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Biotechnological tools in disease diagnosis in animal

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Abstract
Biotechnology is a conventional method in several areas of diagnosis and its application in the field of veterinary practice has only happening to appear with the potential to transform the veterinary field for treatment of animals. The new techniques include the production of more specific antigens by the use of recombination, expression vectors and synthetic peptides. Coupled with the use of monoclonal antibodies, the sensitivity and specificity of a number of traditional types of diagnostic assays have been significantly improved. The improved advanced biotechnological techniques developed for treatment of animal are based on biosensor, proteomics and nanotechnology. These biotechnological applications hold great promise for improving the speed and accuracy of diagnostics for veterinary pathogens after establishing a good method of treatment in humans. However, much developmental work will be required to realise the potential with well-characterized, validated assay systems that provide improved diagnostic capabilities to safeguard animal health. This review has focused on the application of sophisticated biotechnological technique for better future on treatment of animals in veterinary practice.

Keywords: biosensors, biotechnology, immunoassay, nanotechnology, PCR, proteomics

Introduction
Diagnosis of diseases in the veterinary field using tissue/cell culture or antibodies, are adopted by various techniques such as neutralization (Borchers *et al*., 2005; Taniguchi *et al*., 2000) [10, 45], Enzyme-Linked Immunosorbent Assay (ELISA) (Langedijk, *et al*., 2001) [33], Agar Gel Immunodiffusion (Alvarez *et al*., 2010) [4] and Complement Fixation Test (CFT) (Hartley *et al*., 2005; Adone *et al*., 2008) [22, 1]. In the past few years ago onwards, the veterinary diagnosticians has been incorporated new molecular techniques such as the Polymerase Chain Reaction (PCR) and Western blot and improved older techniques through the use of recombinant antigens, monoclonal antibodies and synthetic peptides (Okonko *et al*., 2006) [40]. Nowadays, the molecular diagnostic techniques have better scope for veterinary diagnostics provoking powerful tools through the rapid and specific diagnosis of animal diseases (Jain, 2002) [26]. This paper will review the current and potential uses of biotechnology in veterinary diagnostics.

Improved Immunoassays
Conventional immunoassays for the diagnosis of animal diseases have been based on the detection of antibody to the pathogen of interest, using techniques such as virus neutralisation, enzyme-linked immunosorbent assay (ELISA), complement fixation and agar gel immunodiffusion (Langedijk, *et al*., 2001) [33]. 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 presence of cytopathic effect in cell culture, the haemolysis of red blood cells due to fixation of complement as a result of antibody-antigen interaction, or the colour change of a reaction medium due to the chemical reaction of an indicator enzyme (Tavares *et al*., 2011) [46]. New biotechnological methods such as the cloning of genes, over expression of immunogens, use of expression vectors and peptide synthesis have made the production of specific proteins or peptides that serve

as target antigens or positive control reagents in existing and newly-developed immunoassays possible (Jain, 2002) [26]. The use of these improved antigens can increase the specificity or sensitivity of immunoassays by providing a more defined target for binding antibody and can reduce serial-to-serial (lot-to-lot) variation of test kit performance as a result of using a more homogenous and well-defined antigen to capture antibodies (Jain, 2002) [26]. Molecular techniques also provide opportunities to improve antigens that are used to induce polyclonal or monoclonal antibody production, select monoclonal antibodies that recognise specific target epitopes and purify antisera for specific diagnostic purposes (Svobodova *et al*., 2013) [44]. Use of synthetic peptides (those produced by connecting amino acids together in a specified sequence) for use as positive controls in these assays provides an opportunity to include a specific positive control without the risks involved in producing killed agents (Langedijk, *et al*., 2001) [33]. Recently, the use of transgenic plants to express veterinary pathogen proteins has been shown to be an effective method of producing large amounts of recombinant proteins without the use of animal-origin materials such as foetal bovine serum or eggs (Khandelwal *et al*., 2004) [30]. These techniques have been predominantly applied to the development or improvement of ELISAs and can be used to create highly sensitive screening assays typically designed to detect all infected animals whereas highly specific confirmatory assays was designed to detect only infected animals and reduce the number of false-positive reactions (Langedijk, *et al*., 2001) [33]. The use of synthetic peptides or recombinant antigens in the detection of animals infected with an OIE (World Organisation for Animal Health) listed a animal disease such as classical swine fever (Moennig *et al*., 2003) [36] or foot-and-mouth disease virus (Park *et al*., 2010) [41] or with a zoonotic disease like West Nile virus (Davis *et al*., 2001) [18] or endemic disease like equine infectious anemia virus (Craigo *et al*., 2008) [16], 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 (Davis *et al*., 2001) [18]. 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. This can be accomplished by expressing a single immunogen in a vectored vaccine, or by deleting the expression of a single immunogen in a vaccine, followed by the development of a complementary serological assay (Davis *et al*., 2001) [19].

