Review Article

Application of Molecular and Serological Diagnostics in Veterinary Parasitology

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Abstract | The authors present an update of the molecular and serological techniques used in veterinary parasitology. The application of molecular and serological techniques in veterinary parasitology is increasing worldwide. Several new molecular techniques have been made available for sensitive and specific diagnosis of parasitic diseases. These tests may be either nucleic acid or protein based assays. In nucleic acid based assays, PCR and its variants such as nested PCR, multiplex PCR are the most frequently used tools. The parasitic nucleic acid target sequences are DNA and ribosomal RNA. The DNA sequences provide high specificity for the identification of parasite in biological samples. Real-Time PCR is also increasingly in use since its cost is decreasing. Some other tests such as RAPD, AFLP and RFLP are also used for several parasitic disease diagnoses. Moreover now, complete genome sequencing of many of parasites has been achieved. The complete genome data also provides suitable gene sequence which, when cloned and expressed provides antigens for diagnosis and vaccine preparation. Similarly, protein based serological assays such as ELISA, dot-ELISA, Fluorescence antibody tests have also been used for parasitic diagnosis. However, the use of molecular tools in veterinary parasitology remains restricted to research rather than use in field as diagnostic tool.

Keywords | Parasites, Molecular diagnosis, PCR, RFLP, ELISA

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INTRODUCTION

Biotechnology utilises the cellular and molecular processes to develop products that can improve human and animal lives and health. The modern molecular biology provides us latest technology to combat several diseases. In animal sciences, molecular biology aids in production of transgenic animals, livestock breeding and husbandry, nutrition and better health (García-Sancho, 2015; Ozawa et al., 2015; Pickering et al., 2015). It is also applied for development of innovative diagnostic tools for animal disease control. It allows the livestock rearing at industrial scale through cheaper and reliable means of producing diagnostics and effective vaccines for animal health care management (Abraham and Pal, 2014). Several diagnostic methods using nucleic acid probes, monoclonal antibodies, restriction fragment length polymorphisms have increased the livestock production. These diagnostic methods have also been used for equine and pet animal disease diagnosis and management (Dhama et al., 2013; Dubey et al., 2015; Salim et al., 2014).

Many of animal parasites such as *Toxoplasma gondii, Echinococcus granulosus, Toxocara canis, Trichinella spp.* etc are zoonotic in nature and cause severe disease and induce financial losses to human population (Prasad et al., 2010; Schurer et al., 2013). The international trade of livestock and their products are continuously increasing. However, during movement of live animals and their products, there is possibility of transmission and spread of parasites from one country (or a region) to another. Therefore, OIE (World Organisation for Animal Health) has prescribed certain diagnostic tests for international movement of animals and animal products (Table 1) (OIE, 2013). These are

Table 1: OIE prescribed test for the international trade of animal and its products (OIE, 2013)

Disease	Pathogen	Host affected	Prescribed test(s)
Trichinellosis	*Trichinela* spps.	Human, Rat, Cougars, Pigs and Black bears	Agent identification
Bovine babesiosis	*Babesia bovis* and *Babesia bigemina*	Cattle, Water Buffalo, Deer	Polymerase chain reaction
Theileriosis	*Theileria parva* and *Theileria annulata*	Cattle, African buffalo, Indian water buffalo and Yak	Agent identification, Indirect fluorescent antibody test
Trichomonosis	*Tritrichomonas foetus*	Bovine	Agent identification
Dourine	*Trypanosoma equiperdum*	Equines	Complement fixation test
Equine piroplasmosis	*Theileria equi* and *Babesia caballi*	Horses, Mules, Donkeys and Zebra	ELISA, Indirect fluorescent antibody test

considered as optimal for determining the health status of animals. Thus, prevention of diseases due to these parasites is essential and molecular biology may play a great role in this aspect. The use of molecular tools and knowledge of molecular biology of parasites and their hosts are becoming increasingly important in veterinary parasitology (Tavares et al., 2011). The continuous improvements in molecular and serological tools have played a great role in control of parasitic diseases through developments in antiparasitic drugs and diagnostic reagents. The molecular and serological diagnostic tests are based on identification of either nucleic acid (DNA/RNA) or protein (antigen-antibody). This paper discusses the application of molecular and serological diagnostic in veterinary parasitology.

NUCLEIC ACID BASED ASSAYS

The routine laboratory diagnosis for veterinary parasites involves conventional methods, such as optical microscopy for morphological identification of parasites. However, the conventional methods are not much reliable and have reduced sensitivity and specificity. Moreover, these methods are sometimes not applicable to micro parasites. Therefore, with the advancements in molecular biology techniques, the molecular methods are now more indicated. Several molecular based laboratory techniques (Table 2) such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), loop-mediated isothermal amplification (LAMP), Luminex xMAP-based technology and real-time PCR are used for identification of several parasitc species (Guy et al., 2004; Dunbar, 2006; Quan et al., 2008; Temperley et al., 2009).

Polymerase Chain Reaction (PCR)

PCR utilizes the power of DNA polymerase enzyme to amplify a small number of targeted DNA using specific primers. It can selectively amplify the targeted sequence from a mixture of genomes. PCR acts as a major tool for identification of parasites because it is generally difficult to obtain sufficient amount of parasitic material for conventional assays (Gasser, 2006). PCR can utilize all biological samples such as faeces, blood, skin scrapings, urine and meat for parasitic identification. The detection limit of PCR is higher than conventional method (light microscopy). Therefore, it is useful for detecting low number of parasites in faecal samples (Guy et al., 2004). It can also be used for detection of non-intestinal parasites such as *Leishmania* and *Plasmodium* (Andrade et al., 2005; Antinori et al., 2007). In a recent study, *Toxoplasma gondii* parasite has been genetically characterized using PCR based molecular techniques (Liu et al., 2015). The seven different species of *Eimeria* (*E. acervulina, E. brunetti, E. tenella, E. maxima, E. necatrix, E. mitis and E. Praecox*) have been detected using PCR from Egyptian baladi chickens (Gadelhaq et al., 2015). PCR using cytochrome c oxydase subunit 1 (cox1) gene based identification and subsequently intra-specific variation study was done for *Habronema muscae* in horses in Southern Iran (Rakhshandehroo et al., 2014).

PCR can also be combined with other molecular methods such as RFLP (restriction fragment length polymorphism) or nested PCR to genotype the organisms. In a recent study PCR-RFLP assay was successfully used for identification of *Toxoplasma gondii* (Bacci et al., 2015) and differentiation of *Fasciola hepatica* from *Fasciola gigantica* (Yakhchali et al., 2015). PCR can be modified to multiplex-PCR to detect several pathogens in a single reaction. A multiplex-PCR reaction was validated for amplification of a 340 bp fragment of internal transcribed spacer (ITS-2) of *Fasciola hepatica* rDNA, along with a conserved segment of *Galba cubensis* snail (Alba et al., 2015). Although PCR is much sensitive and specific than conventional methods, it has limitation of being time consuming. It also needs equipment (thermal cycler) and gives qualitative results. Moreover, this technique is still expensive in several countries. In veterinary parasitology, this technique is more used in research purpose.

Real-Time Polymerase Chain Reaction (Real-time PCR or qPCR)

This technique enables the monitoring of PCR amplification in real time along with specificity and sensitivity

