirachtin, nimbolinin, nimbin, sodium nimbinate, salannin and quercetin.	Lesser fecal oocyst counts and reduction in lesion score	Improves weight gain, FCR, reduces mortality	*E. tenella*	Abbas et al. 2006
Beta vulgaris	Betaine	Reduced Oocysts shedding and lesion scores	Enhances FCR, improves organ weight, serum chemistry	Mixed species	Abbas et al. 2017b
Bidenspilosa	Favonoids, porphyrins, quercetin, porphyrins, phenylpropanoids	Lesser fecal oocyst counts and reduction in lesion score	Enhanced Immunity, Survival rate, weight gain	*E. tenella*	Yang et al. 2019
Camellia sinensis	Polyphenolic compounds	are blocked.	Antioxidant properties are demonstrated.	Mixed species	Zhang et al. 2020: Abbas et al. 2017c
Carica papaya	Papain, Vitamin A	Reduced oocysts shedding	Enhanced immunity and improves growth performance	*E.tenella*	Nghonjuyiet al. 2015
Cinnamomum cassia	Cinnamaldehyde	Reduced oocysts shedding	Immunity is boosted. Survival rate, Weight gain	Mixed species	Orengoet al. 2012
Curcuma longa	Curcumins	Inhibits life cycle stages	Increases body weight gain. Shows antioxidative, anti-inflammatory	Mixed species	Abbas et al. 2011
Cyamopsistetragonoloba	Saponins	Reduces the shedding of oocysts	Increases daily body weight while lowering feed conversion ratio. increase	Mixed species	Sánchez-Hernández et al. 2019
Emblica officinalis	Polyphenolics, carbohydrates, amino acids, Tannins, alkaloids (gallic acid, ellagic acid), Emblicanin	Oocysticidal properties and prevents sporulation. Inhibits parasite's life cycle from progressing.	Body weight gain improvement improved cellular and humoral immunity	Mixed species	Sharma et al. 2021
Fomitella fraxinea	Fungal lectin.	Improves the cellular and humoral immunity	It has immunostimulatory properties.	Mixed species	Dalloul et al. 2006

Gallarhois	Methyl gallate and phenolic compounds	Oocyte shedding is stopped, and lesion scores are reduced.	Reduces feed consumption while improving body weight increase. Antibacterial and antiviral properties.	*E. tenella*	Lee et al. 2012
Ganodermalucidum	Glycoproteins, organic acids, Glycosides	Oocyst sporulation is inhibited.	Increases the weight of the carcass. Improves bloody diarrhea.	*E. tenella*	Ahad et al. 2016
Botanical Name	Active Compounds	Anticoccidial Activity	Other Beneficial Effects	Eimeria Species	Reference
Khayasenegalensis	Phenolics and alkaloids	Reduces fecal, lesion scores,	Antioxidant effects	*E. tenella*	Dakpogan et al. 2019
Moringaoleifera	Flavonoids, phenolics, Ascorbic acid, caffeoylquinic acid and kaempferol.	Osmoprotectant Reduces the amount of lipid peroxidation in the intestine.	Enhances body weight gain, reduces mortality, faecal score, Inhibits the production of oocysts.	Mixed species	Ola-Fadunsin and Ademola 2013
Musa paradisiaca	Pectinand flavonoids compounds	Prevents the development of coccidial infections and decreases their reproduction.	Enhances body weight gain, improved FCR	*E. tenella*	Anosa and Okoro 2011
Oleaeuropaea	Maslinic acid, polyphenolic compounds	Damaging impact on oocysts	Improves the anticoccidial, the oocyst, lesion	Mixed species	Debbou-Iouknane et al. 2021
Origanumvulgare	Carvacol and thymol	Damaged life cycle stages of *Eimeria*	Reduces FCR while increasing body weight gain.	Mixed species	Tsinas et al. 2011
Pimpinellaanisum	Methylchavicol, Anethole, anisaldehyde, estragole and eugenol.	Only when combined with A. annua it reduces the extent of oocytes in broiler chickens.	Improves performance by increasing FCR	*E. tenella*	Drăgan et al. 2010
Pinusradiata	Tannins	Reduced oocysts excretion and lesion score	Improves performance by increasing body weight gain More	Mixed Species	Abbas et al. 2017a
Punicagranatum	Corilagin, Ellagic acid and punicalagin	Lessens oocyst output.	Reduces feed conversion ratio while improving intestinal lesions and increasing body weight.	Mixed Species	Ahad et al. 2018
Saccharumofficinarum	Flavones (tricin, luteolin, derivatives)	In vitro inhibitory activity against coccidian oocyst sporulation.	Immunomodulatory antioxidant, Anti-inflammatory, antiviral, antibacterial	Mixed species	Abbas et al. 2015
Salvadorapersica	Alkaloids, Cyanogenic glycosides Vitamin C, salvadourea, tannins, saponins	Inhibits or impairs the incursion, reproduction, and progression of Eimeria parasite species	Anti-inflammatory and antioxidant activities have been reported.	Mixed species	Thagfan et al. 2017
Trachyspermum ammi	Carvacrol and Thymol.	Affecting Eimeria oocyst sporulation (percent) in a dose-dependent manner. Oocyst morphology is affected	Increased body weight and FCR	Mixed species	Abbas et al. 2019a
Tulbaghia violacea	marasmine), bis (methylthiomethyl) methyl disulfide, S - (methylthiomethyl), cysteine sulfoxide	Reduces oocyst formation, host cell death caused by lipid oxidatives	Acts as antioxidant, Improves weight gain and intestinal pathology	Mixed Species	Naidoo et al. 2008
VitisVinifera	Proanthocyanidins, epicatechin and catechin, dimeric, polymeric, trimeric, phenolic acids	Oocyst morphology is defined in some factors such as shape, size, and the amount of sporocysts.	Improves intestinal pathology and weight gain in the chicken's body. Presents antioxidant activity	Mixed Species	Wang et al. 2008: Abbas et al. 2020
Yucca schidigera	Saponins	Oocyst morphology, Excretion is reduced	Enhances productive efficiency (FCR and body weight).	Mixed Species	Hassan et al. 2008
Zingiberofficinale	Oleoresin and Gingerol	Reduced oocysts shedding	Increases the rate of weight gain in the body.	Mixed Species	Ali et al. 2019

due to Eimeria (Abbas et al. 2019b). Botanicals are enriched with antioxidant compounds and are likely to play role in the control of the coccidiosis disease. The beneficial effects against Eimeria are derived from phenolic and flavonoid compounds which attribute to antioxidant activity (Abbas et al. 2020). Many studies have shown that flavonoids have the capacity to act as powerful antioxidants by scavenging free radicals and thus reducing oxidative stress in host caused by Eimeria parasite. Flavonoids having multiple hydroxyl groups act as pro-oxidants. The mechanism of action of flavonoids is conversion of hydroxyl group into pro-oxidant when oxidized by Reactive Oxygen Species (ROS) present in inner cell membrane which leads to late necrosis or apoptosis of damaged cells by eliminating potential mutants (Masood et al. 2013).

