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### BIOTECHNOLOGICAL TOOLS FOR DIAGNOSIS OF EQUINE INFECTIOUS DISEASES

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#### KEYWORDS

Biotechnology

Immunoassay

Equine

Infectious disease

#### ABSTRACT

Rapid diagnosis of infectious diseases and appropriate treatment with in time are important steps that promote optimal clinical outcomes and general public health. Today there is large number of new technologies such as nanotechnology, biosensors, and microarray techniques, are being developed and used as diagnostic tools for equine infectious diseases. Nucleic acid based techniques such as polymerase chain reaction (PCR) have become conventional tools in veterinary research and plays an important role in specific typing determinations as well as for rapid screening of ample numbers of samples at the time of equine disease outbreaks. Other biotechnological techniques are populous to be used in the coming times as they can enhance diagnostic efficacy in less time and cost as compared to conventional techniques. This review focuses on biotechnological tools available for equine diseases diagnosis and its applications hold great promise for improving the speed and accuracy of diagnostics for equine infectious diseases.

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## 1 Introduction

Modern molecular biology provides us newer technology to diagnose and control several equine diseases. It is also used for the development of novel diagnostic tools for equine infectious disease control (Pusterla et al., 2006; Amaya, 2014). Several diagnostic tools such as nucleic acid probes, monoclonal antibodies, restriction fragment length polymorphisms, real time PCR, proteomics, biosensors and nanotechnology have increased the livestock productivity. These methods have been commonly used for equine disease diagnosis and control (Yeh et al., 2010; Johnson et al., 2010; Rakhshandehroo et al., 2014). Several viral and bacterial pathogens such as Japanese encephalitis virus, West Nile virus, Hendra virus, borna virus, equine rabies, *Rhodococcus equi*, *Bacillus anthracis* etc., are causes several serious diseases in equines and induce economic to human population and these are zoonotic in nature (Yeh et al., 2010; Booth et al., 2010; Priestnall et al., 2011; Khurana, 2015).

On various occasion equines are used for various purposes such as ceremonies, riding, sports, draught racing, transport and antitoxin/antibody production, throughout the world (Burnouf et al., 2004). There is possibility of disease transmission and spread at the time of equines movement from one country to another. Therefore, OIE (World Organisation for Animal Health) has enlisted several diagnostic tests for international movement of equines (Table 1) (OIE, 2016). Biotechnology may play an important role in prevention of disease caused by these pathogens.

The correct knowledge of molecular biology of infectious agents and their hosts is very important for controlling the disease (Tavares et al., 2011). Biotechnological and protein based assays can play a main role in equine disease control due to its everlasting developments with the use of developed anti pathogenic drugs and diagnostic chemicals. Even though conventional techniques are still used commonly, recent biotechnological assays have widened the scope of equine diseases detection and give us powerful new techniques for quick and specific identification of equine diseases. This manuscript reviews the current and potential uses of biotechnology tools for equine infectious disease diagnostics.

## 2 Serological assays

Protein based assays are based on antibody and antigen interaction. These types of several assays such as enzyme linked immunosorbent assay (ELISA), falcon assay screening test –ELISA, indirect or direct immunofluorescent antibody tests, immunoblotting dot-ELISA, peptide based-ELISA, complement fixation test, agar gel immunodiffusion and neutralization test are used for equine infectious disease diagnosis. These serological assays are highly sensitive and specific than other techniques like microscopy and it allow clearance of post-therapeutic pathogen.

### 2.1 Enzyme-linked immunosorbant assay (ELISA)

Components of immune system used for detection of immune response against infection in ELISA test. For detection of specific immune response, ELISA assay involves antigen, antibody and enzymes. The antigens are adhered to surface of microtitre plate and antibody specific to the antigen is applied over the surface for binding. It was followed by the conjugation of antibody with an enzyme-Horseradish peroxidase. Further, substrate was added to the plate for producing visible colour change in a reaction mixture. Based on use or not of a secondary antibody, the ELISA test may be either direct or indirect (Figure 1). This test is successfully used for diagnosis of various diseases in equines. Singha et al. (2014) have reported an indirect ELISA using truncated TssB protein for serodiagnosis of glanders.

A sensitive antigen capture ELISA was developed for the detection of secreted NS1 from infected equines with West Nile virus (Macdonald et al., 2005; Chung & Diamond, 2008). Similarly, ELISA has been developed for the detection of EHV-1, EHV-4 (Yasunaga et al., 2000), equine rhinitis virus A (ERAV) (Kriegshauser et al., 2009) and equine rhinitis virus B (ERBV) (Kriegshauser et al., 2008). In the recent studies, ELISA targeting antibodies to the spike (S) of equine corona virus was developed and validated to detect antibodies to ECoV in infected horses (Kooijman et al., 2016).

Table 1 Prescribed test for equine diseases according to OIE, 2016.

Disease name	OIE prescribed tests
African horse sickness	CF, ELISA
Contagious equine metritis	Agent identification.
Dourine	CF
Equine infectious anaemia	AGID
Equine piroplasmiasis	ELISA, IFA
Equine viral arteritis	Agent identification (semen only), Virus Neutralization
Glanders	Complement Fixation

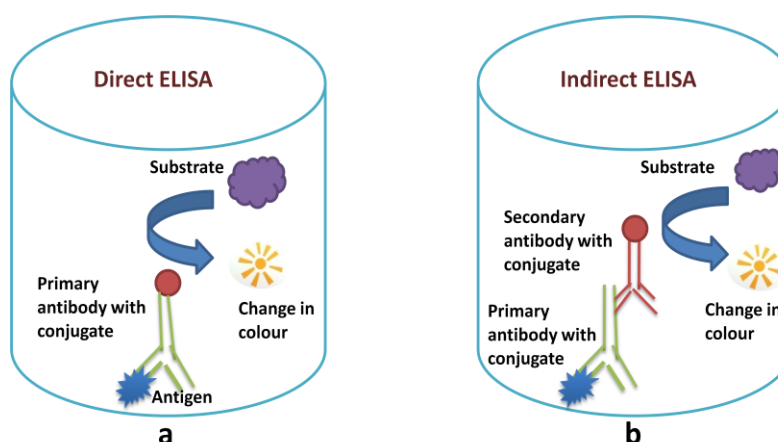


Figure 1 Principle of ELISA test a) Direct ELISA b) Indirect ELISA

## 2.2 Dot-ELISA

Dot-ELISA works on the basis of attachment of small amount of antigen on to a nitrocellulose membrane. Specific antibody is incubated with antigen containing dotted membrane followed by adding of enzyme conjugated anti-antibody. A substrate is added in the last which causes precipitation of a detectable coloured dot on the membrane (Svobodova et al., 2013). It was reported that the dot-ELISA is simple, quick, specific, sensitive, low cost field test that detects minute levels of antibodies much faster than complement fixation test and indirect hemagglutination antibody test (Verma & Misra, 1989; Verma et al., 1990). Dot-ELISA has been used for the sero-diagnosis of glanders (John et al., 2010). By the use of nitrocellulose membrane in this test makes it applicable in the field. This assay is quick and specific in detection of various diseases. It gives us low background as compared to ELISA assay that can easily differentiate between the positive and negative samples.

