

Advanced Techniques in Diagnosis of Livestock Diseases and Zoonotic Pathogens

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Infectious diseases of livestock can cause major economic losses, both to the livestock owners and the country as a whole. Besides, some diseases are often credited to have been associated with public health significance and zoonoses. Zoonoses poses serious burdens of diseases on vast number of people particularly those who are in close contact with domestic as well as wild animals. Establishment of a good diagnostic laboratory for rapid and reliable diagnosis to ensure proper preventive and control measures of infectious diseases in livestock is a necessary step. The most authentic diagnosis of diseases is made by isolation and identification of the pathogen. However, it is time consuming, tedious and sometimes requires living medium. The development of various immunological and molecular techniques has revolutionized diagnostic procedures by providing specific diagnosis or detailed characterization of any pathogen or host pathogen interactions. Some of the recent immunological and molecular techniques employed in addition to the conventional ones for the diagnosis of diseases are discussed in brief.

Immunological techniques

Conventional immunoassays for the diagnosis of diseases have been based on the detection of antibody to the pathogen of interest, using techniques such as virus neutralization, enzyme-linked immunosorbant assay (ELISA), complement fixation and agar gel immunodiffusion. These assays generally rely on the interaction of serum polyclonal antibodies against the agent of interest, followed by the use of a detection system such as the cytopathic effect, haemolysis or colour change of a reaction medium. New biotechnological methods such as cloning of genes, over expression of immunogens, use of expression vectors and peptide synthesis have made possible the production of specific proteins or peptides. The use of these improved antigens can increase the specificity or sensitivity of immunoassays.

The immunological techniques like western blotting, immunofluorescence, immunoperoxidase/enzyme immunohistochemistry, flowcytometry, confocal laser microscopic techniques etc. are routinely used for diagnosis of various diseases or to study the expression profile of various genes.

Fluorescent antibody technique (FAT)

Fluorescent antibody techniques are based on the principle of antigen and antibody interaction. It involves the detection of antigen/protein using the specific antibodies conjugated with fluorescent dye such as fluorescein isothiocyanate (FITC), phycoerythrin (PE) etc. by direct or indirect methods. In the direct method of FAT, primary antibody is labeled with a fluorescent dye whereas in the indirect method (IFAT); secondary antibody raised against the species of origin of primary antibody is labeled with fluorescent dye. In indirect method, primary antibody is specific for antigen while the secondary antibody is specific for the primary antibody. Immunofluorescence techniques can be used for the detection of expressed

protein following transfection and microbial antigen in infected cell culture, tissue sections, clinical samples etc., thus aid in the identification of the protein/pathogen of interest.

Immuno-Peroxidase technique (IPT)

IPT is similar in principle to FAT except that antibody is conjugated to an enzyme e.g. Horse Radish Peroxidase (HRPO). Upon addition to substrate solution that contains diaminobenzidine hydrochloride (DAB) and H_2O_2 , first H_2O_2 is catalyzed by the HRPO to yield nascent oxygen and a molecule of water. Nascent oxygen thus formed oxidizes DAB which forms the brown colour. Thus IPT also offer the detection of expressed antigen in the cultured mammalian cells/tissue sections/impression smears. It has advantages over FAT in the sense that there is no need of fluorescent microscope and slides can be stored for long period without affecting the results. Similar to FAT, IPT is also performed in both direct method and indirect method.

Flow cytometry

Flow cytometry has emerged as a major new technology in veterinary clinical laboratories. Flow cytometers offer rapid and quantitative analysis of a variety of cell types based on cell size, molecular complexity, and antigenic composition. Therefore, flow cytometry complements and extends knowledge that can be obtained by light microscopy. The most prominent uses of flow cytometers are for immunological characterization of lymphomas and leukemias, crossmatching tissues for organ transplants, and counting lymphocyte subpopulations in the peripheral blood infected individuals. Flow cytometric evaluation of cellular proteomics has become an integral part of the laboratory diagnosis and classification of haematopoietic neoplasms. Recent technical advances in lasers, monoclonal antibodies, fluorochromes, and computer-based color compensation algorithms have expanded the usefulness of flow cytometry. Detection of minimal residual disease by flow cytometry in leukaemias and lymphomas is incorporated in many treatment protocols (Dalal, 2007). Flow cytometry analysis is carried out using the instrument known as flow cytometry/fluorescence activated cell sorter (FACS).