A successful example of the power of these improved complementary assay-vaccine combinations is the Aujeszky's disease (pseudorabies) eradication programmes in Europe and the United States of America (Mengeling *et al*., 1997) [34]. Vaccines were developed with a specific gene deletion which prevented the expression of a specific protein. Animals vaccinated with this gene-deletion vaccine do not make antibodies specific to the unexpressed protein, but do make antibodies to the other immunogens produced by the pathogen, resulting in a protective immune response. A complementary ELISA using the specific gene-deleted protein from the vaccine could then detect antibodies from vaccinated and non-vaccinated animals that have been infected with the field strain, but not uninfected vaccinated animals (Bannai *et al*., 2013) [8]. This approach adopted by Mengeling *et al*., 1997 [34] enabled Aujeszky's disease programmes to achieve eradication more rapidly by establishing a vaccinated population of animals in which infection with field strains of virus could be detected.

Mollica *et al*., 2009 [25] illustrated that the use of Western blot assays for highly-specific confirmatory assays has provided diagnosticians with the tools necessary to more accurately detect cross-reactions (a positive reaction from testing the serum of an animal that is not infected with the pathogen). These assays are frequently employed with samples that test positive on screening assays, but come from populations with a low prevalence of animals infected with the target pathogen (Mollica *et al*., 2009) [25]. The Western blot separates the proteins by molecular mass using a gel and detects antibodies to the specific proteins (immunogens); cross-reactive sera usually do not recognise the normal array of specific proteins at the correct molecular masses (Aldridge *et al*., 2008) [2]. In addition to more conventional architectures like the 96- well ELISA plate, new presentations of the immunoassay have been developed, most commonly to provide a single animal test that can be performed in a laboratory that lacks more sophisticated instrumentation or in a pen-side or point-of-sale setting (Taylor *et al*., 2013) [43]. The assays can be designed to detect either antigen or antibody. These individual sample devices frequently embed the same types of reagents used for ELISA-type assays on a membrane that allows the sample to migrate from the application point to the end-point by capillary action (Taylor *et al*., 2013) [43]. As the sample progresses on the membrane, each step of the immunoassay occurs sequentially and a visible line develops at the end-point when the analyte is present. These kinds of assays provide small veterinary practices with individual animal tests that provide data within minutes of sample application (Taylor *et al*., 2013) [43].

Frequently, these assays are designed to use with whole blood, serum or plasma samples or they may be designed to analyse faecal samples for pathogens of interest such as influenza virus (Moore *et al*., 2004) [37]. Recently, commercial assays to detect cell-mediated responses have become available. These include gamma interferon assays for use in primates, cattle, and cervids for the detection of tuberculosis. These assays rely on the 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 sample with viable white cells (Svobodova *et al*., 2013) [44].

Biosensors

A new approach to the detection of either the agent or antibodies is the development of biosensors (Barker, 1987)

[9]. 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 measurable signal (Cruz *et al*., 2002) [17]. Some of the transducer technologies under development include electrochemistry, reflectometry, interferometry, resonance and fluorimetry (King *et al*., 2000) [31].