## 2.3 Fluorescent Antibody Test (FAT)

In Fat assay, antibody is labelled with fluorescent dye, is used in visualization of antigen in a clinical specimens. The antibody conjugated with fluorescent dye and antigen-antibody complex gives a visible glow sign when examined under a fluorescent microscope. The fluorescent dye can be tagged directly with primary antibody which is known as direct fluorescent antibody test or with a secondary anti-antibody known as Indirect Fluorescent Antibody Test (Figure 2). The FAT is used in diagnosis of several equine diseases. This assay was recently investigated for diagnosis of equine leptospiral abortion in mare (Erol et al., 2015). The *sarcocystis neurona* causes a dreadful disease, equine protozoal myeloencephalitis (EPM) in equines. The IFAT was successfully validated for CSF testing for confirmation of EPM in equines (Duarte et al., 2006; Johnson et al., 2013).

The overall specificity and accuracy of IFAT was shown to be better than that of the western blot and modified western blot, which showed its potential to use as a diagnostic assay for detection of EPM caused by *Sarcocystis neurona* (Duarte et al., 2003). The FAT and immune-histo-chemistry (IHC) assay confirmed the presence of Australian bat lyssa virus (ABLV) antigen in horse brain tissues (Shinwari et al., 2014). A FAT assay has been used for the direct identification of bacterial *Helicobacter* on the equine gastric mucosa (Perkins et al., 2012). This technique has been used to describe the spatial distribution of *Helicobacter* species in the stomach of healthy horses to demonstrate the microbiota of normal appearing squamous and glandular mucosa (Burton et al., 2007).

## 2.4 Complement Fixation Test

Complement fixation test (CFT) is an immunological test used for detection of presence of either antigen or antibody in the serum sample. It was generally used for microorganisms which are not easily cultured in research laboratory (Figure 3). Although, several studies have revealed its low specificity and sensitivity for virus detection, it is still used for many equine viral disease diagnoses. CFT is the OIE recommended test for glanders. Due to low prevalence of glanders in equine population it is important to use test with high specificity and sensitivity. CFT was found reproducible and reliable assay for clinical investigation and detection of latent infection of Equine herpes virus 1 (EHV1) (Hartley et al., 2005). A CFT assay has some limitations such as laborious, time consuming and often cross reactivity in nature. The non-specific hemolysis of RBC can be prevented using Potassium Periodate ( $KIO_4$ ). The  $KIO_4$  treatment to horse sera prevented the non-specific hemolysis which helped in determination of precise titers during CF test for EHV-1 diagnosis (Bannai et al., 2013). The CFT and virus neutralisation assays were used for determination of sero-conversion of EHV1 and EHV4 during obtaining acute and convalescent serum samples (Hartley et al., 2005).

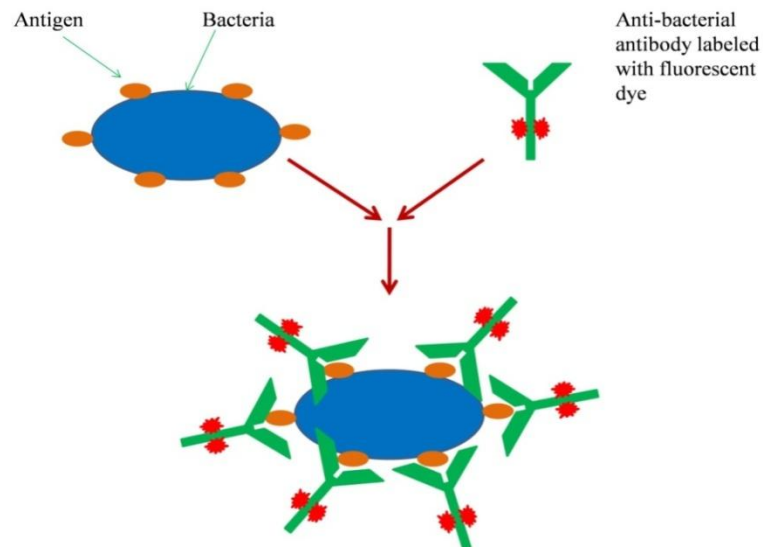


Figure 2 Principle of Direct Fluorescent antibody test (FAT).

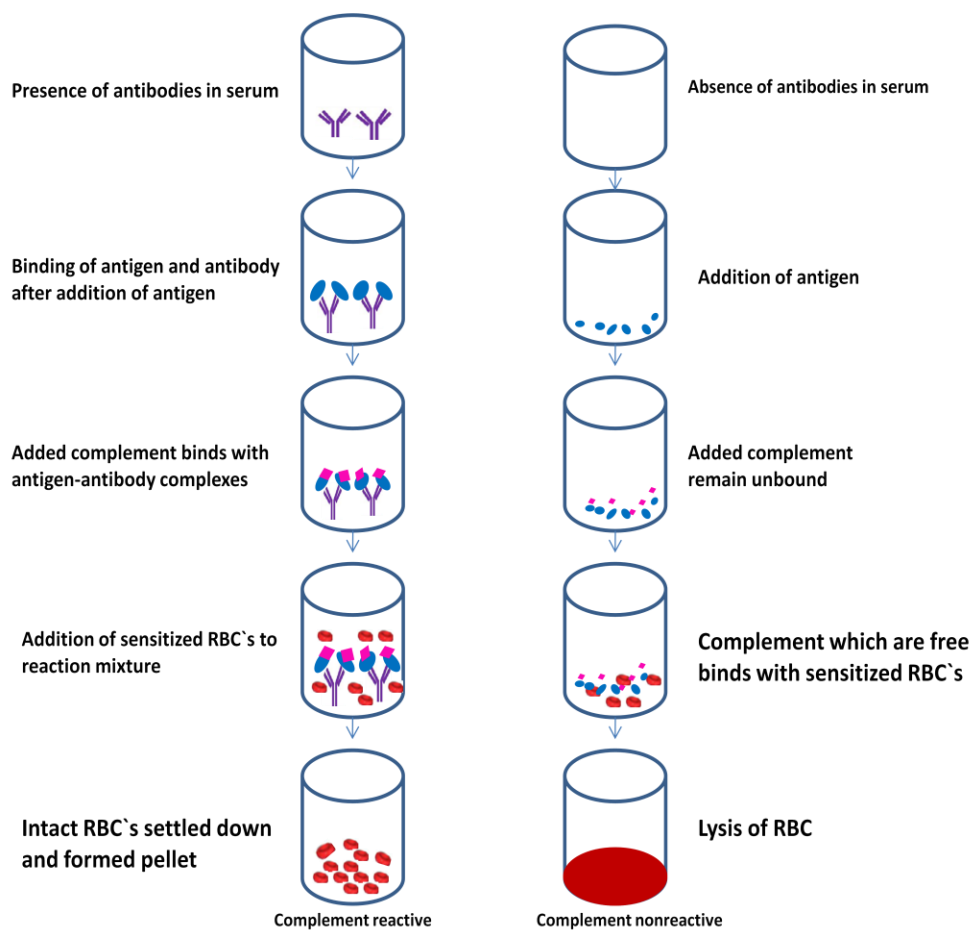


Figure 3 Complement fixation test.

