Molecular diagnosis of parasitic nematodes

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SUMMARY

There is an essential requirement for highly sensitive tools that will differentiate nematode parasites of animals and plants to the species level. For studying host range, genetic variation, virulence and resistance, the availability of well defined populations is vital. Many nematode species cannot be identified with certainty using traditional morphological or morphometric techniques. This is particularly the case for the more accessible developmental stages that, depending on the particular group concerned, live as eggs and larvae in the environment or as micro-filariae that circulate in the blood or inhabit the skin. Morphological identification of these stages requires specialized expertise and is extremely time consuming. Immunological assays have their place in nematode identification but they do not discriminate between current and previous infections, an essential requirement in many epidemiological and prevalence studies. In addition to being highly sensitive, DNA-based methods of detection define present over past infection and are not dependent on the parasite stage. Many types of methodology are available for the detection and definition of nematode DNA. This paper reviews these methods citing examples that have been used with success in the laboratory as well as the field.

Key words: Nematodes, diagnosis, DNA, PCR.

INTRODUCTION

For effective diagnosis, treatment and control of nematode infections, it is essential that individual species can be accurately identified. It is becoming increasingly important that particular species of nematodes can be recognized as a means of monitoring the efficacy of mass-distributed anthelmintic drugs. Where different nematode strains show variation in their pathogenicity or in their ability to acquire resistance to particular anthelmintics, it is necessary to extend beyond species level and identify different strains or isolates of a parasite. Traditionally, nematode identification was based on the delineation of particular morphological features. In many instances, the pre-parasitic eggs and larvae, or circulating immature parasites in the blood, require to be identified as these are the most accessible stages. However, very few of these can be identified morphologically or morphometrically, even to family level. This is compounded by the fact that many of the identification keys that are available for their differentiation are not well illustrated and need to be improved (Lichtenfels, Hoberg & Zarlenga, 1997).

Detection of parasite-specific antibody responses in the serum has been used to identify nematodes. The major disadvantage of this type of methodology is that it is often difficult to discriminate between past and current infections, thereby giving no indication of the present parasitological status of the individual. An example of the problems associated with the detection of antibody responses is provided by the human filarial parasite, *Onchocerca volvulus*, where humoral responses to a cocktail of three parasite-specific recombinant proteins have been examined (Ramachandran, 1993). Although the assay was highly specific and sensitive, it could not discriminate between previous and current infections, nor was it helpful in following infection after chemotherapy since antibody responses remained elevated for years.

Methods for detecting nematode DNA circumvent this problem by allowing clear delineation between current and previous infections. A further advantage is that DNA-based techniques can be applied to most, if not all, life cycle stages of a parasite thereby avoiding identification of particular proteins that are transiently expressed at specific stages during the parasite's life cycle. Many DNA-based methods are available for nematode identification and these have been used widely in studies on nematode parasites of humans, domestic animals and plants. These techniques have proved to be rapid, reproducible and highly sensitive, often detecting DNA down to the level of a single parasite. As many of the methods generate information on species-specific regions of the genome of related parasites, the results also provide invaluable data for phylogenetic analyses.

TYPES OF MOLECULAR ANALYSIS AVAILABLE

Non-polymerase chain reaction methods

DNA hybridization using characterized probes. DNA hybridization has been used extensively for the detection and identification of nematode species. Basically, genomic DNA is immobilized onto a solid support and hybridized with a radioactively or non-radioactively labelled DNA fragment (probe). Next, any unbound probe is washed off at a particular

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stringency leaving only specifically bound probe which can be detected by autoradiography or chemiluminescence. The previously characterized probe can contain sequences that are partially heterologous or completely homologous to those present in the parasite species under study. Detection of particular species is dependent upon the degree of sequence similarity between the sample material and the probe. Often, these probes are isolated from genomic libraries, made from individual nematode species, that have been screened differentially using DNA from homologous and heterologous species. The advantage of DNA hybridization is that it is relatively quick to perform and, if a non-radioactive probe is used, can be carried out directly in the field. The main disadvantage is that it requires a relatively large amount of input DNA of good quality. Nevertheless, genomic DNA hybridization using specific probes has proved to be a useful tool in the identification of nematode species, especially before polymerase chain reaction (PCR) technology was developed.