An example of a commercial application of fluorimetry is the particle concentration fluorescence immunoassay for brucellosis and Aujeszky's disease antibody screening (Nielsen *et al*., 1996) [39]. This technique utilises sub-micron polystyrene particles coated with antigen and placed in a 96-well vacuum plate (Jianrong *et al*., 2004) [28]. Unknown serum and a fluorescent conjugate is added followed by vacuum filtration that removes unbound conjugate. The total particle-bound fluorescence is measured by front-surface fluorimetry (Jianrong *et al*., 2004) [28]. Fluorescence polarisation technology has recently become available for the detection of bovine brucellosis. This technique relies on the use of fluorescence to label antigens or antibodies in a standard preparation (Nielsen *et al*., 1996) [39]. The spin of the labelled molecule is determined using a specialised fluorimeter, the antiserum is then added to the solution, and the spin of the labelled molecule is determined again (Jianrong *et al*., 2004) [28]. If there has been antigen-antibody binding, the size of the labelled molecule will be increased and the spin will be notably slower. This technology offers a rapid detection method, taking only minutes to analyse each sample. Another application of fluorimetry is being adapted to light fibre-technology biosensors (King *et al*., 2000) [31]. Fibre optic biosensors have the potential to do multi-analyte analyses in an automated format. One particular biosensor under development, known as the rapid, automatic and portable fibre optic fluorimeter, has been reported to detect four different analytes in one coupon (test) (King *et al*., 2000) [31]. Potential applications of biosensors include self-contained field use devices for the detection of bioterrorism agents or foreign animal disease agents (Barker, 1987) [9].

Nucleic Acid Diagnostics

The use of nucleic acid-based diagnostics in veterinary medicine has increased exponentially in recent years (Gingeras *et al*., 1990; Hu, 2013) [21, 24]. These techniques have redefined the level of information available for animal disease control programmes. In addition, modifications of nucleic acid detection techniques such as polymerase chain reaction (PCR) have led to the development of rapid, specific assays (Van Brunt, 1990) [47]. The molecular technique with the widest variety and application in veterinary diagnostics is PCR (Van Brunt, 1990) [47]. The strength of this technique is its ability to make millions of copies of a deoxyribonucleic acid (DNA) target (Jain, 2002) [26]. This amplification enables the desired target to be readily detected by other techniques such as electrophoresis and sequencing (Van Brunt, 1987) [48]. Initial use of PCR in veterinary diagnostics was for specific genomic detection; one example is the typing of viral strains such as bovine viral diarrhoea virus. The role of PCR has expanded, and it was used for the rapid and large-scale diagnosis of avian influenza (Alexander, 2004) [3] and Newcastle disease. The detection of viral ribonucleic acid (RNA) by PCR is accomplished by the use of reverse transcription PCR (Hilborne *et al*., 1991) [23]. This assay initially makes a complementary DNA copy of the original viral RNA before final amplification and is more sensitive than the traditional Northern blot method of RNA detection (Jain, 2002) [26]. A potential approach to RNA detection is to use binding stretches of RNA (aptamers). This type of application does not require amplification and is currently used in studies of gene expression in human cells and tissues (Jain, 2002) [26].

Nested PCR refers to the application of a second set of primers targeting a shorter area on the first-stage amplified product (DNA) (Hilborne *et al*., 1991) [23]. Using this approach an increase in the sensitivity of the PCR occurred generating two amplified products for confirmation purposes. This technique has been used to detect a number of agents of veterinary interest including West Nile virus (Johnson *et al*., 2003) [29]. A disadvantage of the nested PCR is the increased risk of cross-contamination due to the opening of amplification tubes to add an additional set of primers. Real-time PCR is the latest improvement in the standard PCR technique to be implemented in veterinary laboratories. This technique is a single-tube, closed assay that greatly decreases the problem of cross-contamination between samples (Johnson *et al*., 2003) [29]. Detection of positive samples is dependent on the amount of fluorescence released during amplification. These fluorescence readings are plotted by computer software and results can be transmitted electronically, eliminating the need for post-PCR reaction analysis by electrophoresis. The development of extraction methods such as the magnetic bead technique (Caldarelli-Stefano *et al*., 1999) [13] has made it possible to use real-time PCR to test large number of samples in a matter of hours during disease outbreaks. Moreover, real-time PCR has adapted for use in veterinary through the use of portable thermocycler and lyophilised reagents (Johnson *et al*., 2003) [29]. This approach may allow for more rapid decision-making during potential disease outbreaks.

