Molecular Techniques Used for Diagnosis of Zoonotic Diseases

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ABSTRACT

Zoonotic diseases are described as diseases that are transmitted between animals and humans through direct or indirect contact or by any other route. Many microorganisms causing recently identified zoonotic infections in humans were initially detected in animals (specifically, wildlife) or products of animal origin. Dogs and cats are the most common pets which can disseminate zoonotic diseases. Rabies, ringworms, Campylobacteriosis, Salmonellosis, and Leptospirosis are the most common diseases that can spread through dogs. All domesticated animals, including poultry, have the potential to harbor germs that cause food-borne diseases. Around 90% of bacteria-related food-borne infections are caused by Salmonella spp. and Campylobacter spp. Molecular biology methods are increasingly being used in small animal veterinary care to diagnose infectious diseases. Understanding of infectious disease agents has improved because of techniques like polymerase chain reaction (PCR), Real-time PCR, Restriction fragment length polymorphism (RFLP), ELISA and CRISPR-CAS. Approximately 50% of bacterial and 87% of viral genomes have been identified so far by different molecular techniques. To subtype organisms beyond phenotypic categories, genotyping techniques such as PFGE, RAPD, REP-PCR, and AFLP are especially applied. As DNA is the starting material, these molecular biology techniques are often frequently referred to as "DNA fingerprinting." In addition to epidemiology, these techniques are also employed in forensic medicine and evolutionary biology research. Compared to phenotyping procedures, genotyping techniques frequently offer better discriminating power. Another way to identify genomic sequences from various microbial communities is through metagenomics. Over the last few decades, metagenomics-based methodologies have been developed to assess, analyze, and utilize biodiversity across a wide range of various environmental niches. Metagenomics has helped characterize the microbiomes in many samples, such as the gastrointestinal tract of various creatures (e.g., feline, canine, human, mouse, and chicken), in addition to the discoveries of viral genomes. As technology continues to evolve, the integration of molecular diagnostics into routine surveillance and healthcare systems holds promise for more effective prevention and control of zoonotic diseases, ultimately safeguarding both humans and animals.

Keywords: Zoonosis, One-Health, RT-PCR, Metagenomics, CRISPR-CAS, Genotyping, Phenotyping.

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1. INTRODUCTION

1.1. ZOONOTIC DISEASES: AN OVERVIEW

Zoonotic diseases are described as diseases that are being transmitted between animals and humans through either direct, indirect contact or by any other route. Many of the microorganisms causing recently identified zoonotic infections in humans were initially detected in animals (specifically, wildlife) or products of animal origin (Van Eeden 2014). Understanding the extra-human reservoirs of these viruses is still essential for figuring out the epidemiology of these zoonotic diseases and potential prophylactic measures. The biological characteristics of the pathogen determine whether it will develop to transmit from person to person. The formation of new diseases can be seen as an evolutionary reaction to the environmental conditions (Gluckman et al. 2007).
Changes brought on by anthropogenic factors include modifications in using agricultural techniques, or, as well as climate change. In order to characterize infectious agents, several methodologies are employed (Ugochukwu et al. 2022). These can be broken down into methods based on direct analysis of microbial protein sequences. Fig. 1 highlights the role of wild animals in transmission and amplification of zoonotic agents.