Western blotting

In western blotting, electrophoretically separated proteins are transferred from a gel to a solid support and probed with reagents that are specific for particular sequences of amino acids (peptide). In case of proteins, they usually are antibodies that react specifically with an antigenic epitopes displayed by the target protein which is attached to the solid support. Western blotting is therefore extremely useful for the identification and quantification of specific proteins in a mixture of protein that are not labeled.

Enzyme-linked immunosorbent Assay (ELISA)

ELISA is a highly sensitive and accurate immunodiagnostic technique in which antibody is conjugated to an enzyme and utilized to detect the presence of an antibody or an antigen in a sample. It can also be used as a tool for quality control checking in various industries. In ELISA, an unknown amount of antigen is affixed to a surface (micro-titer plates or Nitrocellulose paper/Nitrocellulose membrane strips), and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable

signal. Indirect ELISA, Sandwich ELISA, Competitive ELISA are the different types of ELISA technique.

Immunochromatography

Immunochromatography provides a conventional method for detection of antigens in several minutes without special apparatus. The sample is applied on one end of the filter and the microbeads (such as colloidal gold) conjugated with antibody is applied. The antibody binds to microbeads-antigen complex, is trapped on the second antibody that is on the filter, and is easily visualized at the site where the second antibody was fixed.

Newer immunological techniques

Peptide synthesis

Synthetic peptides or recombinant antigens produced by recombinant DNA technology offer many advantages over natural antigens isolated from other biological sources. These advantages include a high purity, high specific activity and consistency. The use of synthetic peptides or recombinant antigens in detection of animals infected with diseases such as classical swine fever, foot -and -mouth disease or with a zoonotic or endemic disease (Langedijk *et al.*, 2001; Davis *et al.*, 2001; Soutullo *et al.*, 2001), reduces the risk involved in the production of the assay and the risk of producing kits with antigen that has not been completely inactivated and, therefore, remains potentially infectious.

Cloning and expressin of specific protiens

The cloning and expression of specific proteins produced by a pathogen have enabled the development of assays that can differentiate vaccinated from non-vaccinated (infected) animals. The genes encoding the specific protein are identified and cloned in appropriate vectors, and these genes/proteins are expressed in bacteria, yeast or eukaryotic systems. The expressed proteins can be easily extracted or secreted and the purified if necessary. These proteins can be used as antigen for more specific diagnosis of diseases.

Detection of gamma-interferons

Recently, commercial assays to detect cell-mediated responses have become available e.g., gamma-interferon assays use for detection of tuberculosis. Presence of gamma-interferon in animals may be an indication of infections by microorganisms such as tuberculosis. The assay rely on detection of gamma-interferon, a cytokine expressed when sensitised immune cells in the blood are exposed to the target agent. These assays rely on the use of host-species specific monoclonal antibodies and require a fresh blood samples with viable white cells.

Biosensors

A new approach to the detection of either the agent or antibodies is the development of biosensors. This type of assay involves the use of a receptor (usually an antibody) for the target pathogen or a disease-specific antibody and a transducer which converts a biological interaction into a measureable signal (Cruz *et al.*, 2002). Some of the transducer technologies under development include electrochemistry, reflectometry, interferometry, resonance and fluorimetry (Barker, 1987). 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. Fluorescence polarization technology has recently become available for the detection of bovine brucellosis (Nielsen *et al.*, 1996).

Proteomics

Proteomics is the study of proteins, including their expression level, post-translational modification and interaction with other proteins, on a large scale. The use of proteomics for the diagnosis of infectious disease is in the infancy but may prove to be of considerable importance. An extremely useful application of proteomics to the diagnosis of infectious disease is the identification of novel diagnostic antigens by screening serum from infected and uninfected individuals against immunoblotted, 2DGE mapped proteomes of infectious agents. Within the veterinary field, proteomics-based research projects are now underway and these will undoubtedly yield novel diagnostic tools for the future. Proteome maps are being derived for a range of veterinary pathogens such as *Brucella melitensis* (Mujer *et al.*, 2002), *Toxoplasma gondii* (Cohen *et al.*, 2002), *Eimeria tenella* (Bromley *et al.*, 2003), *Trypanosoma brucei* (Rout and Field, 2001) and nematodes such as *Haemonchus contortus* (Yatsuda *et al.*, 2003).

