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Advances in Diagnosis of Important Protozoan Diseases: Old and New Approaches

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A B S T R A C T

Protozoa are unicellular parasites which infect a number of animals including human. For diagnosis of protozoa recent research has been focused almost exclusively on molecular based techniques for the identification and quantification of parasite DNA in samples. The development of molecular tools has allowed the diagnosis, as well as the study of the genetic variability of pathogens and the identification of species-specific markers. Paper review details about the recent advance technique in important protozoan parasite in animal

Introduction

Protozoa are unicellular parasites which infect a number of animals including man. As far as animals are concerned, they are causative agents of various diseases in which incur heavy economic losses to livestock industry. So their appropriate diagnosis will be the first step to achieve effective treatment and control of that particular disease.

Diagnosis of protozoan disease began with the advent of microscope as a scientific tool by the Dutch scientist Antony van Leeuwenhoek in the 17th century. Van Leeuwenhoek could detect *Giardia* in his own faecal sample by microscope. The improvement of the microscope from a novelty to a has led to pioneering discoveries concerning protozoa. Today, microscopy is still widely used for the diagnosis of protozoan infections in animals. Then everything seemed to be replaced by serological tests. The various serological tests include IFAT, ELISA, DFA, IFAT etc. Later molecular tools revolutionized diagnostic part of diseases, and have tremendously increased sensitivity as well as specificity.

Diagnosis of a disease starts with the clinic itself where a clinician diagnosis on the basis of history and symptoms of disease, in addition correlating various clinical

parameters to arrive at temporary diagnosis. But for confirmatory diagnosis samples are sending to laboratory. The clinical sample may be evaluated with respect to presence or absence of a parasite or subsequently evaluating immune response against the pathogen.

Laboratory diagnostic methods for protozoan infections it involves

Classic diagnostic techniques which are

Microscope: The most unequivocally diagnosis of protozoan infections is by demonstration of the organism in the blood, bone marrow, cerebrospinal fluid, faeces or urine, mostly in smears and further staining with Romanowsky, applied to diagnose babesiosis, theileriosis or trypanosomosis (Garcia, 1999) which occur in blood.

In the case of gastrointestinal protozoa, the simplest technique is a direct faecal smear with or without further staining (Garcia, 1999), for the identification of trophozoite stages of *Giardia*, *Trichomonas* and *Balantidium* which besides also show movement to aid in identification. Direct smears can also be used to identify the cyst stages of many of these parasites. Further staining is often used to improve the sensitivity of this technique. The greatest disadvantage of smears is its lack of sensitivity; to overcome this, methods for concentrating protozoa from a larger volume of starting material before microscopic examination have been developed, via flotation. The most common flotation solutions used to concentrate protozoan cysts are Sheather's solution and $ZnSO_4$ (Garcia, 1999).

Thick blood smear allows examination of a slightly larger amount of blood than a thin blood smear and is often used in the diagnosis of *Babesia* infections, while a buffy coat method is another concentration technique often used for the detection of trypanosome.

Indirect diagnostic methods: If organisms occur at densities below the sensitivity of the direct method employed or cannot be directly demonstrated in a biological sample due to the life cycle in the host false negative results may be encountered. Numerous serological tests have been developed to indirectly diagnose infections.

Immunodiagnostic – antibody detection

Tests commonly used to detect the presence of antibodies against a specific protozoan include the complement fixation test (CFT), the immunodiffusion (ID), the indirect haemagglutination (IHA), the latex agglutination (LA), the indirect fluorescent antibody test (IFAT), the radio-immunoassay (RAI) and the enzyme-linked immunosorbent assay (ELISA) in blood samples.

A drawback of serodiagnosis is the fact that antibodies persist for long time, even after elimination of the parasite, therefore a positive result does not necessarily indicate the present parasitological status of the host. Moreover, serology is not useful to diagnose acute infections. Cross reactions are also often encountered between closely related parasites resulting in false positive outcome such as trypanosomes. With the development of molecular techniques, it has been possible to develop test that are based on specific subunit proteins/antigens given much greater specificity to these tests for *Babesia*, *Theileria* and *Trypanosoma* (Katz *et al.*, 2000).

