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DIFFERENTIATION OF DORSAL-SPINED ELAPHOSTRONGYLINE LARVAE BY POLYMERASE CHAIN REACTION AMPLIFICATION OF ITS-2 of rDNA

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ABSTRACT: Molecular genetics was used to devise the first reliable diagnostic tool for differentiating morphologically indistinguishable dorsal-spined, first-stage larvae (L1's) and other stages of the nematode protostrongylid subfamily Elaphostrongylinae. A polymerase chain reaction (PCR) assay employing specifically designed primers was developed to selectively amplify DNA of the ITS-2 region of the ribosomal gene. Amplification of the entire ITS-2 region differentiated between larvae of the genera *Elaphostrongylus* and *Parelaphostrongylus,* based on the lengths of fragments produced. Three sets of primers were designed and used successfully to distinguish larvae at the species level. Although it was demonstrated that one primer set in a single PCR assay was capable of distinguishing each of the three *Parelaphostrongylus* spp., a second primer set would be required for confirmation in routine diagnostic use. Two of the three primer sets were capable of amplifying DNA from all six elaphostrongyline species and of identifying *Elaphostrongylus alces* and *Parelaphostrongylus odocoilei.* Although two separate fragments were produced from each *Elaphostrongylus cervi* and *Elaphostrongylus rangiferi,* it was not possible to distinguish these two parasites from each other based on the fragment size. The use of various nematodes, hosts, and fecal controls demonstrated the reliability of the primers for all developmental stages including L1's, third-stage larvae, and adult worms. These primers also have potential for identifying other lungworms as was shown by the amplification of *Umingmakstrongylus pallikuukensis,* the muskox protostrongylid, and *Dictyocaulus* sp. from white-tailed deer. Although this assay may benefit from further refinement, its present design provides researchers, wildlife managers, clinicians, and animal health regulators with a practical tool for the control, management, and study of meningeal and tissue worms and their close relatives.

Key words: Cervids, diagnosis, DNA, *Elaphostrongylus* spp., meningeal worm, *Parelaphostrongylus* spp., polymerase chain reaction, Protostrongylids, tissue nematode.

INTRODUCTION

The subfamily Elaphostrongylinae (Pryadko and Boev, 1971) of the Protostrongylidae contains six nematode species associated with the central nervous system and/or skeletal muscles of cervids. Four of these parasites cause severe neurologic disease and all produce verminous pneumonia (Anderson, 1992; Mason, 1995). Specific diagnosis, however, is problematic. Although it is possible to serologically detect two species as early as three weeks after infection (Ogunremi et al., 1998, 1999), reliable identification of the other elaphostrongylines still requires the study of adult male worms recovered at necropsy. The Baermann beaker method can readily detect first-stage, dorsal-spined larvae (L1's) passed in feces of patently infected hosts (Forrester and Lankester, 1997) but the L1's of all species are similar morphologically (Pybus and Shave, 1984; Lankester and Hauta, 1989). Specific diagnosis of larvae in feces is further confounded by overlapping host and geographic distributions and the occurrence of mixed infections (Prestwood, 1972; Pybus and Samuel, 1981, Lankester and Fong, 1998).

The subfamily includes *Parelaphostrongylus* spp. which are restricted to cervids of the western hemisphere, and *Elaphostrongylus* spp. originally of Old World cervids but introduced elsewhere with translocated stock (Anderson, 1992; Gajadhar

et al., 1994). *Parelaphostrongylus tenuis,* the so-called meningeal worm, so far is known only from eastern North America and causes neurologic disease in cervids, bovids and camelids. *Parelaphostrongylus odocoilei* occurs in wild cervids and bovids of western North America and *P. andersoni* occurs across the entire ranges of white-tailed deer and caribou (*Rangifer tarandus caribou*). *Elaphostrongylus cervi* (tissue worm) occurs in red deer (*Cervus elaphus elaphus*) in Europe, Fennoscandinavia and Asia and has been spread to New Zealand. *Elaphostrongylus rangiferi* occurs widely in reindeer of northern Fennoscandinavia and Russia and has also been spread to caribou in Newfoundland (Canada). A third species, *E. alces* has been reported only from moose (*Alces alces*) of Fennoscandinavia.

