MOLECULAR IDENTIFICATION AND PREVALENCE OF DICTYOCAULUS SPP. (TRICHOSTRONGYLOIDEA: DICTYOCAULIDAE) IN SWEDISH SEMI-DOMESTIC AND FREE-LIVING CERVIDS

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MOLECULAR IDENTIFICATION AND PREVALENCE OF *DICTYOCAULUS* SPP. (TRICHOSTRONGYLOIDEA: DICTYOCAULIDAE) IN SWEDISH SEMI-DOMESTIC AND FREE-LIVING CERVIDS

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ABSTRACT: Lungs of 102 roe deer (*Capreolus capreolus*), 136 moose (*Alces alces*), 68 fallow deer (*Dama dama*), and six red deer (*Cervus elaphus*) were examined during hunting seasons from 16 September 1997 to 1 March 2000. The aim was to determine the species composition and prevalence of *Dictyocaulus* lungworms in these hosts in Sweden. Worms were identified following polymerase chain reaction (PCR) amplification of the internal transcribed spacer of ribosomal DNA (ITS2), followed by hybridization with four species-specific oligonucleotides. In addition, 50 lungworms from five reindeer (*Rangifer tarandus*) from Norway were similarly analyzed. A total of 399 worms were recovered and analyzed representing a range of 29–128 worms per host species. All specimens from roe deer were identified as *Dictyocaulus capreolus,* whereas those from red deer and reindeer were identical with *D. eckerti.* From moose, 73 (81.1%) of the worms were identified as *D. capreolus* whereas 17 (18.9%) were *D. eckerti.* The ITS2 sequence of fallow deer lungworms differed significantly when compared with the ITS2 of *D. viviparus, D. capreolus,* and *D. eckerti.* This indicated that fallow deer in Sweden may be infected with a new genotype of *Dictyocaulus* spp. Consequently, a specific probe designed for the ITS2 from this *Dictyocaulus* sp. hybridized exclusively with samples from lungworms of fallow deer. Interestingly, no *D. viviparus* were found in any of these hosts. The prevalence of infection in each host was as follows: *D. capreolus* in roe deer (14.7%) and moose (10.6%); *D. eckerti* in moose (0.7%) and red deer (33.3%); and *Dictyocaulus* sp. in fallow deer (10.3%). Regardless of lungworm species, the overall prevalence of *Dictyocaulus* spp. in these hosts was 12.2%. Prevalence between male and female animals and among the different age groups did not differ significantly. Finally an enzyme linked immunosorbent assay (ELISA) specific for patent *D. viviparus* infection in cattle was utilized to analyze lung tissue fluids from infected animals. All samples from roe deer, red deer, and fallow deer were negative in the ELISA. However, three out of twelve (25%) samples from moose and 17 of 40 (43%) samples from cattle were positive. This indicated that moose anti-*D. capreolus* antibodies recognized the *D. viviparus* antigen and that anti-cattle immunoglobulin cross-reacted with moose antibodies.

Key words: Cervids, lungworms, *Dictyocaulus* spp., ITS2, molecular identification, ELISA, Sweden.

INTRODUCTION

Populations of wild ruminants in Sweden are composed mainly of roe deer (*Capreolus capreolus*) and moose (*Alces alces*) with estimated numbers of about 1.2–1.6 million and 300,000–400,000 animals, respectively (Cederlund and Markgren, 1987). These cervids are distributed throughout the country except for moose, which are not found on the island of Gotland and are also absent in the most alpine region in northern Sweden. In addition there are substantial numbers of fallow deer (*Dama dama*), red deer (*Cervus elaphus*), and reindeer (*Rangifer tarandus*) in the country. The former two species are free-ranging locally, but most animals are raised in fenced pastures under farmed conditions. Reindeer are only found as semi-domestic herds managed by the Sami people in the northern part of the Scandinavian peninsula.

Nematodes of the genus *Dictyocaulus* cause parasitic bronchitis in a wide range

of wild and semi-domesticated ruminants. Calves in temperate regions are commonly afflicted by the bovine lungworm *D. viviparus* during their first-grazing season (Kassai, 1999). Thus, there has been speculation of the role of wild ruminants as potential carriers for this parasite. For example, Nilsson (1971) suggested that roe deer might act as a reservoir of *D. viviparus* although a prevalence of only 2.9–4.0% was found in Swedish roe deer that were examined between 1966–68. However, this idea has recently been refuted because no *D. viviparus* was identified among 49 individual lungworms from eight naturally infected roe deer (Divina et al., 2000). In addition, lungworms from roe deer did not reach patency nor were any immature worms found in the lungs at necropsy following experimental inoculations of calves with three doses of roe deer-derived third stage larvae (Divina and Höglund, 2002).

