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Advances in diagnostic techniques by using new tools

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Abstract
Innovative diagnostic technology use for early detection of animal pathogens is critical in limiting the economic losses caused by new infectious animal illnesses. Several diagnostic applications are easy to use, cost-effective, highly sensitive, and specific diagnostic procedures with high-throughput detection capabilities are desirable attributes. The enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) remain the most widely used methods for detecting animal diseases around the world. Several serological and molecular technologies, such as lateral flow assays, biosensors, loop-mediated isothermal amplification, and molecular platforms for field-level detection of animal pathogens, have been developed using the principles of ELISA and PCR to achieve higher sensitivity, rapid, detection of animal diseases.

Keywords: Animals, infectious diseases, biotechnology, serology, molecular based technique, nucleic acid-based assays, biosensors, synthetic peptides, diagnostic tests

Introduction
Traditionally, laboratory diagnostics for veterinary pathogens have relied on methods of detecting the pathogen by culture or antibodies, using varied techniques, such as neutralization, Enzyme-linked Immunosorbent Assay (ELISA), agar gel immunodiffusion, and complement fixation. Over the years, veterinarians have incorporated new molecular diagnostic techniques, such as Polymerase Chain Reaction (PCR) and Western blot, as well as improved older techniques by using recombinant antigens, monoclonal antibodies, and synthetic peptides. Despite the routine use of conventional diagnostic assays, new molecular techniques have enabled veterinarians to use new tools for rapid and specific diagnosis of animal disease in real-time.

Immunological and serological techniques
Conventional immunoassays for disease diagnosis are based on the detection of antibodies to the pathogen of interest using methods such as virus neutralization (VNT), enzyme-linked immunosorbent assay (ELISA), complement fixation (CFT), and agar gel immunodiffusion (AGID). These assays are usually based on the interaction of serum polyclonal antibodies to the agent of interest and then use detection systems such as cytopathic effects, hemolysis, or discoloration of the reaction medium.

New biotechnology techniques such as gene cloning, overexpression of immunogens, the use of expression vectors, and peptide synthesis have made it possible to obtain specific proteins or peptides. The use of these improved antigens may increase the specificity or sensitivity of the immunoassay. Advanced Immunological methods include Western blotting, immunofluorescence, immune-peroxidase/enzyme immunohistochemistry, flow cytometry, and confocal laser microscopy. It is commonly used to diagnose various diseases or to study the expression profile of various genes.

Virus Neutralization Assay
A virus neutralization assay is used in conjunction with an infectivity assay, such as the plaque assay described above. This assay detects antibody that is capable of inhibiting virus replication (or in other words, antibody that can neutralize virus infection). Virus neutralization is a specialized type of immunoassay because it does not detect all antigen–antibody reactions. It only detects antibody that can block virus replication. This is important because related groups of viruses may share common antigens, but only a fraction of these antigens are targets of neutralizing antibody. A virus serotype is usually based on virus neutralization (although this is not always specified).

Immunochromatography
Immunochromatography is a traditional method to detect antigens within minutes without special equipment. The sample is applied to one end of a filter, and microbeads (e.g., colloidal gold) are conjugated to the antibody. This element binds to the microbead-antigen complex and is captured by the secondary antibody on the filter, making it easy to visualize in the area where the secondary antibody is immobilized.

Enzyme linked immunosorbent assay (ELISA)
ELISA is the accurate immunodiagnostic method in which an antibody is conjugated to an enzyme and used to detect the presence of an antibody or antigen in a sample. It can also be used as a quality control tool in various industries. In ELISA, an antigen or antibody is attached to a surface (microtiter plate or nitrocellulose paper/nitrocellulose membrane strip) and then washed over the surface to allow specific antibodies to bind to the antigen. These antibodies are enzyme-related, and in a final step, a substance is added that the enzyme can convert into a detectable moiety Signal. Indirect ELISA, sandwich ELISA, and competitive ELISA are other ELISA techniques.

Direct or indirect method
The primary antibody is labeled with a fluorescent dye in the direct FAT method, whereas in the indirect method (IFAT), the primary antibody is labeled. Secondary antibodies generated against the parental antibody species are labeled with a fluorescent dye. In the indirect method, the primary antibody is specific for the antigen, and the secondary antibody is specific for the primary antibody. Immunofluorescence was performed after transfection with expressed proteins and infected cell cultures, tissue sections. Thus, clinical specimens, etc., help identify the protein/pathogen of interest. Immuno-peroxidase method (IPT) IPT is similar in principle to FAT, except that, for example, an antibody is conjugated to an enzyme. Horse-radish peroxidase (HRPO). When a substrate containing di-aminobenzidine hydrochloride (DAB) and H2O2 is added to the solution, the first H2O2 is catalyzed by HRPO to form the resulting oxygen and water molecules. The oxygen produced oxidizes the DAB, turning it brown. Therefore, IPT also detects expressed antigens in cultured mammalian cells/tissue sections/pull smears. It has advantages over FAT in that it does not require a fluorescence microscope and allows long-term slides storage. Like FAT, IPT is performed in both direct and indirect ways.