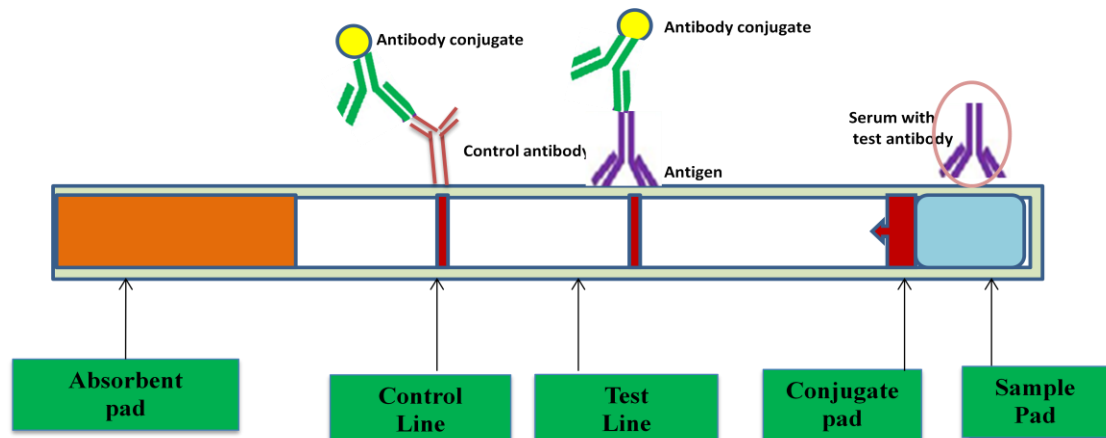


Figure 4 Principle of lateral flow test

### 2.5 Lateral Flow Test (LFT)

This test is also known as a lateral flow immunochromatographic strip assay. LFT is used as diagnostic techniques in medical and veterinary applications. It is an easy and fast assay used for identification of interest target in sample without using any special equipment (Figure 4). This technique can be used for qualitative or specific semi-quantitative identification of many interest targets such as antibodies, antigens and nucleic acid products. The assay indicates a procedural control line which shows that the assay was performed properly and validates the test result. Therefore, presence of two lines gives positive result, while indication of only control line shows negative test in experiment. However, the appearance of no lines or only test line shows invalid result and test must be repeated. This test has been successfully used in diagnosis and detection of various disease associated with equines from biological samples. The recombinant viral capsid protein p26 conjugated to colloidal gold based simple immunochromatographic lateral flow (ICLF) test was validated for specific detection of Equine infectious anemia virus (EIAV) antibodies in equine sera (Alvarez et al., 2010). Similarly, LFT was also used for detection of vesicular stomatitis virus in cattle and horse clinical samples (Ferris et al., 2012).

### 2.6 Virus neutralization test

In virus neutralization tests, serial dilutions of heat inactivated test serum are prepared and poured in a 96 well plate and are incubated with a defined amount (generally 100 TCID<sub>50</sub>) of infectious virus (antigen). After incubation time period, susceptible virus cells are added to the virus-serum mixture and the final serum, virus and cell combination is kept for period of 2-3 days. Depending on the virus, this may be done by microscopic examination of the plate for the indication of viral cytopathic effect (CPE). Serum containing antibodies specific to the virus in target are capable to neutralize the aliquot of virus used in the test line, hence preventing the

infection of cells when added to the plate. At the time of very high concentrations of antibody to the virus in target are present in the test sample, virus neutralization will be occur at high serum dilutions. Whenever, where some or no antibody to the virus is present in the serum sample, it will be able to neutralize the infectious virus at the first dilution used in the test. The result of the test is the target at which the serum sample has been diluted such that it no further neutralizes the entire virus in the test. This dilution indicates the titre of the serum tested. The sero-prevalence of EHV-1 and EHV-9 infections was reported by serum neutralization test (Borchers et al., 2005; Taniguchi et al., 2000).

### 2.7 Agar Gel Immunodiffusion (AGID)

AGID technique is used for the detection, identification and quantification of antibodies and antigens present in biological samples. In this technique, a gel plate is cut to form a series of wells in agar gel. A sample aliquot of interest target is placed in one well, and antibodies are placed in nearby well and the plate was incubated for 48 hours. During incubation time the antigens in the target sample and the antibodies each diffuse out from their corresponding wells. At the point where the two diffusion lines intersect, if any of the antibodies is specific to any of the antigens then they will bind to each other and form a complex. This antigen-antibody complex precipitated gives a thin white line in the gel, helps in the visual identification of antigen recognition. AGID can be used to diagnose Equine Infectious Anemia (EIA) (Beltrao et al., 2015). It detects antibodies against the main capsid viral protein (p26) in horse serum samples and this test is simple, inexpensive and specific to identify EIAV-infected animals (Alvarez et al., 2010).

### 2.8 Peptide based-ELISA

Peptide based-ELISA, a plate based techniques for detecting and quantifying peptides, proteins, hormones and antibodies. In this technique, a synthetic peptide is to be mobilized onto a solid support and complexes with an antibody, linked to an

enzyme. Detection is done by assessing the conjugated enzyme activity after incubation with a substrate to produce a measurable end product (Figure 5). The very important step for the detection strategy is a specific antigen-antibody interaction. Peptide-ELISAs are performed in a 96-well microtiter plate with synthetic peptide in carbonate buffer followed by incubating the plate overnight at 4°C. Now, block the plate with blocking buffer for 1 hour at 37°C followed by addition of freshly prepared diluted primary antibody into each wells and incubate the plate at 37°C for 1 hour. Subsequently, anti-mouse IgG, diluted in 100 µl/well antibody dilution buffer is added with the incubation at 37°C for 30 minutes. In last enhancement solution is added in the plate and incubated at 37°C for 15 minutes. The plate was washed in between each step at least five times with 1X PBST. Read the absorbance at appropriate wavelength with an appropriate time resolved plate reader.

Soutullo et al. (2001) evaluated the performance of an equine infectious an Aemia-ELISA designed with synthetic peptides. This assay could be important to prove for large throughput screening and early detection of equine infectious anaemia (EIA), when the results of the traditional Coggins test are still negative. Recently, a sensitive and specific peptide-based ELISA was developed to determine the sero-prevalance of EHV-1 and EHV-9 (Abdelgawad et al., 2015). For discrimination between serological responses to EHV 1 and EHV4 immunoglobulins-IgG based type specific ELISA was developed (Ma et al., 2013). This technique was also used to discriminate between EHV-1 and EHV-4 glycoprotein E peptides for EHV-1 and glycoprotein G (gG) for EHV-4 (Lang et al., 2013; Yasunaga et al., 1998). Recently, a glycoprotein G based peptide ELISA was developed for detection of equine herpesvirus type 4 (Bannai et al., 2016).