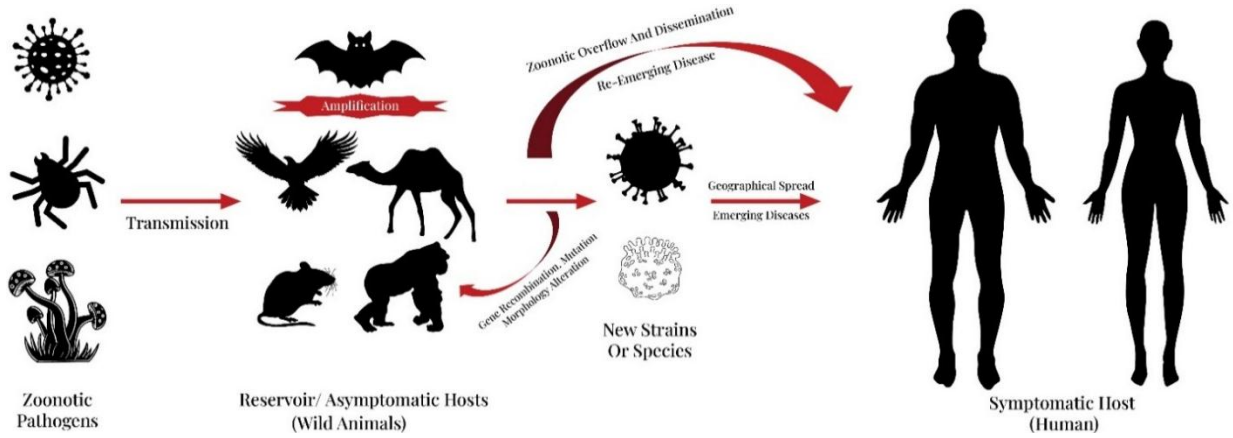

Fig 1: The involvement of the wild animals in the transmission and amplification of etiological agents of emerging and re- emerging zoonosis (Mehmood et al. 2023).

Molecular biology methods are increasingly being used in small animal veterinary care to diagnose infectious diseases. Understanding of infectious disease agents has improved because of techniques like polymerase chain reaction (PCR), Real time PCR, Restriction fragment length polymorphism (RFLP), many of which were created initially for research objectives only. As a result, the diagnostic characterization of these quantitative assays was quickly understood, among these, some are now used to screen for infections in pets and domestic animals (Sellon 2003). Molecular biology approaches rely on the biochemical properties of nucleic acids, which are further determined by their sequencing patterns. The capacity of nucleic acid sequences to bind to one another and produce double-stranded nucleic acid moieties, or denaturation, is influenced by the nucleotide makeup of nucleic acids (Sellon 2003). Denaturation is the separation of the DNA's two identical strands. The sequence of nucleotides also affects

the characteristics of hybridization and determines how susceptible nucleic acids are to be “cut” by restriction enzymes at a particular place in the nucleic acids (Toccafondi 2022).

1.2. IMPORTANT ZOONOTIC DISEASES AND THEIR TRANSMISSION

1.2.1. PET ASSOCIATED TRANSMISSION

Dogs are the most common pets around which can disseminate zoonotic disease. Rabies, ringworms, Campylobacteriosis, Salmonellosis and Leptospirosis are the most common diseases which can spread through dogs. The rabies virus is associated with an infectious viral agent, and the rabies is communicated by the bite or scratch of an infected animal, usually a dog or a bat.
Cats are also associated with the spread of zoonotic diseases like Toxoplasmosis, Round worm and ring worm infections and Cat Scratch disease. Cat-scratch disease is caused by the infection of *Bartonella henselae*. Although the illness is mostly spread from cat to cat, but sometimes can also be spread by fleas and ticks, which can ultimately infect humans.

1.3. BIRD ASSOCIATED TRANSMISSION

The illness, which are mostly spread by infected birds to people through contact, are caused by the Psittacosis also called parrot fever and avian influenza virus and its several variants. Both in established and emerging nations, populations of birds like canaries, finches, sparrows, parrots, and parakeets are growing and these zoonotic diseases are being more prevalent. The majority of zoonotic viruses, which transmit by direct contact and arthropods, are also capable of being carried by these game and ornamental birds. The chickenpox virus, the Newcastle disease virus, *Coxiella burnetii, Coxiella psittaci, Salmonella spp, Listeria monocytogenes, Erysipelothrix rhusiopathiae, and Mycobacterium spp*. are some infectious agents that are present.

1.4. FOOD-ASSOCIATED ZOONOTIC DISEASES

The zoonosis that occurs through the consumption of undercooked meat, improperly boiled milk, and the handling of infected animals without proper protection or taking precautionary measures are included in food-borne zoonosis. *Salmonella spp*. *(Salmonella enterica serovar Enteritidis), Campylobacter spp., Shiga toxin-producing Escherichia coli (STEC)*, and hepatitis E virus are typical food-associated zoonotic diseases. 90% of bacteria-related food-borne infections are caused by *Salmonella spp*. and *Campylobacter spp.* All domesticated animals, including poultry, have the potential to harbor germs that cause food-borne diseases.