Antigen detection

An alternative to improve diagnosis is to specifically detect parasite antigens, rather than host antibodies against the parasite.

Currently, there are several antigen detection tests available for *in vitro* diagnosis of *Giardia* and *Cryptosporidium* in faecal samples. A drawback of many of the diagnostic assays is the lack of standardised reagents resulting in variation in results between laboratories. However, more and more protozoan serological and antigen assays are becoming commercially available.

Nucleic acid-based diagnostics

Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis is a method for characterizing organisms by the relative mobilities under electrophoresis of a large number of intracellular enzymes. Differences in the electrostatic charge and size between homologous enzymes as a result of the underlying variation in the originally transcribed DNA sequence will affect its electrophoretic mobility. Thus, it is possible to relate mobility differences to different alleles at the gene locus for the enzyme in question. Multilocus enzyme electrophoresis has been used to characterize *Trypanosoma* isolates (Barnabé *et al.,* 2000) and *Eimeria* spp. (Shirley, 1975). Multilocus enzyme electrophoresis has many drawbacks; strains with the same enzyme phenotype may in fact have distinct amino acid sequences, the degree of relationship between different phenotypes is not known, putative heterozygous phenotypes are difficult to interpret, it is time consuming and expensive and requires large volume of parasite material.

Southern blot technique

In the Southern blot technique, DNA fragments are digested using one or more restriction enzymes and separated by electrophoresis before being transferred (blotted) onto membrane filters and hybridized with complementary (radio) labeled probes (Southern, 1975). Methods for non-radioactive labelling of DNA probes have also been developed and include the incorporation of reporter molecules, such as biotin (Murasugi and Wallace, 1984), acetylaminofluorenzyl modified guanosine (Tchen *et al.,* 1984) and sulphonated cytidine (Poverenny *et al.,* 1979). Detection of these molecules is with an appropriate antibody or, in the case of biotin, with avidin or strepavidin coupled to a colorimetric, fluorimetric or chemiluminescent signal. Direct cross-linking of probes to enzymes which act as signal generators has also been described (Renz and Kurz, 1984). DNA probes have been developed for the detection of various protozoa in both mammalian hosts and insect/tick vector, including *Babesia* spp., *Theileria* spp. and *Trypanosoma* spp. The first nucleic acid-based detection and characterisation of trypanosomes were done by using the genes coding for trypanosome variable surface glycoproteins (Williams *et al.,* 1982, Majiwa *et al.,* 1985b and Majiwa and Webster, 1987), which can detect the parasite in the tsetse fly vector (Kukla *et al.,* 1987 and Gibson *et al.,* 1988). Ellis and Bumstead (1990) also developed probes that could distinguish between various *Eimeria* spp. A limitation of this technique is that an appropriate probe must be designed to hybridise to the digested DNA fragments and a rather large number of organisms to process.

PCR

Development of the polymerase chain reaction (PCR) in 1985 has revolutionised the diagnosis of infectious diseases in general (Saiki *et al.,* 1985 and Saiki *et al.,* 1988). With PCR, a specific DNA fragment from complex DNA samples can be amplified resulting in many millions of copies of the target DNA molecule. The standard method requires a DNA template, containing the region to be amplified and two oligonucleotide primers

flanking the target region. PCR products can then be visualized by separating them electrophorectically according to size on agarose gels. Since the original description various modifications has been developed to further increase the sensitivity and specificity of the amplification procedure, such as nested PCR, in which the PCR product is subjected to a second round of amplification with a second pair of oligonucleotide primers located internally from the first pair (Dieffenbach *et al.,* 1993). The reverse-line blot assay, which allows for the identification of novel genotypes or species and also allows for the detection of mixed infections has been developed for *Babesia* and *Theileria* infections (Gubbels *et al.,* 1999 and Nagore *et al.,* 2004).