Osmoprotectant

Many botanicals including Beta vulgaris and Camellia sinensis reduce coccidiosis infection by their osmoprotectant activity

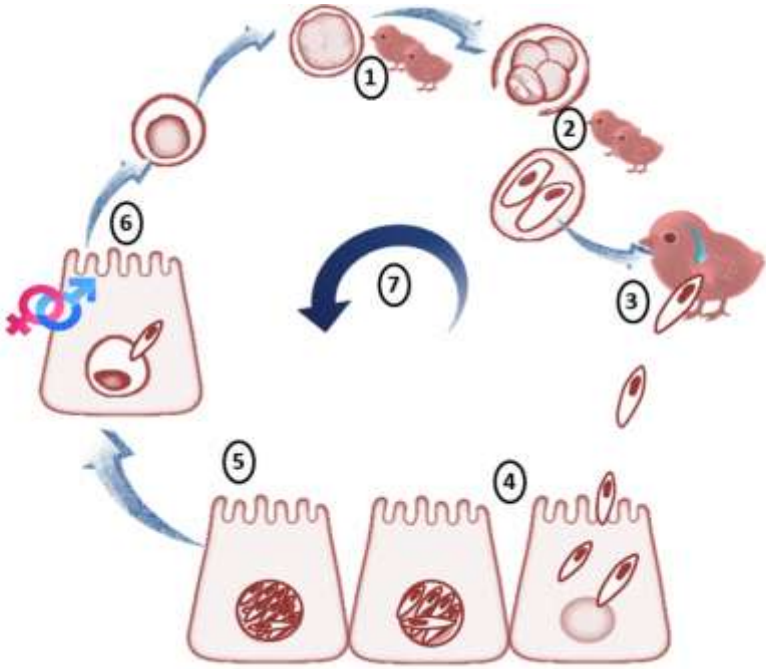

Figure 1: Life Cycle of *Eimeria*. Birds get infected by sporulated oocysts from fecal matter and reproduction occurs in the intestinal cells which leads to damage to the intestinal wall (El-Shall et al. 2022).

by stabilization intestinal cell membranes and immune stimulation thus acting as osmoprotectant against Eimeria parasite (Abbas et al. 2015, 2017a). Plants protect different cell types against chemical and environmental stress (Allen 2003).

Destruction of Life Cycle Stages

The protective effect of botanicals is not only restricted to the intestinal cells, but they also affect and damage life cycle stages of the coccidia including the asexual stages (sporozoites) and sexual life cycle stages (Zhang et al. 2020; El-Shall et al. 2022).

Immunomodulatory and Stimulation of Mucosal immunity

The essential oils of different botanicals such as *Trachyspermum ammi*, *Origanum vulgare* and many others are known for their immunomodulatory effects against parasites by stimulation of mucosal immunity and also known to enhance cellular and humoral immunity against coccidiosis. They are involved in immune stimulation, enhancement by macrophage activity and enhancing antibodies level in infected birds (Abbas et al. 2012a, 2012b, 2017).

Target the Exogenous Phase of Eimeria

Many botanicals such as Artemisia, thyme, clove, and tea tree oils target the exogenous phase as tested in in vitro trials leading to oocyst disruption (Remmal et al. 2011). A pure product extracted from *Artemisia annua* i.e., artemisinin showed a dose-dependent increase of dead oocysts shed in feces, an alteration in the sporulation rate, and a significant reduction of calcium ATPase in macrogamete endoplasmic reticulum, which most likely leads to abnormal oocyst formation (Cacho et al. 2010).

Target the Endogenous Phase of Eimeria

Botanicals also effect the endogenous phases of Eimeria parasite as considerable alterations were observed in sporozoite morphology effecting Eimeria viability and infectivity in an in vitro invasion assay using cumin derived from turmeric plant (Khalafalla et al. 2011). The effect of curcumin was also tested with other phytochemicals including carvacrol (major constituent of Oregano) and Echinacea purpurea extract which also showed immunomodulatory activity (Burt et al. 2013). A similar study was performed using essential oils of oregano and garlic showed strong anticoccidial activity, exhibited a positive effect on intestinal microorganisms in in vitro trial and improved growth performance in in vivo trial. Garlic is also known to have anticoccidial compounds like allicin, propyl thiosulfinate, propyl thiosulfinate oxide, and allicin have been shown to affect the endogenous phase of Eimeria and have anticoccidial efficacy against *E. tenella* (Sidiropoulou et al. 2020).

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CHAPTER 34

RECOMBINANT THERAPEUTICS EXPRESSED IN TRANSGENIC PLANTS WITH POTENTIAL APPLICATIONS IN VETERINARY DISEASES

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INTRODUCTION

The livestock industry has grown up swiftly from the last decade and earned $1.4 trillion annually (Sohaib and Jamil 2017). This industry is continuously evolving due to rapidly increasing demand of livestock products. Therefore, use of veterinary drugs is essential for therapeutic and prophylactic purposes in livestock production to improve growth, productivity, and food safety (Falowo and Akimoladun 2019). Only one segment of this sector i.e., meat production valued at 838.3 billion U.S. dollars in 2020 and forecasted to increase to 1157.6 billion U.S. dollars by 2025. These drugs are globally utilized to enhance the profitability and productivity of modern food animal production by facilitating higher animal densities, earlier weaning, meat quality, cheaper feeds, and carcass yield (Moreno and Lanusse 2017). These life-saving agents include a broad range of natural, synthetic, and semi-synthetic compounds such as, antibiotics, antiparasitics, β-agonist and vaccines (Moreno and Lanusse 2017). Among the antibiotics used in livestock production, commonly consumed are amprolium, penicillin, tetracyclines, streptomycin, tylosin, sulphonamides, aminoglycosides, β-lactams, quinolones macrolides and lincosamides (Landoni and Albarellos 2015; Alhaji et al. 2018) while antiparasitic drugs include anthelmintics or coccidiostats such as stilbenes, nitrofurans, amphenicols, nitroimidazoles, pyrethroids, carbamates and sedatives (Falowo and Akimoladun 2019).

The extensive use of antimicrobial agents leads to continuously increasing antimicrobial resistance. Mostly, scientists believe that improper and immense administration of antimicrobials is a single most significant factor that is responsible for emergence of resistance (Hoelzer et al. 2017). The veterinary researchers have identified that intestinal microbiome of food producing animals can act as a reservoir of resistant bacteria in the society (Graveland 2011; Patchanee 2014; Moradigaravand et al. 2017). However, there is high risk of multi-drug resistant bacterial zoonosis and pose a serious threat to the public health (Zhu et al. 2013; Jans et al. 2017; Lugsomya et al. 2018). At present, the average annual utilization of antimicrobial compounds per-kilogram of animal produced is approximately at >100mg/kg worldwide (Vishnuraj et al. 2016). It has been estimated that almost 80% of the antibiotics consumed in veterinary field are growth promoters, which mostly exceed the amount of total antibiotic consumption in human medical care (Vishnuraj et al. 2016). The antibiotic residues in edible animal products have increased beyond the acceptable level in most of developing countries (Use, 2017). Moreover, many scientific reports revealed that consistently use of antimicrobial agents in enormous amount result in deposition of drug residues in different organs and muscles of animals (Sanz et al., 2015). These residues in edible animal products can cause severe health risks to humans when ingested (Use, 2017). The development of antimicrobial resistance and hypersensitivity reactions are most common outcome in humans (Use, 2017).