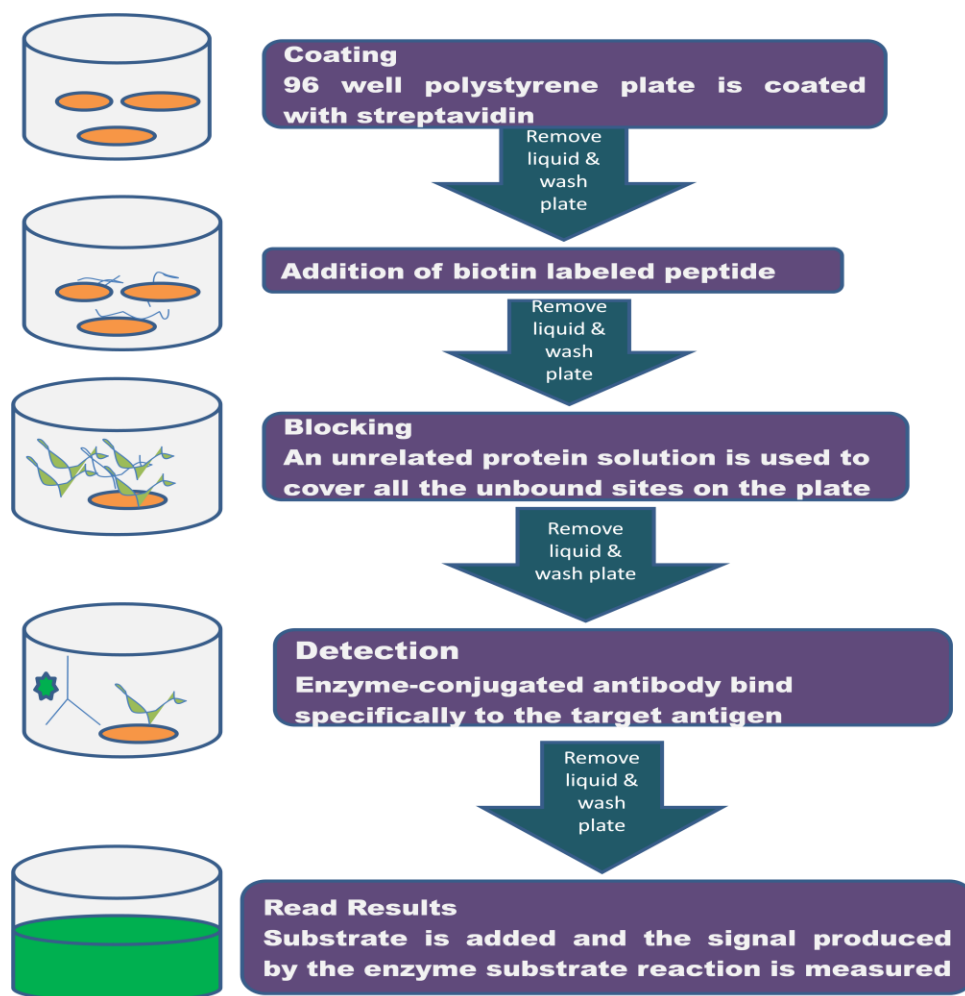


Figure 5 Principle of peptide- ELISA

## 2.9 Nucleic acid diagnostics

Nucleic acid-based detection methods including polymerase chain reaction, reverse transcriptase-PCR, nested-PCR, restriction fragment length polymorphism, amplified fragment length polymorphism, random amplified polymorphic DNA, loop-mediated isothermal amplification, microarray, real-time PCR are used for identification of several equine diseases (Pusterla et al., 2007; Monego et al., 2009; Yeh et al., 2012; Quereda et al., 2000; Larrasa et al., 2002; Eischeid, 2011).

## 2.10 Polymerase Chain Reaction (PCR)

PCR uses the enzyme DNA polymerase that amplifies a small length of targeted DNA using primers which are specific to the target. It will amplify the selected target sequence from a mixture of genome. PCR acts as an important tool for the identification of parasites due to the insufficient amount of availability of antigen and antigen products by using

conventional assays (Gasser, 2006). PCR can utilize almost all kind of biological samples such as meat, blood, urine, skin scrapings and faeces for parasitic infection study. When compared to the conventional method the detection limit of PCR is higher.

Therefore, it is useful for detecting low amount of antigen in suspected samples (Varrasso et al., 2001; Nugent et al., 2006). It can also be used for detection of many equine diseases (Oldfield et al., 2004; Ocampo-Sosa et al., 2007; Pusterla et al., 2007; Letek et al., 2008; Monego et al., 2009). Polymerase chain reaction (PCR)-based diagnostic tests can allow rapid and sensitive detection of equine infectious pathogen (Paxson, 2008). Yeh et al. (2010) developed a duplex reverse transcriptase PCR which is sensitive, specific and very rapid and is useful in both humans and as well as in horses for the simultaneous and differential diagnosis of West Nile and Japanese encephalitis viruses.

Table 2 PCR testing results for a variety of infectious equine pathogens.

Pathogen	PCR test	References
West Nile Virus	RT-nPCR	Johnson et al. (2001)
<i>Strongylus edentatus</i> , <i>Strongylus equinus</i> and <i>Strongylus vulgaris</i>	PCR-RLB	Traversa et al. (2007)
Herpes viruses 4 and 1	PCR	Brendan & Michael (1993)
Equine herpesvirus 1 and 4	Differential multiplex PCR	Carvalho et al. (2000)
Distinguish between EHV-1 and EHV-4	PCR	Wagner et al. (1992)
EHV1, EHV4, EHV2 and EHV5	Multiplex PCR	Wang et al. (2007)
<i>Rhodococcus equi</i>	PCR	Khurana et al. (2015); Pal & Rahman, (2015)
<i>Rhodococcus equi</i>	Multiplex PCR	Chhabra et al. (2015)
Equine arteritis virus	RT-PCR	St-Laurent et al. (1994); Zhang et al. (2008)
<i>Theileria equi</i>	Nested PCR and Nested PCR with hybridisation	Wise et al. (2013)
<i>Babesia caballi</i>	Nested PCR	Battsetseg et al. (2001)
<i>Babesia equi</i>	Nested PCR	Battsetseg et al. (2001)
<i>Streptococcus equi</i>	PCR	Ijaz et al. (2012)
Equine influenza virus	RT-PCR	OIE, (2016)
<i>Leptospira</i> spp.	PCR	Faber et al. (2000)
<i>Salmonella</i> spp.	PCR	Amavisit et al. (2001)
<i>Alternaria</i> spp.	PCR	Dicken et al. (2010)
<i>Emmonsia crescens</i>	Single step PCR	Pusterla et al. (2002)
<i>Lawsonia intracellularis</i>	Faecal PCR	Lavoie et al. (2000)
<i>Corynebacterium pseudotuberculosis</i>	Real-time PCR/ RAPD-PCR	Foley et al. (2004)
<i>Anaplasma phagocytophilum</i>	nPCR	Lee et al. (2015); M'ghirbi et al. (2012)
<i>Streptococcus equi</i>	Species-specific PCR	Javed et al. (2016)
Equine encephalitis virus	RT-PCR	Linssen et al. (2000)
Differentiation of <i>B. mallei</i> and <i>B. pseudomallei</i>	Multiplex qPCR	Janse et al. (2013)
<i>Streptococcus equi</i>	Triplex quantitative PCR	Webb et al. (2013)



Table 3 Examples of Real-time Polymerase chain reaction for the diagnosis of equine infectious pathogens.