Fluorescent antibody technique (FAT)
Fluorescent antibody method (FAT) Fluorescent antibody technology is based on the principle of antigen-antibody interaction. It involves antigen/protein detection using specific antibodies conjugated to fluorescent dyes such as fluorescein iso-thiocyanate (FITC), phycoerythrin (PE), etc.

Flow cytometry
Flow cytometry has become an essential new technology in veterinary clinical laboratories. Flow cytometry provides rapid, quantitative analysis of various cell types based on cell size, molecular complexity, and antigenic composition. Thus, flow cytometry complements and extends the knowledge gained through light microscopy. The most popular flow cytometers are used for immunological characterization of lymphomas and leukemias, tissue matching in organ transplantation, and enumeration of lymphocyte subpopulations in people infected with peripheral blood. Laser, monoclonal antibody, Fluorochrome, and computer color correction algorithms extend flow cytometry capabilities. Many treatment protocols have included detection of minimal residual disease by flow cytometry in leukemia and lymphoma (Dalal, 2007) [3]. Flow cytometry is performed using an instrument known as Fluorescence Activated Flow Cytometry/Sorter (FACS). (McKinnon 2018)

Western blot technique
In Western blotting, molecules transferred from semisolid phase (polyacrylamide gel) to the solid phase (nitrocellulose membrane0nare visualized using specific antibodies. In the case of proteins, antibodies specifically react with antigenic epitopes displayed by the target protein, usually attached to a solid support. Therefore, Western blotting is helpful for identifying and quantifying specific proteins in unlabeled protein mixtures. (Yang 2012) [6]

New immunological methods
Cloning and expression of specific proteins
The cloning and expression of specific proteins produced by pathogens have enabled the development of assays that can differentiate between vaccinated and unvaccinated (infected) animals. Genes encoding specific proteins are identified and cloned into appropriate vectors, and these genes/proteins are expressed in bacterial, yeast, or eukaryotic systems. The expressed protein can be easily extracted, secreted, and purified. This protein can be used as an antigen for a more specific disease diagnosis.

Peptide synthesis
Synthetic peptides or recombinant antigens obtained using recombinant DNA technology have many advantages over natural antigens isolated from other biological sources. These advantages include high purity, high inertness, and high consistency. Use of synthetic peptides or recombinant antigens to detect animals infected with diseases such as classical swine fever, foot-and-mouth disease, zoonotic or endemic (Mustafa 2013) [7] Reduce the risks associated with testing and receiving kits containing antigens that remain potentially infectious because they are not fully inactivated.

Biosensor
A novel approach to detecting agents or antibodies is the development of biosensors. This type of analysis involves using a transducer that converts a biological interaction with a receptor (usually an antibody) for a target pathogen or disease-specific antibody into a measurable signal (Lechuga., 2000) [1]. Some new sensor technologies include electrochemistry, reflectometry, interferometry, resonance, and fluorimetry. Biosensors are frequently coupled to sophisticated instrumentation to produce highly-specific analytical tools, most of which are still in use only for research and development due to the high cost of the instrumentation, the high cost of individual samples analysis, and the need for highly trained personnel to oversee the testing. An example of a commercial application of fluorimetry is the particle concentration fluorescence immunoassay for brucellosis and Aujeszky's disease antibody screening.

Proteomics

Proteomics is a large-scale study involving the expression levels of proteins, their post-translational modifications, and their interactions with other proteins. The use of proteomics to diagnose infectious diseases is at an early stage but can be very important. A handy application of proteomics for diagnosing infectious diseases is the identification of novel diagnostic antigens by screening the sera of infected and uninfected people against immunoblot mapping and 2DGE mapping proteomes of infectious agents. In veterinary medicine, research projects based on proteomics are currently underway and will undoubtedly provide new diagnostic tools in the future.

Gamma interferon detection

Commercial assays have recently become available to detect cell-mediated responses, such as the interferon-gamma test used to detect tuberculosis. Interferon-gamma in animals may indicate infection with microorganisms such as tuberculosis. This assay is based on the detection of interferon-gamma, a cytokine expressed when sensitized immune cells in the blood are exposed to targeted agents. These assays are based on host-specific monoclonal antibodies and require a fresh blood sample with viable white blood cells.