Dawn of Recombinant Therapeutics

The recombinant proteins are gaining much attention worldwide due to its variety of applications. Efficient strategies are utilized to produce high quality proteins in enormous amount with low cost (Palomares et al. 2004). The potential of engineered recombinant proteins are widely explored for the development of therapeutic and prophylactic use (Gifre et al. 2017). These include antibodies, enzymes, cytokines, growth factors and vaccines (Schillberg et al. 2019). These proteins are synthesized in various expression systems depending upon the type of protein. Commonly used expression systems are bacteria, yeast, filamentous fungi, and unicellular algae (Legastelois et al. 2017; Owczarek et al. 2019). All expression systems have their own merits and demerits, and its selection depends upon the protein of interest to be expressed, such as, eukaryotic protein modifications are only possible in eukaryotic expression system because prokaryotic system does not support these modifications (Rai and Padh 2001). Moreover, cell free expression systems are now attracting the attention of scientific community to be utilized for the fast synthesis of recombinant proteins with eliminating the processes of purification (Swiech et al. 2012).

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These proteins are widely utilized due to its dynamic properties. The gene encoding the particular protein is isolated from the respective organism and synthesized in various expression systems. Thereafter, same protein is again injected in the living body e.g., insulin. Therapeutic proteins minimize the issues related to the synthetic and semi-synthetic drugs e.g., antibiotic associated diarrhea, unpleasant taste and reduce absorption from the gut. Appropriate modifications are required to increase specificity, to prolong half-life, and to improve functionality (Gupta et al. 2017). The continuous impressive work by the scientists in the recombinant protein technology have brought multiple therapeutic proteins into clinical applications (Kim et al. 2017). Due to these advancements, demand of recombinant proteins is increasing in the livestock sector as well.

Different Ranks of the Recombinant Therapeutics

Vaccines are very successful method for disease prophylaxis in humans and animals. Most deadly diseases are cured today through the conventional and modern vaccines which significantly decrease the graph of diseases in livestock (Jorge and Dellagostin 2017). Majority of conventional vaccines today in market include live attenuated vaccines, killed vaccines, inactivated vaccines, toxoids and cell membrane compounds (McVey and Shi 2010; Unnikrishnan et al. 2012). Attenuated vaccines are very effective in stimulation of immune response both humoral and cell mediated (Rizzi et al. 2012; da Costa et al. 2015). However, killed and inactivated vaccines are preferred over the attenuated vaccines due to its safety profile in the animal body, but they are less effective to elicit the immune response in the host along with its adverse effects (Cho et al. 2002; Moreira et al. 2016). The main problem associated with the attenuated vaccines is its reversion back to its virulent form after inoculation into the body (Shimoji et al. 2002; Unnikrishnan et al. 2012). Toxoid vaccines are raised against lethal and fatal bacterial and mycotoxins after inactivation through chemical agents (Arimitsu et al. 2004). Toxoids are effective in a sense that these induce reliable humoral immune response but negligible cell-mediated immune response (Jorge and Dellagostin 2017). The widespread use of these prophylactic agents prominently improves animal health. Although, multiple bacterial and viral diseases in animals are efficiently treated with conventional vaccines but they are still expensive to produce and require administration of multiple doses to achieve optimal immune response (Meeusen et al. 2007; Delany et al. 2014). Therefore, it is the necessity of the time to introduce the more immunogenic, safer and economical vaccines, which are more capable to efficiently control and eradicate the animal diseases.

The advancements in next generation sequence technologies and understanding the molecular mechanisms of pathogenesis of various pathogens resulted in the introduction of recombinant veterinary vaccines in the market (Jorge and Dellagostin 2017). It enables the genome and proteome screening with the aid of next generation technologies, which prominently enhance the chances of more appropriate antigen discovery. The vaccines based on the relevant epitopes could successfully evoke the optimal protective immunity in the host (Jorge and Dellagostin 2017). However, impressive progress in the field of genomics initiated the era of 'third generation' of vaccines using the applied technologies such as, reverse vaccinology that open it up in the broad spectrum (Dellagostin 2011; Rappuoli et al. 2014). This approach led to the identification of less abundant and non-identified proteins as vaccines. Furthermore, we can recognize the potential targets for vaccines by eliminating the process of passaging. The next generation vaccines could be multivalent, better safety profile and optimal immune stimulation (Oliveira et al. 2015).

Different types of recombinant vaccines have been designed for livestock animals for preventive purpose. Subunit vaccines are based on small, specific and non-infectious proteins. Therefore, these vaccines are safe and non-replicative immune-modulatory agents (Jorge and Dellagostin 2017). Moreover, we can evoke the immune response against multiple pathogens or multiple serotypes of same pathogen by inoculating the cluster of multiple proteins in one subunit vaccine (Dellagostin 2011). However, major drawbacks of subunit vaccines are moderate immune response and requirement of an adjuvant to produce robust immune response (Jorge and Dellagostin 2017). On the other hand, vector-based vaccines contain the core of pathogen and display multiple membrane bounded antigens (Jorge and Dellagostin 2017). The core is also utilized as a carrier to deliver genes for other pathogens; these antigens will express to stimulate immunogenic response. In contrast, DNA vaccines contain only the template that codes for the antigenic proteins. It not only overcomes the safety issues but also the production of cytotoxic T cells (Meeusen et al. 2007).

Recombinant Therapeutics Producing Plants – Need and Rationale

Plants have been historically used as medicine and lately the trend is shifting back to further explore their potential against bacteria, fungus, and other pathogens (Hamayun et al. 2021; Tariq et al. 2020; Khan and Javaid 2020; Rehman et al. 2020). Scientists are extensively studying the applications of plants related to the production of biopharmaceuticals. The plants are the ultimate source of food and various nutrients for living organisms on earth especially animals and humans. Now, scientists want to merge the biopharmaceutical objectives with their contribution in food manufacturing (Walmsley and Arntzen 2000). Therefore, the potential of plants is utilized for the production of growth factors, enzymes, vaccines, hormones, antibodies and peptide based antibiotic drugs along with the synthesis of essential proteins, primary sugars and vital amines. For this purpose, transgenic plants are developing using various applied techniques. The transgenic plants are preferred to achieve the therapeutic needs due to its enormous production and strengthening antigenicity (Rybicki 2009).

How does it work?

The manufacturing process of plant-based vaccines begin with the selection of gene of interest expressing the particular antigenic determinant. The candidate gene for particular vaccine is cloned in the plant expression cassette that have ability of promoting and terminating expression (Rybicki 2009). Subsequently, the expression cassette is delivered to the plant for synthesis of recombinant protein (Walmsley and Arntzen 2000). The stable or transient transformation occur after successful delivery of expression cassette to the plant carrying the particular gene. The transient gene expression is quick and convenient method but yield of protein is low in amount and production of foreign protein for temporary period (Liew and Hair-Bejo 2015). Contrastingly, the candidate gene is

permanently incorporated in the plant genome which is the principal benefit of stable transgenic expression. The antigenic trait is inherited in the genome, which allows the transfer of desired character over multiple generations (Santi 2009). Thus, mass stocks of transgenic seeds are available for the cultivation of next generation (Joensuu et al. 2008).