The lack of a dependable method of distinguishing elaphostrongyline infections has resulted in restrictions or bans on the translocation of cervids for wildlife management purposes and in the game farming industry. Studies of the impact of infection on wild populations and the natural spread of these parasites also have been impeded. An accurate and convenient method of diagnosing infections is clearly needed globally by wildlife managers, veterinarians, and animal health regulators. The purpose of this study was to investigate the use of molecular genetic techniques for diagnosing patent infections on the basis of L1's present in cervid feces. It has been shown previously that the second internal transcribed spacer (ITS-2) of rDNA contains genetic markers that can be used for species identification among nematodes (Gasser and Monti, 1997). We determined the nucleotide sequence of the ITS-2 region and selected appropriate primers in developing a PCR assay for the genetic differentiation of dorsal-spined L1's of all six recognized species of elaphostrongyline nematodes. Assay specificity also was tested against larvae of other protostrongylid lungworms and *Dictyocaulus* sp.

MATERIALS AND METHODS

Parasites

Various stages of 10 species of nematode parasites were used (Table 1) including eight protostrongylids (all six elaphostrongyline species of cervids), *Muellerius capillaris* of sheep and goats, the muskox lungworm, *Umingmakstrongylus pallikuukensis,* a dictyocaulid of deer (*Dictyocaulus* sp.) and a trichostrongylid (*Haemonchus contortus*) of sheep. The identity of larval stages of parasites used was confirmed by the morphology of adult male worms which were recovered from naturally or experimentally infected hosts (Table 1). The Baermann method was used to isolate first stage larvae (L1's) from the feces of experimentally or naturally infected animals, and a pepsin-HCl digestion procedure was employed to extract third-stage larvae (L3's) of protostrongylids from snails that were experimentally infected with L1's (Gajadhar et al., 1994). L3's of *E. cervi* and *P. tenuis* were produced in the snail, *Triodopsis multilineata,* and *E. alces* was grown in *Arianta arbustorum.* Adult worms were removed from the carcasses of experimentally or naturally infected animals and their identity confirmed by examination of the males. After the initial extraction and concentration of larvae from feces or snails the worms were resuspended in sterile water and individually transferred into fresh suspensions of phosphate-buffered saline (PBS). This procedure was repeated several times until all visible extraneous material was removed. Adult worms were similarly washed several times in PBS. All worms were stored in filtered PBS or water (RNase-, DNase-free) at −70 C until used.

Nucleic acid isolation

Tissues removed from the liver of an uninfected red deer, unexposed snails (*Triodopsis multilineata*), sedimented Baermann supernatant collected from the feces of an uninfected red deer, L1's of *M. capillaris* and L3's of *H. contortus* were each homogenized into a suspension and DNA extracted by a standard phenol-chloroform method (Sambrook et al., 1989). Crude nucleic acid preparations were prepared from samples of various stages of other nematodes previously stored at −70 C (Table 1). Briefly, two 0.25 cm pieces from the anterior portion of adult male worms or 25 larvae (L1's or L3's) were washed three times in sterile water (Sigma, St. Louis, Missouri, USA) and resuspended in 30 μl of 10 mM Tris-HCl, pH 8.3, and 50 mM KCl in a 0.5 ml microfuge tube (BioRad Labs, Hercules, California, USA). Each tube and its contents were boiled

TABLE 1. Origin of nematodes and developmental stages used as a source of DNA to differentiate species of elaphostrongylines by polymerase chain reaction.