The PCR is also used extensively for the genotyping and phylogenetic analysis (relatedness) of veterinary pathogens (Collins *et al*., 2003) [15]. Diagnosis of diseases such as virulent Newcastle disease and highly pathogenic avian influenza is required by the OIE to be based on chicken inoculation and/or the targeted sequencing of PCR product, namely, genes coding for virulence factors, such as haemagglutinin gene (Collins *et al*., 2003) [15].

Non-polymerase chain reaction methods of nucleic acid detection

New methods of nucleic acid amplification have been developed and may eventually be used for veterinary diagnostics. Examples of these methods include the rolling

circle amplification technique and direct signal amplification systems (Zhong *et al*., 2001**)** [49]. These techniques are currently being used in human diagnostics for the detection of human cytomegalovirus and human immunodeficiency viruses; veterinary applications are currently being developed (Beals *et al*., 2010) [10].

Fluorescent *in situ* hybridisation (FISH) is a technique that can localise nucleic acid sequences within cellular material (Min *et al*., 2003) [35]. Peptide nucleic acids are molecules in which the sugar backbone has been replaced by a peptide backbone (Azofeifa *et al*., 2000) [6]. These molecules are perfect mimics of DNA with high affinity for hybridisation that can be used to improve FISH techniques (Azofeifa *et al*., 2000) [7]. A new proprietary, conformationally restricted oligonucleotide known as 'locked nucleic acid' shows promise for hybridisation assays (Nickerson *et al*., 1990) [38]. New techniques utilising locked nucleic acids can better discriminate between correct and incorrect DNA/RNA target sequences and their use looks to be increasing in many applications where oligonucleotide probes are used **(**Van Brunt, 1987**)** [48]. Nucleic acid sequence-based amplification (NASBA) is a promising gene amplification method (Deiman *et al*., 2002) [19]. 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 (Deiman *et al*., 2002) [19]. The entire NASBA process is conducted at a single temperature, thereby eliminating the need for a thermocycler (Chang *et al*., 2012) [14]. The use of this technique has been shown to detect avian and human influenza viruses (Collins *et al*., 2003; Alexander, 2004; Moore *et al*., 2004) [15, 3, 37].

Proteomics

In addition to the use of proteomics to identify and characterise the protein produced by pathogenic agents, proteomic technologies have great potential in veterinary diagnostic applications because they target the patterns of protein expression of the target analyte whether it is viral, bacterial, parasitic, etc (Krah and Jungblut, 2004; Amaya, 2014) [32, 5]. The standard proteomic approach involves the separation of proteins by two-dimensional gels with the staining of the proteins and molecular weight control. This protein 'pattern' or fingerprint is then analyzed by performing image analysis (Krah and Jungblut, 2004) [32]. Proteome maps can be compared in order to find proteins that may be up- or down-regulated due to disease was revaled by Amaya, 2014 [5].

A protein of interest can be cut from the gel and fully characterised using peptide-mass fingerprinting and/or mass spectrometry methods (Amaya, 2014) [5]. In the future, veterinary diagnostics may make use of proteomics to identify or look for known disease markers or patterns with biochip technology (Pernagallo *et al*., 2012) [42] and instrumentation that combines mass spectrometry with other separation chromatography or molecular techniques (Amaya, 2014) [5]. These instrumentations are designed to specifically select, separate by molecular mass, and identify the complex mixture of proteins in a sample, which can then be compared to known samples for diagnostic purposes (Krah and Jungblut, 2004) [32]. For example, serum from a person infected with a mycobacterial pathogen has a unique pattern that can be identified using a specifically-designed biochip and analysis software. This type of technology may be useful for identifying animals infected with agents that do not induce predictable serologic reactions, such as bovine tuberculosis (Bahk *et al*., 2004) [7].