Pathogen	Target gene	Reference
<i>Streptococcus equi</i>	eqbE and SEQ2190	Webb et al. (2013)
<i>Salmonella</i>	<i>ompC</i> gene of <i>S. Heidelberg</i>	Amavisit et al. (2001)
<i>Salmonella spp.</i>	<i>invA</i> gene	Pusterla et al. (2010)
<i>Anaplasma phagocytophilum</i>	16S rRNA	M'ghirbi et al. (2012)
<i>Streptococcus</i>	16S rRNA	Javed et al. (2016)
<i>Streptococcus equi</i>	<i>SeM</i> gene	Javed et al. (2016)
<i>Streptococcus equi</i>	<i>sodA</i> gene	Javed et al. (2016)
<i>Leptospira</i>	<i>ligA</i> and B	Palaniappan et al. (2005)
<i>Corynebacterium pseudotuberculosis</i>	PLD exotoxin gene	Spier et al. (2004)
Equine arteritis virus	EAV ORF7 gene	Balasuriya et al. (2002)
Equine herpes virus 1	Glycoprotein B	Diallo et al. (2006)
Equine herpes virus 4	Glycoprotein B	Diallo et al. (2007)
Equine herpes virus 1	gD and IR6 gene	Goodman et al. (2007)
<i>Strongylus vulgaris</i>	rDNA	Nielsen et al. (2008)
Equine Influenza Virus	Matrix and hemagglutinin gene	Quinlivan et al. (2005)
<i>Pseudomonas syringae</i> pv. <i>aesculi</i>	Gyrase B	Green et al. (2009)
<i>Lawsonia intracellularis</i>	Aspartate ammonia lyase gene	Pusterla et al. (2008)
<i>Theileria equi</i>	18S rRNA	Kim et al. (2008)
Equine infectious anemia virus	<i>gag</i> gene	Cook et al. (2002)
African horse sickness	NS1	Rodriguez-Sanchez et al. (2008)
<i>Babesia equi</i>	<i>ema-1</i> gene	Ueti et al. (2005)
<i>Corynebacterium pseudotuberculosis</i>	Phospholipase D gene	Sharon et al. (2004)
<i>Burkholderia mallei</i>	ISBma2	Janse et al. (2013)

Further, Yeh et al. (2012) developed a diagnostic algorithm which serologically differentiates West Nile virus from Japanese encephalitis virus infection and its validation in field surveillance of horses. Rakhshandehroo et al. (2014) had done an intra-specific variation study for *Habronema muscae* in horses using cytochrome c oxidase subunit 1 gene based identification by PCR technique. *Helicobacter bacterium* infection was reported by Contreras et al. (2007) using the 16S rRNA gene specific PCR in equines.

PCR can also be combined with other molecular methods such as reverse transcriptase or nested PCR to genotype the organisms. RT-PCR has also been developed for Western Equine Encephalitis Virus (WEEV) diagnosis (Linssen et al., 2000; Lambert et al., 2003). RT-PCR assay was successfully used for identification of west nile and japanease encephalitis virus (Lanciotti & Kerst, 2001). A multiplex PCR was designed to amplify herpes simplex virus types 1 and 2, cytomegalovirus, and varicella-zoster virus DNA present in a diverse range of clinical material (Druce et al., 2002). Multiplex PCR assays for the simultaneous identification of varicella zoster virus (VZV), herpes simplex viruses (HSV), CMV, human herpesvirus 6, and Epstein-Barr virus in cerebrospinal fluid (Quereda et al., 2000) and assays for HSV and VZV in mucocutaneous specimens (Jain et al., 2001; Nogueira et al., 2000) and CSF (Read & Kurtz, 1999) have been reported, each with improved utility over existing methods in the diagnostic setting. In a recent study herpesvirus (EHV) type 1 was detected using PCR and neuropathogenic genotype of EHV-1 was identified by DNA sequencing

(McFadden et al., 2016). Various types of PCR are used for testing a Variety of Infectious Equine Pathogens (Table 2).

## 2.11 Random Amplified Polymorphic DNA (RAPD)

It is a type of PCR reaction which amplifies segments of DNA randomly. It uses short primers of nucleotide length varies from 8-12 nucleotides and template DNA for PCR amplification. By resolving the resulted amplified product on agarose gel electrophoresis a semi unique profile pattern can be visualized from a RAPD reaction. Although RAPD is comparatively easy to perform, but it is also a PCR dependent assay so it needs specific PCR protocol to give reproducible result. Any kind of mismatch in template and primer results in complete absence of PCR product and makes it difficult to interpret the results of RAPD. This assay has the potential to play a useful role in genetic analyses of livestock species such as horses (Cushwa & Medrano, 1996). Larrasa et al. (2002) described the development of quick and relevant DNA extraction and RAPD methods that can be used for genotyping *Dermatophilus congolensis* field isolates. Larrasa et al. (2004) reported the molecular typing of *D. congolensis* from horse skin sample by RAPD and pulsed field gel electrophoresis (PFGE) techniques.

## 2.12 Real time PCR

The real-time PCR assay gives us the quantification of several types of biological samples using verities of fluorescent materials such as TaqMan probes, SYBR Green dye and



Scorpion primers (Nado, 2009). The pathogenic nucleic acids from various biological and environmental samples can be quantified to give the information about the extent of infection. The SYBR Green dye based Real-time PCR assays have been validated for many equine diseases from several decades. Table 3 presents an overview of real-time PCR routinely used for the detection of equine pathogens such as bacterial, viral and parasitic pathogens. Although Real-time PCR is excellent in showing sensitive and specific results but it is still uncommon in routine laboratory diagnosis especially in rural endemic areas due to its sophistication. In the Real-time amplification protocols, other procedures such as DNA extraction, choice of primers may cause heterogeneity in results and causes difficulty in standardization of assay (Bretagne & Costa, 2006).

A SYBR Green based assay was developed that could detect 100% of the different WNV target region variants in their study, whereas a TaqMan assay failed to detect 47% of possible single nucleotide variations in the probe-binding site (Papin et al., 2004). Johnson et al. (2010) designed a pan-flavivirus RT-PCR using degenerate primers for the NS5 gene to allow the detection of a range of flaviviruses including WNV. This SYBR Green based RT-PCR was able to detect the WNV however the sensitivity was much lower compared to WNV-specific TaqMan RT-PCR assays (Johnson et al., 2010). SYBR Green has been shown to inhibit the PCR reaction to some extent and melt curve analysis is troublesome by dye

redistribution during melting. Eischeid analyzed and reported about the behaviour of other DNA dyes in Real-time PCR and showed that EvaGreen and SYTO dyes out performed SYBR Green in real-time PCR (Eischeid, 2011).