The plants for vaccine production are grown in plant factory systems instead of conventional soil-based cultivation. Artificial environment is created in plant factory systems to control the CO2 concentration, temperature, humidity, light quality and quantity, and defined hydroponic media (Shim et al. 2019). In contrast to egg-based vaccine production, which requires at least 180 days, the plant-based systems take only 21 days for vaccine production (D'Aoust et al. 2010). However, cultivation of transgenic plants in natural environment demands basic requirements such as, sunlight, water and nutrients for simple and economical propagation. Additionally, harvest and further processing do not need complicated procedures to achieve the final product (Mason et al. 2002). These systems are 10 to 40 times more economical than vaccine production by *E. coli* fermentation (Giddings 2001; Mett 2008) and 140 times cheaper than baculovirus insect-based system (Rosales-Mendoza et al. 2017). Moreover, we can manipulate the glycosylation pathway to produce diversity of similar antigens instead of specific glycosylation (Rosales-Mendoza et al., 2017). These systems also help in the synthesis of cheap and natural vaccines. Thus, plant factory systems are considered as alternative methods for the production of biopharmaceuticals worldwide. Despite its advantages, it requires the more attention of scientists to work out in this domain because only few vaccines have passed the pre-clinical trials and now passing through the clinical trials. The plant-based systems got success to develop the vaccine against most common disease in cloven-hoofed animals i.e., the foot and mouth disease. The VP1 whole coat protein or the antigenic peptides of FMDV are successfully expressed in different transgenic plants like, alfalfa, arabidopsis and potato. In addition, VP1 is also expressed using the plant viral vector such as, tobacco mosaic virus and tobacco leaf curl virus. The leaf extracts were prepared and delivered by ingestion and injection into the intraperitoneal cavity of mice. The mice developed protective immunity and upon the live FMDV challenge, it showed protection against it. Afterwards, experiments were conducted in the swine, the natural host of FMDV. The VP1 immunogenic peptide was inserted in the modified coat of bamboo mosaic virus followed by its infection to *Chenopodium quinoa,* the host for Bamboo mosaic virus. Two doses of 5mg of leaf extract were prepared and inoculated intramuscularly in the pigs. This resulted in the synthesis of anti-FMDV antibodies. Then the pigs were challenged with the live FMDV and after four weeks of booster dose showed complete protection (Liew and Hair-Bejo 2015). Similarly, the same approach was used to produce vaccine against mink enteritis virus and rabbit hemorrhagic disease. In MEV vaccine, the viral VP2 capsid was expressed in black-eyed bean. The short epitope was incorporated in the cotton mosaic virus followed by infection to the plant. Two doses of 1mg leaf extract were injected subcutaneously that developed optimal immunity in the mink (Dalsgaard et al. 1997). Likewise, Vp60 of rabbit hemorrhagic disease was inserted in the potatoes to produce immunity in the rabbits (Castanon et al. 1999).

Apart from livestock vaccines, plant based transgenic poultry vaccines are also under process against major poultry diseases. The infectious bursal disease is highly contagious and deadly disease of young chickens. The VP2 capsid contain two segments, segment A and B (Nick et al. 1976). The strain E gene of segment B contain neutralizing antigenic determinants, which is incorporated in the *Arabidopsis thaliana* (Wu et al. 2004). In another study, gene of attenuated segment A of VP2 was expressed in rice seeds. In oral immunization trial with rice seeds, four consecutive doses of 5g transgenic rice seeds were fed with the interval of one week each (Wu et al., 2007). One dose contained 10mg of VP2 protein and stimulate optimal immunity in the chickens. The chickens remained healthy when infectious IBDV was challenged to it (Mason and Herbst-Kralovetz 2011).

The transgenic plants seem to be excellent alternative source for the production of biopharmaceuticals. The transient expression systems produce rapid synthesis of therapeutic proteins while stable expression requires permanent insertion of genetic element in the plant genome. The transgenic plants grown in the natural systems require only basic plant needs, which are able to produce recombinant proteins. The glycosylation pathway can be manipulated to produce diverse post-translational modification (Shim et al. 2019). Moreover, cheap and enhanced productivity in the plant factory are the prominent advantages in the current economic situation worldwide.

Challenges of Recombinant Therapeutics Production in Plants

Plant based recombinant therapeutics are in high demand due to their low cost, high efficacy, edible property and ease of administration. Host plant system act as a bioreactor for the intended transgenic protein and express it alongside other host proteins. Although plant derived recombinant proteins are holding a promising future yet this system is not completely ideal and risk free. There are certain limitations which hinder the full utilization of plant derived recombinant protein production i.e. (1) selection of plant host (2) limited product yield (3) Safety and health concern (4) Environmental risks.

Selection of Protein and Plant Expression Host

First and most critical step in recombinant protein production is the selection of suitable plant expression system as well as desired protein. This stage is crucial as all the plant expression hosts are not compatible with desired protein or antigen and hence will compromise the expression. Careful selection and use of modern approaches (such as genomics and proteomics analysis) help in development of vaccines for poorly characterize pathogens (Rigano and Walmsely 2005; Sharma and Sood 2011).

Limited Product Yield

In plant based recombinant therapeutics, product yield is of prime importance and defined as grams of product obtained per unit of plant biomass. Although biomass production is scalable in molecular pharming but product expression is low in magnitude and require more attention and efforts to achieve the desired targets. There are various factors behind the yield limitation i.e. genetic elements choice, epigenetic, environmental and biochemical factors alongside downstream processing techniques (Twyman 2013).

Yield Limiting Genetic Factors

Product yield is largely depending upon the transcriptional and translational efficiencies of transgene and require special attention while designing the construct. Choice of upstream and downstream regulatory elements is critical in this regard. Strong promoter alongside other genetic elements is necessary for higher, stable and organ specific transgene expression. A strong constitutive promoter is often required to enhance the transcriptional yield which will further enhance the product (protein) yield. Organ specific promoter is often desirable where transgene product is hindering vegetative growth of host plant or a specific plant organ is intended for harvesting and post-harvest applications. Seed based edible vaccines are an example of it as they are easy to administer and store. Apart from promoter, certain other genetic elements can also be introduced into expression constructs to enhance mRNA stability or to enhance the translation efficiency. These elements are either endogenous elements such as 5' or 3' UTRs (Untranslated regions) or exogenous such as introns, Kozak's consensus sequence etc. (Mitsuhara et al. 1996; Sharma et al. 2008; Lu et al. 2008; Peremarti et al. 2010).

Epigenetic Factors Affecting Yield

As compared to genetic factors, epigenetic effects are independent from DNA sequence. They mainly influence the expression cassette through their position, structure or complexity of the locus. These factors are hard to control as transgene integration is random instead of sequence specific. Due to the above-mentioned limitations, transgene integration into host genome is of prime importance. Surrounding genetic elements as well as integration into a silencing locus (positional silencing) both can influence transgene stability and expression. Sometime multi copy transgene are prone to instability and silencing. But many other instances shows that high copy number is proportional to greater gene expression, hence proving that copy number is not the reason behind silencing but some other factors influence it, such as hairpin loop formation etc. In order to avoid epigenetic silencing, intervening genetic elements (MARs matrix attachment regions) can be introduced in between transgene and surrounding host genetic elements. Transient gene expression (which involves the expression of transgene into non-transgenic plants without integrating into host genome) is often preferred to avoid epigenetic silencing. Although transient expression is short lived due to transgene degradation and environmental stresses but still its ability to give high yield in a short period of time is promising and utilized in large scale vaccine manufacturing to cope the high demand (Topping et al. 1991; Kohli et al. 2003; Datta et al. 2003; Halweg et al. 2005; Kohli et al. 2006; Paul et al. 2013).