The next enhancement of this technology came with the development of real-time amplification. The primary advantage of RT-PCR over conventional PCR is that it provides for high-throughput analysis in a closed system, thus eliminating the problems of cross-contamination. This method can also be used to quantify by exploiting the proportional relationship between the threshold cycle, at which exponential amplification is detected and the starting number of the copies of the target fragment. Various RT-PCR detection chemistries have been developed and applied in the detection of protozoa.

Real-time PCR detection of protozoan parasites (Table 1)

LAMP

Alternative DNA amplification, such as loop mediated isothermal amplification (LAMP) has been applied to protozoa (Alhassan *et al.,* 2007, Karanis *et al.,* 2007, Njiru *et al.,* 2008, Guan *et al.,* 2008 and Karanis and Ongerth, 2009). In this method, six different primers, specifically designed to recognise eight distinct regions on a target gene, with amplification only occurring if all the primers bind and form a product. Unlike PCR, LAMP is carried out at a temperature range of 60–65 °C eliminating the need of a thermal cycler. In addition, the reaction can be carried out without the need of DNA extraction.

Luminex xMAP technology

Luminex is a bead based x MAP technology (multianalyte profiling), a system that combines flow cytometry, flourescent microspheres (beads), lasers and digital signal processing, and is capable of simultaneously measuring upto 100 different analytes in a single sample. It is possible to cover each set of microsphere beads by utilizing a reagent specifically designed for a perticular bioassay. This procedure enables the cpaturing and detection of specific analytes from a given sample. The microspheres can be covalently linked to antigens, antibodies or oligonucleotides, which serve as probes in the assay. Several DNA tests developed in the Luminex platform over the years have been used for identification and genotyping of bacteria, viruses and fungi and may be adopted for parasitological surveys to study the antigenic diversity and for diagnosis of parasitic diseases.

Advancement of detection methods in important protozoan diseases

Piroplasmosis

Piroplasmoses are tick borne infections caused by intra-erythrocytic protozoan parasites belonging to several *Babesia* or *Theileria* species, infecting a wide range of domestic animals worldwide. Piroplasmosis can be diagnosed by the examination of peripheral blood smears or smears from visceral organs (brain/kidney/lung/lymph nodes) stained with Romanovsky-type staining methods, such as

the Giemsa stain. In carrier animals, it is quite difficult, if not impossible, to demonstrate parasites, as the number of parasites fall below detectable levels soon after the acute stages of the disease. While it is possible to differentiate the different *Babesia* species based on their morphology, this is rarely possible in the case of *Theileria* infections.

A number of serological assays are available to detect antibodies in carrier animals. The drawback of serological assays, as described above, is that presence of antibodies only confirms exposure to the parasite in questions and does not indicate acute infection, nor confirms the carrier state. Moreover, many of these tests have been developed in-house at specific laboratories, hence very few are commercially available, with standardised antigens and test procedures, thus making interpretation and comparison between regions difficult. However, serological assays are commonly used for testing animals as requirement for international trade (OIE, 2010). A CFT has been developed to detect antibodies against a variety of *Babesia* and *Theileria* parasites. ELISA test have been developed for various *Babesia* and *Theileria* parasites. Antigens can be crude lysates obtained from infected erythrocytes, soluble extracts from*in vitro* cultures or subunit antigens produced *in vitro* (Katz *et al.,* 2000).

DNA probes have been developed to detect *Babesia* DNA in infected animals, based primarily on sequences of the 18S rDNA gene (Böse *et al.,* 1995). To increase the sensitivity of these techniques PCR reaction is used to amplify specific target sequences (Böse *et al.,* 1995). A reverse-line blot assay has been developed for the simultaneous identification of animals carrying different species of *Theileria* and/or *Babesia* simultaneously. The assay employs one set of primers that specifically amplify the rRNA gene V4 hypervariable region of all *Babesia* and *Theileria* species. The PCR product obtained are then hybridised to a nitrocellulose membrane, onto which different species-specific oligonucleotide probes are covalently linked (Sparagano and Jongejan, 1999). The assay has been used in epidemiological surveys in various countries (Almeria *et al.,* 2002 and Niu *et al.,* 2009).