Parasite	Stage	Extraction site	Host	Type of infection	Original source
Elaphostrongylus cervi	L1	feces	Red deer	Experimental	New Zealand
Elaphostrongylus cervi	L3	foot and viscera	Snail[a]	Experimental	New Zealand
Elaphostrongylus cervi	Adult	skeletal muscle	Red deer	Experimental	New Zealand
Elaphostrongylus alces	L1	feces	Moose	Experimental	Sweden
Elaphostrongylus alces	L3	foot and viscera	Snail[b]	Experimental	Sweden
Elaphostrongylus rangiferi	L1	feces	Caribou	Natural	Newfoundland, Canada
Elaphostrongylus rangiferi	Adult	skeletal muscle	Caribou	Natural	Newfoundland, Canada
Parelaphostrongylus tenuis	L1	feces	White-tailed deer	Experimental	Minnesota, USA
Parelaphostrongylus tenuis	L3	foot	Snail[a]	Experimental	Minnesota, USA
Parelaphostrongylus tenuis	Adult	brain	White-tailed deer	Experimental	Minnesota, USA
Parelaphostrongylus andersoni	L1	feces	Barren-ground caribou	Natural	Northwest Territories, Canada
Parelaphostrongylus andersoni	Adult	muscle	Barren-ground caribou	Natural	Northwest Territories, Canada
Parelaphostrongylus odocoilei	L1	feces	Mule deer	Natural	British Columbia, Canada
Parelaphostrongylus odocoilei	Adult	muscle	Mule deer	Natural	British Columbia, Canada
Umingmakstrongylus pallikuukensis	L1	feces	Muskox	Experimental	Northwest Territories, Canada
Muellerius capillaris	L1	feces	Sheep	Experimental	Maryland, USA
Dictocaulus sp.	Adult	lung	White-tailed deer	Natural	Missouri, USA
Haemonchus contortus	L3	abomasum	Sheep	Experimental	Maryland, USA

[a] *Triodopsis multilineata.*
[b] *Arianta arbustorum.*

TABLE 2. Size (base pairs) of DNA bands determined by agarose gel electrophoresis of PCR amplified parasite DNA using primers targeting the ITS-2 region of the ribosomal gene.

Parasite	Primers			
	NC1, NC2[a] (Fig. 1)	NC2, ECP1R[b] (Fig. 2)	PTP1, PTP2[b] (Fig. 3)	ECP1, PTP2[b] (Fig. 4)
Elaphostrongylus cervi	597	**430**[c], 512	—[d]	—
Elaphostrongylus rangiferi	597[e]	**430**, 512	—	—
Elaphostrongylus alces	581	—	**216**	—
Parelaphostrongylus tenuis	576	—	**228**	**345**
Parelaphostrongylus andersoni	566	—	**226**	—
P. odocoilei	571	—	**231**	**337**
Umingmakstrongylus pallikuukensis	445	725	**194**	—
Muellerius capillaris	ND[f]	—	—	—
Dictyocaulus sp.	ND	779, 837	—	—
Haemonchus contortus	ND	—	—	—

[a] Universal primers for the amplification of the entire ITS-2 region.
[b] Specific primers for the amplification of a portion of the ITS-2 region.
[c] Numbers in bold represent prominent bands.
[d] No DNA amplification occurred.
[e] Data not shown in Figure 1.
[f] Assay not done.

(100 C) for 10 min, allowed to cool for a minimum of 10 min in an ice-water bath at 0 C and centrifuged at 12,000 × g at 4 C for 10 min. Ten milligrams of sterile zirconium beads (0.1–0.15 mM) (Biospec Products, Bartlesville, Oklahama, USA) were added to each sample, followed by vortex at high speed for 2 min. Proteinase K (Sigma) was added to a final concentration of 0.13 mg/ml. Samples were incubated at 56 C for 4 hr, boiled (100 C) for 10 min, cooled in an ice-water bath for a minimum of 10 min and centrifuged at 12,000 × g at 4 C for 10 min. The supernatant portions were used as templates in the PCR reaction.

PCR primers and conditions

The second internal transcribed spacer region (ITS-2) of the ribosomal RNA gene of the six species of *Elaphostrongylus* (*E. cervi* L1, *E. rangiferi* adult, *E. alces* L3) and *Parelaphostrongylus* (*P. tenuis* L1, *P. andersoni* adult, *P. odocoilei* adult) was amplified by PCR using conserved 20-mer oligonucleotide primers NC1-forward: (5′-ACGTCTGGTTCAGGGTTGTT-3′) and NC2-reverse: (5′-TTAGTTTCTTTTCCTCCGCT-3′) derived from the free-living nematode, *Caenorhabditis elegans* (Ellis et al., 1986). The amplified products were electrophoresed on a 1.5% agarose gel and the band of ITS-2 excised and purified with a GeneClean II kit (Bio/Can Scientific, Vista, California, USA) according to the manufacturer's instructions. The purified DNA products were cloned using a cloning kit (TA Cloning Kit; Invitrogen, Carlsbad, California, USA), and forward and reverse M13 primers employed in an automated cycle sequencing to obtain the nucleotide sequences of the clones. The sequences of the six species of protostrongylids were aligned on a computer using commercial software (Align Plus Version 2; Scientific and Educational Software, State Line, Pennsylvania, USA). Primers found to be specific for *E. cervi* or *P. tenuis* were selected from the sequence alignment data. The primers were ECP1: 5′-GGAATCGTCCGTCTATCGTT-3′; ECP1R: 5′-GTCAAAACGATAGACGGACG-3′; PTP1: 5′-CCGTCGAATACATGTCATCC-3′; PTP2: 5′-TCGTCAAGACGATGATTCCC-3′.

