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## Using molecular tools for diagnosis in veterinary parasitology (polymerase chain reaction (PCR), random amplified polymorphic DNA (RADP))

Amandine Comes, Jean Francois Humbert, Jacques Cabaret, Loïc Elard

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Review article

## Using molecular tools for diagnosis in veterinary parasitology

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**Summary** — The development of molecular biology has made available tools that identify parasites which are important in veterinary parasitology. PCR (polymerase chain reaction) is the most frequently used tool. Target sequences are ribosomal DNA and RNA as well as fragments derived from RAPD (random amplified polymorphic DNA). The most studied protozoan genera have been: *Eimeria*, *Babesia*, *Theileria*, *Trypanosoma* and *Cryptosporidium*. *Trichinella* species and ruminant *Trichostrongylid* parasites have been the most investigated helminths. The use of molecular tools for identification in veterinary parasitology, however, remains a research technique rather than one that is currently used in the field for diagnostic purposes.

**diagnosis / veterinary parasitology / molecular tool / polymerase chain reaction (PCR) / random amplified polymorphic DNA (RAPD)**

**Résumé** — Les outils moléculaires pour le diagnostic en parasitologie vétérinaire. Le développement de la biologie moléculaire a permis de mettre au point de nouveaux outils pour le diagnostic des parasitoses animales. Parmi ces outils, la PCR (Polymerase chain reaction) est celui qui est le plus utilisé. Les séquences cibles les plus fréquentes sont les ADN et les ARN ribosomiques ainsi que des fragments obtenus par amorçage aléatoire (RAPD). L'essentiel des travaux chez les protozoaires se rapporte aux genres suivants : *Eimeria*, *Babesia*, *Theileria*, *Trypanosoma* et *Cryptosporidium*. Chez les helminthes, les trichines ainsi que les trichostrongyles gastro-intestinaux ont fait l'objet de nombreuses publications. De l'ensemble de ces travaux, il ressort que l'application de ces outils pour le diagnostic de routine n'est pas encore évidente. En revanche, ces outils de diagnostic seront certainement très utiles à des fins de recherche.

**diagnostic / parasitologie vétérinaire / outil moléculaire / polymerase chain reaction (PCR) / random amplified polymorphic DNA (RAPD)**

\* Correspondence and reprints

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## INTRODUCTION

The tools available in molecular biology have increased enormously over the last ten years and have improved the detection and identification of microorganisms. In the field of human parasitology, for example, investigations of opportunistic diseases in AIDS patients have led to the isolation of polymerase chain reaction (PCR) primers that can be used for the identification of *Pneumocystis carinii* and

*Toxoplasma gondii* (see reviews by Weiss, 1995, and Smits and Hartskeerl, 1995). The large volume of investigations on malaria, due to its significance as a major human disease, has also resulted in the production of PCR primers and probes for the identification of various *Plasmodium* species. In the field of veterinary parasitology, however, the results are much less abundant and most have been published only during the last two years. This is probably due to the exis-

tence of classical techniques of identification (among the Trichostrongyle parasites of ruminants for example) that have been considered to be satisfactory in most cases.

The aim of the present paper is to describe the array of available diagnostic tools for protozoa and helminths that are of particular interest to veterinary parasitology, with attention being given to the strategies that were used to construct them. We have also taken into account articles devoted to species and strain characterization for three reasons. The first one is that a correct diagnosis cannot be achieved without a species and, in future, a strain identification. Thus, the ability to identify nematode strains resistant or susceptible to anthelmintics would lead to a better diagnosis, and therefore better deworming strategies. The second reason is that species characterization with molecular markers might be a first step in the construction of diagnostic tools (see our work on rabbit coccidiosis, Cere et al, in press). The last reason is that a diagnosis could be achieved in a two-step procedure. The isolation and numeration of parasites using 'classical' techniques would constitute the first step. The second step would involve an exact species identification in taxonomic groups using molecular techniques where classical techniques fail to achieve a sufficiently accurate specific identification. For example, the numeration of eggs or larvae of trichostrongyle nematodes of sheep is easily achieved with methods based on faecal examination, but the species identification is very difficult with classical techniques (morphology and morphometry). On the other hand, molecular techniques allow the species identification from eggs or larvae but not the quantification. The combination of the two approaches will permit an efficient diagnosis: species identification and intensity of infection.