Trypanosoma infections

Trypanosomes can be demonstrated microscopically in infected animals by examining fresh or fixed and stained smears prepared from blood or lymph nodes. However, sensitivity of this method is quite low and can only detect $>10^4$ parasites/ml of blood. Although fixed and stained blood/lymph node smears are useful for the specific identification of trypanosomes to the subgenus level, based on morphology and morphometry, its sensitivity is lower than that of fresh blood. Various concentration techniques have been developed to increase the sensitivity of microscopic examination, such as the haematocrit centrifugation technique (also known as the Woo test) with a sensitivity of $\sim 10^3$ parasites/ml of blood. Because of the low concentration of parasites in biological samples, a widely used method is the *in vivo* culture of the parasites by the intra peritoneal inoculation of the samples into mice. The method is often used for the diagnosis of *Trypanosoma evansi* infections, a parasite which is particularly virulent in the mice with results being obtained within 3–5 days. The success of this technique is variable with other *Trypanosoma* species. Because of the lag time in diagnosis and the cost and ethical considerations, this technique is not used for the routine diagnosis.

Use of serological techniques is useful for epidemiological studies. However, considerable antigenic cross-reaction occurs between *Trypanosoma* species, and no

serologically technique is available that will confirm the identification of species. The common tests available include the agglutination, the card agglutination, the CFT, IFAT and the ELISA. As indicated for piroplasms, the production and standardisation of antigens used in these assays remains a constraint.

Since trypanosomes are intravascular parasites and they would release many components, including specific antigens into the blood stream of the infected host, the detection of these antigens has been investigated. A sandwich-ELISA using a series of monoclonal antibodies developed in the late 1980s (Nantulya *et al.,* 1987), which could detect three specific subgenera with high sensitivity and specificity were developed and subsequently applied in the field. However, further field validations showed that these reagents were less sensitive with some cross reaction occurring and maybe ascribed to the fact that hosts contain multiple infections causing errors in the test and this approach has now been abandoned (Eisler *et al.,* 1998).

The first nucleic acid-based detection and characterisation of trypanosomes was based on probes for the variable surface glycoprotein genes (Adams and Hamilton, 2008). No cross-hybridisation occurred with other trypanosome species and it was even possible to distinguish between different groups of *T. congolense*. The 'first generation' of PCR tests relied on species-specific hybridisation probes based on satellite DNA sequences offering increased sensitivity and specificity over other techniques (Majiwa *et al.,* 1985a and Majiwa and Webster, 1987). However, since this test would require a panel of probes to distinguish between the different species in a field sample, it is expensive and several approaches have since been investigated to set up multi-specific diagnosis within a single reaction. One approach is the amplification of the ITS-1 region which enables simultaneous detection of seven Trypanosomes, even in mixed infections (Desquesnes *et al.,* 2001). However, this test lacked sensitivity, especially for *T. vivax*.Cox *et al.,* (2005) increased the sensitivity of this technique by developing nested PCR strategies. Other studies have used generic primers in a semi-nested PCR assay to amplify the variable region of 18S rDNA gene followed by restriction enzymatic digestion. With this restriction fragment polymorphism approach it was possible to distinguish between the important trypanosome species infecting cattle even with mixed infections (Geysen *et al.,* 2003 and Delespaux *et al.,* 2003). With the use of species-specific PCR tests, it is now possible to identify the 11 tsetse-transmitted trypanosome species and subgroups for which there are available primers.

A fluorescent fragment length barcoding method has also been described, which was able to detect and distinguish trypanosomes (with the exception of members of the subgenus *Trypanozoon*) in both laboratory and field experiments (Adams and Hamilton, 2008 and Hamilton *et al.,* 2008) and was reported to be more sensitive than the ITS method. The big drawback of this technique is its cost and the requirement for specialised equipment.

