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MENINGEAL WORM EVOKES A HETEROGENEOUS IMMUNE RESPONSE IN ELK

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ABSTRACT: Meningeal worm (*Parelaphostrongylus tenuis*) is a neurotropic nematode of ungulates in eastern North America. Lack of an effective diagnostic test increases the concern of translocating potentially infected ungulates into western North America, where *P. tenuis* does not occur naturally. In an attempt to identify serodiagnostic molecules, we determined (1) whether elk (*Cervus elaphus*) experimentally infected with *P. tenuis* produce antibodies against infective larvae or adult worms, and (2) if sera consistently recognize antigens that distinguish *P. tenuis* from a common nematode parasite of elk, the lungworm *Dictyocaulus viviparus*. Each of 10 elk were exposed to 15 or 300 infective *P. tenuis* larvae. Serum was collected (0, 41, and 83 days post-exposure and at necropsy) and monitored for antibodies using the enzyme-linked immunosorbent assay (ELISA) and immunoblot. When reactivity of sera with larval *P. tenuis* protein was compared (day 0 versus 83), ELISA values were significantly higher on day 83 for elk exposed to 15 or 300 parasites. Likewise, ELISA values using protein of adult *P. tenuis* were higher for elk exposed to 300 larvae. Immunoblots showed that sera from elk, with adult worms in the central nervous system, consistently recognized the 25–27, 28–30, and 34–36 kDa antigens of infective larvae after 83 days. However, many *D. viviparus* molecules were found to cross-react with antibodies formed against meningeal worm antigens. Use of adult worm proteins for serodiagnosis appears limited, because no protein was consistently recognized by sera collected from elk exposed to 15 larvae. We believe that development of a reliable diagnostic test for meningeal worm requires more research addressing cross-reactivity and detection of *P. tenuis* during the incubation stage.

Key words: Antigen cross-reactivity, *Cervus elaphus*, *Dictyocaulus viviparus*, elk, heterogeneous immune response, lungworm, meningeal worm.

INTRODUCTION

There is a need for reliable diagnosis of the meningeal worm *Parelaphostrongylus tenuis*, in certain ungulate hosts such as elk (*Cervus elaphus*). This is because (1) elk are susceptible to infection with meningeal worm and serious neurologic disease can result (Samuel et al., 1992); (2) some animals survive infection, show few or no clinical signs, and shed variable numbers of parasite larvae in their feces (Welch et al., 1991) and; (3) captive elk on game ranches from eastern North America, which could be infected with meningeal worm because these ranches are within the geographic distribution of the parasite, are currently translocated to game ranches in western North America, where *P. tenuis* does not occur.

Current diagnostic procedures for meningeal worm have met with limited or no success. Application of the Baermann technique to extract parasite larvae from feces is not reliable, even when feces are examined often (Welch et al., 1991). Attempts to diagnose *P. tenuis* by measuring total protein concentration and enzyme activity within the cerebrospinal fluid of domestic goats and white-tailed deer (*Odocoileus virginianus*), show inconclusive results (Drew et al., 1992).

Although the nature of acquired immunity to *P. tenuis* is not clear, it results in production of serum antibodies. Dew et al. (1992) and Duffy et al. (1993), using an enzyme-linked immunosorbent assay (ELISA) with antigen extracts from adult worms, found that parelaphostrongylosis is accompanied by seroconversion in goats and ≥34-wk-old white-tailed deer. Similarly, both goats and deer developed a significant antibody response in the cerebrospinal fluid (Dew et al., 1992). However, the application of this technique cannot un-

equivocally show that elk are free of meningeal worm because of the inability to detect antibodies present during the prepatent period. Moreover, since a diagnostic test for *P. tenuis* needs to be applied to elk that may have concurrent parasitic infections or immunity from previous infections, future research is required to determine if epitopes from common parasites of elk are cross-reactive with *P. tenuis* antigens.

In efforts to address cross-reactivity, Neumann et al. (1994) identified seven antigens from adult worms and two antigens from infective larvae that distinguish *P. tenuis* from two other species of nematodes. That study lead to the current study, to determine if elk, experimentally infected with few (15) or many (300) infective larvae of *P. tenuis,* produce antibodies specific for antigens of adult worms or infective larvae. A subsequent aim was to determine if sera collected between day 0 of infection and death of infected elk, consistently recognize antigens that distinguish *P. tenuis* from another common parasite of elk, the lungworm (*Dictyocaulus viviparus*).