Each PCR reaction consisted of 50 μl reagents containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM each of dATP, dCTP, dGTP, dTTP, 1 μM each of two primers, 1 unit of Taq polymerase and template DNA (10 ng of extracted DNA or 5 μl of supernatant from a crude nucleic acid mixture) prepared from species of nematodes (Table 2). Reagents, template DNA and individual PCR reaction tubes were kept on ice during PCR setup. A "master mix" of reagents, excluding template DNA, was prepared and 45 μl dispensed to each PCR reaction tube and 5 μl of template DNA or sterile, RNase-DNase-free water (negative control) (Sigma) was added to each reaction tube. Each reaction mixture was overlaid with 50 μl of mineral oil (Perkin Elmer, Foster City, California, USA). The PCR reaction was carried out in a thermocyler (Perkin Elmer 480; Applied Biosystems Canada

Inc., Mississauga, Ontario, Canada); each PCR reaction tube was incubated at 95 C for 10 min and then subjected to 35 cycles of the following steps: 95 C for 1 min, 60 C for 1 min, 72 C for 1 min. The PCR reaction tubes were then incubated at 72 C for 10 min prior to being held overnight at 4 C.

PCR products were separated on a 1.5% agarose gel prepared in 1 × TBE buffer pH 8.3 (Sigma). The gels were stained for 30 min in a 0.5 μg/μl solution of ethidium bromide in 1 × TBE buffer, pH 8.3, and photographs were taken using a Polaroid MP-4 camera (Polaroid Corp., Cambridge, Massachusetts, USA). The size of the amplified DNA fragments were calculated by measuring the relative positions of the amplified bands and marker standards or by using the AplhaImager 2000 computerized gel documentation system (Alpha Innotech Corp., San Leandro, California, USA).

RESULTS

Amplification of the ITS-2 rDNA from each of *E. cervi, E. rangiferi, E. alces, P. tenuis, P. andersoni, P. odocoilei* and *U. pallikuukensis* using conserved primers NC1 and NC2 produced a single nucleotide fragment (Fig. 1 and Table 2). When PCR products were separated out on a 1.5% agarose gel, the single amplification bands for *Elaphostrongylus* spp. (581–597 bp) were slightly larger than those of *Parelaphostrongylus* spp. (566–576 bp), but size differences between species in each genus were not readily distinguished by band position alone because the band positions appeared similar (Fig. 1). Sequences of the ITS-2 region of all species of the genera *Parelaphostrongylus* and *Elaphostrongylus* showed a high degree of homology (GenBank, accession number pending). Potential species-specific primers were generated from sequence areas with minimum homology, particularly in the 3′ end. Several primer combinations were evaluated for their ability to distinguish between *Elaphostrongylus, Parelaphostrongylus* and other nematodes.

Amplification of genomic DNA from *E. cervi* and *E. rangiferi* using primers NC2 and ECP1R resulted in a 430 bp band and a less intense satellite band of 512 bp (Fig. 2). Two faint bands of 779 and 837 bp