#### TECHNICAL CHOICES AND SCREENING OF TARGET SEQUENCES

On rare occasions (9 references out of 43), detections were based on the use of a hybridizing probe with highly repeated sequences that did not require amplification. These techniques of using hybridization without previous amplification are less frequently employed since the development of PCR. We will nevertheless mention them in this paper.

Actually, the vast majority of diagnostic procedures use PCR (37 articles out of 43) as a first step in the detection of the very small amount of DNA present. This amplification is performed either on known sequences (PCR with a primer size greater than or equal to 20 bases that hybridize to known sequences) or randomly (random amplified polymorphic DNA (RAPD) with a primer size of 10 bases). The PCR products are directly stained with ethidium bromide or cut by a restriction enzyme and then stained with ethidium bromide (PCR-Restriction fragment length polymorphism [PCR-RFLP]) or transferred to a membrane for hybridization with a radioactive probe.

Three types of sequences are used as PCR targets: (i) ribosomal DNA or RNA (13 references out of 43) that present highly repeated sequences and thus permit a sharp detection level. These sequences are generally highly preserved, which may hinder any identification of phylogenetically closely related species; (ii) sequences chosen after cloning, either for their specificity or for the fact that they code for an antigen surface protein specific to the species candidate for identification (7 references out of 43). Specificity is thus high in this case, whereas sensitivity is lower than might normally be expected from the use of repeated sequences; and (iii) sequences obtained after random primer amplifica-

tion (15 references out of 43). The use of RAPD furnishes numerous fragments that are potentially interesting for diagnosis. When these fragments incorporate highly repeated sequences, a high level of sensitivity can be achieved. As a stand-alone, however, RAPD is a poor technique for identification.

## IDENTIFICATION OF PROTOZOA

### *Eimeria spp*

This genus has undergone a great deal of investigation as its morphological characterization has often been misleading and a real need exists for the reliable identification of the particular species harboured by chicks, rabbits, sheep, goats and cattle. Three papers (Cere et al, 1995; MacPherson and Gajadhar, 1993; Procnier and Barta, 1993) describe the use of RAPD which provide evidence of characteristic fragments of species or even isolates. These fragments are not really helpful for diagnosis because, when several species are found within a studied sample, the fragments obtained after amplification are different from those observed in cases of single-species infection. Single-species fragments might be of diagnostic interest under particular conditions (Cere et al, in press) as we have demonstrated for the *Eimeria* species of rabbits. This new technique is primarily based on RAPD (fig 1) to identify a fragment of potential use for diagnosis. The fragment obtained is cloned, then labelled with digoxigenin (Boehringer), and its extremities are sequenced. Finally, from the obtained sequences, two primers (20 bases) are chosen in order to amplify this fragment by PCR. We can detect, using PCR-hybridization with the labelled probe, less than ten oocysts of a particular

species directly from 1–5 g of faeces, even in multispecific infections. Berriatua et al (1995) developed a similar procedure using PCR-hybridization of repeated sequences on sheep coccidia and achieved a sensitivity of less than ten purified oocysts. Another technique (Stucki et al, 1993), based on the use of a 5S ribosomal RNA repeat unit, was also very efficient (up to two purified oocysts) for the detection of *E tenella*, a chicken parasite.