Table 2: Comparison of different molecular and serological assays

Molecular assays			
Diagnostic assays	**Advantages**	**Limitations**	**Examples of application in veterinary parasitology***
PCR	Specific and sensitive. Small quantity of starting DNA (just from one cell) can also be amplified. Old or degraded samples can be used	Small amounts of contaminating DNA from other sources can also be amplified. Time consuming method. Qualtitative results.	*Toxoplasma gondii* (Liu et al., 2015), *Eimeria* spp. (Gadelhaq et al., 2015)
Real-time PCR	Simple, fast and automatized amplification system. Low risk of cross-contamination. Quantitative method.	High costs. Technical expertise requirements.	*Sarcocystis* spp. (Moré et al., 2013), *Leishmania* spp. (Ceccarelli et al., 2014)
LAMP assay	Highly sensitive and specific. Can discriminate single nucleotide difference. Rapidly amplify nucleic acid under isothermal condition. No need of thermal cycler. Available for small laboratories and especially suitable for remote areas. No need for post amplification processing. Result can be evaluated in real time through visualization by naked eyes. Easy distinction between positive and negative results.	High sensitivity level contributes the carry over contamination. Partially purified or impure DNA may not allow for amplification Affected by the amplification time.	*Echinococcus multilocularis* (Ni et al., 2014), *Eimeria* spp. (Barkway et al., 2015)
Luminex	Simple, fast, and inexpensive. Not require prior knowledge of DNA sequence. Shortened the result time by liquid bead array approach, with smaller sample requirements. The flexible multiplexing in the range of 1 to 100 analysts facilitates reduced cost and labour of reaction.	Designing of multiplex reactions is a challenge. Needs costly instrument.	*Cryptosporidium* spp. (Bandyopadhyay et al., 2007), *Giardia duodenalis,* (Li et al., 2010)
RAPD	Minimal cost of reaction. No prior knowledge of genome sequence. Amenable to automation of reaction.	Large quantity of pure DNA sample is needed. Low reproducibility between laboratories.	*Taenia solium* (Bobes et al., 2010), *Echinococcus granulosus* (Sharbatkhori et al., 2010)
AFLP	Allows a quick scan of whole genome for polymorphisms. No prior sequence information is needed. Extremely useful in creating quick genetic maps. Can produce highly informative fingerprint of entire parasitic genome. Highly reproducible.	Needs computer for big data analysis. In genetic mapping of organism, AFLPs cluster at the centromeres and telomeres of chromosome.	Differentiation of *C. parvum* into two genotypes, and *Leishmania* to visceral and cutaneous leishmaniasis (Kumar et al., 2010)
Probe-hybridization	Fast, sensitive, specific and direct detection method for parasite. Independent of immunocompetence or host previous clinical history. Parasites do not need to be viable. Can be automated.	Extensive, multistep. Low sensitivity and specificity. May show false-negatives due to PCR inhibitors. May show false positives from carryover contamination.	*Giardia duodenalis* (Weissenböck et al., 2011), *Theleria annulata* (Saravanan et al., 2011)
RFLP	Differentiate closely related parasites. Simple to perform.	Large quantity of purified DNA sample is needed.	*Toxoplasma gondii* (Quan et al., 2008), *Theileria* spp. (Zaeemi et al., 2011)

Microarray	Highly useful in new gene discovery and study of gene expression. Can be used for parasitic disease diagnosis and in drug discovery and toxicology.	Detect only previously known organisms. Lack of standardization (low reproducibility). Inadequate computer based tools for result analysis. Probe length may interfere with results. Relatively costly than other assays.	*Theileria* and *Babesia* spp. (El-Ashker et al., 2015), *Fasciola. hepatica* (Wesołowska et al., 2013), *Giardia duodenalis* (Dreesen et al., 2012)
Genome sequencing	May help in identification and characterization of new parasites. Environmental changes that can mediate the resistance development to chemotherapy in parasites may be identified and moderated. Develop personalized medicine. Identification of genes misregulation and allows selection of best treatment.	Expensive. Needs technical expertise. Needs high computational power and softwares. A lot of generated information is unusable at present (the roles of several genes are still unknown or incompletely understood). Policies and security measures to maintain the privacy and safety of sensitive genomic information (of human) is still a challenging task.	*Dirofilaria immitis* (Godel et al., 2012), *Schistosome* spp. (Berriman et al., 2009)
Serological assays			
Diagnostic assays	**Advantages**	**Limitations**	**Examples of application in veterinary parasitology***
ELISA	Rapid and convenient assay. Antigens of low or unknown concentration can be detected. Used in wide variety of tests of several pathogens. Safe.	Generally monoclonal antibody is used for confirmatory diagnosis which is costlier. Negative controls may indicate positive results if blocking solution is ineffective. Enzyme/substrate reaction is for short duration; hence reading must be taken as soon as possible.	*Trichinella zimbabwensis* (Ludovisi et al., 2013), *Toxoplasma gondii* and *Neospora caninum* (Xu et al., 2012)
FAST-ELISA	Performed with minimal equipment. High sensitivity, short assay time, and ease of operation. Ideal for field studies.	False positive results.	*Fasciola spp.* (Farghaly et al., 2009), *Taenia* spp. (Ko and Ng, 1998)
LIPS	Rapidity and accuracy in detecting pathogens. Low background as compared to ELISA.	No specific limitation.	*Strongyloides stercoralis* (Anderson et al., 2014; Buonfrate et al., 2015)
RIA	Highly specific and sensitivity.	Radiolabeled reagents and produce severe radiation hazards. Requires special laboratory and trained staff to handle radioactive material. Requires special arrangements for storage and disposal of radioactive material.	*Babesia bovis* (Kahl et al., 1982), *Trypanosoma congolense* (Mutayoba et al., 1994)
IFAT	Highly sensitive and specific. Used on pathogens that can't be easily cultured. Allows viewing of labelled cells in natural environment. Allows visualization of multiple cell types in one sample simultaneously.	Cross reactions with other antigens. Must run simultaneous control to assure no false positives or negatives.	*Babesia bovis* and *Babesia bigemina* (Terkawi et al., 2012), *Babesia* and *Theileria* spp. (Nayel et al., 2012)

CFT	Screen large number of samples at a time. Can be automated with relatively simple and inexpensive equipments. Shows increased specificity with reproducible result.	Not much sensitive and cannot be used for immunity screening. Time consuming and labour intensive assay. Non-specific binding of complement may produce false positive results.	*Trypanosoma evansi* (Cauchard et al., 2014), *Trypanosoma equiperdum* (OIE, 2013; Luciani et al., 2013)
LFT	Cheaper and easy to prepare. Stable over a wide range of environmental conditions and very long shelf life. Requires small sample volume with simple and user friendly operation. Takes few minutes. High potential of commercialization.	Mostly qualitative or semi-quantitative. Reproducibility varies from batch to batch. Cross reactivity cannot be ruled out. Once sample is applied to the strip, capillary action cannot be increased or decreased. Analysis time is also dependent on nature of sample i.e. viscosity, surface tension.	*Leishmania infantum* (Castellanos-Gonzalez et al., 2015), *Echinococcus granulosus* (Khalilpour et al., 2014)

* The examples are so numerous; we are presenting here just some relevant and recent examples.

of original PCR assay. The Real-time PCR assay provides quantification of the sample using several fluorescent agents such as TaqMan probes, SYBR Green dye and Scorpion primers (Ndao, 2009). The parasitic nucleic acids from various biological and environmental samples can be quantified to provide information about the intensity of infection. The SYBR Green dye based Real-time PCR assays have been validated for several parasitic species such as *Cryptosporidium*, *Trypanosoma*, *Leishmania* and *Mecistocirrus digitatus* (Gasser, 2006; Lalonde and Gajadhar, 2011; Subhadra et al., 2013; Ceccarelli et al., 2015). The gastrointestinal infection of nematode in small ruminants was successfully detected using Real-time PCR (Roeber et al., 2015). A Real-time PCR assay has been developed for *Toxoplasma gondii* quantification (Lin et al., 2000). Real-time PCR assay can easily detect differential gene expression due to different strains of parasites in tissues. Genetically distinct *T. gondii* strains in mice reveal clear differences in modulation of host immunological and pathophysiological response in peripheral lymphocytes and brain tissue in a strain specific manner (Jia et al., 2013). The Real-Time PCR is rapid, specific, sensitive and quantitative technique to detect parasite in clinical samples. Different studies of leishmaniosis such as animal models, vectorial capacity, diagnosis, drug efficacy have been investigated using Real-time PCR (Talmi-Frank et al., 2010). The treatment of *Leishmania* infected dogs using meglumine antimoniate and allopurinol was monitored using quantitative PCR (Manna et al., 2008). A new version of real-time PCR i.e. multiplex real-time PCR was used for identification of *Sarcocystis spp*. in cattle in a single reaction (Moré et al., 2013). Although Real-time PCR assay has shown excellent specificity and sensitivity, the introduction of this method in routine laboratory diagnosis especially in rural endemic areas is still uncommon. The Real-time PCR amplification protocols, DNA extraction, choice of primer sets may cause diversification in results and lead to difficult in standardization of assay (Bretagne and Costa, 2006).

