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Review Article

Molecular Tools for Diagnosis of Latent Haemoprotozoan Diseases of Livestock.

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Haemoprotozoan diseases such as Theileriosis and Babesiosis, present a serious challenge to the exotic and cross-bred cattle and buffalo population. Diagnosis of these diseases often poses a serious problem. Identification of the parasite in the blood and other tissues of the infected animals is the conventional method used for the diagnosis of these diseases. In these methods characteristic staining reaction with certain dyes like Romanowsky's stains are frequently used for these purpose. Stained bodies in the tissues and cells measuring between 0.5 to 20mm in size help in the detection of the organism hence the result obtained are often presumptive. The most common technique of diagnosis of haemoprotozoan infection involves observing pear or ring-shaped and amoeba-like forms of the parasite in the erythrocytes of the blood, stained according to the Giemsa or Wright's method. Sometimes, in cases of low parasitaemia, infected cells can remain undetected. Moreover, the identification of different species of *Babesia* and *Theileria*, on the basis of morphology is not only difficult but also erroneous. The inoculation of laboratory animals with blood from suspected cases of babesiosis is time consuming because the first symptoms occur after 10 days. Carrier animals, in which low numbers of erythrocytes remain infected, are important contributors to the transmission of the infection by tick bite. Hence, detection of piroplasms in carrier animals is very important to control the infection. However, detection of piroplasms by microscopy is not easy and it is generally not possible to distinguish pathogenic species from non-pathogenic species that may occur simultaneously within the same host. This is specially true in low grade infection when only a few organisms are present in the circulation. It is therefore, a number of serological tests are used as a tool for diagnosis of these diseases. Serological tests, which are commonly used for the diagnosis of haemoprotozoan diseases are Fluorescent Antibody Test (FAT), Indirect Fluorescent Antibody Test (IFAT), Complement Fixation Test (CFT) etc. Serological tests like Radioimmuno Assay (RIA), and Enzyme Linked Immunosorbent Assay (ELISA) have become more popular because of their high sensitivity. Immunological and serological methods are characterized by their high specificity and sensitivity. Although serological tests can be used to detect circulating antibodies, cross-reactivity with antibodies directed against other species of piroplasms has been reported (Kuttler, 1981; Papadopoulos *et.al*, 1996a; and Papadopoulos *et.al*, 1996b). Moreover, antibodies tend to disappear in long-term carriers, whereas piroplasms persist. Therefore, animals with a negative serological test can infect ticks and be the source of the infection for other animals. Another drawback with the serological tests is that the antibodies can still be detected years after recovery even though the parasite is not present in the circulation. Again a false positive result may occur with the serum sample obtained from such animals. Most of serological tests employ crude parasite antigen and /or polyclonal antiserums as a test reagent. Such reagents generally produce poor specificity and lack uniformity in results. It is therefore the traditional methods have been complemented or even ousted by the molecular

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ones. New developments in molecular biology have generated exciting possibilities for improved diagnosis of parasitic diseases. Molecular diagnosis of haemoprotozoan diseases involves several PCR-based diagnosis procedures, which help in the identification of the parasite up to the species or even strain level (Figueroa *et.al*, 1993; Birkenheuer *et.al*, 2003; Rampersad *et.al*, 2003 and Criado-Fornelio *et.al*, 2003). Though parasite antigens for serodiagnosis can be produced *in vitro* gene cloning and expressio0n and peptide synthesis, the nuclear hybridization techniques offer a vastly improved approach for identification of parasites in the tissue specimens of infected hosts as a means of diagnosis. Furthermore, the advent of the polymerase chain reaction technique has made it possible to increase the sensitivity of nuclear hybridization techniques, through amplification of target DNA sequences of the parasites in test material, by *in situ* synthesis of these sequences prior to hybridization with the diagnostic probe. Finally, through the use of monoclonal antibody technology, it is possible to design highly specific and sensitive serological assays, as well as assays for parasite antigen detection in tissue fluids and in the excreta of infected hosts, as a means of diagnosis. However, increased sensitivity and specificity can be achieved by combining PCR with a specific hybridisation by means of reverse line blot (RLB), a macroarray that is also capable of identifying mixed infections (Gubbels *et.al*, 1999; Georges *et.al*, 2001; Nagore *et.al*, 2004a, 2004b and Schnittger *et.al*, 2004). Application of these tests in the diagnosis of haemoprotozoan infections are being discussed below.