One parasite species that has been characterized extensively by DNA hybridization is *Onchocerca volvulus*. Onchocerciasis is a major cause of morbidity in Tropical Africa, the Middle East and Central and South America, with 1% of those infected becoming blind (reviewed by Bradley & Unnasch, 1996). Until relatively recently, definitive diagnosis of this disease was dependent upon the detection of whole parasites, either as adults in skin nodules or as microfilariae in the skin or eye. The main disadvantage of these techniques was that they were rather insensitive, especially in areas of low transmission of the parasite. Furthermore, adult worm detection was of no use for the identification of prepatent infections. It is essential that these parasites be detected in order to monitor their transmission, especially in areas where control programmes are in operation. Initially, attempts to improve diagnosis of onchocerciasis were based on the detection of specific antibody responses in the serum or the use of characterized antibodies as reagents to detect parasite products in body fluids. More recently, DNA probes have been developed for the detection of *Onchocerca* and these have been extremely useful in examining the presence of different strains of the parasite and in distinguishing this species from other filarial parasites in the insect vector. The initial approach relied on differential screening of genomic libraries made from different parasite species (Harnett *et al*. 1989; Meredith *et al*. 1989) and strains (Erttmann *et al*. 1987). Using this technique, a number of *O. volvulus* DNA probes were isolated that showed varying degrees of specificity; some probes recognized all species present in the genus, others were *O. volvulus*-specific, whilst others identified particular strains of this species (Erttmann *et al*. 1987; Harnett *et al*. 1989; Meredith *et al*. 1989). Several of these probes were derived from a sequence family of short repeats of around 150 base pairs (bp) which was designated O150 (Meredith *et al*. 1989). There was variation within the repeats with some being present in all *Onchocerca* species, while others were specific to *O. volvulus* with some repeats unique to particular strains of this parasite. Unfortunately, these probes were not always sufficiently sensitive to detect the epidemiologically most important stages of the parasite, especially as it proved difficult to extract DNA from larvae and skin microfilariae in a form suitable for genomic hybridization. Subsequently, the sequences derived from these probes have been used to design primers for use in more sensitive PCR-based assays for the detection of *Onchocerca* (see below).

Haemonchus contortus, *Ostertagia circumcincta* and *Trichostrongylus colubriformis* are pathogenic nematodes found in the gastrointestinal tract of sheep. Difficulties in identifying these trichostrongylid nematodes at the pre-parasitic stages has limited their effective diagnosis and control. DNA hybridization techniques have been developed to identify these parasites at the egg stage in faecal samples; for example, a novel repetitive sequence found in *T. colubriformis* DNA was cloned and used as a probe to distinguish the eggs of this parasite from those of the abomasal species, *H. contortus* and *O. circumcincta* (Callaghan & Beh, 1994*a*). Similarly, a genus-specific repetitive sequence was isolated from a *H. contortus* genomic library (Callaghan & Beh, 1994*b*), while, in another study, 4 genus-specific probes were developed for *Ostertagia*, *Haemonchus*, *Cooperia* and *Oesophagostomum* (Christensen, Zarlenga & Gasbarre, 1994). These probes were derived from partial genomic libraries of representatives of each of these genera after each had been screened differentially using heterologous and homologous genomic DNA. Although not species-specific, these probes detected as few as 25 eggs of each genus when used in dot blot assays. Later, the sequences derived from these probes were used to design primers that were used in the PCR to identify individual species present within each of these genera (Gasser *et al*. 1995).

Many important species of plant parasitic nematode have been characterized using DNA hybridization. For example, members of the economically important root knot nematode genus, *Meloidogyne*, have been identified to the species level using DNA based probes. Of the 50 or so recognized *Meloidogyne* species, most agronomic damage is thought to be inflicted by *M. arenaria*, *M. incognita*, *M. javanica* and *M. hapla* and so it is important to be able to distinguish these species from the less pathogenic root knot nematodes. In Southern blot experiments, a *Sty*I satellite DNA probe, previously isolated from *M. hapla*, gave hybridization signals

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only with the homologous species (Piotte *et al*. 1995). This probe also discriminated amongst different populations of *M. hapla* and was used successfully on preparations of nematodes that had been squashed on slides, thus reducing the need for prior DNA extraction. In these experiments, *M. hapla* could be distinguished from *M. chitwoodi* using a single female worm isolated from root tissues (Piotte *et al*. 1995). *M. arenaria*, the peanut root knot nematode, is a particular problem in South East USA. A 900 bp fragment, containing a random repeat region of 28 bp, was generated following differential library screening (Baum, Lewis & Dean, 1994) and was used in the dot blot system to distinguish this species from other *Meloidogyne* species in nematode egg masses. In other studies, as few as 6 larvae of *Globodera pallida*, a potato cyst nematode, could be distinguished from related nematodes using a species-specific DNA fragment in dot blot assays (Marshall & Crawford, 1992). Single cysts of other *Globodera* species have been differentiated by dot blot assay using probes identified following differential screening with DNA from various members of this genus (Stratford *et al*. 1992). Identification of the plant parasitic nematode *Pratylenchus* was also investigated by dot blot assay: an intron within the major sperm protein gene contained regions that were species-specific and a probe derived from this region was used to distinguish individual *Pratylenchus* species (Setterquist *et al*. 1996). Primers were designed from the sequence within this intron and these identified individual species by PCR (Setterquist *et al*. 1996).