Still, *D. viviparus* has been recorded from a range of other hosts besides cattle in the Nordic countries. Host records include various wildlife ruminants such as roe deer (Nilsson, 1971), reindeer (Christenssen and Rehbinder, 1975; Kummeneje, 1977) and fallow deer (Guildal, 1962). According to the latest revision by Gibbons and Khalil (1988), the genus *Dictyocaulus* contains six species of which cattle are regarded as the primary hosts for *D. viviparus*, and cervids as primary hosts for *D. eckerti*, which originally was described from reindeer in Russia (Skrjabin et al., 1954). However, morphology has limited use in specific identification of *Dictyocaulus* spp. from cattle and wild cervids because of overlap in characteristics previously considered to be species specific (Divina et al., 2000).

In recent years, molecular studies showed that lungworms from fallow deer are genetically distinct from other species in the genus (Epe et al., 1996, 1997; Schnieder et al., 1996; von Samson-Himmelstjerna et al., 1997). Furthermore, a novel genotype of *Dictyocaulus* was identified as a result of a nucleotide sequence analysis of second internal transcribed spacer (ITS2) from lungworms of wild cervids in Sweden (Höglund et al., 1999) and then described as *D. capreolus* (Gibbons and Höglund, 2002). Subsequently, species-specific oligonucleotide probes were applied and it was shown that Swedish cattle were exclusively infected with *D. viviparus*, whereas moose was infected with a mixture of *D. eckerti* and *D. capreolus*. Interestingly, we only detected the latter species in Swedish roe deer (Divina et al., 2000).

The current study was undertaken to further determine the species composition of *Dictyocaulus* lungworms and their prevalence in the most common wild and semi-domestic ruminant hosts in Sweden.

MATERIALS AND METHODS

Collection and identification of lungworms

During consecutive hunting seasons between 16 September 1997 to 1 March 2000 (Table 1), local hunters and veterinarians submitted lungs of roe deer, moose, fallow deer, and red deer. Lungs were mainly from central and southern Sweden (Fig. 1). They were examined for lungworms as described earlier (Höglund et al., 1999; Divina et al., 2000). Total DNA was extracted from frozen individual lungworms using a QIAmp Tissue Kit (QIAGEN, Hilden, Germany). DNA was also extracted from 50 ethanol-preserved lungworms from five reindeer in Tromsö, Norway received in 1999. Prior to DNA extraction from these samples, they were flushed in distilled water for 10 min. Internal transcribed spacer was amplified from DNA samples by polymerase chain reaction (PCR). For PCR, 50 μl volume reactions were set up for each sample containing 10 mM Tris (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs (Amersham Biosciences, Uppsala, Sweden), 1 μM of forward and reverse primer, respectively, 1 unit AmpliTaq DNA polymerase (Applied Biosystems, Foster City, California, USA) and 3 μl of template DNA sample. The thermocycler settings included an initial denaturation step for 2 min at 94 C followed by 45 sec at 94 C, 20 sec at 55 C for the annealing step and 30 sec of extension at 72 C. After 30 cycles, the last extension was prolonged for 5 min at 72 C.

After PCR amplification, each amplicon was probed with species-specific digoxigenin-la-

TABLE 1. Molecular probing of polymerase chain reaction (PCR)-amplified *Dictyocaulus* spp. Internal transcribed spacer (ITS2) region with digoxigenin-labeled oligonucleotides using the filter hybridization assay.

Host	Number of probed PCR-positive samples	Probe and number of positive hybridizations[a]			
		OP 108 (D. viv)	OP 109 (D. eck)	OP 110 (D. cap)	OP 220 (D. sp)
Roe deer	102	0	0	102	0
Moose	90	0	17	73	0
Fallow deer	29	0	0	0	29
Red deer	128	0	128	0	0
Reindeer	50	0	50	0	0
Total	399	0	195	175	29

[a] D. viv=*Dictyocaulus viviparus*; D. eck=*Dictyocaulus eckerti*; D. cap=*Dictyocaulus capreolus*; D. sp=new *Dictyocaulus* sp.