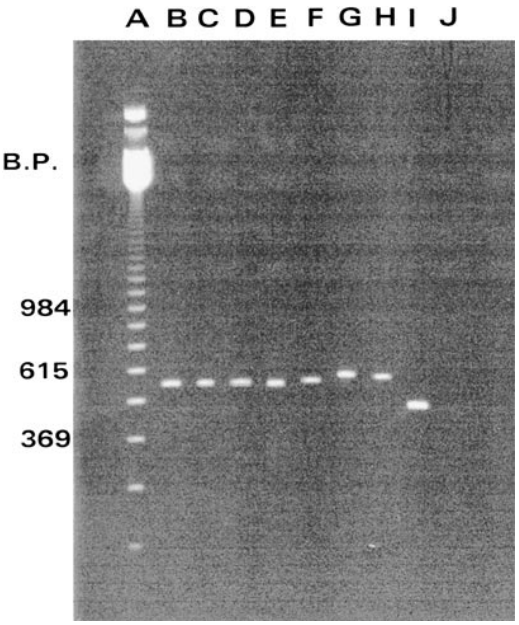

FIGURE 1. Ethidium bromide stained agarose gel of the ITS-2 region amplified by PCR using universal primers. The bands indicate amplified DNA products from *Parelaphostrongylus tenuis* (576 bp), *Parelaphostrongylus andersoni* (566 bp), *Parelaphostrongylus odocoilei* (571 bp), *Elaphostrongylus cervi* (597 bp), *Elaphostrongylus alces* (581 bp), *Umingmakstrongylus pallikuukensis* (445 bp). Lanes A = molecular weight markers in base pairs (bp), B = *P. tenuis* L1, C = P. tenuis L3, D = P. tenuis adult, E = *P. andersoni* L1, F = *P. odocoilei* adult, G = *E. cervi* L1, H = *E. alces* L3, I = *U. pallikuukensis* L1, J = control without DNA template.

were amplified from *Dictyocaulus* and a single 725 bp band from *U. pallikuukensis.* No amplification bands were seen with any of the *Parelaphostrongylus* spp. or other nematodes tested with primer NC2 and ECP1R. Primers PTP1 and PTP2 amplified *P. tenuis, P. andersoni, P. odocoilei, E. alces* and *U. pallikuukensis* (Fig. 3). *P. tenuis* and *P. andersoni* each produced a similar single band (228 and 226 bp), *P. odocoilei* had a band of 231 bp, *E. alces* a 216 bp band and *U. pallikuukensis* a band of 194 bp. Primers ECP1 and PTP2 amplified only *P. tenuis* and *P. odocoilei* (Fig. 4); a single band of 345 bp was produced with *P. tenuis,* whereas *P. odocoilei* generated a 337 bp band.

The results reported in this study were

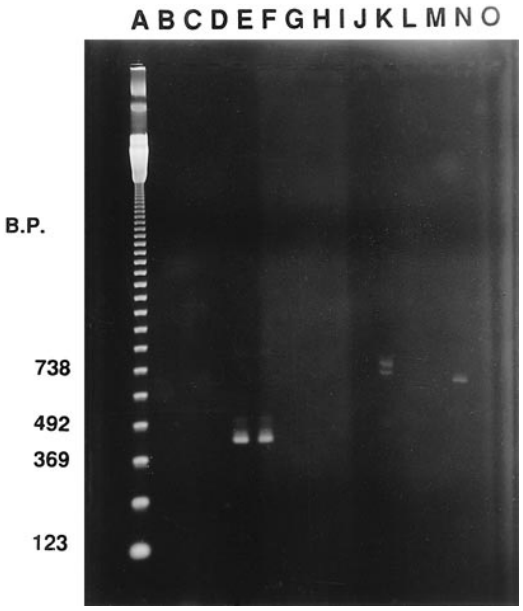

FIGURE 2. Ethidium bromide stained agarose gel of PCR products amplified using primers NC2 and ECP1R. Bands indicate the presence of products from *Elaphostrongylus cervi* (431, 512 bp), *Elaphostrongylus rangiferi* (431, 512 bp), *Dictyocaulus* sp. (782, 728 bp) and *Umingmakstrongylus pallikuukensis* (699 bp). Lanes A = molecular weight markers in base pairs (bp), B = *Parelaphostrongylus tenuis* L1, C = *Parelaphostrongylus andersoni* adult, D = *Parelaphostrongylus odocoilei* L1, E = *E. cervi* L1, F = *E. rangiferi* L1, G = *Elaphostrongylus alces* L1, H = Baermann filtrate, I = snail tissue, J = deer tissue, K = *Dictyocaulus* sp. adult, L = *Haemonchus contortus* L3, M = *Muellerius capillaris* L1, N = *U. pallikuukensis* L1, O = control without DNA template.