Techniques for Detection of Nucleic Acids

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a compassionate and reliable molecular method for the *in vitro* amplification of one or more copies of DNA fragments. Millions of copies of DNA segments can be obtained from a single copy of a DNA template in less than 23 hours. Most PCR methods can amplify DNA fragments of 10-40 kb in size. This method involves denaturation (94-95 ºC) of the double-stranded DNA under investigation followed by hybridization (50-65 ºC) of specific short oligonucleotides (primers) with specific segments of the target genome (complementary sequence) followed by extension (72 ºC) Taq polymerase. Repeat this process for 25 to 40 cycles to obtain the amplified gene. The amplified DNA segments are electrophoresed on a 0.8-2% agarose gel and stained with ethidium bromide Visualization with UV light (Sambrook and Russell, 2001). Specific organisms' serotypes, genotypes, and pathogenic types can be identified using specific primer pairs. Multiplex PCR is used to detect more than one target gene. Reverse transcription PCR (RTPCR) is used to detect RNA or cDNA copies in microorganisms (such as RNA viruses).

Real-time PCR

Real-time PCR is the latest improvement in standard PCR methods to quickly and accurately diagnose disease onset. Real-time PCR requires less manipulation, is faster and more specific than conventional PCR techniques, has a closed-tube format that reduces the risk of cross-contamination, and is highly sensitive and specific, maintaining qualitative efficiency and providing quantitative information. Detection of positive samples depends on the amount of fluorescence emitted during amplification. It can quantify the DNA or RNA content of a given sample. PCR is also widely used for genotyping and phylogenetic analysis of veterinary pathogens. In many cases, Real-Time PCR has proven to be more sensitive than conventional reference methods. (Heim *et al*., 2003; Weidmann *et al*., 2003).

Polymerase spiral reaction (PSR)

PSR is a novel nucleic acid sequence amplification-based technique, which can amplify target templates under the isothermal condition (60–67 °C) with outstanding rapidity and high sensitivity and specificity. (Liu *et al*., 2015)

DNA microarray

The microarray contains more than 20,000 different known DNA, each of which is coated on a glass slide to form an array. In microarray diagnostics, known DNA as a target is immobilized on a glass slide, and unknown DNA is labeled in a liquid state to create a probe. Pathogen detection in microarray analysis is limited only by the amount of target DNA in the matrix. Microarray analysis has great potential for investigating diseases of unknown etiology, diseases in which more than one pathogen may be present, and diseases requiring subtyping. The most significant advantage of microarray analysis when searching for pathogens is that hundreds of pathogens can be viewed simultaneously with one probe. Microchip slides. Microarrays can be combined with PCR amplification to increase the sensitivity of pathogen detection.

DNA probes

When the DNA probe hybridizes, DNA obtained from a sample suspected of containing the pathogen ("unknown") binds to highly characterized DNA ("known" DNA) previously obtained from the pathogen of interest. For example, in conventional DNA probing, an unknown target DNA (or RNA) is immobilized on a solid surface. The known DNA applied to the target (labeled/stepped probe) is in the liquid phase. Bound probes can be detected by adding specific enzyme-binding molecules/substances that produce color or light (chemiluminescence). The number of probes used limits the detection of pathogens by this method.

Application of Newer Techniques in disease diagnosis

Nucleic acid sequence-based amplification (NASBA)

NASBA is a promising method for gene amplification. This isothermal method consists of a two-step process in which initial enzymatic amplification of the target nucleic acid occurs, followed by detection of the resulting amplicon. The entire NASBA process is performed at one temperature, eliminating the need for a thermal cycler. This method has been shown to detect avian and human influenza viruses. (Moore *et al.*, 2004) [5].

Fluorescent in situ hybridization (FISH)

FISH is a technology that allows the localization of nucleic acid sequences in cellular material. Peptide nucleic acids, molecules in which a peptide backbone replaces the sugar backbone, are ideal DNA simulators with high hybridization affinity that can be used to improve FISH techniques (Stender, 2003).

Nanotechnology

Nanotechnology is broadly defined as systems or devices related to nanometer-scale features (one billionth of a meter). The small dimensions of this technology have led to nanoarrays and nanochips as test platforms (Jain, 2005) [2]. One advantage of this technology is the potential to analyze a sample for an array of infectious agents on a single chip. Applications include the identification of specific strains or serotypes of disease agents or the differentiation of diseases

caused by different viruses but with similar clinical signs. Another facet of nanotechnology is the use of nanoparticles to label antibodies. The labeled antibodies can then be used in various assays to identify specific pathogens, molecules, or structures. Examples of nanoparticle technology include gold nanoparticles, nano barcodes, quantum dots, and nanoparticle probes (Zhao *et al*., 2004). Other nanotechnology includes nanopores, cantilever arrays, nanosensors, and resonant light scattering. Nanopores can be used to sequence DNA strands as they pass through electrically charged membranes (Emerich *et al*., 2003) [4]. Although nanotechnology is still in the research stage, it is expected that it will be possible to diagnose endemic diseases in veterinary animals by applying nanotechnology in the future.