Thus, DNA based probes have been developed for many different types of nematodes and these have been used extensively in defining parasite populations and in detecting particular nematode species in epidemiological studies. Many of these probes have been superseded, however, by more sensitive PCR-based techniques.

Restriction fragment length polymorphism (*RFLP*) *analysis*. Restriction endonucleases are enzymes of bacterial origin that recognize and digest specific DNA sequences which are of 4–6 bp in length. Variation between species can be detected when there is a DNA polymorphism at the restriction site or where there is a larger mutation that alters the length of the DNA between sites. To detect these differences, the digestion products are size separated through a gel matrix. Often, the digested DNA is transferred to a solid support by Southern blotting and detected using a labelled probe. Following this, the number and pattern of DNA fragments produced is compared between organisms. Although this is an indirect indication of sequence variation, it is a relatively easy technique to perform and a large number of individuals or loci can be examined simultaneously. A disadvantage is that, unless laborious restriction mapping is performed, one can only surmise that co-migrating bands in different species are homologous. When linked with the PCR (see below), this is a very useful technique for identifying nematodes.

Although superseded by PCR-linked RFLP analysis, straightforward RFLP analysis using genomic DNA has been effective in the characterization of several nematode species or populations. For example, genomic RFLP analysis was used for the identification of different populations of *Meloidogyne* (Carpenter *et al*. 1992). Traditional morphological methods had been unsuccessful in distinguishing different populations of *M. arenaria*, even though these populations differ greatly in their reproductive capacity and aggressiveness. Total genomic DNA isolated from eggs of different populations was subjected to RFLP analysis using the restriction enzyme, *Eco*RI, and probed by Southern hybridization with previously characterized probes directed against single copy and interspersed multicopy genes. One probe, when hybridized to the parasite DNA, displayed an interspersed repetitive pattern and the RFLPs distinguished one population ('Govan') from the other 2 isolates. This work demonstrated the utility of genomic RFLP analysis for distinguishing closely related populations of parasites within the same species. To test the usefulness of these repeated sequence probes for detecting the presence of nematode DNA in DNA samples from roots inoculated with varying numbers of nematodes, dot blot hybridization was performed. This analysis showed that the homologous population could be detected down to a level of 30 nematodes per root sample (Carpenter *et al*. 1992).

Polymerase chain reaction-based approaches

PCR has revolutionized molecular genetic studies, not least the development of DNA-based identification techniques. From limited amounts of material, particular regions of the genome can be selected and amplified for subsequent cloning and/or sequencing. The PCR consists of repetitive cycles of DNA denaturation, primer annealing and extension by a thermoresistant DNA polymerase. The PCR amplifies DNA exponentially so that, after 30–40 cycles, 'millions' of copies of the original fragment are available for subsequent manipulation. The main advantages of PCR-based methods are that they are highly sensitive, can use DNA of relatively inferior quality and are not very labour intensive. The limitation is that some sequence information is required in the first instance to allow the design of primers for amplification. Nevertheless, as complete homology of primers is not essential, these can be designed from a closely related organism or from regions of sequence conserved across a range of

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organisms. Technically, the main drawback of PCR is the amplification of contaminated DNA, especially when highly degenerate primers are used. PCR can be used for both diagnostic purposes and for phylogenetic analysis.

PCR-linked RFLP. This is a modification of the RFLP method outlined above. In essence, the region of DNA that is of interest is amplified by PCR using specific primers and the amplification products are digested with one, or a cocktail of restriction endonucleases. The digestion products are separated by gel electrophoresis and the fragment patterns compared between individuals or species. Often, the products of digestion do not have to be hybridized with a specific probe, which makes this method less time consuming and less expensive than the hybridization protocols identified above. Furthermore, because it is a PCR-based technique, only nanogram amounts of template DNA are required. Many nematode species have been characterized in this manner and the results of these studies have shown that PCR-RFLP can distinguish both inter- and intra-specific variation.