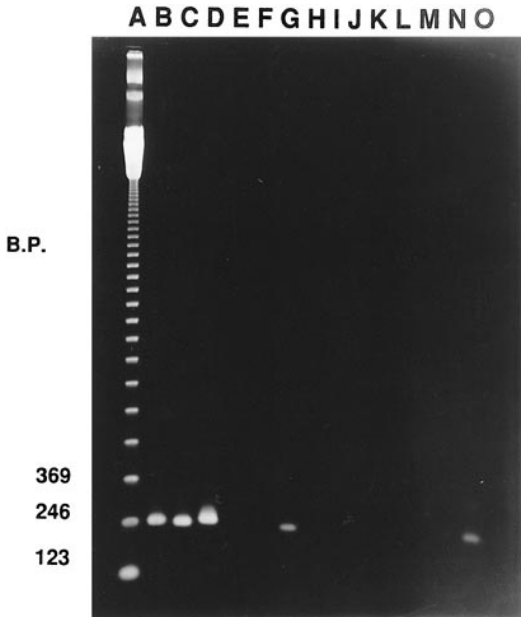

FIGURE 3. Agarose gel electrophoresis of PCR products amplified using primers PTP1 and PTP2. Products appear as bands and were amplified from *Parelaphostrongylus tenuis* (246 bp), *Parelaphostrongylus andersoni* (246 bp), *Parelaphostrongylus odocoilei* (260 bp), *Elaphostrongylus alces* (237 bp), and *Umingmakstrongylus pallikuukensis* (220 bp). Lanes A = molecular weight markers in base pairs (bp), B = *P. tenuis* L1, C = *P. andersoni* adult, D = *P. odocoilei* L1, E = *Elaphostrongylus cervi* L1, F = *Elaphostrongylus rangiferi* L1, G = *E. alces* L1, H = Baermann filtrate, I = snail tissue, J = deer tissue, K = *Dictyocaulus* sp. adult, L = *Haemonchus contortus* L3, M = *Muellerius capillaris* L1, N = *U. pallikuukensis* L1, O = control without DNA template.

consistently achieved in at least three repeats of each PCR assay described, and the results did not vary when multiple samples of L1, L3 or adult stages of various nematodes (see Table 1) were used as a source of DNA for the PCR reaction. DNA extracted from control fecal Baermann filtrate, snails and red deer were not amplified in any of the PCR assays.

DISCUSSION

The use of DNA to differentiate morphologically indistinguishable larvae of all of the known species of elaphostrongylines has been accomplished for the first time in this study. Although it has been shown previously that the ITS-2 region of the rDNA can be used to identify various species of nematode larvae (Hoste et al., 1995; Gasser and Monti, 1997), this is the first report of genetic differentiation of protostrongylid nematodes. Genetic comparison using only the ITS-2 region has been sufficient for species differentiation within various groups of organisms (Wesson et al., 1993). This is possible, partly because of the low intraspecies variation within the ITS-2, and any differences within groups of organisms are representative of species level differences. Similar characteristics were observed amongst the protostrongylids investigated in this study.

Using universal primers, the two genera

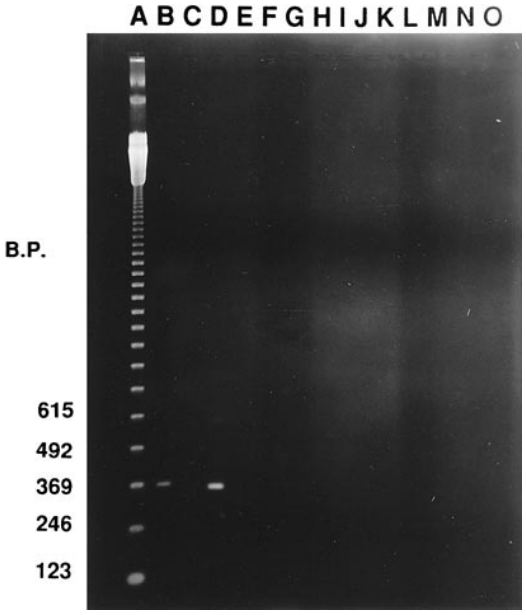

FIGURE 4. Agarose gel electrophoresis of PCR products using primers ECP1 and PTP2. Bands indicate amplified DNA from *Parelaphostrongylus tenuis* (369 bp), and *Parelaphostrongylus odocoilei* (361, 603 bp). Lanes A = molecular weight markers in base pairs (bp), B = *P. tenuis* L1, C = *Parelaphostrongylus andersoni* adult, D = *P. odocoilei* L1, E = *Elaphostrongylus cervi* L1, F = *Elaphostrongylus rangiferi* L1, G = *Elaphostrongylus alces* L1, H = Baermann filtrate, I = snail tissue, J = deer tissue, K = *Dictyocaulus* sp. adult, L = *Haemonchus contortus* L3, M = *Muellerius capillaris* L1, N = *Umingmakstrongylus pallikuukensis* L1, O = control without DNA template.