Yield Limiting Biochemical and Environmental Factors

Expression and accumulation of recombinant protein in plants is depending upon both intracellular as well as external factors. Biochemical species within cells (include proteases, free radicals, pH and salt etc.) often interfere with recombinant protein, limiting its accumulation and stability. Through experimental investigations, it is learnt that secretory pathway of protein synthesis is more feasible in terms of protein folding and post translational modification as compare to cytosolic protein synthesis. A common approach in this regard is the attachment of a signal peptide to target recombinant protein into ER and Golgi bodies from where they will be either stored into vacuole or apoplast (Schillberg et al. 1999; Vitale and Denecke 1999).

External factors (such as nutrients, heat, pH, light etc.) can also interfere in plant growth ultimately affecting expression of recombinant protein. Nitrogen availability is critical in plant metabolism as it pivotal in amino acid biosynthesis. In order to ensure uniform growth, plants are grown under controlled condition and all growth requirements are monitored regularly (Fischer et al. 2012; Twyman 2013).

Downstream Processing or Harvesting Issues

Downstream processing or harvest intended for the extraction of recombinant protein is critical in both plants derived as well as conventional expression systems. Innovative separation strategies are being utilized to overcome this issue such as co-extraction of proteins with lipid fraction (Oleosin platform) followed by endo-proteolytic cleavage. Other strategies include expression of recombinant proteins into edible parts of plant such as seed or fruits and consume directly. Despite of their promise, edible recombinant proteins havecertain limitations such as dosage determination, antigen selection, efficacy, quality control and regulatory issues (Paul and Ma 2011).

Health and Safety Concern

Plant derived recombinant proteins pose some health and safety concerns which need to be considered while utilizing the plant-based systems. One of the safety concerns is the development of hypersensitivity (allergy) especially against orally administrated vaccines and therapeutics. Certain post translational modification such as N glycosylation and administration of vaccines with adjuvants may cause hypersensitivity issues. In order to produce plant derived protein of biopharmaceutical use, manufacturing facility should be well equipped and follow the guidelines of regulatory authorities. Stringent quality control management which includes Good Agriculture Practices (GAP) and Good Manufacturing Practices (GMP) is the mandatory and should be primary responsibility of manufacturer. Implementation of GMP standards is a huge challenge which can be addressed through in process monitoring, skilled workers and by the proper design of the production facility (Cox et al. 2012; Guan et al. 2013; Sato et al. 2014).

Environmental Risk

Escape of the transgene into environment is the biggest concern in genetic engineering. Many GMO varieties utilize the toxic or resistance genes which if escape can cause some serious problems. Molecular pharming of transgenic plant alongside the non-transgenic varieties can contaminate the non-GMOs and confer them toxic or resistance properties. During the production of recombinant proteins, transgene might escape and contaminate the normal food chain; this will result in safety and health issues (allergic reactions). Majority of recombinant protein production utilizes the antibiotic resistance gene markers; hence imparting the resistance issues in bacteria and other microorganisms (WHO 1992).

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CHAPTER 35

ADVANCEMENT IN AQUACULTURE SYSTEM BY APPLYING BIOTECHNOLOGICAL TOOLS

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INTRODUCTION

The usage of technology has always been a desire of humans to make life easier. Seafood is in high demand all across the world. Sufficient natural harvesting does not appear to be a viable option for meeting rising demand. Several natural ocean and freshwater fisheries are being fished to their limits, according to worldwide consensus. Aquaculture might assist to fulfil rising demand and biotechnology can help to enhance aquaculture output significantly. The application of current biotechnological methods to promote production of aquatic species offers significant promise not only to satisfy demand but also to improve aquaculture. Fish farming is in high demand and biotechnology can assist to supply that need. Within the last few decades, developments in biotechnology have offered the equipment needed to manipulate genes and chromosomes in live beings artificially. The existence of transgenic fish and shellfish is a hot issue in marine research because of the promise for increased productivity that this technology may provide (Zbikowska 2003; Dunham 2004). Researchers are looking for alleles that will boost the synthesis of natural fish growth hormones and innate defensive chemicals which are utilized by sea creatures to combat microbial diseases. Gene editing is now making a major impact to coastal population growth while also posing considerable problems. It believes that current biotechnologies ought to be utilized in conjunction with traditional technologies instead of as a replacement. Genetic manipulation and bioengineering have enormous promise for improving the production of aqua cultured fish. Aquaculture is in high demand, and biotechnology can assist supply that need. Aquaculture, like other bio-engineered foods, would be heavily controlled prior to getting allowed on the market. Biotechnology in aquaculture is also environment friendly. Biotechnology may help to satisfy the demands of a rising and highly industrialized society over the next century if implemented fully with some other techniques in the field of food, farm products, and services. Only a thorough study and learning foundation in the biology, breeding, physiology, pathology, biochemistry, and genetics of the transformed organism can result in effective biotechnology implementation. But without the need for a continuous commitment to fundamental research, the benefits promised by emerging technologies will not came to realization. Use of artificial hormonal steroids in induced breeding, transgenic fish, gene banking, uniparental and polyploidy population are all possible biotechnology applications in aquaculture. This is either the prevailing biotechnology which has piqued the interest of the people because it has the potential to pose a significant impact on the global market. Genetic manipulation is a fundamental aspect of modern biotechnology. Genetic manipulation is not a biotechnology within itself, but rather a biotechnological approach that has evolved over years of fundamental study in cell and molecular biology. A gene may now be found, extracted, cut out, inserted and altered. Genetic engineering is the term for this type of genetic alteration. Food, agricultural production, forestry, fish farming, livestock farming, and horticulture are just a few of the areas where genetic modification has improved our awareness of living organisms and allowed us to adapt that expertise to our lives and activities (Opabode and Adebooye 2005; Ezeonu et al. 2012). The increased public need for fisheries and the degradation of natural resources have prompted researchers to look at how biotechnology may help increase the production of fishery, resulting in aquaculture being a burgeoning topic of research (Billington and Hebert 1991). In fisheries, biotechnology helps researcher to search and integrate good characteristics in marine species to boost productivity and profitability. Researchers are looking for genomes which might boost the

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synthesis of aquatic animal growth and natural defensive proteins which marine organisms utilize to combat microbial illnesses.

Importance of Aquaculture

Living beings have recognized the value of water as a resource since it covers 70% of the earth's surface. As a result, fish farming, primarily for the production of food, is one of the most intensively utilized sectors in terms about the use of freshwater resources, as compared to just utilizing terrestrial land. Aquaculture is the practice of raising, reproducing, and harvesting aquatic animals and plants in controlled aquatic settings such as seas, lakes, rivers, ponds, and streams. Aquaculture has the potential to significantly boost and try to guarantee worldwide supply of food in environmentally and socially responsible ways. Aquaculture is among the most resource-efficient and ecologically friendly ways to generate protein for humans when compared to rearing of land animals like cows and pigs. Aquacultured seafood can also contribute to the achievement of the United Nations' Sustainable Development Goals (SDGs). It's a lot more effective, which means the seafood producers can increase yields with less feed. Because less feed and energy are required in the manufacturing of food, the procedure is also less expensive. It conserves resources and even allows for the production of additional food, resulting in secure stockpiles and reduced environmental stress. Individuals and organizations all around the globe, benefit from aquaculture because it provides economic possibilities and quality jobs.

Relationship Between Aquatic Life and Ecosystem

The phrase "aquatic environment" refers to everything in the bodies of water to the coastal waters that surround them, to large lakes (especially landlocked saline open sea), to lakes and ponds and the small marshes and swamps that are frequently found nearby. Water is found in huge amounts in all living species, and existence as we know it would not be conceivable without the unique qualities of such water (Barnes and Mann 1991).