Individual species of the intestinal parasite, *Strongyloides*, have been characterized by PCR-linked RFLP (Ramachandran, Gam & Neva, 1997). Several human and dog isolates of *S. stercoralis* were compared, along with one species that infects monkeys, *S. fulleborni*, and another that infects rats, *S. ratti*. DNA extracted from these species was subjected to PCR using primers designed to amplify the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) and an adjacent portion of the 23S-like ribosomal RNA gene. The PCR product sizes varied amongst the three *Strongyloides* species and restriction digestion of the *S. stercoralis* strains enabled them to be differentiated. PCR-linked RFLP studies have been used to differentiate the human intestinal parasites *Oesophagostomum bifurcum* and *Necator americanus* which exist together in parts of Togo and Ghana but cannot be differentiated morphologically at the pre-parasitic stages (Romstad *et al*. 1997). Again, the ITS region and its flanking sequences in the rDNA were analysed by PCR-linked-RFLP using several restriction endonucleases. Following digestion individually with different endonucleases, a consistent fragment size difference was obtained between the products from DNA from the 2 species. There was no detectable intraspecific difference in the size of the PCR products obtained, nor was there significant variation in restriction patterns within each species. Other hookworms have been characterized by PCR–RFLP of the ITS region and the adjacent 5·8 rRNA gene with unique PCR-RFLP patterns produced for each of the 3 species, *Uncinaria stenocephala*, *Ancylostoma caninum* and *A. ceylanicum* (Gasser, Stewart & Speare, 1996).

Several nematodes of veterinary importance have been characterized using PCR-linked RFLP analysis. One large group of parasites that have been subjected to PCR-RFLP are the equine strongyles. These are a complex group of over 50 nematodes that live in the equine large intestine and, similar to many of the other species mentioned above, these cannot be differentiated morphologically at the egg or larval stages. From a veterinary viewpoint, it is important to be able to discriminate between infections with these species as they have distinct life cycles and pathogenic effects. A portion of the ITS region of the rDNA from 16 different strongyle species was amplified using conserved primers and the PCR products were digested separately using 6 endonucleases yielding characteristic RFLP patterns for each of the 16 species (Gasser *et al*. 1996). Similar studies have been performed on nematode species that inhabit the ruminant gastrointestinal tract (Gasser *et al*. 1994). Using four different restriction endonucleases, 6 nematode species were differentiated and no intraspecific variation was detected. Ascaridoid parasites of the dog and cat have been subjected to characterization by PCR-linked RFLP. These parasites, particularly *Toxocara canis*, are important zoonoses and pose a threat to human health. PCR-RFLP analysis of the ITS-2 region of the rDNA was used to differentiate *T. canis*, the most likely parasite to cause a serious zoonosis, from *T. cati* and *Toxascaris leonina* (Jacobs *et al*. 1997). In addition, these parasites were differentiated from other species of ascaridoids that can be found in human tissues. Subsequently, primers were designed to unique regions of the ITS-2 for the 3 species named above and these were used successfully to discriminate them directly by PCR (Jacobs *et al*. 1997).

In addition to parasitic nematodes of animals, the PCR-linked RFLP technique has been performed to distinguish species of root knot nematodes. The ITS region was amplified by PCR in several species of *Meloidogyne* (Zijlstra, Uenk & van Silfhot, 1997). Using three endonucleases, individual species were detected when they represented a minimum of 5% of the parasite mixture. This assay is being developed for analysis of field isolates that are submitted for routine analysis.

Random amplified polymorphic DNA (*RAPD*). This technique is a variation of the standard PCR in which primers of arbitrary sequence are used to amplify fragments of the genome (Williams *et al*. 1990; Welsh & McLelland, 1990). The arbitrary primers are short and anneal at positions of highest homology under particular PCR conditions. If the primers anneal in relatively close proximity, with their 3′ ends directed towards each other, on opposite strands of the DNA, a double strand will be amplified. For a given DNA template, a number of