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Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a laboratory technique evolved by Dr. Kary B. Mullis, in the year 1985 for which he has been awarded Nobel Prize in the year 1993. PCR's popularity comes from its ability to specifically amplify a target DNA sequence more than a million fold within several hours. Furthermore, PCR is so sensitive that a single DNA molecule can be amplified out of complex mixtures of genomic sequences and visualized as a distinct band on an agarose gel. The PCR is an *in vitro* method for enzymatic synthesis of specific DNA sequences, using oligonucleotide primers that hybridize to opposite strands that flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of a annealed primers by DNA polymerase results in the exponential accumulation of specific fragments. These newly synthesized fragments can then serve as template in the next cycle, approximately doubling the number of target DNA sequences in every cycle. Theoretically after every 20 cycles of PCR about a millionfold (2^{20}) amplification occurs. In a simplified way it can be said that PCR merely involves combining a DNA sample with oligonucleotide primers, deoxynucleotide triphosphates and thermostable *Taq* polymerase in a suitable buffer, then repeatedly heating and cooling the mixture for several hours until a desired amount of amplification is achieved. The PCR product then can be analysed by several methods, such as Dot blots, Southern hybridization, and Gel electrophoresis.

Polymerase Chain Reaction (PCR) or PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) is the most promising diagnostic tool and one of the most reliable tools for the detection and identification of protozoan parasites but for the efficacy of the test, selection of appropriate genetic markers of the parasite DNA is extremely important. Different molecular targets were tested for PCR and genes encoding the rRNA of the small ribosomal subunit were found to be the most useful. Within the ribosome, regions conserved in evolution can be distinguished, i.e. having the nucleotide sequences similar to the majority or all parasite species and to others closely related to them. Such an organisation of the gene enables the design of primers complementary to conserved sites for PCR and which can detect a large group of related organisms. In case of *Babesia*, the gene encoding the β-tubulin protein is being widely used as molecular marker for the accurate identification of the parasite. There are two introns within this gene; the first one exhibits much variability with regard to length as well as to the nucleotide sequence. Therefore, the PCR products are of varied lengths depending on the *Babesia* species.

However, these differences are too small for the identification of some species and so, confirmatory methods that extend the duration of the diagnosis are essential. The other genes whose sequences can be used as molecular targets for the detection and differentiation of *Babesia* species are genes encoding the Heat Shock Proteins, in particular, hsp70. However, the hsp70 gene is largely conserved in its nucleotide sequence even between non-related organisms. Therefore, this method, based on the amplification of the whole genome or its fragments, applies mainly to molecular phylogenetic analysis. In conclusion, the selection of a genetic marker for PCR is very important for the sensitivity of this technique.

Selection of a genetic marker for PCR

Now most pathogens transmitted by ticks, such as *Theileria* spp. and *Babesia* spp. the genome of these parasites has been completely sequenced. From the established pool of genetic markers used in detecting these and many other protozoans, the most commonly used are fragments of genes encoding rRNA for the small ribosomal subunit, because they are present in the genome of every eukaryotic organism. In *Babesia* genome three combinations of genes encoding the ribosomal RNA exists. They are present in the following order: 18S-ITS1-5, 8S- ITS2-28S. The 18S rDNA gene (18S rDNA, ssu rDNA) encoding rRNA of the small ribosomal subunit is one of the most frequently used molecular marker in the diagnostic and epidemiological studies of *Babesia* parasites (Figueroa *et.al*, 1993). The size of this gene is different in different species of the parasite and contains between 1720 and 1770 bp. There exist conserved sequences, i.e., with sequences of nucleotides identical in most or in *all Babesia* species and also in species closely related to them. Such an organisation of the gene enables the designing of primers complementary to the conserved sites in PCR and the detection of the DNA of a large group of related organisms. The identification of different *Babesia* species will be possible only if the obtained product is digested with restriction enzymes. Another possible way to differentiate species is the nested PCR method, because the gene encoding 18S rRNA also includes variable sites. In *Babesia*, as well as in other *Eukaryota* the gene harbours eight variable regions numbered V1 to V5 and V7 to V 9 (region V6 occurs in *Prokaryota*). The biggest and the most changeable region is the fragment of the V4 gene comprised of a sequence of 300 bp. Designing primers for nested PCR, complementary to genes unique in terms of their sequences enables the amplification of products characteristic for some *Babesia* species or even strains within this species. Furthermore, a total sequencing of this gene, with its conserved flanking regions bookending the variable interior will, after amplification and sequencing enables comparison with sequences already existing in GenBank and the precise identification of different species (Zahler *et.al*, 2000). Persing *et.al* (1992) were the first to use PCR for the diagnostics of human babesiosis. Krause *et.al* (1996) conducted a blinded study of the sensitivity and specificity of the PCR-based test with primers B1 and Bab4 complementary to the gene fragment (238 bp) encoding the 18S rRNA of *Babesia microti*. They examined patients with babesiosis and an asymptomatic group residing in a region in southern New England (USA) where babesiosis is enzootic. They found that PCR was as sensitive and specific as the use of Giemsa-stained blood smears and inoculation of hamsters. In another study where PCR was applied as the method of diagnostics of babesiosis, Aktas *et.al* (2005) used a pair of primers complementary to the sequence of the gene encoding the ss rRNA of *Babesia ovis* isolated from sheep in eastern Turkey. They obtained a product characteristic only for this species. In order to assess the sensitivity of PCR, they used several dilutions of DNA samples (from 10^{-1} to 10^{-9}). The PCR was sensitive enough to detect parasite DNA from a dilution of 10^{-6} with 0.00001% parasitemia. Moreover, at the same time they carried out observations of blood smears under light microscopy and only four samples were positive for *Babesia ovis* whereas in PCR 21 samples were shown to be positive. The authors concluded that PCR can significantly simplify the diagnostics of babesiosis when the infectious factor is not evident or when serological tests are falsely negative. With the aim of developing the *molecular* diagnostics of babesiosis, Birkenheuer *et.al* (2003) carried out