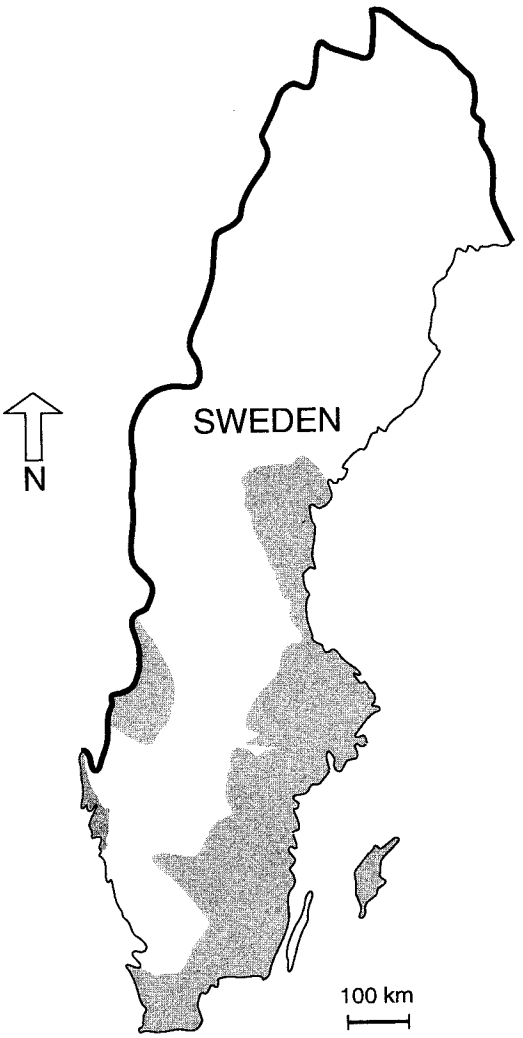

FIGURE 1. Map of Sweden; shaded areas show where lung samples of Swedish free-ranging and semi-domestic cervids were collected.

belled oligonucleotides probes (Table 2) using filter hybridization and chemiluminenscence detection procedures previously described (Divina et al., 2000).

DNA sequencing

Internal transcribed spacer amplicons were either sequenced directly or after cloning into the pGEM-T vector (Promega, Madison, Wisconsin, USA) as previously described (Divina et al., 2000). BigDye chemistry (Applied Biosystems) was used for the DNA sequencing reactions and the samples were analyzed on ABI377 DNA sequencer (Applied Biosystems).

Enzyme-linked immunosorbent assay (ELISA)

Tissue fluids from infected lungs were collected during gross examination, or 50 g of lung tissue was obtained and stored overnight in 50 ml of phosphate buffered or normal saline solution at about 4 C. Tissue fluid was collected into centrifuge test tubes and centrifuged at 3,000 rpm for 5 min, supernatant was collected and stored at about −20 C until analyzed by an ELISA kit (Ceditest®, ID-DLO, Lelystad, The Netherlands) designed for the serologic detection of patent *D. viviparus* infection in cattle. Similar samples collected from 40 lungworm infected cattle lungs from various abattoirs in central and southern Sweden were also included in the test for comparison. Results with a seropositivity above 15% in the Ceditest® are positive (Cornelissen et al., 1997).

Statistical evaluation

Prevalence of infection in each host species and distribution of lungworms by host age and sex were determined. Frequency distribution of infections based on sex and age of the host was analyzed using the G-test for independence (Sokal and Rohlf, 1973). The Fisher's exact test (Epi Info v. 6.04b, Centers for Disease

TABLE 2. Species-specific digoxigenin-labeled oligonucleotide probes (OP) used in the molecular identification of *Dictyocaulus* species in Sweden.

Nematode	Oligonucleotide probe sequence	Code
D. viviparus	5′-GAAGACGATATAAGGCAG-3′	OP 108
D. eckerti	5′-TAGCAGTACACATACATA-3′	OP 109
D. capreolus	5′-TAAGAACGGCGGTAATAT-3′	OP 110
Dictyocaulus sp.	5′-CGGATAGCATACATATGTGC-3′	OP 220

Control and Prevention, Atlanta, Georgia, USA) was used to analyze the ELISA result. Statistical tests were considered significant at the $P \leq 0.05$ level.