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different sized fragments will be produced depending on the template sequence, the primer sequence and the PCR conditions. The PCR products are separated by electrophoresis to generate a fingerprint of bands. This technique has been used to obtain nematode strain- and species-specific fingerprints. The main advantages of the RAPD technique are that no prior sequence information is required for primer design, radioisotopes are not necessary and the method is quick to perform. There are, however, several drawbacks. Often, RAPDs are not very reproducible since the slightest alteration in the PCR conditions or components will alter the banding pattern that is produced. Secondly, amplification results in the production of completely uncharacterized regions of DNA that may, in fact, represent rapidly evolving sequences that would be of no long-term use for species or strain differentiation. Furthermore, if one is attempting to identify unique sequences for development of species- or strain-specific primers or probes, there is no guarantee that these sequences are absent in non-amplified parts of the genome of the heterologous species. One would have to screen for these sequences in heterologous species by testing the primers on DNA from all known species or isolates. As the annealing temperature used in RAPD is low and the primers are random, any contaminating DNA will be amplified to produce a separate fingerprint that would totally confuse the analysis and thus the technique requires pure DNA from the target species. This method should be used with great caution, especially where there is any opportunity for contamination by host or any other parasite material. Nevertheless, many workers have used this method to develop fingerprints for the parasites that they study.

One of the earliest studies using RAPD-PCR on nematodes was performed on several species from a range of genera, these included; *Meloidogyne* species, *Globodera* species and *Trichinella* species (Chacon *et al*. 1994). Although RAPD profiles were obtained that allowed *M. incognita* to be differentiated from *M. hapla*, no differences were detected in the banding patterns that were generated from DNA from *M. javanica*, *M. arenaria* and *M. graminicola*. In this study, the patterns produced from 4 separate races of *M. incognita* were indistinguishable, although analysis of different *Globodera rostochiensis* isolates showed intraspecific differences. Eight isolates of *Trichinella* were examined using this technique and 4 different fingerprints were identified that corresponded to 4 *Trichinella* clusters previously characterized by isoenzyme analysis. Thus, the level to which the RAPD-PCR can discriminate between nematodes is dependent on the particular parasite group that is under study. RAPD-PCR analysis has been taken one step further where bands that had been amplified uniquely from *M. hapla* and *M. chitwoodi* were cloned and sequenced (Williamson *et al*. 1997). Subsequently, the sequences were used to design 2 sets of primer pairs that were used together in a 'multiplex' PCR to identify single juveniles of both species in mixed populations (Williamson *et al*. 1997). RAPD patterns have also been used to characterize *Globodera* species (Roosien *et al*. 1993). Various sources of parasite material were tested in this study and the results showed that fingerprints could be obtained from crude homogenates and whole juvenile worms not subjected to prior DNA extraction, emphasizing the straightforward nature of this technique in the absence of host material.

RAPD-based approaches have been used to identify strains within a nematode species that differ in virulence. An arbitrary primer has been identified that distinguished 2 isolates of soybean cyst nematode that had similar virulence, from a separate geographical isolate that was less virulent (Li *et al*. 1996). From this marker, a probe was developed that was used successfully in a dot blot assay. Other RAPD analyses have not been quite so successful. For example, RAPD-PCR was performed on 8 different ruminant nematode species, as well as morphs of each species. However, species identification was confused by the high level of intraspecific variation (Humbert & Caberet, 1995).

Identification of nematodes using PCR with specifically designed primers. The advantage of having defined primers over arbitrary primers is that the PCR can be performed at high stringency without the need to purify the parasites or their DNA. Primers have now been designed for the detection and identification of many nematode species (and strains) either to amplify species-specific products or to generate a product that differs in size between related species.

Loa loa is an important filarial parasite that affects humans in tropical areas. By designing specific primers from the repeat regions of the gene, 15r3, which codes for the *L. loa* 15 kDa polyprotein antigen, DNA was amplified specifically from blood lysates of occult infected individuals, i.e. people who did not have detectable microfilariae in their blood (Touré *et al*. 1997). A 396 bp fragment was detected in patients from different geographical areas and the species-specificity of the PCR was confirmed using blood from people known to be infected with other filarial species. This assay has been extremely useful for detecting this parasite in endemic areas where many individuals have occult infections.

Primers have been designed for the identification of different *Dirofilaria* species. These are important mosquito-borne parasites that infect domestic and wild animals. The most important species are *D. immitis* and *D. repens* that infect dogs. Very rarely, these cause a zoonosis and develop through to the adult stages to produce a circulating microfilaraemia in humans (Nozais, Bain & Gentilini, 1994). These species can only be identified on the basis of adult