A promising development is the application of the LAMP method for *Trypanosoma* detection. Recently this technique has been adapted to detect African trypanosomes. A number of primers have been described for the detection of *T. brucei*, *T. congolense*, *T. vivax*, *Trypanosoma gambiense* and *T. evansi* (Kuboki *et al.*,2003, Njiru *et al.,* 2008 and Thekisoe *et al.*,2007).

Cryptosporidium infections

It is recognised as an important waterborne infection. Currently more than 20 species are recognised, with many more genotypes being identified. The majority of these species tends

to be host specific and is not considered pathogenic to the immuno-competent hosts. The exception is *Cryptosporidium parvum*, which infects a wide variety of domestic and wild animals, including humans, and is often the primary cause of diarrhoea in newborn animals. Among domestic ruminants, newborn kids are the most susceptible species, followed by calves and lambs. Although some studies have indicated that *C. parvum* occurs less frequently in sheep, others have shown that the failure to detect C. parvum may have been due to the preferential amplification of the dominant species in mixed infections (Xiao, 2010).In the 1970s *Cryptosporidium* was first recognized as an important aetiological agent in newborn calf diarrhoea complex (Pohlenz *et al.*, 1978). Initially, diagnosis of the infection was based on demonstrating the organism in histological sections. Henriksen and Pohlenz (1981) investigated differential staining of the organism in faecal smears, by using techniques routinely used in the bacteriological laboratory and discovered that acid-fast techniques were suitable differentiating stains. The Ziehl–Neelson staining technique was the first staining technique to be used routinely. The technique is based on the principle that oocysts can be stained with carbol-fuchsin and retains the dye during the decolourising step with acid alcohol. Var-ious other direct staining method have been developed, such as Auramine-O (Casemore *et al.*, 1985); DMSO carbol-fuchsin (Pohjola, 1984); Kinyoun (Ma and Soave, 1983) and safranin-methylene blue (Baxby *et al.*, 1984). Direct staining is still widely used today for the demonstration of oocysts in faecal matter. However, generally the sensitiv-ity of these techniques is low, hence, due to their small size and paucity in some samples, oocysts may be overlooked or confused with yeast cells. The use of fluores-cently labelled monoclonal antibodies directed against the oocysts wall of *Cryptosporidium* oocysts has been reported to achieve higher specificities and sensitivities (Jex *et al.*, 2008). Commercially FITC-mAbs, directed against *Cryptosporidium* oocyst wall, are available and routinely used for the detection and enumeration of *Cryptosporidium* oocysts in faecal and environmental samples (Smith, 2008). Indirect methods to detect *Cryptosporidium* antigens in faecal samples have also been developed and a number of them are available commercially in an ELISA format. These copro-antigen assays have been developed for detecting *C. parvum* antigens in faeces, although when applied to animal faecal samples results can be variable and some-times less sensitive than routine microscopic approaches (Johnston *et al.*, 2003).

Given the limitation of these staining techniques and inability to discriminate between species or genotypes, various molecular methods have been developed. Fluo-rescent labelled oligonucleotide probes targeting variable regions of the ribosomal RNA can be used to detect oocysts in environmental samples (Smith, 2008). These probes, however, do not distinguish among species and geno-types. This has lead to the development of various PCR and nested PCR approaches targeting different genetic loci. The most common loci used for the specific identification of *Cryptosporidium* is the 18s ss rRNA gene; *Cryptosporidium* oocysts wall protein (cowp), 70 kDa heat shock protein (HSP70) and 60 kDa glycoprotein (gp60) (Smith, 2008).Specific enzymatic digestion of the products (fragment polymorphism) or sequencing is then used to identify species or genotypes. Boulter-Bitzer *et al.*, (2007) reviewed additional genetic markers with potential for diagnosis and population genetic studies. Real-time PCR has also been developed to quantify and differentiate species and genotypes of *Cryptosporidium* in animal, human and environmental samples (Monis and Giglio, 2006). Mini- and microsatellite typing and gp60 sequencing have been used to

subtype *C. parvum* and *Cryptosporidium hominis* (Smith, 2008). Studies in recent years have identified a number of *Cryptosporidium* species and genotypes in sheep faeces. The most frequently described species, apart from *C. parvum*, include *Cryptosporidium bovis*, *Cryptosporidium xiaoi* (=*C. bovis*-like genotype) and *Cryptosporidium ubiquitum* (=*C. cervine* genotype) (Ryan *et al.,* 2005; Mueller-Doblies *et al.,* 2008; Robertson *et al.,* 2010).