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semi-nested PCR to detect and differentiate the DNA of *Babesia gibsoni*, *Babesia canis* and *Babesia vogeli* in canine blood samples. They designed pairs of primers to amplify an approximately 340-bp fragment of the 18S rRNA genes from *B. gibsoni*, *B. vogeli* and *B. canis* but not mammalian DNA. The authors emphasized that in the diagnostics of babesiosis, the determination of the species, subspecies and even genotype that caused the babesiosis in dogs is very essential, because virulence, prognosis and response to medicines against *Babesia species* may be different.

Another *molecular* marker that allows the precise identification *of Babesia* species is the gene encoding the β-tubulin protein (component of microtubules). This gene occurs in the cells of all organisms. Studies have shown that in this species, the β-tubulin gene is approximately 1 350 bp in length and that it encodes a protein whose size is 440 amino acids. There are two introns within this gene and the first one exhibits large variability in terms of length as well in its nucleotide sequence (Caccio *et.al*, 2000). The method is based on the specific amplification of this variable fragment and uses degenerate primers complementary to the conserved sites of this gene. The PCR products are of different length depending on the *Babesia* species (from 310 bp in *B. microti* to 460 bp in *B. caballi*); their length is determined by the presence of the intron, which varies in length (Caccio *et.al*, 2000). Identification of the species may be carried out through direct analysis of the length of amplicons after PCR, the product together with internal primers (nested PCR), or by using the RFLP protocol. The RFLP procedure, which uses restriction enzymes, allows the generation of a band pattern characteristic for only one *Babesia* species (Caccio *et.al*, 2000). Other gene sequences may be used as the molecular target for DNA detection and for differentiating *Babesia* species are genes encoding the Heat Shock Proteins (hsp 70). The HSP proteins are a group of proteins, which are activated, and biosynthesized during different kinds of cell stress and they occur in all living organisms (Yamasaki *et.al*, 2007). Their role lies in the regulation of the vital functions of cells, for example, the controlling of cell divisions by connecting cells in complexes together with other cell proteins. A main polypeptide of this family is the hsp 70 protein – the most conserved and most commonly occurring one among all of these proteins. Among the protozoans of *Babesia* genus, a sequence of gene encoding this protein has been so far described in several species, i.e., in *B. microti*, *B. bovis*, *B. rodhani* and *B. gibsoni*. Among these species, the length of this sequence that encodes a protein of about 650 amino acids varies slightly and is in the region of about 1 940 bp. It is already known that in *B. microti* only one copy of the hsp 70 gene is present. The hsp 70 gene shows a large con-servatism in its nucleotide sequence even among non-related organisms. Therefore, this method based on the amplification of a whole gene or its fragments finds its application mainly *in molecular* phylogenetic analysis.