1.5. ENVIRONMENT-ASSOCIATED ZOONOTIC DISEASES

The coronavirus illness (COVID-19) produced by the severe acute respiratory syndrome coronavirus 2 (*SARS-CoV-2*) has affected humanity and has a huge impact on human existence worldwide. This zoonotic pandemic seems to be the worst disease epidemic recently (Mishra et al. 2021). The expanding human population and anthropogenic activities, which are also closely connected to the current and other recent zoonotic disease epidemics, have terribly harmed the ecosystem. Although the causes of zoonotic pandemics vary, most contributing environmental factors include fragmentation of the living environment, deforestation of habitat, loss of biodiversity, intensive livestock farming, unchecked urbanization, pollution increase, drastic changes in climate, and the trade and consumption of bush meat are among the main factors that contribute to their emergence and spread (Perera 2021).

As seen in the *West Nile virus* (WNV) in North America, where an adaptive mutation resulting in temperature variability efficiently propagates the virus at elevated temperatures. The climate-driven changes such as precipitation, dust, ozone disruption and temperature may bring genetic instability in the evolutionary structure of viruses (Kruse et al. 2004). Studying MERS-CoV revealed that between April and August, 56.1% of positive cases occurred, and that conditions that were favorable to the virus' survivability included high temperatures, high UV indices, moderate wind speeds, and low relative humidity (Altamimi and Ahmed 2020).
It was discovered that, in the instance of H5N1 transmission, environmental parameters like temperature, relative humidity, and wind speed altered migratory bird behavior, increasing avian flu outbreaks in particular locations of Egypt (Allan 2017).

1.6. WILDLIFE ASSOCIATED ZOONOSIS

Dermatophytosis, sometimes known as "ringworm," is a fungal skin disorder that causes scaly, circular areas of hair loss in both humans and animals (Lunestad et al. 2007).
External parasites like fleas, ticks, lice, and mites can occasionally be spread by handling infected bedding or being near an infected animal. Leptospirosis and tularemia, which are bacterial diseases that plague mice and rabbits in the wild, are sporadic infections that wild predators who hunt or drink contaminated water may occasionally get (Lunestad et al. 2007).

1.7. WATER ASSOCIATED ZOONOTIC DISEASES

Many protozoan and helminth parasites are transmitted through the environment, with water, soil, and food playing a particularly important role. In addition to being substantial contributors to protozoan waterborne infections, *Cryptosporidium spp.* and *Giardia spp.* are major causes of diarrheal sickness in humans globally. Examples of zoonosis that spread through watery channels include *SARS, E. coli* O157:H7, and *Salmonella* (Blancou 2003).
In areas with water scarcity, overhead and subsurface storage tanks are crucial and a mandatory way of water storage. Water is kept in overhead tanks and pumped from the tanks into residences as needed to guarantee a constant residential supply. The growth of *N. fowleri* amebae in tap water and water from mosques may have been aided by water storage in tanks. Summertime temperatures can exceed 44°C, which raises the water temperature in the above tanks. We discovered water with temperatures as high as 34°C, which may help *N. fowleri* amebae grow into infectious forms. The cause of infection, *N. fowleri* multiplication in storage tanks, is most likely explained by slime, filth, and high ambient temperatures (Yoder et al. 2012).

1.8. COMMONLY USED MOLECULAR ASSAYS FOR DETECTING INFECTIOUS AGENTS

Some important and commonly used purely molecular or partially molecular methods are mentioned below.

1.9. PCR (POLYMERASE CHAIN REACTION)

PCR and RT-PCR are the molecular diagnostic methods most frequently employed to identify infectious diseases (Mullis and Faloona 1987). The PCR is used to identify DNA initially, making it particularly helpful for detecting infectious agents that employ DNA as their main genetic component to detect infectious agents, including bacteria and viruses (Fig. 2). In case of these viruses, RNA could be used as their major genetic material and cannot be recognized by end-point PCR. For this purpose, reverse transcription (RT)

can be used to create a copy of the infectious agent's DNA, which is called cDNA (Mullis 1990). Reverse transcriptase encourages the development of a DNA molecule from an RNA template, in contrast to transcription, which typically creates messenger RNA from a DNA template. The entire process is known as RT-PCR. A PCR may be performed after the creation of cDNA (Condron et al. 2013).