Luminex is able to distinguish the species *C.hominis* and *C. parvum* using oligonucleotide specific probes for the ML-2 regions of each species, without the need for DNA sequencing. This method provides results in about five hours, being faster and less expensive than PCR followed by DNA sequencing. Luminex also prove upto be 100% specific and more sensitive than direct immunoflourescence (DFA), a method routinely employed to identify species of *Cryptosporidium* and *Giardia.*

Coccidian infections

Eimeria spp. are apicomplexan protozoan parasites that infect a wide variety of domestic animals. and often is an infection of significant economic importance. Coccidiosis is diagnosed by demonstrating presence of the oocysts during microscopic examination of faecal samples following sugar/salt concentration, in conjunction with clinical signs and the typical macroscopic lesions and location of these lesions seen during post mortem examination.

Species identification is based on the morphology and morphometrics of sporulated oocysts. Identification is based on the size, shape and presence of characteristic elements, such as the polar cap, the micropyle, the colour characteristic of the oocysts wall, the number of sporocysts and sporozoites and the presence or absence of oocysts and sporocyst residual bodies. Even then, diagnosis can be difficult and would require an expert to differentiate between species. Most research has been done on improving the diagnosis on Eimeria spp. infecting poultry. *With Southern blot analysis-RLFP, E. tenella, E. acervulina or E. necatrix were differentiated* (Ellis and Bumstead 1990). *By* Field inversion Pulsed-field gel electrophoresis (FIGE), molecular karyotype of five species of Eimeria from chickens (Pasternak, 1991). RAPD screening for *E. acervulina, E.tenella and E.maxima,* using 150 different primers, some 110 specific bands were observed. Also specific bands for different species strains could be observed, maximum observed for *E.acervulina.*

PCR methods have been developed to detect Eimeria spp. by using ITS-1 or ITS-2 sequences (Woods *et al.,* 2000; Gasser *et al.,* 2001; Lew *et al.,* 2003; Haug *et al.,* 2007) or sequence characterized amplification regions (SCARs) (Fernandez *et al.,* 2003). Woods *et al.,* (2000) described a polymerase chain reaction-linked restriction fragment length polymor-phism (PCR-RFLP) approach targeting the second internal transcribed spacer (ITS-2) to characterize six Eimeria spp. However, the ITS sequences show some variability both within a genome as well as between species and strains (Cantacessi *et al.,* 2008).

Toxoplasmosis

Toxoplasma gondii is a tissue cysts forming coccidian parasite that infects most warm-blooded animals; in utero infection can result in foetal death in humans, sheep and goats. The demonstration of oocysts in cat faeces is possible by using standard flotation techniques, but definitive diagnosis usually require sporulation of oocysts and followed by bioassay in mice to distinguish them from other closely related coccidian species (Dubey and Beattie, 1988).

Table.1 Real-time PCR detection of protozoan parasites

Detection chemistry		**Examples of protozoa for which can be applied**	**References**
Intercalating dyes (SYBR Green I)	Fluorescence when bound to dsDNA, but not when free in solution	*Cryptosporidium parvum*	Widmer *et al.*, (2004)
		Leishmania	Nicolas *et al.*, (2002)
		Trypanosoma brucei	Becker *et al.*, 2004
TaqMan probes	Fluorescence following hydrolysis by Taq DNA polymerase	*Cryptosporidium*	Higgins *et al.*, (2001),Keegan *et al.*, (2003)
		Giardia	Bertrand *et al.*, (2004)
		Toxoplasma gondii	Jauregui *et al.*, (2001)
		Theileria parva	Papli *et al.*, (2011)
Fluorescence resonance-energy-transfer (FRET) assay	Energy transfer between donor fluorophore and reporter fluorophore at 3′ and 5′ ends, respectively, of 2 different probes. Fluorescence is detected only when the probes hybridize adjacent to each other on the target DNA.	*Toxoplasma gondii*	Simon *et al.*, (2004)
		Cryptosporidium parvum	Limor *et al.*, (2002)