### 2.13 Probe Hybridization

Fragments of DNA or RNA usually around 100-1000 bases length used to detect the presence of nucleotide sequences that are complementary to the probe sequence called hybridization probe and this probe hybridizes to single-stranded nucleic acid sequence (Wetmur, 1991). Due to the nucleotide base complementarity between the target and probe, the nucleotide sequence of probe allows pairing of probe and the target (Figure 6). The labelled probe is then hybridized to the target RNA (Northern blotting) or ssDNA (Southern blotting) immobilized on a membrane or in situ. The probe is tagged with a molecular marker of either radioactive (P32, I125 etc.) molecules or non-radioactive fluorescent molecules to detect the hybridization (Digoxigenin). The probe hybridization based assays have been used for diagnosis of equine infections such as equine arteritis virus (Balasuriya et al., 2002; Westcott et al., 2003). The probe hybridization assay is relatively easy to perform. EHV-1 virus strain was reported by means of Southern blot and dot-blot hybridization (Morris & Field, 1988). The probe hybridization assay was confirmed and the sensitivity was inferior to classical techniques such as virus isolation (Morris & Field, 1988).

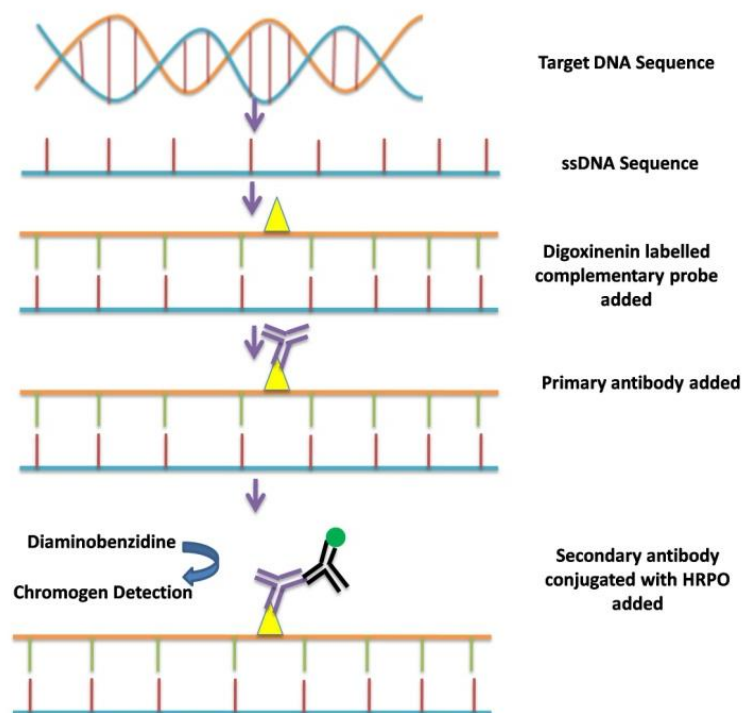


Figure 6 Principle of probe hybridization

## 2.14 Microarray

The nucleic acid microarray technique is a collection of microscopic nucleic acid spots attached to a solid glass surface. Picomoles of specific nucleic acid sequence are present on each spot called probe (Bumgarner, 2013). Probes are allowed to hybridize labelled target nucleic acid (cDNA or cRNA/anti-sense RNA). This probe-target hybridization can be detected and also can be quantified by silver, fluorophore or chemiluminescence-labeled targets (Figure 7). This technique is also used to measure the expression levels of many expressed genes of same or different species simultaneously. Microarray has also been used for diagnosis of equine disease detection. Equine-specific microarray has been used to estimate gene expression in laminitis (Noschka et al., 2009) and articular cartilage repair (Mienaltowski et al., 2009). A recent study using microarray technology on placental tissues identified a >900-fold upregulation of mRNA encoding the cytokine interleukin-22 in chorionic girdle, which is the first time IL-22 has been reported in any cells other than immune cells (Brosnahan et al., 2012). On the basis of whole genome single nucleotide polymorphism (SNP) analysis of all available Venezuelan equine encephalitis viruses (VEE) antigenic complex genomes, verifies that a SNP-based phylogeny accurately captured the features of the phylogenetic tree based on multiple sequence alignment, and reported a high resolution genome wide SNP (Gardener et al., 2016).

## 2.15 Loop-mediated isothermal amplification (LAMP)

LAMP is a nucleic acid amplification procedure that works under a unique amplification principle; involves two steps: these are cyclic or non-cyclic phase (Ushikubo, 2004; Parida et al., 2008). The non-cyclical step precedes the cyclical phase of amplification (Parida et al., 2008). It involves the four primers as well as the *Bst* DNA polymerase with strand displacement activity, play a role in this first stage of LAMP reaction. The

cyclical step builds upon the product of the non-cyclical step which involves two outer primers along with the *Bst* DNA polymerase. The loop primers might be involved in the cyclic step when six primers are used (Nagamine et al., 2002). LAMP assay due to its unique properties has provided a powerful diagnosis of various pathogens (Notomi et al., 2000). LAMP technique amplifies nucleic acid at a very faster rate along with maintaining high specificity, sensitivity and efficiency (Parida et al., 2008). The most inventive feature of this technique is the simplicity of its protocol (Figure 8), and the low cost of overall amplification. Alhassan et al. (2007) developed a LAMP method for diagnosis of equine piroplasmosis.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) has been developed and it should be applicable to detect the equine rotavirus infection in molecular laboratories (Nemoto et al., 2010). Equine influenza virus was also reported using LAMP assay (Nemoto et al., 2011; Nemoto et al., 2012). Novel LAMP methods was developed specific to the pathogenic bacteria found in equine secondary pneumonia, namely, the *Bacteroides-Prevotella* group, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia* and *Staphylococcus aureus* (Kinoshita et al., 2015). Two different LAMP assays targeting *Escherichia coli* (Hill et al., 2008) or *Pseudomonas aeruginosa* (Goto et al., 2010) were used by Kinoshita et al. (2015), on clinical respiratory specimens and a high accordance was found between the results of the LAMP assays and bacterial culture. Use of these LAMP assays could enable rapid detection of pathogenic bacteria and swift administration of the appropriate antimicrobials. In this way, it is possible to concurrently perform LAMP assays to detect both the primary and secondary causative pathogens of lower respiratory bacterial infections in horses in only 60 min with the naked eye; this will make it possible to institute appropriate antimicrobial therapies more quickly in horses with secondary bacterial pneumonia (Kinoshita et al., 2015).