### *Trypanosoma spp*

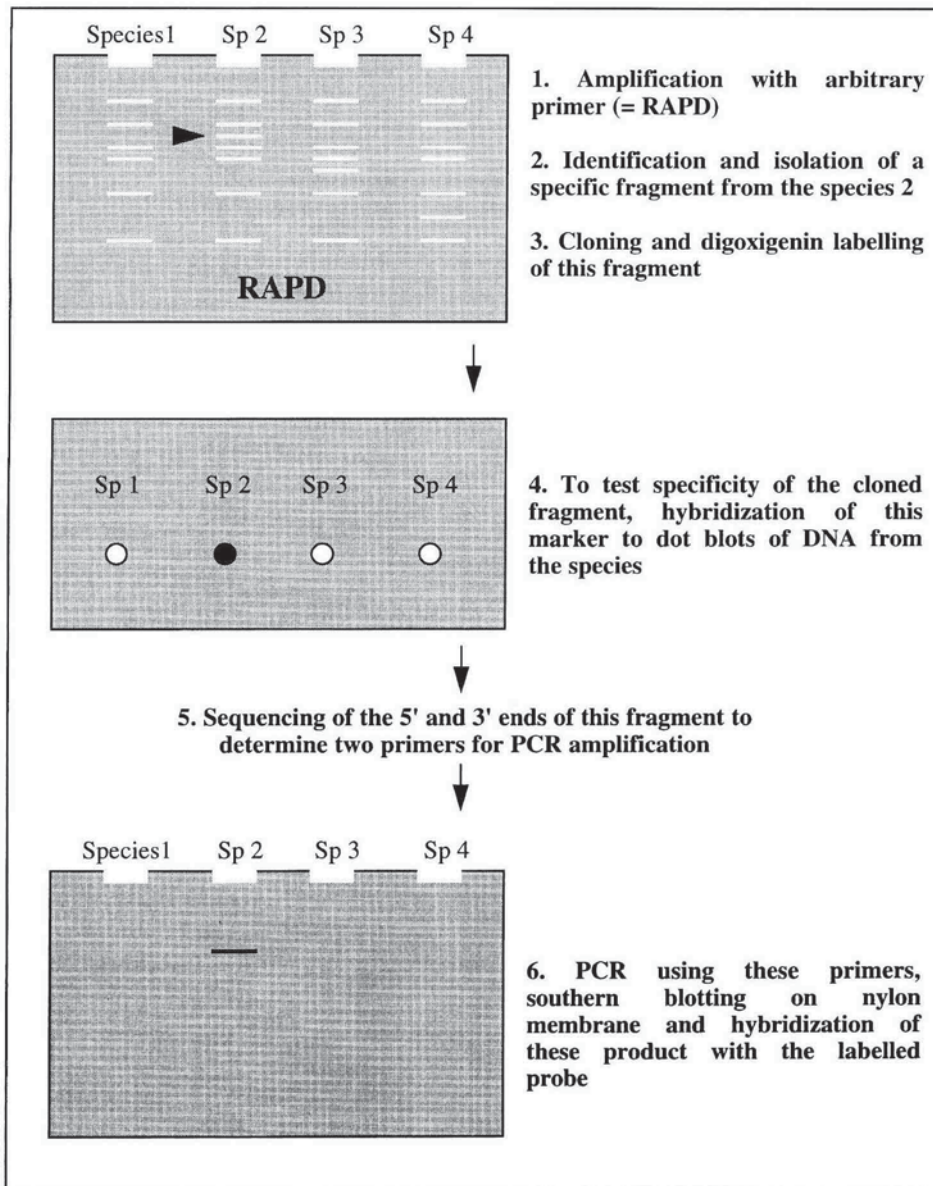
In two studies (Dirie et al, 1993; Waitumbi and Murphy, 1993), fragments characterizing species or isolates of *Trypanosoma*, a parasite of ruminants and pigs, were found using RAPD. It was, however, necessary to purify the parasites from the host blood before amplification and the method needs further refinements before being used as a diagnostic tool.

### *Theileria spp*

Three papers discuss the use of molecular tools for this parasite which affects ruminants, mostly bovines. Tanaka et al (1993) utilized primers and a probe derived from a gene encoding a 32-kDa intraerythrocytic piroplasm surface protein of *T sergenti*. Their method was sufficiently sensitive to detect four parasites per microlitre of blood with a 10- $\mu$ L sample. D'Oliveira et al (1995) reported the detection, using PCR, of *Theileria annulata* in blood samples of carrier cattle. The assay employed primers specific for the gene encoding the 30-kDa major merozoite surface antigen of this species. The specificity and sensitivity of this PCR assay are very good (three parasites per microlitre of blood). Allsopp et al (1993) developed another technique based on PCR using

oligonucleotide probes which detected small subunit ribosomal RNA sequences (srRNA). These probes were efficient at discriminating between the six different

species harboured by cattle. One investigation has increased the accuracy of the identification of isolates or strains using RAPD (Bishop et al, 1993).