Loop-Mediated Isothermal Amplification (LAMP)

LAMP is an extremely sensitive and specific method of nucleic acid amplification which can discriminate single nucleotide differences. In LAMP assay, six different sequences of target genes are identified by a set of four primers (Figure 1). The DNA polymerase used (*Bst* DNA polymerase) in LAMP assay can work under isothermal condition with high specificity and low sensitivity to inhibitors in sample (Paris et al., 2007). It amplifies the DNA only when all primers bind to targeted DNA. The results can be seen directly by adding SYBR green, HNB or Calcein dye. Therefore, electrophoresis is not required which also reduces the time for interpretation of result (Njiru, 2012). This technique can synthesize 20 µg of DNA from 25 µL of reaction volume within one hour of time (Mori et al., 2001). Moreover, a novel OmniAmp DNA polymerase has been validated for LAMP assay of DNA and RNA both (Chander et al., 2014). Earlier, this technique was used for diagnosis of many human and animal viral and bacterial pathogens. However, now this method is also validated for many parasites such as *Plasmodium spp*., *Cryptosporidium spp*., *Taenia spp*., *Trypanosoma spp*., *Fasciola hepatica*, *Schistosoma spp*., *Toxoplasma gondii*, *Theileria* spp., *Echinococcus spp* and *Babesia spp*. (Paris et al., 2007; Nkouawa et al., 2010; Kumagai et al., 2010; Abbasi et al., 2010; Ai et al., 2010; Lau et al., 2010; Wang et al., 2010; Ni et al., 2014). It can also be used for identification of parasites in their vectors. *Plasmodium spp*. and *Dirofilaria immitis* have been detected in mosquitos using this technique (Aonuma et al., 2009). Also, it could detect the par

asites in intermediate host of *Schistosoma* i.e., miracidium can be detected after first day of snails' exposure (Abbasi et al., 2010). In a recent study, LAMP assay was used for diagnosis of seven chicken *Eimeria* species (Barkway et al., 2015). The LAMP assay is ten times more sensitive than conventional PCR in identification of *Fasciola spp*. in snails and stool samples. The multiplex LAMP (mLAMP) assay was standardized for simultaneous identification of bovine protozoan parasites i.e., *Babesia bovis* and *Babesia bigemina* (Iseki et al., 2007). Although LAMP is simple and low cost DNA amplification technique, it has some limitations. The LAMP assay product is not easily degraded hence, chance of carry over contamination may exist (Bai et al., 2011). The impure or partially purified DNA as template may not amplify properly (Deguo et al., 2008). It is also affected by the amplification time limit. The LAMP products usually take 60 minute to 120 minute for amplification and negative control may get amplified at 180 minute (Francois et al., 2011). LAMP is also less versatile than PCR because it is not useful for cloning reactions. Moreover, the multiplexing assays using LAMP are less developed than PCR.

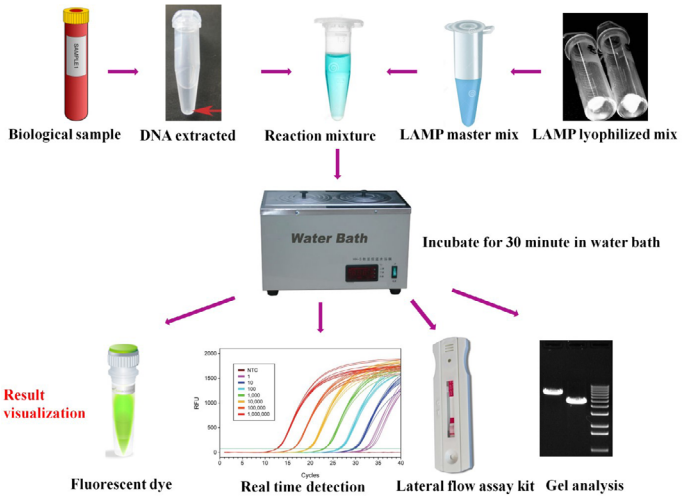

Figure 1: Principle of LAMP assay

Luminex

It is a bead-based xMAP (multianalyte profiling) technology which combines fluorescent microspheres (beads), flow cytometry, lasers and digital signal processing technology (Figure 2). It has the capability of simultaneous analysis of 100 different analytes in a single tube (Luminex, 2010). The 100 distinct sets of Luminex tiny beads (microspheres) coated with specific reagent of particular bioassay allow the detection of specific analytes in a sample. In Luminex compact analyser, lasers excite the internal dyes and identify each microsphere beads. To validate the result several readings are taken on each bead set. Thus bead based xMAP assay allow rapid and precise multiplexing of up to 100 unique assays within a single reaction. The several DNA assays for Luminex platform have been developed for identification of fungi, viruses and bacteria such as *Candida* spp., *Mycobacterium spp.*, *Escherichia coli*, *Salmonella* spp., *Campylobacter jejuni*, *Trichosporon spp.*, *Listeria monocytogenes* (Cowan et al., 2004; Diaz and Fell, 2004; Das et al., 2006). This technique was used for study of *Cryptosporidium spp*. (Bandyopadhyay et al., 2007). Although *Cryptosporidium hominis* and *C. parvum* are very similar, they can be differentiated by a single nucleotide in microsatellite region-2 (ML-2) by Luminex assay. This difference can also be easily detected by DNA sequencing. However, sequencing is time-consuming, laborious and expensive. Luminex assay is more sensitive and specific than direct immunofluorescence (DFA) in diagnosis of *Cryptosporidium* and *Giardia* species. Moreover, DFA cannot differentiate *C. hominis* and *C. parvum* (Bandyopadhyay et al., 2007). The Luminex assay can also be allied with nested PCR to increase sensitivity and specificity for the diagnosis of *Giardia duodenalis*, *Cryptosporidium* spp. and *C. parvum* (Li et al., 2010). Although the Luminex assay shows several advantages such as shortened time to result, reduced cost and labour by multiplexing up to 100 analytes in a single reaction, it has certain limitation of designing multiplex PCR reactions and cost and requirement of Instrument.

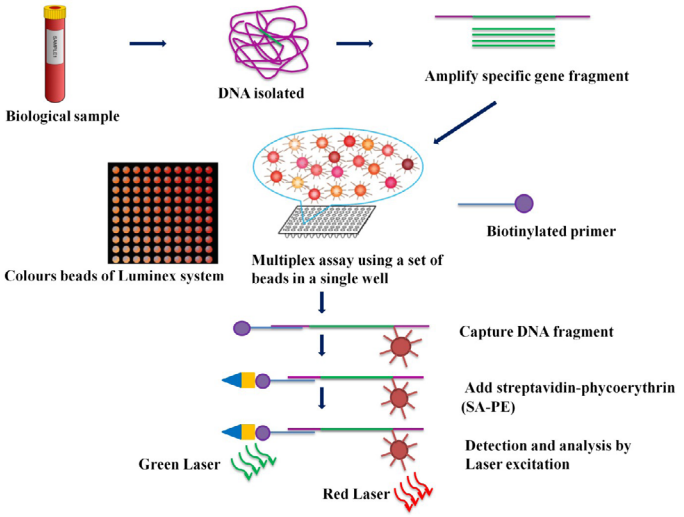

Figure 2: Principle of Luminex assay

Random Amplified Polymorphic DNA (RAPD)

It is a PCR reaction which amplifies random segments of DNA. It uses several arbitrary, short primers of 8-12 nucleotides in length and a large template of DNA for PCR amplification. By resolving the resulting amplification patterns on agarose gel electrophoresis a semi unique profile can be visualized from a RAPD reaction. Several studies on nematodes of human and plant origin have showed the high efficiency of RAPD marker in identification and differentiation of parasites at species level (Martinez et al., 2003). RAPD is also useful for study of genetic differences in population of microorganism because it reveals polymorphism in non-coding region of genome (Jain et al., 2010). It is successfully used for differentiation of *Leishmania*, *Plasmodium* and *Trypanosoma spp*. (Hajjaran et al.,

2004; Alimoradi et al., 2009).

RAPD is increasingly used in parasitology due to its fast, simple and inexpensive nature since it does not require prior knowledge of the DNA sequence or DNA hybridization (Alimoradi et al., 2009). This technique was used for differentiation of endemic strains of *Wuchereria spp.* in south East Asian countries (Nuchprayoon et al., 2007) and *Leishmania* in Sudan (Hamad et al., 2010). In Mexico, RAPD marker was used for genetic diversity study of *Taenia solium* in pigs (Bobes et al., 2010). In Iran, it was used for *Echinococcus granulosus* genotyping of cattle, sheep, goats and camel origin (Sharbatkhori et al., 2010). Although RAPD is easy to perform, it is notoriously PCR dependent assay and needs specific PCR protocol to give reproducible result. The primer and template mismatch may also result in total absence of PCR product and make difficult to interpret RAPD results.