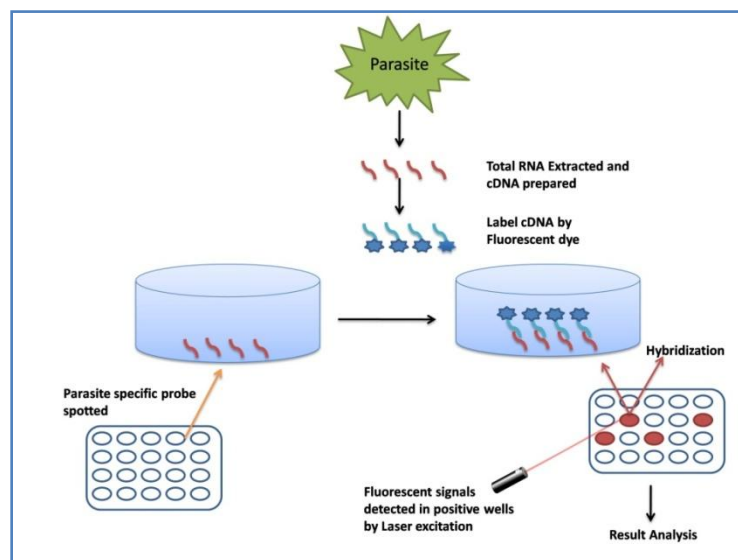


Figure 7 Principle of microarray assay.

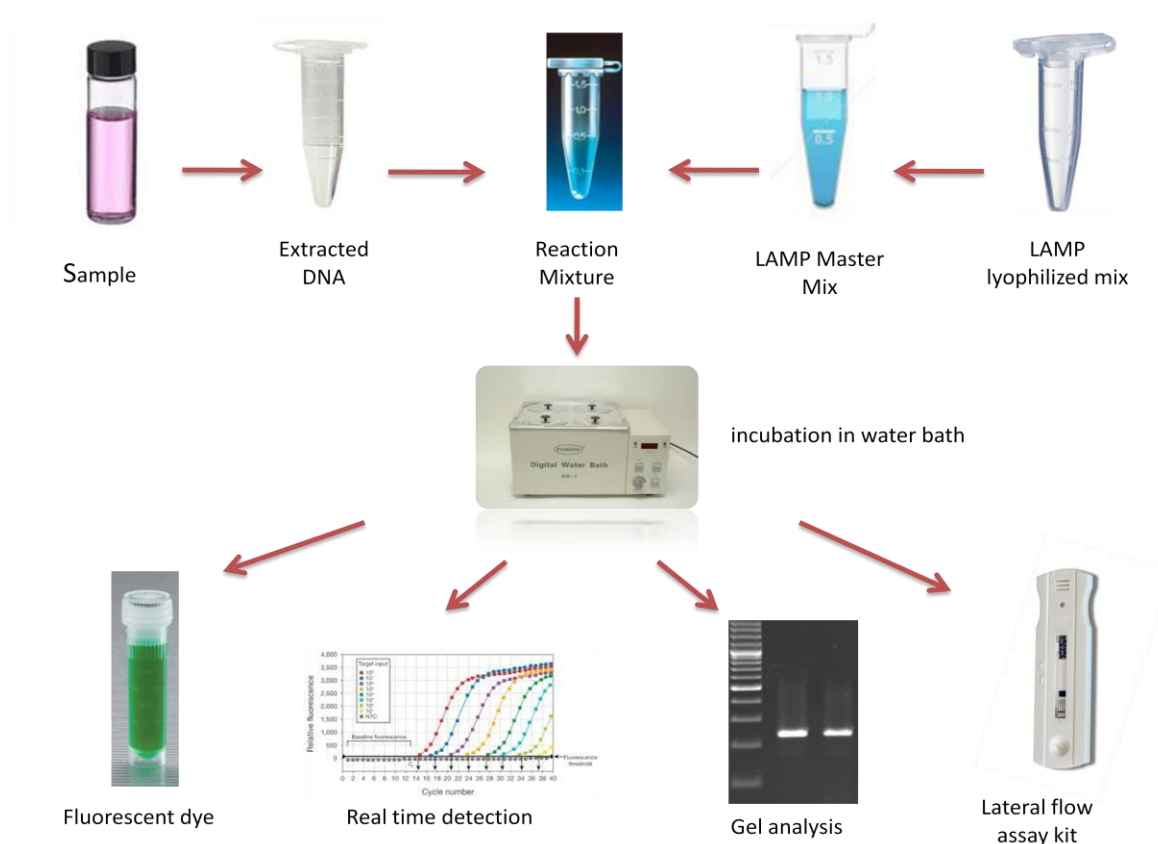


Figure 8 Principle of LAMP.

### 2.16 Sequence analysis study

Whole genome sequencing is a process that gives the complete DNA sequence of an organism's genome at a single time. High-throughput genome sequencing technologies have largely been used as a research tool and are currently being introduced in the clinics (Van et al., 2013; Gilissen, 2014; Nones et al., 2014). Genome sequencing of the domestic horse and subsequent advancements in the field of equine genomics have led to an explosion in the development of tools for mapping traits and diseases and evaluating gene expression (Finno & Bannasch, 2014). In 2011, whole-genome sequencing of an individual American quarter horse mare was performed using massively parallel paired-end sequencing (Doan et al., 2012). Several single-gene disorders in quarter horses, such as polysaccharide storage myopathy (McCue et al., 2008; Tryon et al., 2009), hyperkalemic periodic paralysis, glycogen branching enzyme deficiency (Rudolph et al., 1992), and hereditary equine regional dermal asthenia (Ward et al., 2004; Finno et al., 2009) has been reported due to whole-genome sequencing of an individual American quarter horse mare. A high-quality draft assembly was constructed and additional sequence were provided by the inclusion of bacterial artificial chromosome end sequences from a related male thorough bred horse (Leeb et al., 2006). Kinoshita et al., (2014) reported the genera *Bacteroides* and *Prevotella*

especially *B. fragilis* and *P. heparinolytica* are dominant anaerobes in lower respiratory tract infection in horses.

### 3 Biosensors

Biosensor is an advanced technique for the detection of either the antigen or antibodies. This assay involves the use of a receptor (mostly an antibody), a disease specific antibody and a transducer that converts a biological interaction into a measurable signal (Cruz et al., 2002). These biosensors are frequently coupled to sophisticated instrumentation to produce highly-specific analytical tools, most of which are still in use only for the research and development purpose due to the high cost of instrumentation, high cost of individual sample analysis, and the need for highly trained persons to oversee the testing. Fibre optic biosensors have the potential to do multi-analyte analyses in an automated format. Portable fibre optic biosensors, has been reported to detect four different analytes in one coupon (King et al., 2000). Biosensors can be used as self-contained field devices for the detection of foreign animal disease agents. West Nile virus was detected using biosensors and microfluidic systems, a linear, 15 amino acid fragment of domain III of WNV was successfully used as an antigen on an amperometric immunosensor (Ionescu et al., 2007). Neng et al. (2010) reported that, a surface enhanced Raman scattering immunoassay was shown to be highly sensitive for the

detection of anti-WNV immunoglobulin. Hu et al. (2004) developed a genetically biotinylated single chain fragment variable antibody (scFv) against Venezuelan equine encephalitis virus (VEE). Patrick et al. (2014) studied the evolution of equine influenza and the origin of canine influenza with the help of biosensor.