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worm morphology which requires surgery to locate the worms in the heart and pulmonary blood vessels. Primers have been designed for use in the PCR to discriminate these species. These primers have been used to detect *D. immitis* microfilariae in the blood of infected dogs, as well as L_3 from infected mosquitoes (Scoles & Kambhampati, 1995). A separate set of primers has been designed that enable this species to be discriminated from *D. repens* (Favia *et al*. 1996). A 'multiplex' PCR has been developed to enable *D. immitis* to be differentiated from *Wuchereria bancrofti* in their common vector, *Aedes polynesiensis* (Nicolas & Scoles, 1997). This assay has a sensitivity of 1 pg and 10 pg, equivalent to 0·1 L_3 and 1 L_3 per pool, for *W. bancrofti* and *D. immitis*, respectively. *Oesophagostomum dentatum* and *O. quadrispinulatum* are morphologically similar parasites that are found in large intestine of pigs. Primers were designed from ITS-2 sequence obtained from both these species and used successfully to differentiate them at the L_3 stage (Newton *et al*. 1997). These primers are used routinely to monitor the purity of larval cultures of *Oesophagostomum* and to confirm the identity of larvae derived from the faeces of infected pigs.

Different species of potato cyst nematode have been distinguished using primers designed from previously characterized regions of the ITS rDNA (Bulman & Marshall, 1997). PCR primers based on the sequences of *G. rostochiensis* and *G. pallida* have been used to identify these species in mixed populations of potato cyst nematodes.

A variety of nematodes has been identified based on size polymorphisms detected using primers that amplify different sized products from individual species within a groups of parasites. For example, size differences in amplified intergenic spacer rDNA has been demonstrated in *Meloidogyne* species where polymorphisms were detected between *M. chitwoodi*, *M. hapla* and *M. fallax* (Petersen & Vrain, 1996). This was also demonstrated by Zijlstra (1997) who showed fragment size polymorphisms in the amplified ITS-2 region between the 3 aforementioned species and also *M. incognita*. Similar work has been performed with sheep nematodes where part of the β tubulin gene was amplified with specific primers in a range of nematodes (Roos & Grant, 1993). Discrimination of *H. contortus* from *T. colubriformis* eggs was based on the size of the amplicon that was obtained.

Identification of nematodes by PCR linked to hybridization with specific oligoprobes. The PCR has been used to increase the sensitivity of previously developed oligonucleotide probes. For example, lack of sensitivity was one of the main problems associated with the O150 probes that were developed for the detection of different species or strains of *Onchocerca*. By comparing the DNA sequences of the 18 known examples of the 150 bp repeat present within the O150 DNA of *Onchocerca* species, 2 populations of degenerate oligonucleotides were designed that amplified DNA from all members of this genus (Meredith *et al*. 1991). Since results had demonstrated that the O150 family was restricted the *Onchocerca* genus, only DNA from these parasites could serve as templates for these O150 primers (Meredith *et al*. 1991). Parasite DNA samples that proved positive by O150 PCR were thus ascribed to the *Onchocerca* genus and could then be classified to a particular species or strain by hybridization with the specific probes. This approach was sufficiently sensitive to allow single infective larvae or microfilariae to be classified (Meredith *et al*. 1991). Since the whole O150 family was amplified in the PCR step, DNA samples could be divided, after amplification, into aliquots so that an individual DNA sample could be tested with a number of O150 based probes thus speeding the characterization of each sample. Results from field samples showed that the O150 PCR, coupled with subsequent hybridization, was more sensitive than skin snip sampling for the detection of infection with *O. volvulus* (Zimmerman *et al*. 1994). Moreover, it was observed that classification based on the strain-specific DNA probes correlated almost exactly with the observed disease pattern, i.e. with the development of blindness, allowing the different probes to be used to determine the pathogenic potential of a particular parasite population (Zimmerman *et al*. 1992).

Although PCR and subsequent probe hybridization can be performed without the use of radioactive materials, this technique is relatively time-consuming and requires equipment that is not always easily accessible in the field. To overcome these problems, an ELISA-based detection system for the O150 PCR products has been developed (reviewed by Nutman *et al*. 1994). As many laboratories use ELISA-based technology to measure serum antibody responses, this makes the assay more amenable to field studies. Basically, parasite DNA is amplified using 1 biotinylated primer and 1 unbiotinylated primer and the resulting double-stranded products (including the biotin-labelled negative strand, its complimentary unbiotinylated strand and the unincorporated primers) are bound to streptavidin-coated wells of a microtitre plate. The unbiotinylated strand is removed during a denaturation step with sodium hydroxide. Detection of the bound biotinylated PCR products is initiated by hybridization with a species- or strain-specific fluorescein-labelled oligonucleotide probe (positive strand). Hybridization of the fluorescein-labelled probe is performed under stringent conditions that are developed for each probe. In the case of *O. volvulus* this is approximately 15 min (at 37 °C). After washing under stringent conditions, an alkaline phosphatase-conjugated antibody which has been raised against fluorescein is added. Fol-