Amplified Fragment Length Polymorphism (AFLP)

It is a technique which allows detection of DNA polymorphism without the knowledge of actual DNA sequence. This technique utilizes the PCR to selectively amplify the restriction fragments of digested genomic DNA (Savelkoul et al., 1999). The entire analysis completes in four steps: restriction endonuclease digestion of DNA, ligation, amplification and polyacrylamide gel analysis. The presence or not of DNA fragments in gel reveals the polymorphisms. This method is highly advantageous because of its reproducibility, its ability to search entire genome for polymorphisms and possibility of being used against parasites without any prior genetic information (Bonin et al., 2007; Buttow et al., 2010). AFLP has been used for differentiation of *C. parvum* into two genotypes, and *Leishmania* to visceral (VL) and cutaneous leishmaniasis (CL) (Kumar et al., 2010). However, AFLP has some of the limitations such as it is difficult to develop locus specific markers from individual genome fragments of a species.

Probe Hybridization

The hybridization probe is a 100-1000 bases long fragment of DNA or RNA used for detection of presence of nucleotide sequences complementary to probe sequence. The probe hybridizes to single-stranded nucleic acid sequence (Wetmur, 1991). The nucleotide sequence of probe allows probe-target base pairing due to nucleotide base complementarity between the target and probe (Figure 3). The labelled probe is hybridized to the target RNA (Northern blotting) or ssDNA (Southern blotting) immobilized on a membrane or hybridised *in situ*. To detect hybridization, probe is tagged with a molecular marker of either radioactive (P^{32}, I^{125} etc.) molecules or non-radioactive fluorescent molecules (Digoxigenin). The probe hybridization based assays have been used for diagnosis of animal parasites. The *in-situ* hybridization assay for detection of *Giardia duodenalis* trophozoites in intestinal canine, feline and porcine samples were developed (Weissenböck et al., 2011). A dot-blot based probe hybridization assay for detection of *T. annulata* was developed in India (Saravanan et al., 2011). The probe hybridization can also be used for disease surveillance in vector population. It was used for detection of as little as 10 pg genomic DNA of third stage larva (L3) of *Dirofilaria immitis* from mosquito vector (Watts et al., 1999). The probe hybridization assay is relatively easy to perform. However, it has creation limitations such as it is difficult to differentiate between species when using 16S rRNA sequences due to similarity. Moreover, nucleic acid sequence data base is constantly becoming larger thus the possibility of a random hybridization between a specifically designed probe and an unknown target organism cannot be ruled out.

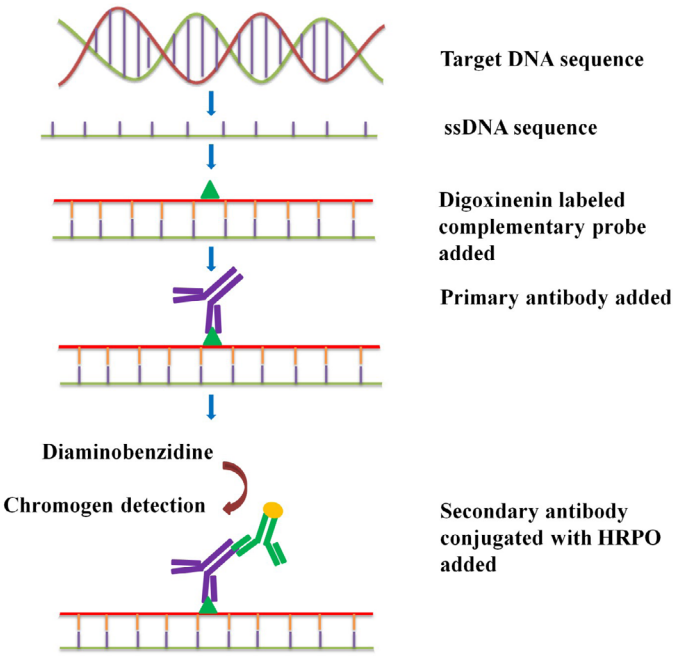

Figure 3: Principle of probe hybridization assay

Restriction Fragment Length Polymorphism (RFLP)

This technique utilizes the specific restriction enzyme to digest the genomic DNA sequence in small fragments followed by separation on gel electrophoresis. The gel electrophoresis showed different patterns of nucleic acid fragment which help in identification of parasite species or genotype. It allows the detection of multiple genotypes in a single reaction. The RFLP is a widely used technique for *Toxoplasma gondii* genotyping (Quan et al., 2008). RFLP analysis was used for characterization of several species of *Cryptosporidium* such as *C. hominis, C. parvum, C. meleagridis, C. canis* and *Cryptosporidium* of rabbit and cervid origin isolated from human paediatric patients (Molloy et al., 2010). It was also used for differentiation of *Theileria lestoquardi, T. ovis,* and *T. annulata* in sheep (Zaeemi et al.,

2011). Despite its many benefits, the RFLP analysis is slow and tedious process than the newer DNA analysis techniques. Moreover, it requires relatively larger sample size than other forms of nucleic acid analysis techniques.

Microarray

The nucleic acid microarray is a collection of microscopic nucleic acid spots attached to a solid glass surface (microscope slide). Each spot consists of picomoles of specific nucleic acid sequence called probes (Bumgarner, 2013). The probes are allowed to hybridize labelled target nucleic acid i.e., cDNA or cRNA (anti-sense RNA). Probe-target hybridization can be detected and quantified using silver, fluorophore or chemiluminescence-labeled targets (Figure 4). This technique is used to measure expression levels of several genes of same or different species simultaneously. Microarray has also been used for diagnosis of animal parasites. DNA microarrays along with PCR and gene sequence analysis technique have been used for molecular identification of *Theileria* and *Babesia* in cattle in Egypt (El-Ashker et al., 2015). Microarray analysis can also be used to monitor the efficacy of vaccines in immunized animals. It was used to monitor the level of vaccine efficacy against *F. hepatica* parasite by monitoring the expression of cytokines levels in immunized and challenged rats (Wesołowska et al., 2013). Microarray can also be used to study the host-parasite interactions. The host-parasite relationship of *Giardia duodenalis* in calf's small intestine was studied using high density oligo microarray system using global gene expression in small intestine after infection by *Giardia duodenalis* (Dreesen et al., 2012). The DNA microarray hybridization assay can also be used for screening of samples for specific single nucleotide polymorphisms (SNPs). Although, Microarray is an effective technique for

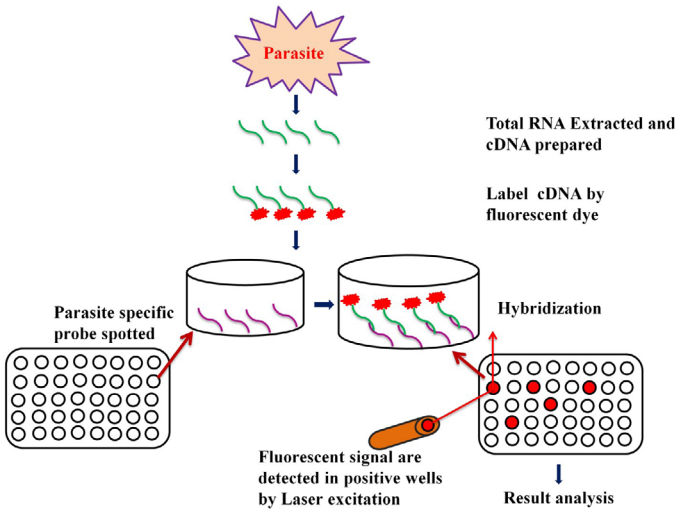

Figure 4: Principle of Microarrays assay

diagnosis of several pathogens, it is still a costly method and moreover, the complete genome based microarray chip is not available for many of the animal parasites.