RESULTS

The number of probed PCR-amplified ITS2 samples that were positive after hybridization is shown in Table 1. The *D. eckerti* probe hybridized with all red deer and reindeer ITS2 samples, whereas the *D. capreolus* probe reacted with all samples from roe deer. However, both species occurred in moose where 17 (18.9%) and 73 (81.1%) of the probed lungworms were identified as *D. capreolus* and *D. eckerti*, respectively. However, only one moose of 14 harbored a mixed infection of *D. capreolus* and *D. eckerti*. Of the 399 worms that were analyzed no *D. viviparus* was found in any of the hosts examined.

None of the original set of three probes reacted with the PCR samples from the worms of fallow deer. We therefore sequenced the ITS2 amplicon from one worm. The sequence analysis suggested that the worms from fallow deer represented a new genotype of *Dictyocaulus* sp. Using the direct sequencing approach we could not completely resolve the ITS2 sequence, as there appeared to be regions of sequence heterogeneity. This observation was confirmed after cloning the ITS2 fragment from a single fallow deer lungworm and sequencing five individual inserts. Two short stretches of sequence variation were identified. In the first, the number of ATGT repeats varied and in the second region the sequence ATTATGCT occurred as tandem repeat in three of the clones. More importantly was the observation that the ITS2 sequence from fallow deer lungworm differed significantly from ITS2 of lungworms from the other hosts. With the aid of the sequence data we designed a new probe (OP220) specific for ITS2 of *Dictyocaulus* from fallow deer. In the subsequent analysis OP220 hybridized exclusively with ITS2 samples from all fallow deer lungworms.

All lung and tissue fluids from lungworm infected roe deer, red deer, and fallow deer were negative in the ELISA. However, three of 12 (25%) samples from moose were positive with 19–25% seropositivity. In cattle, 17 of 40 (43%) samples were positive with 17–100% seropositivity. The probability of correctly identifying true positives in the two species did not differ $P=0.49$.

The number of animals examined and prevalence of *Dictyocaulus* spp. in the different cervid hosts based on the hybridization assay are shown in Table 3. Regardless of lungworm species, total prevalence of infection was 12% of which about 9% could be attributed to *D. capreolus*-infection. Of six red deer examined two were infected. All five reindeer from Norway had *D. eckerti*. All were less than 1 yr old.

Table 4 presents the prevalence of *Dictyocaulus* spp. based on sex and age of the host. No significant differences were found between the number of infected male and female animals (G value$=4.81<X^2_{0.05[2]}=5.99$) or among the different age groups (G value$=6.27<X^2_{0.05[3]}=7.82$) in each host.

DISCUSSION

The lungworm *D. eckerti* was originally described from reindeer from the Tobolsk

TABLE 3. Prevalence of *Dictyocaulus* spp. in Swedish cervids.

Host	Number of lungs examined	Species	Number of positive animals	Prevalence (%)	Examination period
Roe deer	102	*D. capreolus*[a]	15	14.7	16 Sep 1997–1 Feb 2000
Moose	136	*D. capreolus*	13	10.6	
		D. eckerti	1[b]	0.7	13 Oct 1997–11 Jan 2000
Fallow deer	68	*Dictyocaulus* sp.	7		1998–16 Feb 2000
Red deer	6	*D. eckerti*	2		4 Apr 1999–1 Mar 2000
Total	312		38	12.2	

[a] Sensu Gibbons and Höglund 2002.
[b] In mixed infection with *D. capreolus.*

tundra in 1931 (Skrjabin et al., 1954). Since then, this species has been reported in a range of other cervids both elsewhere in Asia and Europe (see Gibbons and Khalil, 1988; Jansen and Borgsteede, 1990; Bienioscheck et al., 1996). Divina et al. (2000) first reported occurrence of *D. eckerti* in Sweden in moose. We found this species in monospecific infections in red deer and reindeer as well as in mixed infections with *D. capreolus* in moose. With the exception of moose, *D. capreolus* was exclusively found in roe deer. It seems that the host range of *D. eckerti* is wider compared to *D. capreolus.* The locality where the *D. eckerti*-positive moose originated was from an area that also had a substantial population of wild red deer. The source of the *D. eckerti* infection in moose may be red deer rather than the other way around.