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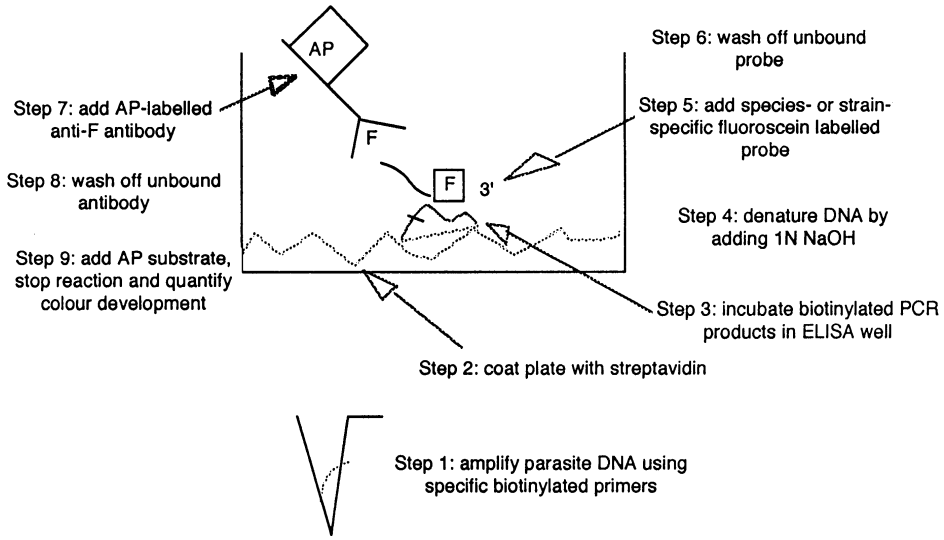


Fig. 1. Schematic diagram of the steps involved in the ELISA-based detection method of PCR products. Adapted from Nutman *et al*. 1994. AP = alkaline phosphatase substrate, F = fluorescein.

lowing incubation and washing, a substrate for alkaline phosphatase is added for 1–2 h and the colour change quantitated. This substrate step can be shortened further by using an enzyme amplification system. The assay is shown schematically in Fig. 1.

This assay has been optimised for the detection of *O. volvulus* DNA using a fluorescein-labelled oligonucleotide probe designated OVS-2 (Zimmerman, Toe & Unnasch, 1993). The length of time used to perform the assay was short, 2·5–3·5 h, when compared with standard hybridization methods. The specificity of this detection system was demonstrated using a biotinylated positive strand primer alone with fluorescein-labelled OVS-2 proble; using the O150 biotinylated negative strand primer alone with fluorescein-labelled OVS-2 probe; using the O150 biotinylated negative strand primer with genomic DNA extracted from other filarial parasites and using skin snips from individuals who had never been exposed to filarial parasites (Zimmerman *et al*, 1993). Tests using known concentrations of input plasmid DNA containing 12 copies of the O150 repeat indicated that the ELISA-based assay was approximately 10 times more sensitive than standard hybridization by slot or Southern blot.

Cost is probably the major factor that will determine if this approach will be used widely in the field. Excluding the thermal cycler, ELISA reader and labour costs, and using reagent prices found in standard laboratory catalogues, Nutman and colleagues (1994) estimated that this assay run, in duplicate, could be performed for US$0·80 per sample. The microtitre plate format is extremely flexible as it can be automated for large volume throughput or used for just a few samples and because the readout is numerical, quantitation can be performed easily and linked to a computer. This system can be adapted for the detection and/or quantification of any target DNA for which sequence data is available. In fact, this system has been used for the detection of *Brugia malayi* (Williams *et al*. 1993) and *W. bancrofti* (McCarthy *et al*. 1996; Nicolas & Scoles 1997) DNA. The O150 system has been tested in the field. In Ecuador it was used to identify infected individuals with levels of skin microfilariae that were not detectable by standard parasitological methods (Nutman *et al*. 1996). In addition, the assay was used as a diagnostic aid to indicate when microfilariae reappeared within a population of people treated previously with anthelmintic (Nutman *et al*. 1996).