Detection of Nucleic Acids

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a highly sensitive and reliable molecular technique to amplify a single or few copies of a piece of DNA under *in-vitro* condition. Million copies of DNA segment can be obtained from a single copy of DNA template within 2-3 hours. Most PCR methods can amplify DNA fragments between 10 - 40 kb in size. This method involves denaturing (94-95°C) of double stranded DNA to be investigated followed by hybridization (50- 65°C) of specific short oligo-nucleotides (primers) to a specific segment (complementary sequences) of the targeted genome which get extended (72°C) with the help of Taq polymerase. This process is repeated for 25- 40 cycles to obtain the amplified genes. The amplified DNA segment is electrophorized in 0.8- 2% agarose gel; stained with ethidium bromide and visualized under UV light (Sambrook and Russell, 2001). Serotypes, genotypes and pathotypes of a particular organism can be identified by using specific primer pairs. Multiplex PCR is used to detect more than one target genes. Reverse Transcription-PCR (RT-PCR) is used for detection of RNA or a cDNA copy of the RNA of microorganisms (e.g. RNA viruses).

Real time PCR

Real time PCR is the latest improvement in the standard PCR technique that enables rapid and specific diagnosis of disease outbreaks. Real time PCR require less manipulation, is more rapid and specific than conventional PCR technologies, has a closed-tube format therefore decreasing risk of cross-contamination, is highly sensitive and specific, thus retaining qualitative efficiency, and provides quantitative information. Detection of positive samples is dependent on the amount of fluorescence released during amplification. It can be used to quantify DNA or RNA content in a given sample. The PCR is also used extensively for the genotyping and phylogenetic analysis of veterinary pathogens. In many cases, the real-time

PCR assays have proved to be more sensitive than existing reference methods (Heim *et al.*, 2003; Weidmann *et al.*, 2003)

Diagnosis by DNA probes

In DNA probe hybridization the DNA, derived from sample suspected of containing a pathogen (the 'unknown'), bind with highly characterized DNA derived in advance from a pathogen of interest (the 'known' DNA). In conventional DNA probing the unknown DNA (or RNA), the target, is immobilised on a solid surface e.g. a filter; and the known DNA (labelled/staged probe) which is applied to the target, is in the liquid phase. The bound probe can be detected by addition of specific molecules/substances linked to an enzyme that generate colour or light (chemiluminescence). Detection of pathogen by this method is limited by the number of probes used.

DNA microarray technology

A microarray comprises 20,000 or more different known DNAs, each being spotted onto glass slides, to form the array. In microarray diagnosis the known DNA that is the target, immobilised on a glass slide, and the unknown DNA, in the liquid phase, which is labelled to make a probe. In microarray analysis, the detection of pathogen is limited only by the number of target DNAs on the array. Microarray analysis has great potential when one is investigating diseases of unknown aetiology, diseases where more than one pathogen might be present and when subtyping is required. The great advantage of microarray analysis in searching of pathogens is that hundreds of pathogens can be looked simultaneously when probing a single microarray slide. To enhance sensitivity in pathogen detection, microarrays can be coupled with PCR amplifications.

Newer Techniques

Fluorescent in situ hybridization (FISH)

FISH is a technique that can localize nucleic acid sequences within cellular material. Peptide nucleic acids, molecules in which the sugar backbone has been replaced by a peptide backbone, are perfect mimics of DNA with high affinity for hybridization that can be used to improve FISH techniques (Stender, 2003).

Nucleic acid sequence-based amplification (NASBA)

NASBA is a promising gene amplification method. This isothermal technique is comprised of a two-step process whereby there is an initial enzymatic amplification of the nucleic acid targets followed by detection of the generated amplicons. The entire NASBA process is conducted at a single temperature, thereby eliminating the need for a thermocycler. The use of this technique has been shown to detect avian and human influenza viruses (Collins *et al.*, 2003; Moore *et al.*, 2004).