within the subfamily Elaphostrongylinae, were clearly separated by their ITS-2 region lengths. This genetic distinction between the two genera confirms the traditional classification based on details of the male genitalia and the former geographic separation of these parasites between the Old and New Worlds (Anderson, 1992; Carreno and Lankester, 1993). It would be interesting to compare the nucleotide lengths of the ITS-2 of other protostrongylids from these two geographic regions and elsewhere to determine patterns which may or may not be consistent with present classification schemes at the genus level. Amplified products from the universal primers suggest that these primers are unsuitable to distinguish individual species within the genera *Elaphostrongulus* and *Parelaphostrongylus.*

Sequence data generated from the six species of elaphostrongylines provided the basis for designing PCR primers. The availability of only incomplete sequence data of the ITS-2 of these parasites severely limited the potential for designing species-specific primers. The universal primers for the ITS-2 and the four custom-designed primers were tried in various combinations to develop a PCR assay that could serve to distinguish any of the elaphostrongyline parasites. There was not sufficient nucleotide heterogeneity among species to distinguish all of the elaphostrongylines tested with one set of primers or to use a unique set of primers for each species of elaphostrongyline. Nevertheless, all parasites tested were amplified by at least one set of primers and most nematodes could be differentiated by one or two PCR amplifications. The generation of a second minor DNA band by primer set NC2/ECP1R (Fig. 2) occurred when some parasites were tested. The appearance of this second band does not affect the reliability of the results, as in all cases, conclusions were based on the presence or absence of the dominant primary band which was unaffected by the second band. Furthermore, this primer set is not required for differentiating among the three species of *Parelaphostrongylus.* Multiple bands have been reported previously when ITS-2-based primers were used to amplify other nematodes (Gasser and Monti, 1997; Gasser et al., 1997). The presence of secondary bands may be due to an over-abundance of template DNA or to heteroduplex formation. Reducing the amount of DNA used in the PCR assay or slightly increasing the annealing temperature to avoid any misamplification should eliminate secondary bands.

Both *E. cervi* and *E. rangiferi* were amplified with only a single set of primers and were discernible from other nematodes, but not from each other. It is interesting

to note that *E. alces,* the only other member of the genus, was not amplified with this primer set, but instead by the same set that amplified all *Parelaphostrongylus* spp. and *U. pallikuukensis.* The lengths of DNA amplified from this region were indistinguishable for *P. tenuis* and *P. andersoni,* but were useful for separating *E. alces* and *P. odocoilei* from the other nematodes. This and one other primer set independently produced fragments from *U. pallikuukensis* and could be used to identify this parasite.

Using the single primer set ECP1/PTP2, the L1's of all three species of *Parelaphostrongylus* were distinguishable. A single fragment (345 bp) was amplified from *P. tenuis,* and a 337 bp fragment was generated from *P. odocoilei,* but no products were produced from *P. andersoni.* The absence of a DNA product when dorsal-spined larvae of a *Parelaphostrongylus* sp. is PCR assayed with this primer set indicates the presence of *P. andersoni.* This can be confirmed by using primer set PTP1/PTP2 to produce a single 226 bp fragment.

For most practical purposes, using this test to identify elaphostrongyline larvae in cervid feces is simplified somewhat by existing knowledge of the geographic distribution of the six recognized species. Concerning samples from much of North America, only the three *Parelaphostrongylus* spp. have to be separated using two sets of primers. An exception is in Newfoundland where caribou are known to have both *P. andersoni* and *E. rangiferi,* in which case the universal primer set will suffice. In Eurasia and Fennoscandinavia, three *Elaphostrongylus* spp. are thought to occur and the one primer set so far available only distinguishes *E. alces* from the other two species.