1.10. RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

Restriction sites provide restriction enzymes with the ability to "cut" DNA. Because DNA nucleotide sequences specify the restriction site that is "fetched" by a certain restriction enzyme, each restriction enzyme detects and cuts at a unique nucleotide sequence (Miller et al. 1994). For a given restriction enzyme, which is used to cut DNA, several restriction sites may exist inside a specific DNA segment from a certain human or organism. It is a known fact that when restriction enzymes are employed to cut DNA that has been obtained from an organism and that part of DNA is then placed and visualized via gel electrophoresis. Differences in fragment lengths, or polymorphisms, are caused by differences in DNA sequence between the restriction sites. Variations in the restriction fragment lengths during gel electrophoresis influence the migratory properties of the fragments, allowing comparison between species (Huang et al. 2015). The more restriction enzymes that are employed to cut a certain DNA sample, the more patterns there may be to investigate. Restriction fragment length polymorphisms (RFLP) are frequently unique to certain individuals or populations of closely related species, which is why RFLP

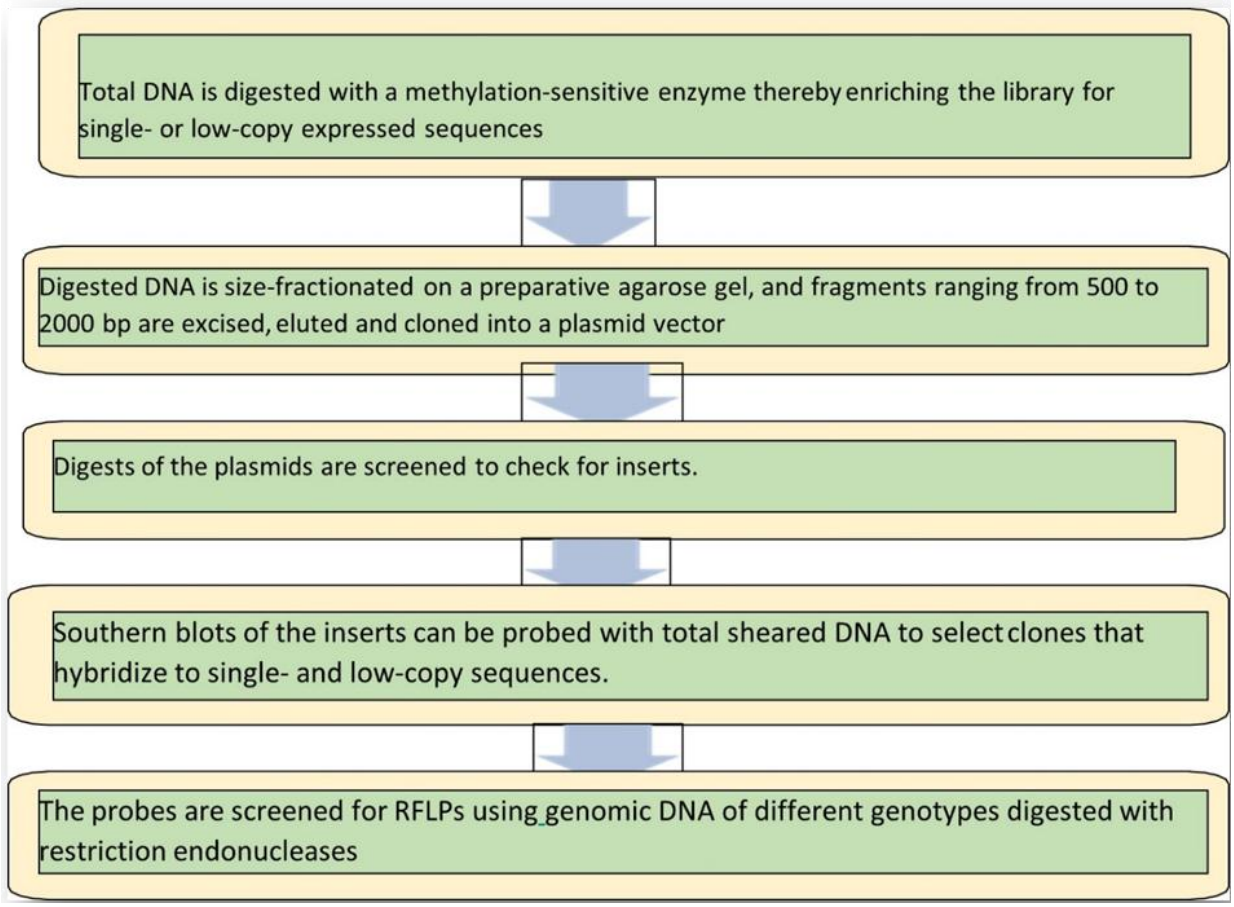

Fig 2: The step-by-step process of the Polymerase Chain Reaction.

analysis is sometime also known as “DNA fingerprinting” (Allen et al. 1990). Comparing variations between an unknown and known pattern can help identify novel or distinct organisms linked to the identified agent (Masiga and Turner 2004). RFLP analysis can be used to precisely identify the amplicon produced by the PCR-based techniques (Alevizos et al. 2001). However, RFLPs have been proven to be less effective as a solo diagnostic technique for detecting infectious illnesses in small animals when compared to PCR-based testing. RFLP is a very effective tool for detecting how closely related or distantly related pathogenic organisms are in a particular animal or group of animals (Walter et al. 2012). The presence of different pathogens in populations, or in the case of chronic illnesses, even within the same person, maybe discernible when RFLP patterns are compared. *Bartonella henselae* variations were found in cats with chronic infection using RFLP analysis; this discovery may point to a possible source of persistent infection (Huang et al. 2015). Genome mapping, forensics, paternity testing, genetic disease diagnosis, and variation analysis are common uses for RFLP probes.