Microarray technology, originally developed for the mapping of genes, is being used to detect a wide variety of veterinary pathogens (Butcher, 2004) [12]. Specific oligonucleotides are bound to small solid supports such as glass slides, silicon chips or nylon membranes. Extracted DNA or complementary DNA is labelled with a fluorescent dye and then hybridised with the microarray (Butcher, 2004) [12]. Specific patterns of fluorescence are detected by a microarray reader which allows the identification of specific gene sequences found only in the veterinary pathogen of interest. This technology has the potential to identify the presence of agents of interest at the serotype or subspecies level, or to differentiate agents that cause similar clinical signs, for example, vesicular lesions (Butcher, 2004) [12].

Nanotechnology

The term 'nanotechnology' is broadly defined as systems or devices related to the features of nanometre scale (one billionth of a metre) (Emerich and Thanos, 2003) [20]. This scale of technology as it applies to diagnostics would include the detection of molecular interactions (Emerich and Thanos, 2003) [20]. The small dimensions of this technology have led to the use of nanoarrays and nanochips as test platforms (Jain, 2003) [27]. Advantage of nanotechnology has the potential to analyse a sample for an array of infectious agents on a single chip.

Applications include the identification of specific strains or serotypes of disease agents, such as the identification of specific influenza strains, or the differentiation of diseases caused by different viruses but with similar clinical signs, such as vesicular viral diseases (Moore *et al*., 2004) [37]. Many research groups are considering the use of chip assays that detect a number of agroterrorism agents in each sample. Small, portable platforms are being designed to allow pen-side testing of animals for diseases of concern. Another facet of nanotechnology is the use of nanoparticles to label antibodies (Jain, 2003) [27]. These labelled antibodies can then be used in various assays to identify specific pathogens, molecules or structures. Examples of nanoparticle technology include the use of gold nanoparticles, nanobarcodes, quantum dots (Cadmium selenide) and nanoparticle probes (Jain, 2003) [27].

Additional nanotechnologies include nanopores, cantilever arrays, nanosensors and resonance light scattering (Emerich and Thanos, 2003) [20]. Nanopores can be used to sequence strands of DNA as they pass through an electrically-charged membrane. Cantilever sensors are comprised of a thin, flexible beam made of silicon coated

with DNA that can bind to a selected target sequence (Jianrong *et al*., 2004) [28]. These sensors can be placed in a microarray format to detect numerous DNA targets in a single sample using different wavelength-emitting detection molecules for each target.

Nanosensors are comprised of a nanowire that is coated with any biological recognition substance (Emerich and Thanos, 2003 [20]. After chemical interaction with target analyte, a change in electrical conductance of the nanowire can be measured. Resonance light-scattering technology is based on the use of nano-sized metallic particles (Jianrong *et al*., 2004) [28]. These particles can scatter light when illuminated so that specific levels of intensity and differing emitted colours can be easily detected, differentiated and quantified. This can greatly increase the sensitivity by which biological molecules can be measured (Emerich and Thanos, 2003) [20]. Nanotechnology is still primarily in the research stage, with the focus on the detection of bioterrorism agents (Jain, 2003 [27].

Conclusion

While veterinary diagnostics are still primarily comprised of time-tested, traditional diagnostic techniques, a profound change has occurred in recent years with the introduction of new biotechnological assays. These new biotechnologies include the production of more specific antigens by the use of recombination, expression vectors and synthetic peptides. Coupled with the use of monoclonal antibodies, the sensitivity and specificity of a number of traditional types of diagnostic assays have been significantly improved. Various forms of PCR have become routine diagnostic tools in veterinary laboratories not only to make specific typing determinations, but also to rapidly screen 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. While not yet implemented in veterinary laboratories, nanotechnologies hold the promise of screening for numerous pathogens in a single assay. Their small size may make nanotechnology the choice for mobile, pen-side testing. Biotechnology and its applications hold great promise for improving the speed and accuracy of diagnostics for veterinary pathogens. Much developmental work will be required to realise the potential with well-characterized, validated assay systems that provide improved diagnostic capabilities to safeguard animal health.

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