#### 4 Nanotechnology

The systems or devices which are related to the features of nanometre scale are broadly defined as nanotechnology. This scale of technology as it applies to diagnostics would include the detection of molecular interactions. The tiny dimensions of this technology led a basement to the use of nanoarrays and nanochips as test platforms (Jain, 2003). The potential use of this technology is to analyse a sample for an array of infectious agents on a single chip. Many research groups are considering the use of chip assays that detect several agroerrorism agents in each sample. Small, portable platforms are being designed to allow pen-side testing of animals for diseases of concern.

The use of nanoparticles to label antibodies is another facet of nanotechnology. These labelled antibodies can be used in various assays to identify specific pathogens, structures or molecules. The use of gold nanoparticles, nanobarcode, quantum dots and nanoparticle probes are the examples of nanotechnology (Yguerabide & Yguerabide, 2001). Nanopores, nanosensors, resonance light scattering and cantilever arrays are some of the additional nanotechnologies and it is anticipated that many of the specific nanotechnologies will eventually be applied to the diagnosis of endemic veterinary diseases in the future. Klier et al. (2012) reported about an aerosol formulation of biodegradable, biocompatible and nontoxic gelatine nanoparticle-bound CpG-ODN2216, to treat equine recurrent airway obstruction in a clinical study.

#### 5 Proteomics

Proteomics is the new emerging field to isolate and characterize the protein produced by various etiological agents. Different bacterial, viral as well as parasitic proteins can be targeted with the help of this technology. Hence, proteomics has potential applications in veterinary diagnostics. The usual approach of proteomic involves separation of the proteins with the help of two dimension gel electrophoresis and staining them with appropriate protein marker. The protein 'pattern' is different in different species; hence it can be recognized as a fingerprint. It is then analyzed by performing image analysis (Krah & Jungblut, 2004). Proteins that are up- or down-regulated due to disease are compared and find by using proteome maps. A protein of interest can be cut and taken out from the gel and purified. This purified protein can be further fully characterized using peptide-mass fingerprinting and/ or mass spectrometry methods. Veterinary diagnostics may make use of proteomics to identify or look for known disease markers or patterns with biochip technology and instrumentation that combines mass spectrometry with other separation chromatography or molecular techniques in the

future. These instrumentations are designed to specifically select, separate by molecular mass, and identify the complex mixture of proteins in a sample, which can be compared to known samples for diagnostic purposes.

In equine medicine, proteomics is been used in the diagnosis of different metabolic as well as orthopedic diseases which show some of the alteration in the expression levels of marker proteins (Amaya, 2014). In the proteomic marker analysis conducted in biopsy samples of horse muscles, it was found that three significantly increased proteins: alpha actin, tropomyosin alpha chain and creatine kinase M chain (CKM). CKM was represented by multiple spots probably due to posttranslational modification, one of which appeared to be unique for tying-up suggesting that altered energy distribution within muscle cells is part of the disease etiology (Freek et al., 2010a). In another study they have identified, 20 differential spots representing 16 different proteins. Evaluation of those proteins complies with adaptation of the skeletal muscle after normal training involving structural changes towards a higher oxidative capacity, an increased capacity to take up long-chain fatty acids, and to store energy in the form of glycogen.

Intensified training leads to additional changed spots. Alpha-1-antitrypsin was found increased after intensified training but not after normal training. This protein may thus be considered as a marker for overtraining in horses and also linked to overtraining in human athletes (Freek et al., 2010b). In another study, which was conducted on the proteomics, study of cerebrospinal fluid, a total of 320 proteins were confidently identified across six healthy horses, and these proteins were further characterized by gene ontology terms mapped in UniProt, and normalized spectral abundance factors were calculated as a measure of relative abundance and these results provide an optimized protocol for analysis of equine CSF and laid the basement for future studies involving the CSF study of equines in the context of pathogenic disease states (Carolyn et al., 2014). The analysis of osteoarthritis and osteochondrosis conducted by Elisabetta et al. (2012) has identified some putative protein markers which can be further tried for the definitive early diagnosis of osteoarthritis in the horses. A highly sensitive proteomic comparison together with insightful data mining enabled us to identify proteins and pathways involved in early OA which could aid the development of early OA diagnostic markers and therapeutics (Peffer et al., 2012). In case of a very unpredictable disease of equines, laminitis identification and measurement of novel protein biomarkers present in blood that predict the onset and resolution of laminitis would both aid clinical management of at-risk equine patients and shed light on underlying mechanisms with the intent of developing novel preventive strategies and therapeutic approaches (Joseph et al., 2008).

#### Conclusion

A profound change has been occurred in recent years in veterinary diagnostics with the introduction of new biotechnological assays which completely changed the

scenario of time-tested, traditional diagnostic techniques of veterinary disease diagnosis. These new biotechnological methods, includes the production of more specific antigens by the use of recombination, expression vectors and synthetic peptides. When 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 a routine diagnostic tool in veterinary laboratories for rapid screening of large number of samples during disease outbreaks to develop prevention and control measures and also to make specific typing determinations for research purpose.

Other technologies are likely to be widely adopted in the future as they demonstrate the ability to improve the diagnostic capabilities while reducing the time and, perhaps cost associated with more conventional technologies. Proteomics has the potential to look at the broader picture of protein expression for a pathogen of interest or by infected animals and it may lead to a special niche of veterinary diagnostics. Nanotechnologies hold the promise of screening numerous pathogens in a single assay, while not yet implemented in veterinary laboratories. Nanotechnology has become the choice for mobile and pen-side testing of animal diseases due to its small size and easy handling. Biotechnology and its applications hold the great promise for improving the speed and accuracy of diagnostic tests for veterinary pathogens. Much developmental work will be required to realise the potential with well-characterised, validated assay systems that provide improved diagnostic capabilities to safeguard animal health.

Traditionally, pathogens were detected by microscopic and other conventional methods of various biological samples. Later on several molecular and serological assays have been employed for diagnostic purpose. These assays are shown highly effective and sensitive results for the detection of parasites regardless of the type of infection and sample. Among the various available techniques, some are used for treatment monitoring along with the diagnosis of parasites. Thus they became a useful tool in the clinical decision making process. The molecular and serological methods are also useful in vast epidemiological studies, because they are also involved in the geographical distribution study of parasites, genetic diversity of populations, susceptibility of infections and mutations in parasites. Detailed knowledge about the genetic characteristics, morphology and behaviour of parasitic disease in the affected population is provided by the molecular tools. Although, the cost of molecular diagnosis is higher than the conventional methods, they are highly used in veterinary clinical diagnosis, epidemiological studies and treatment monitoring of animals. The suitable molecular tests showing rapid, sensitive, accurate and reliable result and which can detect all or most targeted pathogens in a multiplex amplification system should be developed. Moreover, for faster surveillance strategies and monitoring of parasitic epidemiology automated technology should be developed to process the large number of serum samples for antibody

detection. Recently, advanced software tools and the computing power for bioinformatics analysis of parasitic large genome size data is a need of modern molecular diagnosis. The major challenge regarding development of new technologies is to optimize and evaluate the tools for control and eradication programs of parasitic diseases. So it will help in the development of newer technologies to a level of analytical sensitivity which will be appropriate for testing of clinical samples directly without previous processing.

### Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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