1.11. ELISA (ENZYME-LINKED IMMUNOSORBENT ASSAY)

The catalysis capabilities of enzymes are used in enzyme immunoassays (EIAs) to identify and measure immunological responses. Clinical research makes use of the heterogeneous EIA method known as the enzyme-linked immunosorbent test (ELISA) (Aydin 2015). In this kind of assay, a reaction component is covalently or nonspecifically attached to the surface of a solid phase, such a microtiter well. The separation of bound and free-labeled reactants is made easier by this connection (Alhajj and Farhana 2023).

Adding a little quantity of sample or calibrator containing the target antigen (Ag) and letting it interact with a solid-phase antibody (Ab) is the most common application of the ELISA method. After washing, an enzyme-labeled antibody is added to make a solid-phase Ab-Ag-Ab enzyme “sandwich”. Specific antibodies in a sample may also be evaluated using an ELISA technique, which binds an antigen rather than an antibody to a solid phase. The second reagent is an antibody that has been enzyme- labeled and is specific to the analyte (Shah and Maghsoudlou 2016). ELISA methods have also been extensively used to identify virus- and autoantigen-specific antibodies in serum or whole blood.

A 96-well polystyrene plate with a protein-binding layer is the most typical form used for ELISA testing (Alhajj and Farhana 2023). The test requires a substrate or chromogen, analyte or antigen, coating antibody or antigen, buffer, washing, and primary and/or secondary detection antibodies, depending on the kind of ELISA to be performed (Baker 1995). An antibody that binds to the target protein with precision is used for the primary detection. A secondary detection antibody connects an enzyme-conjugated primary antibody to an enzyme-conjugated primary antibody (Alhajj and Farhana 2023). Four main general steps are performed in an ELISA immunoassay. (i) Coating (with either antigen or antibody). (ii) Blocking (typically with the addition of bovine serum albumin (iii) Detection. (iv) Final reading of detected antigen or antibody.