Nanotechnology

Nanotechnology is broadly defined as systems or devices related to the features of nanometer scale (one billionth of a metre). The small dimensions of this technology have led to the use of nanoarrays and nanochips as test platforms (Jain, 2003). 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. Example of nanoparticle technology includes the use of gold nanoparticles, nanobarcodes, quantum dots and nanoparticle probes (Santra *et al.*, 2004; Zhao *et al.*, 2004). Additional nanotechnologies include nanopores, cantilever arrays, nanosensors and resonance light scattering. Nanopores can be used to sequence strands of DNA as they pass through an electrically- charged membrane (Emerich *et al.*, 2003). Nanotechnology is still in the research stage but it is anticipated that nanotechnologies will eventually be applied to the diagnosis of endemic veterinary diseases in the future.

DNA Based Typing of Microorganisms

Random Amplification of Polymorphic DNA (RAPD)

In RAPD analysis, unknown target sequences in a genomic DNA are amplified by PCR employing primers with arbitrary sequences (10 bp primers). Many fragments with varying size may be amplified that can be observed in agarose gel and dendogram analysis is done with the help of computer softwares. These results can be used for study of genetic variability among closely related genotypes.

Restriction fragment length polymorphisms (RFLP)

This DNA- based method is used to distinguish between isolates of closely related pathogens, whether they are viruses, bacteria, fungi or parasites. The RFLP approach is based on the fact that the genomes of even closely related pathogens are defined by variation in sequence. The RFLP procedure consists of isolating the target pathogen, extracting DNA or RNA (with subsequent reverse transcription to DNA) and then digesting the nucleic acid with one of a panel of restriction enzymes. The individual fragments within the digested DNA are then separated within a gel by electrophoresis and visualized by staining with ethidium bromide. Ideally each strain will reveal a unique pattern, or fingerprint. The results can be further analysed with the help of computer softwares.

PCR-RFLP

PCR-RFLP is a modification of the basic RFLP technique whereby the polymerase chain reaction (PCR) is incorporated as a preliminary step. The PCR method is used to amplify a specific region of the genome (known variable sequence between pathogens), which then serves as the template DNA for the RFLP technique. This new combination (PCR-RFLP) offers a much greater sensitivity for the identification of pathogens and is especially useful when the pathogen occurs in small numbers or is difficult to culture.

Pulsed field gel electrophoresis (PFGE)

The limitation to separate very large DNA molecules by standard gel electrophoresis techniques can be overcome with this new technique, called pulsed field gel electrophoresis (PFGE). In PFGE an alternating voltage gradient is applied which facilitates the differential migration of large DNA fragments through agarose gels by constantly changing the direction of the electrical field during electrophoresis. The development of PFGE expanded the range of resolution for DNA fragments by as much as 2 orders of magnitude. PFGE has been successfully applied in subtyping of many pathogenic bacteria among other applications such

as cloning of large plant DNA, construction of physical maps, genetic fingerprintings, etc. This technique is time consuming and require high-level of skill.

Conclusion

Diagnosis of infectious diseases of livestock and zoonotic pathogens primarily comprised of traditional diagnostic techniques. However, in the recent years a profound change has occurred with the introduction of new biotechnological assays. These new assays include the production of more specific antigens by the use of recombination, expression vectors and synthetic peptides. Application of these assays coupled with the use of monoclonal antibodies, the sensitivity and specificity of a number of traditional diagnostic assays have been significantly improved. Various forms of PCR have become routine diagnostic tools in veterinary laboratories for specific typing as well as rapid screening of large numbers of samples during disease outbreaks. Proteomics has the potential to look at the broader picture of protein expression by a pathogen of interest or by infected animals and may lead to a special niche of veterinary diagnostics. Though it is not yet implemented in veterinary laboratories, nanotechnologies hold the promise of screening for numerous pathogens in a single assay. Nanotechnology may be the choice for mobile or pen side testing for disease diagnosis in future. Biotechnology and its applications hold great promise for improving the speed and accuracy of diagnostics for veterinary pathogens and much developmental work will be required to provide improved diagnostic capabilities to safeguard animal health.

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