Biotechnological Application in Aquaculture

Biotechnology in Fish Breeding

According to Bhattacharya et al. (2002), GnRH (Gonadotropin-Releasing Hormone) has become the most effective biotechnological tool for triggering fish breeding. GnRH is the primary responsible and fundamental activator of the reproductive cascade in all vertebrates. It's a decapeptide that can cause the hypothalamus to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). It was initially identified from pig and sheep hypothalamus (Schally et al. 1973). Since then, just one version of GnRH has been discovered as the single polypeptide responsible for both the secretion of LH and FSH in all terrestrial animals, including humans. Nevertheless, twelve GnRH variations have been already structurally characterized in non-mammalian species (excluding the guinea pig), with seven or eight distinct forms extracted from marine animals (Powell et al. 1994; King and Millar 1995; Jimenez-Liñan et al. 1997). The recent development in GnRH discovery were made by Carolsfeld et al. (2000) and Robinson et al. (2000). A variety of synthetic equivalents have been developed based on structural variants and bioactivities, one being the salmon GnRH alternative, which is widely used nowadays in fish breeding and sold publicly with the name "Ovaprim" across the globe. In fact, unless the hormone stimulates them, the majority of commercially significant culturable fish in landlocked water do not reproduce. The advancement of GnRH technology has now effectively enabled induced breeding of fish (Halder et al. 1991).

Transgenesis

Transgenesis provides a wonderful chance, enabling aquatic scientists that can change as well as improve the biological characteristics of commercially significant fishes, shellfish, and crustaceans. External gene/DNA is introduced into the host's genetic material which results among its permanent retention, propagation, and activation. The procedure has already been used to a variety of aquatic species with tremendous results. Palmiter et al. (1982) were among the first to develop transgenic mice by inserting metallothionein-in human growth hormone fusion gene (mT-hGH) in mice eggs, leading to a remarkable increase in growth. This sparked a flurry of genetic manipulation experiments in commercially significant species, notably fish. Zhu et al. (1985) in China generated the very first transgenic fish, claiming transitory transcription in presumptive transgenic organisms but provided no scientific proof for transgenic incorporation. Substantial growth increase has been shown utilizing this strategy notably in Salmonids (Devlin et al. 1994). Hew et al. (1995) revealed that rise in size is remarkable, averaging four to six times than that of the reference, with many animals as large as ten to thirty times that of the control. An improvement of fish resilience to low temperatures has become a focus of fish transgenic studies for some times (Fletcher et al. 2001). Many fishes are stressed by cold-water conditions, and just a few that are able to tolerate temperature range below 0°C. In frigid regions, this is really a big issue in aquaculture. Certain fishes generates a signal that contain large quantities of serum antifreeze proteins (AFP) or glycoproteins (AFGP), that substantially lower the subzero temps by inhibiting ice crystal development. For a long time, researchers in Canada have been studying the isolation, characterization, and control of these antifreeze proteins, notably in the Atlantic Ocean bream *Pleuronectes americanus*. As a result, the genes for winter flounder liver antifreeze protein was effectively incorporated into the genomic sequence of Atlantic Salmon, where it was linked into the germ line and subsequently transient response to the progeny F3 where it is being produced particularly in the liver (Hew et al. 1995). The production of lines with this mutation would've been extremely beneficial in aquaculture production in areas where minimum temperature frequently approach such fish' biological limitations.

The advancement of embryonic stem cell (ESC) technology is definitely the most intriguing technique also for advent of transgenic fish farming. Because the cells are undeveloped and totipotent, they may be altered in laboratory and then reintroduced into embryonic cells to participate towards the recipient's genetic structure. Then it would make it easier to add or remove traits in a stable fashion (Melamed et al. 2002). Whilst great advancements have been made in multiple research centers across the globe, there are still a number of issues to be tackled until recombinant hatchlings for farming

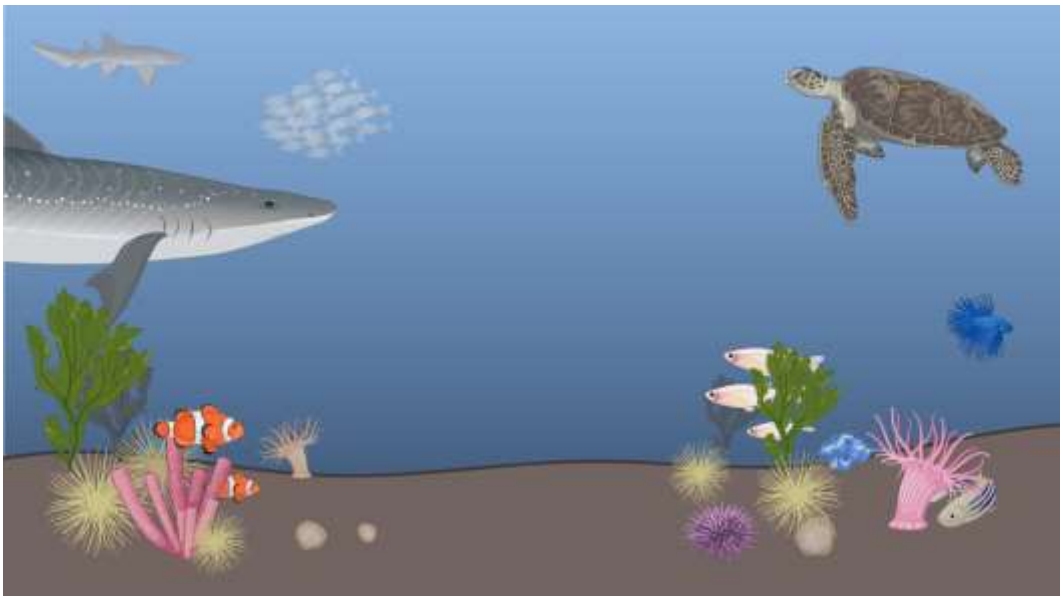

Fig. 1: The Aquatic Environment.