1.12. CRISPR-CAS

Approximately 50% of bacterial and 87% of archaeal genomes have so far been identified by different molecular techniques (Cui et al. 2017). In 1987, researchers found the repetitive 29-nt and 32-nt regions in the *E. coli* genome. In 2005, it was discovered that the CRISPR-Cas system is a component of an adaptive immune system in bacteria or archaea (Mojica et al. 2005). After the first invasion by the invader, the host cell memorizes the genetic composition of alien species by integrating small amounts of the invader’s foreign gene into its own genome. Some diagnostic techniques based on the CRISPR-Cas system only use the binding qualities of the target’s sequence without depending on the subsequent cleavage activity,

despite the fact that the primary goal of the CRISPR-Cas system is to cleave the target gene while also searching for a target based on the target's sequence (Guk et al. 2017). The majority of these techniques employ deactivated Cas9 (dCas9) proteins, which are proteins that have lost the capacity to break dsDNA due to two amino acid changes (Guk et al. 2017). The CRISPR-Cas system has developed into an exciting and effective diagnostic tool by using both the sequence-specific target binding and cleavage and the target-specific trans-cleavage. The trans-cleavage activity makes the CRISPR-Cas complex more appealing and improves detection sensitivity since it may attach to a target several times. Since the CRISPR-Cas system primarily targets nucleic acids, several nucleic acids associated with diseases have been successfully discovered and analyzed. These include methylation DNA, mRNA, miRNA, SNPs, and genomic DNA.

1.13. DETECTION OF DISEASES CAUSED BY MAJOR ZOONOTIC AND LIVESTOCK PATHOGENS: GENOTYPING METHODS

In order to subtype organisms beyond phenotypic categories, genotyping techniques are especially applied. As DNA is the starting material, these molecular biology techniques are often frequently referred to as "DNA fingerprinting." In addition to epidemiology, these techniques are also employed in forensic medicine and evolutionary biology research. Compared to phenotyping procedures, genotyping techniques frequently offer better discriminating power. These methods are also anticipated to have improved throughput, repeatability, and type ability, albeit, in some circumstances, these expectations might not always be met (Tasie and Gebreyes 2020). Amplification, restriction digestion, hybridization, and/or sequencing are some of the main molecular technologies that are frequently combined in genotyping procedures. Certain genotyping techniques are applied to zoonotic and animal illnesses. A list of the most popular techniques in veterinary medicine may be found. Each genotyping technique has distinct properties depending on the size of the genome it may be applied to, the primary molecular approaches it uses, the complexity level, and other elements (Tasie and Gebreyes 2020).

1.14. PFGE

PFGE, a macro restriction genotyping method that has been available since 1984, is one of the most widely used methods for detecting bacteria linked with cattle, particularly foodborne diseases (Schwartz and Cantor 1984). This technique was initially used to describe the fungal *Saccharomyces cerevisiae*. The basic idea behind this technique is to use a rare-cutter restriction endonuclease to perform restriction digestion on an intact genome.

1.15. RAPD (Randomly amplified polymorphic DNA)

Another genotyping technique that is often used in research on zoonotic diseases and cattle is (RAPD), also known as randomly primed PCR. The approach was initially explained in 1990 (Williams et al. 1990). As its name suggests, RAPD aims to amplify random targets located throughout an organism's DNA. Even without prior knowledge of the nucleic acid sequence of the organism being researched, this method may be used very easily. This technique is among the least expensive to set up in laboratories and is straightforward to teach (Williams et al. 1990).

1.16. REP- PCR

Comparatively less commonly used than the methods mentioned above, repetitive palindromic PCR (REP-PCR) is a subtyping approach, based on recognition of repetitive elements found in a variety of eukaryotic

and prokaryotic genomes. REP-PCR was initially reported as a technique used in infectious disease research based on repetitive elements published in 1984 (Williams et al. 1990).

1.17. AFLP

This method has been used to map the genomes of eukaryotic organisms. A modified version of the AFLP has been used in several infectious disease research with relevance to food safety and zoonotic risk. The AFLP test works by amplifying genomic regions that have been digested by two restriction enzymes, one common cutter (commonly MseI) and one unusual cutter (often EcoRI).
Therefore, this method may be used to amplify a random fraction of DNA fragments that are characteristic of the genome (Tasie and Gebreyes 2020). The most recent addition to the genotyping techniques currently gaining popularity for epidemiologic research is whole-genome sequencing (WGS). The technique is only sometimes utilized in highly developed nations and only in situations of high value food-borne illness outbreaks for trade enforcement and other regulatory objectives, therefore its utility in veterinary epidemiology is still in its infancy (Tasie and Gebreyes 2020). These applications may be seen, for instance, in the investigation into the *Salmonella serovar Bareilly* outbreaks in two states of the United States that were linked to scraped tuna imported from India (Riess et al. 2016) and the *E. coli* O104:H4 epidemic in Germany (Schiebahn et al. 2015).