However, cats only excrete oocysts for a short period after primary infection and, therefore, various serological methods have been developed to detect humoral antibodies in exposed cats and other animals. The CFT was the first sero-logical tests to be developed to detect antibodies in exposed individuals (Warren and Sabin, 1942); this was followed by the Sabin–Feldman dye test (Sabin and Feldman, 1948), which showed to be a very sensitive test. However, as live tachyzoites are used in this procedure, which could potentially pose a danger to the operator, other tests have been developed. These include the immunofluorescent antibody test, the direct agglutination test, the latex agglutination test and the modified agglutination assay (Buxton, 1998). Various ELISA methods using crude, fractioned or recom-binant antigens have been developed (Dubey, 2009). The modified agglutination test still appears to be the most sensitive and specific of all the serological tests available. Both animal inoculation and in vitro culture methods have been used to demonstrate *T. gondii* in cases of abortion. However, these techniques are slow and expensive and rely upon submission of fresh material to the diagnos-tic laboratory and, therefore, they are not routinely used (Buxton, 1998). Immunohistochemical techniques, allowing for the visualisation of *T. gondii* in tissue sections are often used in the diagnosis of abortions (Buxton, 1998; Dubey, 2009). To overcome the limitations of the serological tests, var-ious PCR, nested PCR and real-time PCR techniques have been developed to detect *T. gondii* DNA in samples (Switaj *et al.,* 2005; Gutierreza *et al.,* 2010). However, the PCR diagnosis is not standardised and no consensus on the primers/DNA targets to be amplified exists. Primers are generally based either on the 18S rRNA-, P30-, B1-genes, 529 bp repeat fragment or the AF146527 element. PCR test amplifying genes with high copy numbers in the genome are more sensitive. Recently, Zhang *et al.,* (2009) described a LAMP method for the detection of *T. gondii*, by using the 529 bp repeat element of *T. gondii*, and it was found that the assay was slightly more sensitive than the equivalent PCR.

Immunostimntating complexes (iscoms) were first described by Morein and coworkers. Iscoms are spherical, cage-like structures with a diameter of 30-4Omn, composed of the saponin adjuvant Quil A, cholesterol, phospholipids and proteins. They are formed by hydrophobic interactions and the first iscoms contained viral membrane components of amphipathic character. Since then, a number of methods have been developed to prepare iscoms containing hydrophilic proteins, recombinant antigens and peptides and iscoms containing a wide variety of proteins of viral, bacterial and parasitic origin have been prepared. As the name implies, iscoms are effective in presenting antigens to the immune system and their main area of use has been as adjvants and carriers of immunogens. They have been known to enhance the uptake and internalisation of antigen, increase the major histocompatibility complex class II (MHC II) expression on antigen basting cells, stimulate interleukin 1 (IL-l) pmduotion, activate T-helper cells and cytotoxic T cells and generate a potent antibody response.

Another application of iscoms is their use as antigen in immunoassays. The iscom concept is then used as a tool to select for amphipathic antigens, such as surface membrane proteins of micro-organisms, thus decreasing the number of internal proteins that might cause problems with non-specific binding and cross-reactivity. Serological methods utilising iscom antigen preparations have so far been used for the diagnosis of infections with the coccidian parasites Toxoplasma gondii and Neospora caninum.