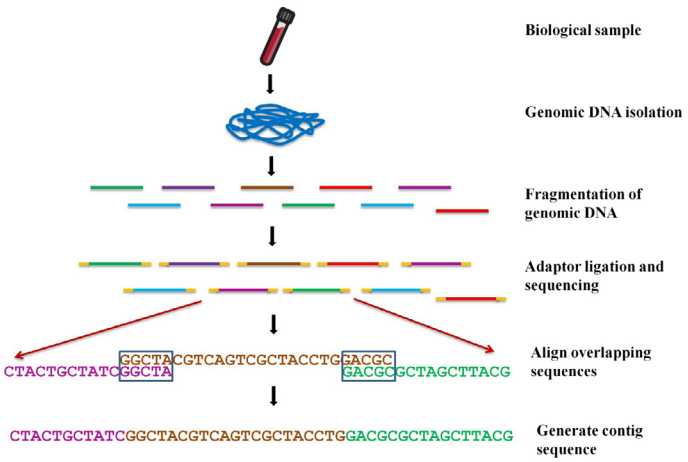

Figure 5: Basic principle of next generation sequencing technologies

GENOME SEQUENCE STUDY OF ANIMAL PARASITES

The veterinary parasitology have utilizes the traditional immunological and empirical drug screening methods for development of vaccines, diagnostic tools and drugs. However, these conventional approaches have some limitations such as inadequate efficacy of vaccines, cross reactive of parasitic diagnosis methods and drug resistance of parasites (Sutherland and Leathwick, 2011; Sharma et al., 2015). The drug resistance against many well-known antiparasitic drugs threatened the livestock production in several parts of the world (Sutherland and Leathwick, 2011). In the last few years, the gene sequencing and complete genome sequencing is becoming popular in identifying the new pathogens and its potential target genes. In complete genome sequencing the whole genomic DNA is cut in smaller fragments, sequence the fragments individually and then assemble the fragmented sequence to get whole genome sequences (Figure 5). There is several complete genome sequencing platforms have been developed in recent past (Box 1). The whole genomes of several human and animal parasites were sequenced (Table 3). The human *Schistosoma* genome project (Berriman et al., 2009) may provide useful information for research on parasites such as *Leishmania, Plasmodium* and *Fasciola hepatica.* These three species can contaminate humans. However, *Plasmodium* in animals is not very important (except monkeys); they are studies in the frame of comparative pathology. Similarly, the genome sequence of heart worm of dog (*Dirofilaria immitis*) provides new insight in the molecular diagnosis tools for veterinary parasites (Godel et al., 2012). The whole genome sequencing is highly essential because it will provide the necessary information for subsequent identification and functional analyses of the newly discovered genes. Moreover, the amino acid sequence and subsequently protein

Table 3: Complete genome sequencing of animal and human parasites

Parasites	Relevant host	Organisation involved in genome sequencing	Year of completion of project
Giardia intestinalis	Humans	Multicenter collaboration	2009
Toxoplasma gondii	Mammals and birds	J. Craig Venter Inst., TIGR, UPenn.	2008
Eimeria tenella	Fowl	The Wellcome Trust Sanger Institute	2007
Giardia lamblia	Humans	Karolinska Institutet, Marine Biological Laboratory	2007
Leishmania braziliensis	Humans	Sanger Institute, Universidade de São Paulo, Imperial College	2007
Leishmania infantum	Humans	Sanger Institute, Imperial College and University of Glasgow	2007
Trichomonas vaginalis	Humans	TIGR	2007
Babesia bovis	Cattle	-	2007
Entamoeba histolytica	Humans	TIGR, Sanger Institute and the London School of Hygiene and Tropical Medicine	2005
Theileria annulata	Cattle	Wellcome Trust Sanger Institute	2005
Theileria parva	Cattle	TIGR and the International Livestock Research Institute	2005
Trypanosoma brucei	Humans	Sanger Institute and TIGR	2005
Trypanosoma cruzi	Humans	TIGR, Seattle Biomedical Research Institute and Uppsala University	2005
Cryptosporidium hominis	Humans	Virginia Commonwealth University	2004
Cryptosporidium parvum	Humans	UCSF and University of Minnesota	2004
Neospora caninum	Cattle, sheep, goats, horses and dog	Wellcome Trust Sanger Institute	-
Plasmodium falciparum	Humans	Malaria Genome Project Consortium	2002
Plasmodium yoelii yoelii	Rodents	TIGR and NMRC	2002
Ascaris suum	Pigs	University of Edinburgh/Sanger Centre	2000
Haemonchus contortus	Sheep, cattle, goats, deer, camels	University of Edinburgh/Sanger Centre	2000
Trichinella spiralis	Mammals	University of Edinburgh/Sanger Centre	2000

structure responsible for host specificity, pathogenesis, virulence, required for parasite survival in host and environment may be identified and targeted.

Mitochondrial genomes of *Eimeria* species are highly conserved. However, the three CDS region (COI, COIII and CytB) in mitochondrial genome, possess sufficient sequence variability for differentiating poultry *Eimeria* species. Thus the mitochondrial CDS regions of *Eimeria* species make it highly suitable for use in diagnostic assays as well as potential genetic marker for molecular epidemiology and phylogenetic study (Ogedengbe et al., 2014). The genome sequence information is readily used for BLAST (Basic Local Alignment Search Tool) search of NCBI (National Centre of Biotechnological Information) (Box 2) and phylogenetic analyses (Box 3) which indicate the origin of animal parasite and may also provide information about its zoonotic migration and adaption to humans. The genomic basis of adaptation of *Plasmodium falciparum* to human hosts was studied using complete genome sequencing of closely related chimpanzee parasite *Plasmodium reichenowi*. The genome organisation of most virulence associated multigene families such as hyper variable *var* genes, is broadly conserved in these two species (Otto et al., 2014). However, rif and *stevor* genes were found in smaller subset in *Plasmodium falciparum* whose products are expressed on erythrocyte surface of infected hosts (Otto et al., 2014). The complete genome sequence study of animal parasites is a need for modern nucleic acid based diagnostic assays. However, complete genome sequence study of only few animal parasites have been studied so far due to its costly nature and technical expertise required.

PROTEIN BASED ASSAYS

These assays are categorised into antibody and antigen detection assays. There are several protein based assays such as enzyme-linked immunosorbent assay (ELISA), dot-ELISA, Falcon Assay Screening Test ELISA (FAST-ELISA), indirect or direct immunofluorescent antibody (IFA or DFA) tests, immunoblotting, complement fixation test (CFT) are used for parasitic disease diagnosis (Table 2). These tests are more sensitive and specific than microscopy and allow post-therapeutic parasitic clearance.

Box 1: Next-Generation DNA Sequencing Technology

Next-generation sequencing (NGS) technology is also known as high-throughput sequencing used for combined description of several modern sequencing technologies such as: Roche 454 sequencing, Illumina (Solexa) sequencing and Ion torrent: Proton / PGM sequencing etc. These technologies have started a new era for molecular study of parasites by sequencing nucleic acid much more quickly and cheaply than Sanger sequencing. In traditional Sanger sequencing single DNA fragment of 700-800bp is sequenced individually. Whereas, in NGS, a large number of short reads of DNA are sequenced in a single stroke. The input DNA sample is cleaved into short fragments (reads) using either ultrasonication or enzymatic degradation method. The length of fragments usually depends on biochemistry of particular sequencing technology used.

Roche 454 sequencing

The Roche 454 sequencing technology can sequence up to 1kb of DNA or RNA read length. The reads are allowed to join with adaptors to the ends and annealed to beads as one fragment per bead. Then PCR is used for amplification of fragments using adaptor specific primers by PCR. Thus each bead will be covered with several PCR copies of single fragment. The beads are then placed in well of a slide containing DNA polymerase and sequencing buffers in such a way that each well will contain a single bead. Finally one of the four NTPs is poured on the slide. The nucleotide is added to the sequence read. If the nucleotide base repeats, then more number of nucleotide will be added. The addition of base releases a light signal which is detected to identify the beads on which nucleotide is added. This NTP mix is washed away and the process repeated with next set of NTPs and signal is detected. The signal density in wells determined the nucleotide sequence.

Illumina sequencing

The 100-150bp fragments (reads) are ligated to adaptors and annealed to a slide having complementary strands of adaptors. Then PCR is used for amplification of each read to generate many copies of the same read and finally the reads are separated into single strands for sequencing. In sequencing mixture, fluorescently labelled nucleotides with colour corresponding to nucleotide base and DNA polymerase are used. The sequencing mixture also has a terminator, which allows addition of one base at a time. The fluorescent signal generated at each read location is captured which indicate the addition of nucleotide base. The same process is repeated by adding one nucleotide at a time and fluorescent signal is captured every time. Finally the computers used to construct a sequence according to fluorescent signals captured at each nucleotide base.

Ion Torrent: Proton / PGM sequencing

The Ion torrent sequencing is based on principle that the addition of dNTP to a DNA strand releases an H^+ ion. Like other NGS technologies the DNA segment is fragmented in ~200bp size. Adaptors are ligated to fragment and amplified by emulsion PCR by putting one fragment onto a single bead. The beads are then placed into a single well of slide and flooded with a single species of dNTP at a time, along with buffers and polymerase. The addition of nucleotide will release the H^+. The release of H^+ will reduces the pH. The pH changes will determine the addition of number and type of nucleotide bases. The dNTPs are washed away, and the same process is repeated through remaining dNTPs types. The change in pH will determine how many nucleotide bases were added with each cycle.