1.18. VIRAL ZOONOTIC DISEASES: MOLECULAR DIAGNOSIS

1.18.1. INFLUENZA VIRUS

To ascertain if the influenza A virus is present in the samples or not, preliminary influenza screening tests are carried out. Depending on the animal species being tested, specific H and N subtyping is carried out if a test for the type A influenza virus is positive. All samples that test positive for the type A influenza virus undergo testing for the H5 and H7 subtypes of the influenza virus, which can evolve into viruses that are particularly lethal in poultry. The OIE-approved avian influenza reference laboratories, such as the National Veterinary Service Laboratories of the USDA conduct confirmation testing on those H5- or H7-positive sample (Tasie and Gebreyes 2020).
A common technique for identifying influenza is (RRT-PCR), which focuses on the (M) gene, is a virus. The positive samples are subsequently subjected to the H5 and H7 tests and a few RRT-PCRs for selected N subtypes. Additionally, partial genome sequencing and/or WGS are used as confirmatory testing. To compare with previously identified HPAI virus sequences, the H protein gene is often partly sequenced first. If the genetic sequence matches one, the sample is assumed to contain an established HPAI virus and is sent to the OIE. Thanks to recent developments in sequencing technology, epidemiologic surveillance typically employs WGS in combination with phylogenetic analysis (Tasie and Gebreyes 2020).

1.19. HPAI VIRUS (HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS)

For advanced examination of influenza virus, phylogenetic analysis based on partial- or full-genome sequencing is now the method of choice. As the two main surface proteins, H and N, control the pathogenicity, responsiveness to vaccination, and zoonotic potential of avian influenza viruses in addition to other crucial characteristics (Suh et al. 1999). As a result, the H gene and, to a lesser extent, the N gene, have received the greatest focus throughout sequencing. However, more full-genome sequencing is being done as a result of recent developments in sequencing technology, a decline in sequencing costs, and the

comparatively modest size of viral genomes (approximately 14,000 nucleotides) (Wu et al. 2011). Comprehension of the adaptability or interspecies transmission of the H5N1 virus has increased thanks to genetic research of particular amino acid changes in the viral genome (Kanekiyo et al. 2013). Editing the viral genome became possible with the development of the influenza virus's reverse genetics system, which produces a virus from a full-length cDNA copy of the viral genome.

1.20. IBDV (INFECTIOUS BURSAL DISEASE VIRUS)

IBDV has two double-stranded RNA genomic sections (Dobos et al. 1979). Young chicks' immune systems are weakened when this virus infects them (Giambrone et al. 1978). Despite the fact that there are two known serotypes, only serotype 1 can be dangerous. It is essential to create a molecular diagnostic test that can only identify viruses of serotype 1 as a result. Additionally, (Ismail et al. 1990) has shown that immunizing hens against just one antigenic subtype of serotype 1 viruses fully protects them from immunizing against a second antigenic subtype. It is known that the hypervariable sequence region of the capsid surface protein VP2 controls the antigenic subtype of IBDV. By amplifying the VP2 variable region using RT-PCR, these viruses can be found.

1.21. TICK-BORNE DISEASES: MOLECULAR DIAGNOSIS

Zoonotic and veterinary diseases caused by ticks pose a severe hazard to the health of humans and/or animals (Merle et al. 2014). Domestic dogs, or *Canis familiaris*, are maintained as pets all over the world and cohabitate with people in homes where they serve as hosts for diseased ticks that could be taken inside (Sellon 2003). These ticks might be dangerous to our health if a child, an elderly person, or anyone with compromised immune system is attacked (Dantas-Torres and Otranto 2014). In order to prevent and manage zoonotic disorders, it is essential to study neglected zoonotic diseases in companion dogs and the vectors that spread infections. This is because dogs are often considered to be "sentinels" for diseases that may affect humans in an endemic environment (Cardoso et al. 2016). The tick-borne diseases *brucellosis, rickettsiosis, anaplasmosis,* and ehrlichiosis are all thought to be re-emerging around the globe and are all caused by various species of the genus *Brucella* and certain members of the order *Rickettsiales (Rickettsia, Anaplasma, and Ehrlichia)*, (Zintl et al. 2003). There have been at least 13 new human infections linked to Rickettsiales that have been transmitted by ticks in mainland China during the past 30 years. Since its first description, *Anaplasma ovis* has been found in China and other countries (Song et al. 2018).