Enzyme-linked immunoassay (ELISA) has proved a useful technique for demonstration of antibodies to a variety of antigens. Since it enables rapid determination and titration of antibodies, it is well suited for serological surveys. The use of ELISA for demonstration of antibodies to *T. gondii* and *N. caninum* has not, however, been without problems. The major problems encountered have been high background absorbances and non-specific reactions. This might, at least partly, be due to cross-reactivity with related coccidian parasites. In conventional ELISA systems, water-soluble antigens are used to capture the antibodies to be detected. Such antigen preparations are obtained by disruption of the organisms by repeated freeze-thawing and sonication. The solution is centrifuged and the supernatant collected for use in ELBA. These preparations contain a large number of antigens, the majority probably of intracellular or cytoplasmic origin. However, both the sensitivity and specificity of *T. gondii* assays based on intracellular antigens have been questioned and it has been suggested that a serology based on surface membrane antigens should be more species-specific Consequently, different approaches to enhance the amount of membrane molecules in the ELISA antigen have been used. This includes utilising of e.g., purified membrane proteins membrane proteins incorporated into ISCOMS recombinant antigens and chemically fixed whole parasites.

Iscoms are built up from 10-12 nm subunits formed by Quil A and cholesterol. In the presence of phospholipids these subunits are assembled into cage-like structures into which proteins can be incorporated. Regardless of the size of included proteins, the iscoms are virtually all of the same size, 35-40 nm, and have a sedimentation coefficient of about 19s. In a typical iscom, 60-70% of the weight is Quil A, l and 15% is cholesterol and phospholipid, and 10-l 5% is protein. The preparation comprises solubilisation of the amphipathic protein to be incorporated with a non-ionic detergent, and subsequent addition of Quil A, cholesterol and phosphatidyl choline. The iscoms are formed by hydrophobic interaction; and the protein is readily incorporated when the detergent is removed by gradient centrifugation or dialysis. Unincorporated Quil A and protein can be separated from the iscoms by centrifugation through a sucrose gradient. Iscoms without proteins can also be formed and are then called iscom matrix.

The iscom ELISA was also adapted for bovine sera, and was found to be more specific than a crude antigen ELISA. The ISCOM ELISA has also been used for screening in herds where seropositive animals have been found (Bjorkman *et al.*, 2000).

Using mAb, four of these antigens were identified as the major surface antigens SAGI, SAG2, SAG3 and a 6-kDa surface antigen P35 (Lunden, 1994). Using mAb antibody to bovine IgGl as secondary antibody used in the ISCOM ELISA, is that it cross reacts with immunoglobulins from other ruminants. Therefore, the ISCOM-ELISA can also be used to analyse sera from, e.g., water buffalo and sheep.

In conclusion, microscopy still remains the gold standard procedure for the diagnosis of many proto-zoan infections in animals, but the specific identification requires skilled and experienced personnel. Immunoassays, detecting antibodies or specific protozoan antigens, have been developed but often lack sensitivity and specificity due to close relationship between many protozoa.S erological tests are often applied in the international trade to prevent the introduction of protozoan parasites into areas or farms, where the respective diseases do not occur. Recent research has focused almost

exclusively on molecular based techniques for the identification and quantification of parasite DNA in samples. The development of molecular tools has allowed the diagnosis, as well as the study of the genetic variability of pathogens and the identification of species-specific markers. Frequently, the bottleneck/difficulty for an effective molecular diagnostic procedure is not the PCR amplification of the genomic DNA, but rather the preparation of the DNA. The environmental stages of many of the protozoa have thick walls that are resistant to chemical and mechanical forces; for example, effective techniques are needed to rupture the oocysts wall of coccidian species before DNA extraction. The inhibitors present in faecal material also have negative effect on sensitivity, because of the loss of material due to the purification steps needed. Major challenge remains to develop specific, fast and cheap diagnostic test for the identification of protozoan parasites in animals that could still be applied to remote areas with limited infrastructure. The development of molecular tools has allowed the diagnosis, as well as the study of the genetic variability of pathogens and the identification of species-specific markers. Opinion differ on most appropriate targets to use and there are very few diagnostic kits available making comparison between laboratories difficult Major challenge remains to develop specific, fast and cheap diagnostic test for the identification of protozoan parasites in animals that could still be applied to remote areas with limited infrastructure. Future research needs to focus on robust, cheap field diagnostic assays

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