This type of methodology has also been used for the identification of nematodes of veterinary importance. PCR with group-specific primers combined with hybridization with species-specific oligonucleotide probes was used to characterize small strongyle (cyathostome) species that inhabit the large intestines of horses. The intergenic spacer region of the rDNA was subjected to PCR using primers that had been developed to amplify DNA from all members of this subfamily (Kaye *et al*. 1998). The PCR products were cloned and sequenced for 16 species of cyathostome and, using the sequence data, species-specific oligonucleotide probes were designed for 4 species and used in hybridization studies to discriminate each of these species from all the others. These probes are currently being tested in the PCR-based ELISA.

Identification of nematodes using PCR-linked single strand conformation polymorphism. When large numbers of samples have to be analysed, mutation scanning approaches such as PCR-linked single strand conformation polymorphism (SSCP) offers an alternative over conventional techniques such as PCR-RFLP or direct sequencing, both of which are time-consuming. PCR-linked SSCP is a relatively

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straightforward technique that has the potential to discriminate between DNA fragments differing by a single base and is thus useful in differentiating closely related organisms. The underlying principle is that the electrophoretic mobility of a single stranded DNA molecule in a non-denaturing gel is dependent upon its size and structure, i.e. conformation. The conformation is related to the primary nucleotide sequence such that a mutation at a particular site in the sequence can change the conformation of the molecule and so alter its mobility during electrophoresis. The advantage of this technique over other fingerprinting methods is that the primers used can be designed to amplify across a particular group, for example genus or subfamily, of parasites and thus the PCR can be performed at high stringency, minimizing the possibility of co-amplification of contaminating DNA. PCR-linked SSCP has been used to characterize several nematode species of veterinary importance and was performed on amplified ITS-2 DNA (Gasser *et al*. 1997). In these studies, parasite species that could not be discriminated by gel electrophoresis of PCR-generated ITS-2 products, were distinguished by PCR-linked SSCP. The patterns were reproducible for an individual parasite but there were low levels of intraspecific variation.

TAXONOMY

Over time, mutations accumulate in both coding and non-coding regions of the genome, the rate depending on the tolerable rate of mutation. Non-coding regions and introns evolve more rapidly as they are unlikely to be highly constrained by function, while nucleotides that encode for proteins, particularly those encoding amino acids at the active sites of enzymes or at protein binding sites, are indefinitely conserved. Mutations may arise in these regions but as preservation of function is likely to be vital to the survival of the organism, these mutations are rarer than those in non-coding region. Because of these pressures, different genes and particular segments within a gene will evolve at different rates. Hence, when developing a phylogenetic framework for a particular group of nematodes, it is important to select a region that is diverging at a suitable rate. A compromise must be reached between regions of low variation, where resolution of individual species may not be obtained, to regions of high variation, which may lead to convergences and parallelisms that would obscure the true genetic relationship. By examining previous studies that have investigated genetic relationships at a similar level, appropriate genes or regions of genes can be selected that will provide sufficient phylogenetic data to resolve the group of nematodes under study.

This said, the phylogeny of the Nematoda is extremely complex and a detailed description is beyond the scope of this particular review. There are few obviously homologous morphological characters in nematodes to produce a consistent evolutionary framework and there is no informative fossil record on which to base a molecular clock. As in many other organisms, the genes encoding the rRNA subunit have proved to be useful in taxonomic studies of nematodes as they include regions with varying rates of evolution from the extremely conserved (for example, the small subunit gene) to the more variable (for example, the intergenic spacer regions). The most extensive phylogenetic study of animal parasitic nematodes so far was based on analysis of the small subunit rDNA of 53 different taxa (Blaxter *et al*. 1998). The results of this study suggested that convergent morphological evolution within Nematoda was extensive and that the higher level classification currently available will need revision. This work identified 5 major clades within Nematoda and each of these contained representatives of species that were parasitic. This lead to the conclusion that animal parasitism may have arisen independently on at least four occasions and that plant parasitism has occurred on 3 separate occasions.

CONCLUSIONS

In recent years there have been many advances in the identification of parasitic nematodes, especially since the development of PCR-based techniques that have increased the sensitivity of detection. These advances have enabled the rapid characterization of many different nematode species and, more relevant to diagnostics, in the environment or in the fluids of infected hosts. Although many of the assays are still largely experimental tools for use in laboratory studies of parasite prevalence and epidemiology, it is likely that, with further development, many of these assays will be utilized for the detection and characterization of parasites on a routine basis in the field. Especially promising is the PCR-based ELISA method, developed for the detection of different filarial parasites, as this is a cost effective, highly sensitive and relatively easy technique to perform. Preliminary field studies have indicated that routine use of this type of assay in the field is a realistic option.

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