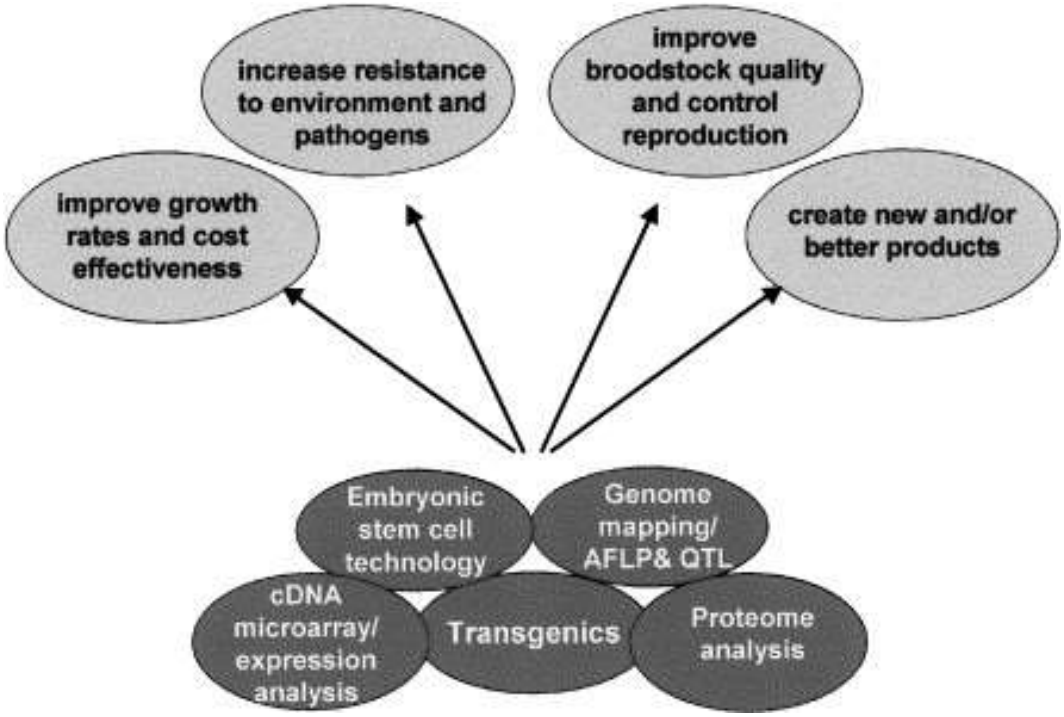

Fig. 2: An illustration of several genetic and genomic technological tools (dark-shaded) and their prospective farming uses (light-shaded).

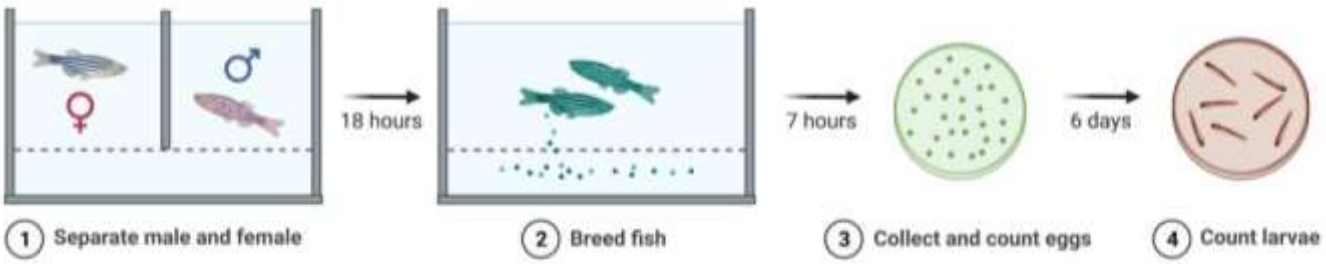

Fig. 3: Fish breeding procedure

may be successfully commercialized. Numerous significant technological discoveries are necessary to fully fulfill the capabilities of mutant fish biotechnology in aquaculture. There are (i) highly accurate bulk genetic manipulation methods (ii) focused transfection techniques such as embryonic stem cell gene transfer (iii) adequate regulators to guide gene

transcription at optimum levels at the appropriate phases of growth (iv) Genetic mutations for desirable features in aquaculture and its other uses found. (v) Genetically modified fish security and ecological consequences. Employing various transgenic approaches, researchers are attempting to enhance the specific genes of farmed fish. Employing various transgenic approaches, scientists have attempted to enhance the specific genes of farmed fish. Scientists are attempting to create fish that will be fatter, mature quicker, much more effective at turning their diet into musculature, diseases resilient, can withstand lower oxygen concentration, and can withstand cold temperatures. In recent times, tremendous progress has been made in the breeding programs of Tilapia utilized in farming. For instance, by the employment of mono-sex and selective breeding procedures (Beardmore et al. 2001).

Remodeling of Chromosomes

In farmed fish species, chromosomal sex modification methods were used widely to create polyploidy (triploidy and tetraploidy) and autosomal chromosomal transmission (gynogenesis and androgenesis) (Lakra and Das 1998; Pandian and Koteeswaran 1998). These procedures are significant for improving fish progeny because they give a quick method for pubertal ablation, sex selection, mutant survival enhancement, and cloning. Many animals are heterozygous, which means their somatic cells have two full chromosomal sets. For aquaculture marine species, chromosomal sex modification methods have been utilized frequently to create polyploidy (i.e. triplody and tatraploidy) and uniparent chromosome transmission (gynogenesis and androgenesis) (Baroiller et al. 1999). Such procedures are significant for improving fish reproduction because they give a quick method for gonadal sterility, sex selection, offspring survival enhancement, and cloning. The most successful approach for creating infertile salmon for farming and marine control is artificial triploidy (Bongers et al. 1994). Animals with polyploidy have one or perhaps more extra chromosomal pairs, raising the overall the number of ploidy to triple in triploids, quadruple in tetraploids, and so forth. The much more successful approach for creating barren fish for farming and fishery control is artificial triploidy. Tetraploid mating strains might help fisheries by making things easier to create massive groups of sterile tetraploid fish using straightforward interploidy combinations among tetraploids with diploids (Chourrout et al. 1986; Guo et al. 1996). Whilst tetraploidy has indeed been generated in a variety of freshwater fish species genera, tetraploid survival has already been limited in most cases (Rothbard et al. 1997). Strict regulations and a lack of customer acceptability of hormone-treated fish items may hinder the adoption of synthetic hormones. Triploidy can really be generated by subjecting eggs to physical or chemical treatment soon after conception to prevent the second polar body from extruding, since sister chromatids fail to attach appropriately throughout the first stage of meiosis, triploid fish are likely to be infertile (Purdom 1983; Thorgaard 1983; Ihssen et al. 1990). Thermal stress (warm or cold), surface tension shock, or drugs such as ancolchicines, cytochalasin-B, or nitrous oxide are all used to induce triploidy in egg cells. Teraploids and diploids could also be crossed to generate triploids. Tetraploid initiation is achieved by fusing oocytes containing healthy sperm and treating the diploid zygote to thermochemical intervention in order to prevent the first mitotic phase. Gynogenesis is the stage of animal growth in which only feminine chromosomes are passed along. Fish breeders are particularly interested inside the creation of gynogenetic swimmers since a substantial rate of inbreeding may be achieved in a couple of generations. In fishes exhibiting feminine homogamety, gynogenesis can also be employed to establish overwhelmingly female colonies and expose the sex - selective processes in fish. Regarding gender reversal research, it is more practical to employ entirely female gynogenetic inbred lines (rather than regular hetero descendants) (Gomelsky et al. 2000). Androgenesis is a technique that might be used in fisheries for economic purposes. It could also be used to create homozygous fish strains and to regain lost genomes from cryopreserved male gametes.