**Fig 1.** Description of the RAPD methodology used for the isolation of a species-specific marker and construction of a PCR-hybridization assay.

### **Babesia spp**

Figuerola et al (1994) set up a PCR-based diagnostic assay to detect *B bovis* in chronically-infected cattle. The target sequence was a gene encoding a 60-kDa merozoite surface protein. The level of sensitivity was high as the PCR product was detected in blood samples containing approximately three *B bovis*-infected erythrocytes (20  $\mu$ L of packed cells with a parasitemia of 0.000 001%). Carson et al (1994) used RAPD to reveal markers of species and isolates for *B bovis* and *B bigemina*.

### **Cryptosporidium spp**

Awad-El-Kariem et al (1994) used amplification by PCR of an 18 srRNA fragment, followed by digestion with restriction enzymes, to identify three species of *Cryptosporidia*: *C parvum*, *C muris* and *C baileyi*. Another study (Johnson et al, 1993) compared the efficacy of two molecular techniques (srDNA and hybridization of repetitive DNA probes) with a classical technique (immunofluorescence assay, IFA) to detect the presence of *Cryptosporidia*. The best compromise between sensitivity and rapidity of response was found when PCR was used. Webster et al (1993), using primers adapted to *C parvum*, obtained good sensitivity, as samples of only 20 purified oocysts were able to be detected. When this PCR is coupled with an immunomagnetic particle separation of faecal samples, 80–90 oocysts per gram of bovine faeces can be detected. This represents an increase in sensitivity over conventional diagnostic methods based on faecal examination (Webster et al, 1996).

### **Other protozoa**

A specific DNA probe and PCR amplification were proposed by Ho et al (1994) to detect *Trichomonas* infection in cattle. Wheeler et al (1990) demonstrated that a PCR test developed for the detection of *Toxoplasma gondii* in human and murine tissues can be used for the diagnosis of ovine toxoplasmosis. Good results were obtained when detecting *Perkinsus marinus* in the oyster *Crassostrea virginica* by Marsh et al (1995); the parasites were identified and the level of infection was semi-quantified for the first time.

## **IDENTIFICATION OF HELMINTHS**

### **Cestodes**

*Echinococcus multilocularis* is the only species to be studied thus far due to its zoonotic significance. Gootstein and Mowatt (1991) characterized this species using PCR-hybridization. Bretagne et al (1993b) established efficient diagnostic tools using another target sequence (*U1 snRNA* gene), and could thus detect as little as one egg in 4 g of fox faeces.

### **Trematodes**

The detection of five larval stages of *Fasciola hepatica* within intermediary snail hosts was possible with a probe derived from an rRNA sequences (Shubkin et al, 1992). Utilization of the second internal transcribed spacer (ribosomal RNA) of adult flukes (Adlard et al, 1993) permitted the identification of three species of Fasciolidae: *F hepatica*, *F gigantica* and *F magna*.