Random Amplification of Polymorphic DNA (RAPD)

In RAPD analysis, an unknown target sequence of genomic DNA is amplified by PCR using random sequence primers (primer 10bp). Many fragments of different sizes can be amplified, which can be observed on an agarose gel, and dendrogram analysis is performed using computer software. These results can be used to study genetic variation between closely related genotypes.

Restriction fragment length polymorphisms (RFLP)

This DNA-based method differentiates isolates of closely related pathogens, such as viruses, bacteria, fungi, or parasites. The RFLP approach is based on the fact that sequence variations also determine the genomes of closely related pathogens. The RFLP procedure consists of isolating the target pathogen, extracting DNA or RNA (reverse transcription into DNA), and then digesting the nucleic acid using one panel of restriction enzymes. Individual fragments of digested DNA are separated from the gel using electrophoresis and visualized by staining with ethidium bromide. Ideally, each strain will show a unique pattern or fingerprint. Results can be further analyzed using computer software.

Pulsed-field gel electrophoresis (PFGE)

PFGE is the standard method used for typing bacteria. It is also an invaluable tool for epidemiologic studies and genetic mapping of microbial and mammalian cells, facilitating the development of large-scale insertional replication systems such as bacterial and yeast artificial chromosomes.

PCR-RFLP

PCR-RFLP is a modification of the primary RFLP method in which the polymerase chain reaction (PCR) is performed as a preliminary step. PCR amplifies specific regions of the genome (known variable sequences between pathogens), which serve as template DNA for RFLP. This novel combination (PCR-RFLP) provides much higher sensitivity to identify pathogens and is particularly useful when pathogens are few or difficult to culture.

Conclusion

Diagnosis of infectious and zoonotic pathogens in livestock mainly consists of traditional diagnostic methods. However, in recent years some significant changes have occurred with new biotechnology tests. These new assays include the production of more specific antigens using recombinant expression vectors and synthetic peptides. The use of these assays in conjunction with monoclonal antibodies has significantly increased the sensitivity and specificity of many existing diagnostic assays. Various forms of PCR have become standard diagnostic tools. Rapidly screen large numbers of samples during disease outbreaks and veterinary laboratories for specific types. Proteomics can see a broader picture of protein expression by a pathogen of interest or an infected animal and fill a unique niche in veterinary diagnostics. Although nanotechnology has not yet been implemented in veterinary labs, it can screen multiple pathogens in a single test. Nanotechnology could be the future of choice for mobile or portable testing to diagnose disease. Biotechnology and its applications have great potential to increase the speed and accuracy of veterinary pathogen diagnosis, and any developments will be needed to provide enhanced diagnostic capabilities to protect animal health.

References

1. Lechuga LM, Calle A, Prieto F. Quim Anal. 2000;19:54-60.
2. Jain KK. Role of nano biotechnology in developing personalized medicine for cancer. Technol Cancer Res Treat. 2005;4:407-16.
3. Dalal. Clinical applications of molecular haematology: flow cytometry in leukaemias and myelodysplastic syndromes J Assoc Physicians India. 2007;55:571-3.
4. Emerich. Nanotechnology and medicine Expert Opin Biol Ther. 2003;3(4):655-63. doi: 10.1517/14712598.3.4.655.
5. Moore C, Clark EM, Gallimore CI, Corden SA, Gray JJ, Westmoreland D. Evaluation of a broadly reactive nucleic acid sequenced based amplification (NASBA) assay for the detection of noroviruses in faecal material. J Clin Virol. 2004;29:290-296.
6. Yang, Ping-Chang, Mahmood, Tahrin. Western blot: Technique, theory, and troubleshooting. North American Journal of Medical Sciences. 2012;4(9):429-434. doi:10.4103/1947-2714.100998. ISSN 1947-2714. PMC 3456489. PMID 23050259.
7. Mustafa AS. *In-silico* analysis and experimental validation of Mycobacterium tuberculosis -specific proteins and peptides of Mycobacterium tuberculosis for immunological diagnosis and vaccine development. Med Princ Pract. 2013;22(Suppl 1):43-51. doi: 10.1159/000354206. Epub 2013 Aug 31