A. ovis has previously been found in ticks or blood samples from cattle and wild animals (Wang et al. 2020). Only *Rh. turanicus* from pet dogs were used in this investigation to screen for A. ovis, and all other blood samples from pet dogs came back negative. The prospect that ticks, dogs, and even people might get A. ovis is raised by a report that an isolate from a stray dog in Henan was shown to be highly similar to the strain discovered in a human in Cyprus (Cui et al. 2017).

1.22. EMERGING MOLECULAR DIAGNOSTIC TECHNIQUES

1.22.1. METAGENOMICS

Another way to identify genomic sequences from various microbial communities is through metagenomics. These techniques have been used for research and diagnostic purposes. Over the last few decades, metagenomics-based methodologies have been developed in an effort to assess, analyze, and

utilize biodiversity across a wide range of various environmental niches (Karesh et al. 2012). Even with diagnostic capabilities, metagenomics methods have grown in prominence in clinical trials. Through the isolation of microorganisms and the acquisition of axenic cultures, conventional diagnostic (cultivation-dependent techniques) detects pathogen species, strains, and serotypes of interest in independent colonies (Karesh et al. 2012). Because of this, metagenomics is used to study microbiomes and viromes, which cannot be cultivated in a laboratory setting. This enables diagnostic analysis of pathogen bacteria using culture-independent approaches (Quiroz-Castañeda et al. 2018).

The first crucial stage in the metagenomics study is the extraction of high-quality DNA. When studying microbiomes and virome from human or animal samples, a significant amount of human or animal DNA is frequently recovered (Quiroz-Castañeda et al. 2018). This method was initially created to analyze the microbial genomes found in environmental samples, but in the last 10 years, its use has been expanded to characterize novel infections that affect both humans and animals. Additionally, metagenomics has been utilized to describe microbiomes and virome from various tissues and organisms and has a significant influence on public health (Quiroz-Castañeda et al. 2018).

Studies on zoonotic illnesses and animal microbiota are becoming more common thanks to metagenomics and high-throughput sequencing methods. Since cloning techniques are not necessary, these methods produce millions of short sequences reads (about 150 pb) and make analysis easier. To explain the diversity and dynamics of bacterial, viral, and fungal species in tissues and samples taken from various animals, metagenomics is a potent and valuable approach (Gereffi and Sturgeon 2013). Metagenomics has helped characterize the microbiomes in many samples, such as the gastrointestinal tract of various creatures (e.g., feline, canine, human, mouse, and chicken), in addition to the discoveries of viral genomes. These investigations have identified taxonomic groups with zoonotic potential.

1.23. LAMP (LOOP-MEDIATED ISOTHERMAL AMPLIFICATION)

The LAMP method is based on traditional PCR, but unlike that method, it uses a DNA polymerase with high strand displacement activity, four primers that recognize six to eight distinct regions of the target DNA, and results in a stem-loop structure of the DNA that makes it easier to repeat rounds of amplification. By using a DNA-binding dye, such as SYBR green, (Gereffi and Sturgeon 2013) one may identify the release of pyrophosphate that occurs after the synthesis of the target DNA stem-loop. LAMP is more sensitive than traditional PCR since it can produce up to 109 copies of products in less than an hour (Rule et al. 2021).

1.24. NASBA AND TMA (NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION)

Unlike PCR, isothermal amplification techniques like NASBA and TMA often use a variety of mRNAs as the target sequence. These methods are the industry standard for gonorrhea and chlamydial infection diagnosis. Another isothermal amplification technique is HDA, (Gerace et al. 2022) which divides dsDNA into two single strands that can be used as a template for fresh DNA synthesis.

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