### **Nematodes**

The majority of the investigations concern trichostrongyles or *Trichinella* spp. In order to characterize the *Trichinellae* species, two strategies were followed. The first one was based on the utilization of repeated sequences. Dupouy-Carnet et al (1991) using PCR, identified *T spiralis* in mouse. Dick et al (1992) using other primers could identify pig isolates with a good level of sensitivity. It was possible to identify an isolate from a single larva, following digestion or in situ in the muscle tissue. Recently, several references in the literature have demonstrated the utility of RAPD in identifying *Trichinella* species (Bandi et al, 1993a, b, 1995; Dupouy-Camet et al, 1994; Tighe et al, 1994). These results represent only preliminary steps in the construction of a reliable diagnostic tool. Among the Trichostrongyles, Beh et al (1989) demonstrated that it was possible to utilize repeated sequences in order to identify the different species without using PCR. Christensen et al (1994a) isolated other repeated sequences that were able to discriminate between four genera and were capable of detecting as few as 25 eggs. A specific *Haemonchus placei* probe has been isolated (Christensen et al, 1994b). Other papers refer to the diagnosis of these species based on ribosomal DNA or RNA, or RAPD fragments. Zarlenga et al (1994) as well as other studies from Australia (Campbell et al, 1995; Chilton et al, 1995; Gasser et al, 1994; Hoste et al, 1995; Stevenson et al, 1995) have shown the utility of ribosomal DNA or RNA for strongyle identification. Identification is possible on a single egg. We have found other specific markers using RAPD in our laboratory (Humbert and Cabaret, 1994; Jacquiet et al, 1995). From these specific markers we isolated probes in order to identify three species of *Haemonchus*

either as adult or infective larvae. Similar work was done by Chandrasekharan et al (1994), who described the utilization of a repeated sequence to identify *Dirofilaria repens*. A single filaria in infected dog blood samples and a single third stage larvae in mosquitoes have been detected.

### **CONCLUSION**

PCR has obviously been the major molecular biological technique for identification in the last few years. The ability to detect very small quantities of target material and the absence of the need to use radioactive elements are two of the advantages of PCR compared with hybridization techniques. It is not, however, evident that its use will spread to routine diagnosis in veterinary laboratories. There are several drawbacks to the routine use of such a technique. First of all, contamination of the laboratory environment has to be rigorously controlled and this implies numerous controls for quality diagnoses. The enormous amplification achieved by PCR implies that a very small contamination may appear significant. Means of avoiding contamination have been presented by Altwegg (1995) and Carrino and Lee (1995). The first suggestion relates to the organization of the laboratory space for the different stages of diagnosis. DNA and RNA extraction, and the analyses of PCR products (by electrophoresis for example), should be processed in different parts of the laboratory, with specific materials restricted to each process. The second suggestion relates to the use of Uracil DNA Glycosylase (UDG) for the preamplification sterilization of the PCR products (Longo et al, 1990). During PCR, dUTP are incorporated in the synthesized fragments. Before the following PCR, the amplification mixture should be systematically treated with the



enzyme (UDG) that cuts the ADN fragments in which dUTP are incorporated. Any contamination resulting from a previous amplification may then be avoided. For the detection of contaminants, negative controls must be realized at each step of the PCR preparation (eg, isolation of the sample, extraction of the DNA). PCR can fail due to the inhibition of specific amplification (false negative). Thus, the use of positive controls is necessary to increase confidence in negative PCR results. These controls are realized with one fragment which is coamplified with the fragment of interest (Cone et al, 1992). Bretagne et al (1993a) demonstrated in their diagnosis of *Toxoplasma gondii*, the interest of having such an internal control incorporated in the PCR mixture.

The cost of diagnosis is the second drawback of PCR. Although PCR identification costs are being progressively reduced, they remain higher than those of immunological reactions (immunofluorescence, etc) and histological procedures.

A final drawback is the absence of quantification. One unique work (Marsh et al, 1995) did attempt to estimate the intensity of parasitisation. In the case of strongyle infections, intensity of parasitisation is estimated by classical techniques (faecal egg counts for example) and PCR is only of particular use for the identification of the strongyle species either from the eggs or larvae. The identification of blood parasitosis is much more complex and requires the quantification of parasite DNA before it can constitute a real alternative to classical diagnosis. Competition between an added control nucleic acid (internal control) and the native nucleic acid of interest could be exploited in order to quantify the PCR signal (Gilliland et al, 1990).

The use of molecular biology tools for parasite identification will therefore continue to be used in research activities (from

taxonomy to ecology or epidemiology) rather than for day-to-day diagnosis in veterinary laboratories. These tools will probably be of interest primarily in the diagnosis of zoonotic diseases such as *Trichinellosis*, *Echinococcosis* and *Cysticercosis*.

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