Box 2: BLAST (Basic Local Alignment Search Tool)

BLAST is an algorithm for comparing biological sequences of nucleotides and amino acid. It is one of the most reliable bioinformatics tool for sequence search and comparison. The BLAST algorithm was developed by Altschul et al. (1990). It is online available on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Zhang et al., 2000). It utilises a much faster heuristic method for sequence search. BLAST finds similar sequences by determining short sequence match between the two sequences rather than comparing entire sequence. This process of finding initial match is called seeding which leads to local alignments by BLAST. In BLAST analysis the new or unknown sequence (query sequence) is searched against target sequence in database. Usually, the query sequence is much smaller in size than the database. The input sequence format in BLAST is either in FASTA, GenBank or weight matrix and output format can be in a variety of formats such as HTML, plain text, and XML. However, NCBI offers default output format as HTML. The BLAST output result of NCBI shows graphical format showing hits found and in tabular format sequence identifiers such as total score, max score, E value, query cover, percent identity and alignment sequence accession number. There are several formats of BLAST are available on NCBI web page such as BLASTn (Nucleotide-nucleotide BLAST), BLASTp (Protein-protein BLAST), PSI-BLAST (Position-Specific Iterative BLAST), BLASTx (Nucleotide 6 frame translation-protein), tBLASTx (Nucleotide 6-frame translation-nucleotide 6-frame translation), tBLASTn (Protein-nucleotide 6-frame translation) and megaBLAST (Large numbers of query sequences) for different purposes. The BLASTn and BLASTp utilize the direct comparisons of nucleotide and protein sequences without translations. Hence they are most commonly used in bioinformatics calculation. However, for study of coding DNA sequences tBLASTn, tBLASTx and BLASTx produces more accurate and reliable result because protein sequences are more conserved than nucleotide sequences evolutionarily. The BLAST programme is used for several purposes such as identifying species, establishing phylogeny, locating domains, DNA mapping and comparison etc. BLAST is also used as a part of several other algorithms which require sequence matching such as Bioedit, Mega etc.

Enzyme-linked immunosorbent assay (ELISA)

ELISA test uses components of the immune system to detect immune responses against pathogens. The ELISA test involves antigen, antibody and an enzyme to detect specific immune response. The antigens are attached to polystyrene microtiter plate surface and a specific antibody is applied over the surface to bind the antigen. The antibody is conjugated with an enzyme (HRPO- Horseradish peroxidase). Finally a substrate is added to produce a detectable colour change in reaction mixture. Based on use or not of a secondary antibody, the ELISA test may be either direct or indirect (Figure 6). This test is successfully used for diag

Box 3: Phylogenetic tree

A phylogenetic tree is a branching diagram which shows the evolutionary relationship of different biological taxa. All the taxa joined together in a tree construction have descended from a common ancestor. Phylogenetic trees are constructed using several computational phylogenetic methods. The ClustalW programme (sequence alignment method) creates phylogenetic tree using distance based simpler algorithm. Distance-matrix based methods such as neighbour-joining or UPGMA calculate genetic distances between sequences by multiple sequence alignments. They are simple to implement, but do not reveal correctly the evolutionary model. Other simple method is maximum parsimony which implies an inherent evolutionary model called parsimony. The other advanced methods use Bayesian Framework for optimality criterion of maximum likelihood between sequences and apply an explicit model of evolution for phylogenetic tree calculation. Phylogenetic trees are constructed with basic assumption that characteristics of an organisms changes in lineages over time, organisms are related with their descent through a common ancestor and branching or bifurcating of lineage-splitting occur over time. Phylogenetic trees are of several types such as rooted, unrooted and bifurcating tree.

Rooted tree

It is a tree with unique node which shows most recent common ancestor of different taxa of the tree. The edge lengths in some trees usually interpreted as evolutionary time estimates. Each node is known as taxonomic unit. The internal nodes are usually known as hypothetical taxonomic units because they cannot be directly observed. Rooted trees are useful in various areas of biological sciences such as study of systematic and comparative phylogenetic and bioinformatics.

Unrooted tree

The unrooted tree reveals the relationship of leaf nodes without showing information about their ancestry. It does not require the ancestral root for tree construction. They can be generated from rooted trees by simply omitting the root. The inferring the root of an unrooted tree needs some means of identifying ancestry of taxa. This is usually done by introduction of outgroup in input data. This leads to the root in between outgroup and the rest of the taxa of the tree (Maher, 2002).

Bifurcating tree

Both rooted as well as unrooted trees may be either bifurcating or multifurcating in nature. They may be either labelled or unlabeled. The rooted bifurcating tree has two descendants arising from each interior node whereas; the unrooted bifurcating tree appear as unrooted binary tree i.e., a free tree having three neighbours at each internal node. However, rooted multifurcating tree have more than two taxa at some nodes and unrooted multifurcating tree consists of more than three neighbours at the some nodes. The labelled tree shows specific values to its leaves, whereas an unlabeled tree defines only topology.

Special tree types

Apart from above trees certain other tree types are also used for phylogenetic analysis.

Cladogram

It only represents a branching pattern. Its branch spans do not show information about time or relative amount of character change taken place.

Phylogram

It is a phylogenetic tree where branch spans are proportional to the amount of character change taken place.

Dendrogram

It is a term usually used for representation of a phylogenetic tree in diagrammatic format.

Limitations

The phylogenetic trees may not accurately represent the evolutionary history of gene or species. In fact, they show only a scientific hypothesis for further study by other improved methods. In most of the time very large number of taxa with huge sequence data is used for tree construction. Thus suitable algorithm with huge computing power is a prerequisite.

nosis of several parasitic diseases in both humans and animals. The humoral immune response against *Trichinella zimbabwensis* in Nile crocodiles were detected using indirect ELISA tests (Ludovisi et al., 2013). Similarly, specific ELISA assays have been developed for diagnosing *Cyathostomum catinatum, Anoplocephala perfoliata* and *Strongylus vulgaris* in horses (Andersen et al., 2013). The indirect-ELISA test showed high seroprevalence of 13.71% and 15.07% for *Toxoplasma gondii* and *Neospora caninum* respectively in dairy cows in China (Xu et al., 2012). ELISA can also be used for disease surveillance. The high prevalence rate of *Trypanosoma brucei* in pigs was detected by a monoclonal antibody based antigen-ELISA (Ogunsanmi et al., 2000). There are several types of ELISA test such as dot-ELISA, FAST-ELISA etc, which are increasingly in use in veterinary parasitology.

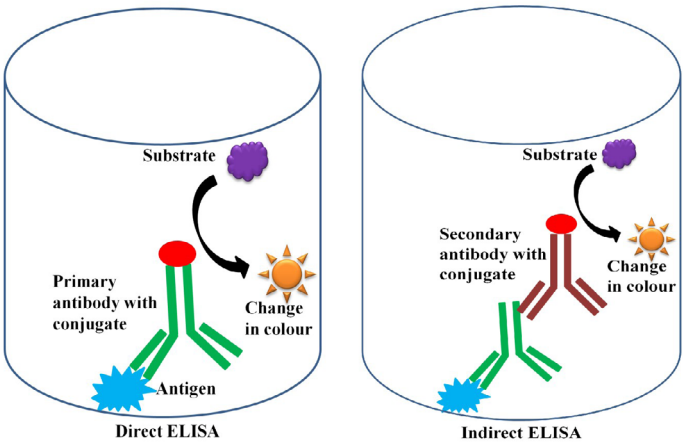

Figure 6: Principle of ELISA test

Dot-ELISA

In dot-ELISA, a nitrocellulose membrane is used for attachment of small amount of antigen. The dotted membrane is incubated in antigen specific antibody followed by enzyme conjugated anti-antibody. Finally a chromogenic substrate is added which causes precipitation of a visible coloured dot on the membrane (Svobodova et al., 2013). This technique is fast cost-effective and easily interpretive. Dot-ELISA has been extensively used in diagnosis of several animal and human parasitic diseases such as helminths: schistosomiasis, trichinosis, fascioliasis, cysticercosis, echinococcosis, toxocariasis, protozoan: trypanosomiasis, toxoplasmosis, amebiasis, babesiosis, cutaneous and visceral leishmaniasis etc. (Pappas, 1988). The Dot-ELISA has also been used for detection of *Haemonchus contortus* (Prasad et al., 2008), *Theileria equi* (Kumar et al., 2008), Fas*ciola gigantica* (Kumar et al., 2008), *Trypanosoma brucei* (Courtioux et al., 2005) and *Trypanosoma cruzi* (Carrasco et al., 2005). In recent past years several studies have demonstrated the use of dot-ELISA for detection of animal parasites such as *Fasciola gigantica* (Kumar et al., 2008), *Haemonchus contortus* (Prasad et al., 2008), *Neospora caninum* (Ahmad et al., 2011). The dot-ELISA has greater sensitivity and specificity. Finally, the use of the nitrocellulose membrane makes this assay applicable in the field.