Ano *et.al* (2001) tested the sensitivity of the nested PCR protocol carried out on the basis of the gene fragment encoding 18S rRNA in dogs experimentally infected and in naturally infected patients. They found that visualization of the product after the first-round of PCR was poor in both groups and that only after the second round was there a clear band on the agarose gel. The authors checked the sensitivity of the described nested PCR protocol by using different dilutions of blood samples and found that the result was positive at 0.0001% parasitemia. The sequences of the 18S rDNA gene have also been used to differentiate species of piroplasms occurring in dogs with RFLP (Restriction Fragments Length Polymorphism) protocols (Jeffries *et.al*, 2003). The enzyme used for the restriction of the nested PCR product which was carried out to intensify the signal has allowed the differentiation of different *Theileria and Babesia* sp. from each other. The authors carried out a sensitivity test for the discussed PCR-RFLP protocol and found out it to be high, as it was possible to use it at a parasitemia level of 2.7×10^{-7}% (when the amount of DNA template is smaller than 1 or 2 molecules and when DNA is isolated from full blood collected into EDTA). Moreover, the application of a filter paper containing blood samples, significantly enhances

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the detection of piroplasms and can be used for routine screening of animals for blood protozoan infections for an epidemiological studies.

Real – Time PCR

Real-time PCR which was developed in early 1990s (Higuchi *et.al,* 1992) is a type of quantitative PCR which measures the amount of cDNA or mRNA in a sample, either from a population of cells (tissue or cell culture), or recently even from a single cell. The main advantage of the real-time PCR over conventional PCR is that it allows high throughput analysis in a close tube format and does not require handling after the amplification. The principle of the Real-time PCR is to incorporate a specific, intercalating dye (e.g: ethidium bromide) into the PCR to measure the change in fluorescence after each cycle using a digital camera and a fluorometer attached to the reaction tube (Higuchi *et.al,* 1993). Real-time is used commonly to determine the expression of a gene's mRNA, and its expression levels (copy number of mRNA) during certain conditions such as treating cells with a drug. Real-time PCR can be used to compare normal (control) samples to disease samples, giving an idea as to expression changes, which occur with pathogenesis. Real-time PCR due to its sensitivity is also used in the detection of parasite in the blood. The technique has been successfully used in the diagnosis of Cryptosporidium, Leishmania and Trypanosoma parasites successfully (Nicols *et.al,* 2002 and Becker *et.al,* 2004).

Microchip Electrophoresis

The application of microfabrication technology to microchip electrophoresis (ME) has been increasing in the interdisciplinary field in analytical chemistry. ME separation is significantly faster than conventional gel electrophoresis, and is usually completed in 10 to 200 seconds and consumes only a few microliters of reagents. Sangmin *et al.*, (2004) conducted the first successful demonstration for the diagnosis of bovine theileriosis in the electrophoretic microchip after amplifying the target 816-bp DNA using only 200nL of whole blood. They suggested that a combination method using whole blood PCR and ME for the diagnosis of haemoprotozoan diseases would be a very simple and ultrafast methodology for use in a clinical diagnostic laboratory. King *et.al* (2005) evaluated a novel strategy for fast diagnosis by microchip electrophoresis (ME), using programmed field strength gradients (PFSG) in a conventional glass double-T microfluidic chip. The ME-PFSG allows for the ultrafast separation and enhanced resolving power for target DNA fragments. These results are based on electric field strength gradients (FSG) that use an ME separation step in a sieving gel matrix (poly-ethylene oxide). The gradient can develop programmed shapes FSG over the time. The PFSG method could be easily used to increase separation efficiency and resolution in ME separation of specific size DNA fragments. Compared to ME that uses a conventional and constantly applied electric field (isoelectrostatic) method, the ME-PFSG achieved about 15-fold faster analysis time during the separation of 100 bp DNA ladder. The ME-PFSG was also applied to the fast analysis of the PCR products, 591 and 1191 bp DNA fragments from the 18S rRNA of *Babesia gibsoni* and *Babesia caballi* and 816 bp DNA fragments from the 18S r RNA of *Theileria buffeli* (Kim and Kang, 2005).

Reverse Line Blot macroarray (RLB)

Sanmartin *et.al* (2006) for the first time reported the detection of *Babesia* as well as *Theileria* spp. using Reverse line blot macroarray techniques in subclinical and carrier animals. The technique allowed the simultaneous detection and identification of different bovine *Theileria* and *Babesia* species using oligonucleotide probes whose specificity has been previously determined (Gubbels *et.al,* 1999). Moreover, the combination of a generic *Babesia* and *Theileria* PCR targeting the V4 region of the 18S rRNA gene and a hybridization with specific probes provided high sensitivity (Nagore, 2004a). Since detection of the parasite in Giemsa-stained blood smears is the technique that has been traditionally used for diagnosis of piroplasmosis, whenever possible RLB and microscopy examination were performed in parallel. Besides, positive microscopy allowed us to identify animals with parasitaemia. Subsequently this technique was used by Salih *et.al* (2007) who used this technique in the

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