Improved Disease Tolerance in Fish

A variety of vaccines targeting viruses and bacteria have indeed been formed for commercial fish farming. Some of them were traditional vaccinations made from dead microorganisms, however a new generation of immunizations made up of protein subunit vaccines, genetically modified organisms, and DNA vaccines is now being developed. Biotechnological technologies including molecular screening techniques, vaccinations, and immunostimulants are rapidly growing for enhancing disease resistance in marine species throughout worldwide. When it comes to viral illnesses, pathogen prevention is crucial. In this situation, a quick approach for detecting the pathogen is required. Therefore in this field, scientific technologies like genetic markers and polymerase chain reaction (PCR) have a big future. For a variety of infections that afflict fish and crustacean, gene probes and PCR-based detection approaches have been devised (Karunasagar and Karunasagar 1999). Vaccination against diseases is a typical technique for prevention. Although the immune response of shrimp is yet underdeveloped, scientific technologies can aid in the synthesis of molecules that can boost such responses. Latest research has demonstrated some bacterial substances like lipopolysacharides, peptidoglycans, and glucans which can trigger the generic defense mechanism (Itami et al. 1998). Glucan and levamisole are two immunostimulants that have been shown to improve phagocytic activity and specific immune reactivity in fish (Sakai 1999). Health issues are indeed a serious roadblock to aquaculture growth. Vaccines targeting bacterial and viral pathogens have now been obtained in the commercial fish aquaculture sector. Traditional screening for immune function and biochemical tests of microorganisms for characterization and identification are both employed to enhance animal wellness using genetic biotechnologies. DNA-based technologies are increasingly being employed to describe various pathogen's genera and types. Genomic probes may be designed to test for particular infections in tissues, entire individual, and perhaps even water and soil samples after the culprit has been identified. In several regions, such approaches are really being utilized to identify viral illnesses in marine shrimp as well as bacterial and fungal pathogens in fisheries (Subasinghe and Bondad-Reantaso 2006; Subasinghe 2009). The successful prevention as well as cure of farmed fish illness necessitates the availability of quick, accurate, and extremely susceptible diagnostic assays. To address these issues, bioassays and DNA-based screening approaches, as well as polymerase chain reaction (PCR) amplification approaches, have been developed.

Vaccines

Vaccines and immunostimulants can be given as feed supplements, dissolved in water, or injectable within the case of relatively larger cultured animals such as fish and poultry. To prevent animals from infections, genetically modified vaccines are indeed being investigated. Inside the realm of vaccinations and immunostimulants for farmed fish, technological advancements are really useful. These make it possible to perform disease-prevention strategies such as immunization or immune defense strengthening. Fish vaccines became an accepted, verified, and economical strategy for managing various contagious illnesses in farmed animals across the globe within last 2 - 3 decades (Subasinghe 2009). Several vaccines are being widely marketed for fish infections, such as for furunculosis (Aeromonas salmonicida), and many others in progress, such as viral hemorrhagic septicemia (VHS), are presently available. Vaccines not only lower the illness intensity but also minimize the use of antibiotics, leaving no remnants in the product or ecosystem, and therefore do not cause microbial resistance (Subasinghe and Bondad-Reantaso 2006).

Gamete Cryopreservation or Gene Banking

It is characterized by the fact that a really low temperature slows or incapacitates a cell's physiological and metabolic activity, allowing it to survive for an extended length of time. Livestock farming have also adopted that cryopreservation of fish spermatozoa (milt) technique. Cryopreservation is a process that involves the long-term preservation and retention of biomaterial at extremely low temperatures, typically -196 °C (liquid nitrogen temperature). Blaxter (1953) documented the very first accomplishment in keeping fish sperm at low temperatures by fertilizing Herring (Clupea herengus) oocytes using frozen thawed sperm cells. Mostly all farmed fish species' sperm cells have now been cryogenically preserved (Lakra 1993). Cryopreservation fixes the issue of male maturation prior to female maturation, allowing for selective breeding and stock management, and provides for survival (Harvey 1996). The gene pool stocks are used to create the majority of plant types. The marine gene bank, on the other hand, suffered from the reality that only the male gametes of finfishes can be cryopreserved now at the moment, with no feasible method to preserve female oocytes and embryos. Nevertheless, previous findings by Diwan and Kandasami (1997) and Subramonium and Newton (1993) upon this chilling of shrimp embryos appear encouraging. As a result, it is important that gene banking of cultured and cultivable aquatic species be completed as soon as possible.

Bioinformatics

The use of digital technology to the administration of biological data is known as bioinformatics. Biological and genetic data is collected, stored, analyzed, and integrated using computers. Bioinformatics has brought several advances to the biomedical sciences, including algorithms for creating, maintaining, and accessing sequence databases. Bioinformatics aims to create an extensive list of genomes and nucleotide sequences. Proteomics, in opposition to genomics, aims to investigate the proteins that are described. Proteins are involved in both physiological and pathological processes in a cell or organism, and proteomics describes the whole catalogue of proteins in relation to in vivo factors. Proteomics is a method for studying biosciences that complements genomics.

Bioinformatics Tools

Bioinformatics tools are software applications that are used to store, retrieve, and analyze datasets in order to collect required data. These tools come in a variety of shapes and sizes.

Tools for Homology and Similarity

The concept homology refers to a parallel evolutionary development between two attributes. Homologous genes are those that have diverged from either a single ancestor and are similar. This methodology could be used to make comparisons between unique reference sequences with uncertain structure and function and database sequences with defined structure and function.

Protein structural Analysis

This package of tools helps you to measure structures to databases of known structures. Since structurally homologs happen to associate functionalities, a protein's activity is much more entirely a result of its own form than that of its sequencing.

Protein sequence Analysis

This software suite enables you to perform more in-depth research on any known sequences, such as phylogenetic analysis, genotyping detection, hydropathy areas, CpG islands, and configurational errors.

Recent Biotechnological Advancement in Aquaculture

Surrogate Broodstock Technology

Surrogate broodstock technique entails the creation of donor-derived gametes in foster parents, as well as the transplantation of donor animals' spermatozoa into sterile receivers (surrogates) of the same or similar species (Yoshizaki and Yazawa 2019). While using this approach, Okutsu et al. (2007) and Takeuchi et al. (2004) were able to efficiently form masu salmon (Oncorhynchus masou) producing rainbow trout (Oncorhynchus) mykiss reproductive cells. There are two key phases in surrogate broodstock technology: (i) Collection and fortification of germline stem cells (GSCs), the progenitors of gametes, and (ii) implantation of GSC into barren receivers (Jin et al. 2021).

By use of surrogate bloodstock technology has significant prospects to expand the breadth and effectiveness of genetic modification studies in farmed fish. The development of CRISPR/Cas genome editing tools has advanced gene and genome function studies through the production of animals and cell lines containing exact focused mutations. In farmed fish, this has often been achieved by pronuclear inoculation of genome editing agents in early-stage embryos, which has been effective in attaining gene deletion inside the founding (F0) individuals (Jin et al. 2021).

Further Perspectives

It is essential to urge individuals globally to create and adopt suitable techniques and hazard assessment standards for food biotechnology research and to assure the healthiness and quality of food chain. Appropriate bio-safety rules, hazard evaluation of biotechnological products, method and techniques for monitoring usage and observance are essential to assure that there would be no detrimental impacts on the surroundings or even for humans. Significant ecological risks from emerging technologies of biotechnology, notably including genetically modified organisms (GMOs), have created worries that in the lack of sufficient laws, multinational businesses in affluent nations may exploit underdeveloped countries as test ground for their goods. A few of the important ecological dangers involve plant - parasitic nematodes (Altman 1999). Improved fertility in physiologically acceptable wild species can come via genetic leakage from genetically modified organisms.

Conclusion

Biotechnological research and development is rapidly expanding. Biotechnology has been increasingly important for the overall growth of aquaculture, agribusiness, especially changed people's life in past few years. Biotechnology has given us technological skills and incredible capacity to develop genetic variants and genetic mutations in crops, livestock and in aquaculture. The use of biotechnology in the fishing industry is indeed a fairly new activity. Nonetheless, it appears to be a good place for increasing seafood output. In addition to stimulating in protecting the natural environment, the use of biotechnological techniques has the potential to change modern farming practices. This chapter highlights the present state of transgenesis, chromosomal editing, the use of synthetic hormones in fish breeding, biotechnology in health management, and gene banking.

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