Falcon Assay Screening Test-ELISA (FAST-ELISA)

FAST-ELISA uses synthetic and recombinant peptides to detect antibody responses to antigens (Hancock and Tsang, 1986). This method was used for diagnosis of fasciolosis (Hillyer et al., 1992; Farghaly et al., 2009), schistosomiasis (Maddison, 1987) and taeniasis (Ko and Ng, 1998). However, this technique has some drawbacks as other antigen-antibody based assays. The recombinant LiHsp83 protein based diagnosis of canine serum sample using FAST-ELISA showed ninety percent of sample as seropositive for *Leishmania infantum*. The assay indicated the Hsp83 protein as potent immunogen for diagnosis of canine leishmaniasis (Angel et al., 1996). Antibodies raised against a parasite protein may cross react with proteins of several other parasites (Angel et al., 1996). Therefore this assay is not much in use for diagnosis of parasites.

Luciferase Immunoprecipitation System (LIPS)

It is a modified ELISA-based assay. The specific antibodies in serum are identified by light production (Burbelo et al., 2005). The antigen of choice is fused with an enzyme reporter Renilla luciferase (Ruc) which is expressed as a Ruc-fusion in mammalian cells (Figure 7). The Ruc-fusion protein extract is incubated with protein A/G beads and test serum. The Ruc-antigen fusion along with antibody complex immobilizes on the A/G beads. The antibody can be quantitated by measurement of light production after addition of coelenterazine substrate (Burbelo et al., 2005). This method is rapid and accurate in detection of pathogen. It produces low backgrounds as compared to ELISA which enhances the accuracy of separation between positive and negative samples (increases sensitivity and specificity). The LIPS assay has been successfully used for diagnosis of *Strongyloides stercoralis* using recombinant antigen from infected sera samples (Anderson et al., 2014; Buonfrate et al., 2015).

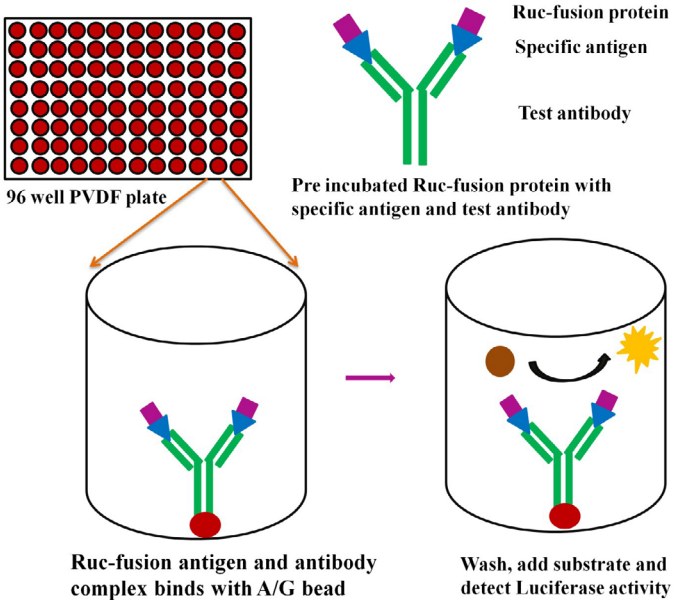

Figure 7: Principle of Luciferase immunoprecipitation system (LIPS)

Radioimmuno Assay (RIA)

RIA is used for detection and quantitation of antigen in a sample using specific antibodies. It is very sensitive and specific. It can detect as low as few picograms of antigen using antibodies of high affinity. This assay is based on competitive binding, where a radioactive antigen competes with a non-radioactive antigen for a fixed number binding sites on antibody (Figure 8). The RIA has been used for diagnosis of parasitic diseases of human and animals. A solid phase radioimmunoassay was developed for detection of *Plasmodium falciparum* antigen in blood sera (Mackey et al., 1980) and *Babesia bovis* in infected bovine RBC (Kahl et al., 1982). The RIA assay demonstrated that *Trypanosoma congolense* infection in rams lead to reduced secretion of testosterone (Mutayoba et al., 1994). However, RIA is not much preferred because of its obvious drawbacks such as radiation hazard for laboratory workers and difficulty in disposal of radioactive waste material.

Fluorescent Antibody Test (FAT)

In FAT, antibody is tagged by fluorescent dye which is used for visualization of antigen in clinical specimens. The immunoglobulin conjugated with fluorescent dye in antigen-antibody complex produce visible glow when examined under a fluorescent microscope. The fluorescent dye can be tagged directly with primary antibody (direct flu

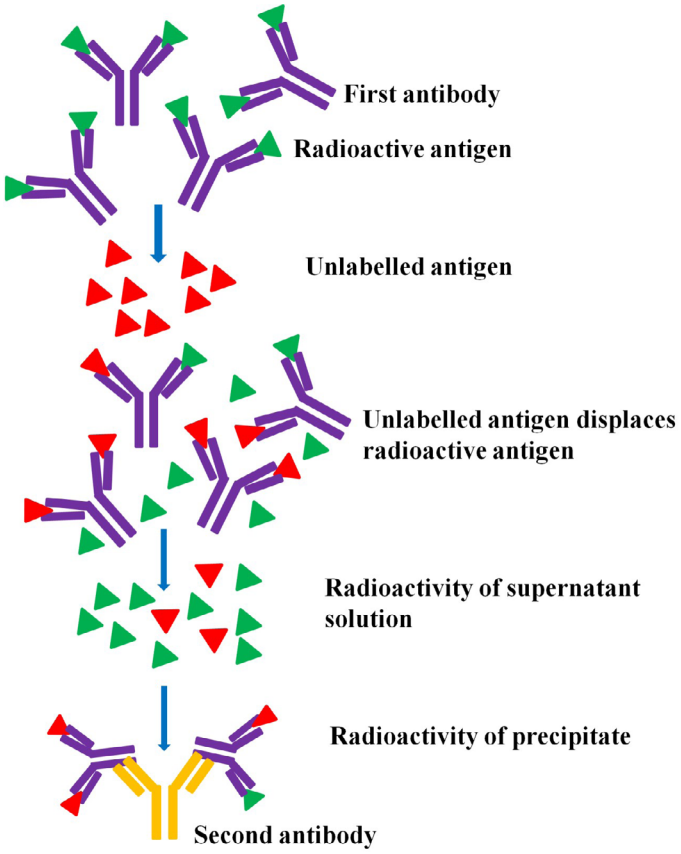

Figure 8: Principle of Radioimmuno assay

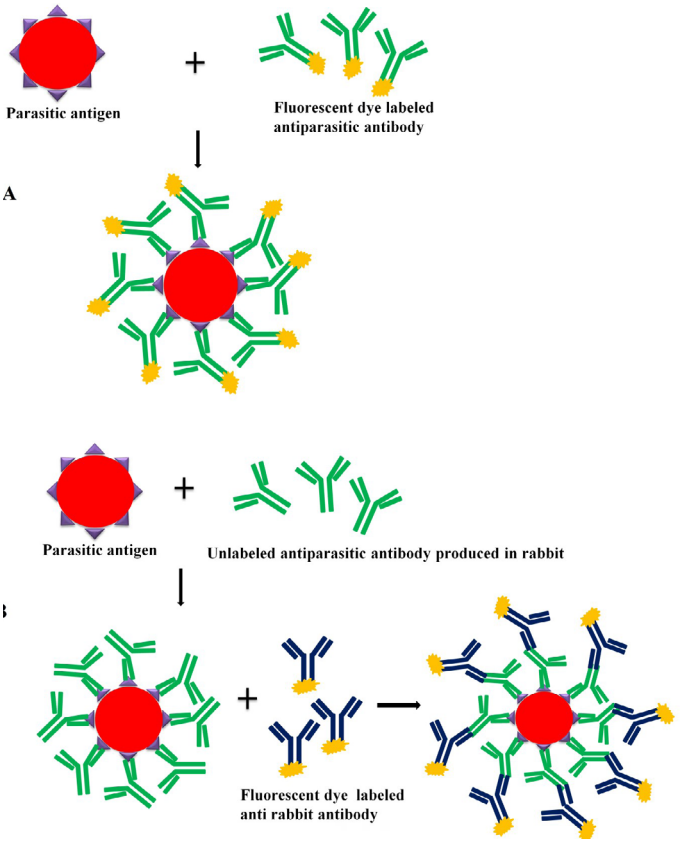

Figure 9: Principle of immunofluoresenct antibody test (IFAT).
A: Direct Fluorescent Antibody Test and **B**: Indirect Fluorescent Antibody Test

orescent antibody test) or with a secondary anti-antibody (Indirect Fluorescent Antibody Test) (Figure 9). The FAT is used in diagnosis of several parasitic diseases.