Dictyocaulus eckerti evolved in a subartic and cold climate (Skrjabin et al., 1954). Accordingly, this species is probably adapted to harsh climatic conditions where opportunities for larval transmission are less than optimal. This may explain why the host range of *D. eckerti* is wide, because it will increase opportunities for larval transmission and accordingly the survival of this parasite. In contrast, the ITS2 amplicons of lungworms from fallow deer only reacted with the *Dictyocaulus* sp. probe (OP220). This indicates that this host harbors a lungworm that is genetically distinct from both *D. eckerti* and *D. capreolus.* Sequence analysis of cloned amplified ITS2 region from the fallow deer lungworm and of the ITS2 from *D. eckerti* supported the hybridization data, even though the existence of individual clone variations reflected within species variations in the ITS2 region also was observed. The variation in the ITS2 sequences of fallow deer observed in this study was similar to that earlier reported for *D. eckerti* in moose (Divina et al., 2000). Taken together the present results further confirm the

TABLE 4. Prevalence (%) of *Dictyocaulus* spp. in Swedish cervids according to sex and age of host.

Sex and age of animal		Host and number of infected animal (%)[a]			
		Roe deer	Moose	Fallow deer	Red deer
Sex	Male	5 (4.9)	5 (3.7)	5 (7.4)	0
	Female	7 (6.7)	5 (3.7)	1 (1.5)	0
	ND[b]	3 (2.9)	3 (2.2)	0	2 (33.3)
Age	<1 year	11 (10.8)	6 (4.4)	5 (7.4)	1 (1.7)
	≥1 year	2 (2.0)	6 (4.4)	2 (2.9)	0
	ND	2 (2.0)	1 (0.7)	0	1 (1.7)

[a] (Number of infected animal/total number of animals examined) × 100.
[b] ND=not determined.

suggestion that cervids are afflicted with more than one species of *Dictyocaulus* (Höglund et al., 1999).

The evolutionary mechanisms responsible for the occurrence and persistence of at least three genetically distinct variants of *Dictyocaulus* lungworms among Swedish cervids are unknown. Thus the biogeography of various genotypes in the genus *Dictyocaulus* and how these are distributed in different hosts are unknown. Obviously, further studies are required before we can draw more definite conclusions and we have to take into account that host movements and/or artificial translocations may disturb the patterns observed.

Some cervids are susceptible to infection with *D. viviparus* under experimental condition. However, based on the present results it seems that natural cross-transmission are unlikely to occur in Sweden as shown by the complete absence of this species in all of the wildlife species examined. The higher prevalence obtained in the present study than that of Nilsson (1971) shows that lungworm infections in Swedish wildlife have increased since that time. There were about 200,000 roe deer in Sweden during the 1960s, so the population has increased 6–8 times. Our data support the earlier conclusion (Nilsson, 1971) that prevalence did not differ between male and female animals.

Cross-reacting antibodies to the *D. viviparus* antigen were detected in lung tissue fluids from moose indicating that the ELISA kit can also be used for detecting patent *D. capreolus* infection. However, the sensitivity of this serologic test was low. Only 43% of the tissue fluid samples from infected cattle lungs reacted in the ELISA when tissue fluids were analyzed instead of serum samples. The proportion of ELISA-positive samples was also much lower in moose compared to what was obtained in cattle. Consequently, despite cross-reactivity between antibodies to *D. capreolus* in moose and *D. viviparus* in cattle ELISA was not sensitive enough to be used for monitoring of lungworm infection in moose.

In conclusion, this study shows that Swedish cervids are infected by at least three genotypes of *Dictyocaulus* spp. lungworms, one of which (*D. capreolus*) is a newly described species (Gibbons and Höglund, 2002). In addition, a genetic variant of *Dictyocaulus* was identified that occurred as a mono-specific infection in fallow deer. It was genetically different in its ITS2 region from *D. eckerti,* which has been known to infect fallow deer in other parts of Europe. In light of these findings, there may be a need to review the taxonomy of the genus. We also further confirm absence of *D. viviparus* in cervids host indicating that they are not source of lungworm infection for cattle in Sweden. From our studies, we will like to suggest that previous reports of *D. viviparus* in Swedish wildlife are erroneous. This may also be true for other reports of *Dictyocaulus* in other countries (see for example: Mason, 1985; Pybus, 1990). Thus, the species composition of lungworm in wildlife in other countries should be examined using molecular methods as well. This would help clarify the complexity of lungworm evolution as well as the epidemiology of this genus of parasites.

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