Caution is warranted, however, because of uncertainties regarding the complete geographic distribution of some species. For example, it has been suggested that *P. andersoni* may have a circumpolar distribution and occur in reindeer of Eurasia as well as in caribou and white-tailed deer across North America (Lankester and Hauta, 1989). It must also be confirmed that *E. rangiferi* has not spread with caribou from Newfoundland to mainland Canada (Lankester and Fong, 1989). Nonetheless, the suite of tests described here makes it practical for the first time to investigate these possibilities. It also can be used to investigate the possible existence of different genotypes of recognized elaphostrongyline species. For example, the greater pathogenicity of the isolates of *E. cervi* in farmed cervids in central and eastern Asia may be attributable to strain differences (Anderson, 1992). Biotype differences may also exist in *P. odocoilei* (Pybus et al., 1984) and in *P. andersoni* (unpubl. data) that can now be investigated using DNA technology.

It is possible that this PCR assay may be useful in identifying infections in cervids that harbor more than one species of elaphostrongylines and for investigating possible parasite interaction. In North America, wild white-tailed deer may have either *P. tenuis* or *P. andersoni,* and caribou may have either *P. andersoni* or *E. rangiferi.* Although mixed infections are possible, the extent to which they occur in nature has yet to be determined. It has been suggested, for example, that *P. andersoni* may exclude *P. tenuis* from infecting white-tailed deer in the extreme southeastern United States through cross-immunity (Lankester and Hauta, 1989; Davidson et al., 1996). Cross-infections of *Elaphostrongylus* spp. between their respective usual hosts in Fennoscandinavia are possible experimentally (Halvorsen et al., 1989; Stéen et al., 1997) and an investigation of their frequency in the wild is now possible.

In North America, this PCR assay would be of particular value to wildlife managers, clinicians and animal health regulators in identifying animals infected with pathogenic elaphostrongylines. White-tailed deer with patent *P. tenuis* infections could be identified and managed accordingly to limit the spread or translocation of the

parasite. It should also be possible to use this assay to confirm the cause of neurologic signs in wild moose with *P. tenuis,* but only in those animals that survive infection long enough to pass larvae in their feces. Wapiti can develop patent infections of *P. tenuis* and are capable of spreading the infection; individuals may or may not show neurologic signs (Samuel et al., 1992). A diagnosis of cerebrospinal elaphostrongylosis (CSE) in caribou caused by *E. rangiferi* can be confirmed using this test by identifying larvae in feces. Dorsal-spined larvae found in imported red deer and wapiti stock can be screened for patent infections of *Elaphostrongylus* spp. which is pathogenic to native North American cervids (Gajadhar and Tessaro, 1995).

A differential PCR diagnosis for other species of protostrongylids may be necessary to increase the utility of the test in particular areas. In North America, principal concerns are with *Varestrongylus alpenae, Protostrongylus colburni* and *Orthostrongylus macroti* in cervids and *Muellerius* and *Protostrongylus* spp. in ovids. Similarly in Eurasia, *Varestrongylus* spp. (Synonym: *Bicaulus*) commonly occur with elaphostrongylines in cervids. Depending on the known presence of particular elaphostrongylines, the use of primer sets tested here will confirm the presence or absence of the genera *Muellerius* and *Dictyocaulus.* The genus *Varestrongylus* presently can be distinguished microscopically from elaphostrongylines by its shorter length and from *Protostrongylus* spp. by the absence of a dorsal spine. Nevertheless, diagnostic efficiency could be improved in future by using additional nucleotide sequence data for modified or newly designed primers.

The primers described in this study were used successfully to identify protostrongylid nematodes regardless of the developmental stage of the parasite. This allows for the application of the primers in various types of studies and applications. The identification of protostrongylid species using L1's recovered from the feces of cervids or L3's recovered from mollusc intermediate hosts would greatly facilitate epidemiological studies and various field investigations aimed at controlling these parasites and preventing disease. As well, genetic characterization by species-specific primers should help to confirm or revise the classification within the elaphostrongylines and closely related protostrongylids.

The results reported in this paper represent a major advancement in the identification of elaphostrongyline nematodes. However, caution should be used in applying these primers for routine testing. Field validation involving elaphostrongyline parasites of various hosts in different geographic regions should be performed to ensure consistent results, and the utility of these primers on samples containing mixed species should be confirmed. Some refinements of the PCR primers or conditions may be required. Although PCR technology has become a rather routine research tool, its application in diagnostic tests requires that PCR assays be conducted in properly equipped laboratories by experienced personnel and that appropriate controls and other quality assurance measures are used.

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