An indirect fluorescent antibody test (IFAT) was validated for detection of *Toxoplasma gondii* infection in southern sea otters (*Enhydra lutris*) (Miller et al., 2002). The *Neospora* antigens were detected successfully from spontaneously aborted foetal fluids of bovine foetuses using IFAT (Barr et al., 1995). The IFAT shows positive result in carrier state animal to hemopathogens when blood smears could be negative. For example, IFAT of horses and donkeys serum sample showed a higher percentage (6%) of positivity for trypanosome infection than thin smear staining by Giemsa stain (2%) (Nadeem et al., 2011). Similarly, the negative blood sample by microscopic examination of blood smears showed 18.36% and 21.74% prevalence of *Babesia bovis* and *Babesia bigemina* by IFAT (Terkawi et al., 2012). Similarly, IFAT showed 15.82% and 20.89% prevalence of *Babesia* and *Theileria* spp in cattle in Egypt (Nayel et al., 2012). The *Neospora caninum* and *Toxoplasma gondii* specific IgG antibodies were detected using IFAT in dogs in Brazil (Mineo et al., 2001). The *Sarcocystis neurona* specific antibodies in serum and cerebrospinal fluid of horses had been detected using IFAT assay (Duarte et al., 2003). IFAT has also been used for diagnosis of *Trypanosoma equiperdum* and *Babesia canis* (Furuta et al., 2009; OIE, 2013).

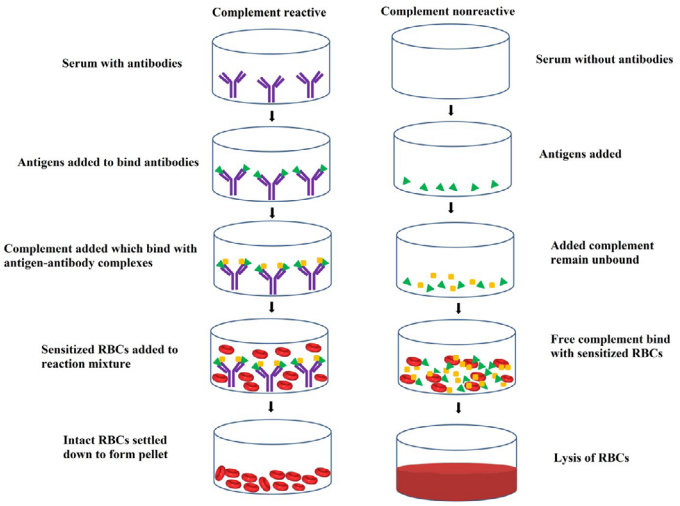

Figure 10: Principle of Complement fixation test

Complement Fixation Test (CFT)

CFT is an immunological test used for detection of presence of either antigen or antibody in serum sample. It was used mostly for microorganisms which are not easily cultured in laboratory (Figure 10). Although, the critical study revealed its poor sensitivity and specificity in detection of parasites, it is still used for parasitic disease diagnosis. The CFT was found reproducible and reliable assay for *Trypanosoma equiperdum* sera sample (OIE, 2013; Luciani et al., 2013). This test is used to confirm the clinical evidence

and to detect latent infection of *Trypanosoma equiperdum*. Therefore it is used as recommended test by OIE (OIE, 2013). However, the specificity of this test was found less in *Trypanosoma evansi* diagnosis (Cauchard et al., 2014). The IFAT was also found more sensitive than CFT for diagnosis of equine piroplasmosis caused by *Babesia caballi* (Ogunremi et al., 2008) and *Theileria equi* (Ogunremi et al., 2007). The CFT has some of the critical limitations such as time consuming, labour intensive and often non-specific (cross-reactivity) in nature.

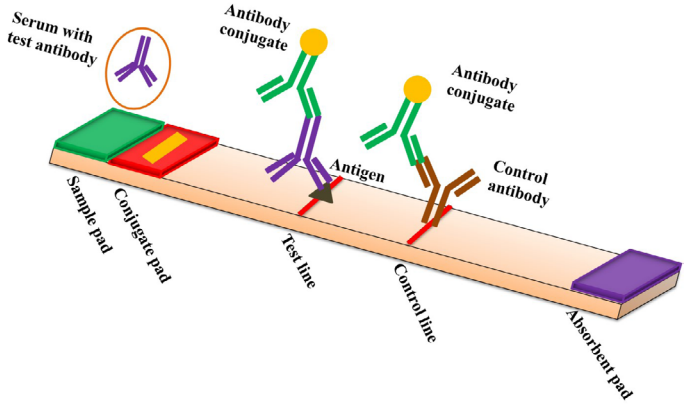

Figure 11: Principle of Lateral flow test

Lateral Flow Test (LFT)

LFT is also known as lateral flow immunochromatographic strip (ICS) assay. It is used for diagnostics purpose in medical and veterinary applications. It is a simple assay used for detection of a target analyte in sample without any specialized equipment (Figure 11). However, it is supported by specialised reading equipment in many laboratories (Yetisen, 2013). It can be used for specific semi quantitative or qualitative detection of many analytes such as antibodies, antigens and nucleic acid amplification products. The assay includes a procedural control line which is used for validation of test result. Therefore, appearance of two lines indicates positive result, while appearance of only single control line shows negative test result. However, the appearance of no lines or only the test line shows invalid result and must be repeated. The LFT assay has been successfully utilised in diagnosis of many animal and human parasites from various biological samples. A novel lateral flow card (TS-Card pork) assay was standardised for the serological detection of *Trichinella* infection in pigs (Patrascu et al., 2001). A rapid LFT assay for detection of *Theleria annulata* infection from field samples has been validated which delivers results within 10 minutes (Abdo et al., 2010). The LFT can also be coupled with other assays to enhance its sensitivity. It was combined with isothermal recombinase polymerase amplification (RPA) to detect *Leishmania infantum* (Castellanos-Gonzalez et al., 2015). The combined RPA-LF assay showed sensitivity similar to real time-PCR for detection of *Leishmania infantum* spiked in canine blood sample, which was equivalent to 40 parasites/mL (Castellanos-Gonzalez et al., 2015). Similarly this assay was also used for diagnosis of several human parasites such as *Echinococcus granulosus*, fasciolosis (*F. hepatica* and F. gigantica), *Giardia lamblia* and *Cryptosporidium parvum* (Khalilpour et al., 2014; Martínez-Sernández et al., 2011; Abdel- Hameed et al., 2008).

Although, several antigen-antibody based assays for parasitic immunodiagnostics have been developed, they have some of the serious limitations. The antigen-antibody cross-reactions leading to misleading false positive diagnosis are a serious problem in regions where several parasites are enzootic. The experimental results have also been too variable due to several types of antigen (e.g., adult worm, egg, crude, recombinant purified) preparations and non-standardized test procedures used. Moreover, many parasitic infections such as *Echinococcus* cysts (Moro and Schantz, 2009) do not elucidate antibody response significantly. In this case antibody based detection methods may not be a reliable assay.

CONCLUSION AND FUTURE PERSPECTIVE

Traditionally, the parasites are detected by microscopic and other conventional methods of various biological samples. However, later on several molecular and serological assays have been employed for this purpose. These assays are shown as highly effective and sensitive for detection of parasites regardless of the type of infection and sample. Among the various techniques available, some are used not only to diagnosis of parasites, but also in treatment monitoring. Thus they became a useful tool in the clinical decision making process. The molecular and serological methods are also useful in epidemiological studies, because they are also involved in study of geographical distribution of parasites, genetic diversity of populations, susceptibility to infections and mutations in parasites. The molecular tools also provide detailed knowledge on genetic characteristics, morphology and behaviour of parasitic disease in the affected populations. Although, the cost of molecular diagnosis is higher than conventional methods, they are increasingly used in veterinary parasitology for clinical diagnosis, epidemiological studies of parasites and treatment monitoring of patient.

The suitable molecular tests showing rapid, sensitive, accurate and reliable result and which can detect all or most targeted parasitic pathogen 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. Similarly, advance software tools and 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 disease. However, it will help in development of newer technologies to a level of analytical sensitivity which will be appropriate for testing of clinical samples directly without previous processing.

COMPETING INTEREST

All authors declare that they have no conflict of interest.

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AUTHOR'S CONTRIBUTION

Ranjan K collected the information and wrote the manuscript, Minakshi P and Prasad G made critical correction in manuscript

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