MATERIALS AND METHODS

Parasites

Snails, *Triodopsis multilineata,* were reared (Gray et al., 1985a) and exposed to first-stage larvae of *P. tenuis* (original source, white-tailed deer, Rachelwood Wildlife Research Preserve, Pennsylvania, USA) (Gray et al., 1985b). Approximately 60 days after exposure, third-stage larvae were recovered from the snails by digestion using an artificial digest solution (1% pepsin, 0.75% NaCl, 0.02 M HCl) at 37 C for 2 hr (Neumann et al., 1994). Larvae were separated from snail tissue, washed in phosphate buffered saline, and used to infect the ungulate host or for immunological analysis of parasite antigens. Antigen extracts of adult worms were prepared from *P. tenuis* and *D. viviparus* nematodes that were obtained from experimentally infected white-tailed deer or elk.

Hosts

Use of animals in this study was approved by the University of Alberta Biosciences Animal Care Committee (protocol #89803). Ten elk, approximately 7-mo-old, acquired from local sources were maintained at the Ellerslie Research Station (University of Alberta, Edmonton, Alberta, Canada). Elk were housed in an outdoor cement enclosure with pelleted alfalfa-based ration and grass hay and water provided *ad libitum.* Prior to infection with *P. tenuis*, all elk were without clinical signs of disease.

Animals were randomly divided into two groups of five. Using the method of Samuel et al. (1992), each elk in one group was exposed to 15 infective larvae of *P. tenuis* while each elk in the other group was exposed to 300 larvae. A modified Baermann technique (Welch et al., 1991) was used to determine the number of nematode larvae present in feces that were collected weekly (1 wk before infection and concluded on day of necropsy). Feces were examined for larvae of *P. tenuis* and *D. viviparus* weekly from day −7 to day 41 of infection, every 3 days from day 41 to day 104, then daily thereafter. Fecal analysis and necropsy results for *P. tenuis* included in this study was reported as part of another study that addresses survival of elk and implications of translocation (Samuel et al., 1992).

Elk were anesthetized by intramuscular injection of approximately 0.5 mg/kg xylazine hydrochloride (Haver-Lockhart Laboratories, Shawnee, Kansas, USA) and bled on day 0 (negative serum), 41, and 83, and at necropsy. After allowing the whole blood to clot, it was centrifuged (1780 × *G*) and the supernatant collected. The serum was then stored at −80 C until needed.

Five elk with neurologic signs were anesthetized 60 to 111 days post-exposure (DPE) with xylazine hydrochloride as described above and killed with intravenous injection of 100 mg/kg sodium pentobarbital (M. T. C. Pharmaceuticals, Mississauga, Ontario, Canada) when they could not rise or stand unaided. The remaining animals were killed 104–154 DPE. As previously described, a detailed necropsy was performed on all elk. Particular attention was given to the lungs (i.e., palpitation for nodules, opening of airways, and isolating larvae from macerated lung tissue using the Baermann technique) (Samuel et al., 1992).

Antigen

Soluble extracts of larvae and adult worms were prepared as described (Neumann et al., 1994). The Pierce micro BCA protein assay reagent kit (Pierce, Rockford, Illinois, USA) was used for the spectrophotometric determination of parasite protein concentration.

ELISA

The ELISA was used for detection of antibodies against *P. tenuis* or *D. viviparus* in elk exposed to meningeal worm. Approximately 0.05 μg of *P. tenuis* (adult or larvae) or *D. viviparus* soluble protein was dispensed into the wells of a 96-well microtiter plate. Between all incubations, the wells were washed three times with Milli-Q-water and blocked for 10 min with blocking buffer (0.17 M H_3BO_3, 0.12 M NaCl, 0.05% Tween 20, 1 mM ethylenediaminetetraacetic acid, 0.025% bovine serum albumen, 0.05% NaN_3, pH 8.5). Three incubations followed (2 hr each at room temperature): elk serum (1:50), rabbit anti-elk antiserum diluted 1:100 (Neumann et al., 1994), and goat anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate diluted 1:1500 (Bio-Rad Life Sciences Division, Richmond, California, USA). Following the last wash, p-nitrophenyl phosphate solution was added to each well. Thereafter, optical densities were determined using a microplate autoreader (Bio-tek Instruments Inc., Winooskyi, Vermont, USA) set at 405 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Solubilized parasite extracts were separated by SDS-PAGE under reducing conditions as reported (Neumann et al., 1994). Briefly, 7.5 μg of protein from each sample was lyophilized and the precipitate was dissolved in sample buffer (59.5 mM Tris-HCl [pH 6.8], 5.7% SDS, 9.5% glycerine, 9.5% 2-β-mercaptoethanol, 0.02% bromophenol blue). The solution was heated at 95 C for 5 min and applied to the gel. The samples were electrophoretically separated on 1 mm 5% stacked 7.5–20% (or 5–15%) gradient SDS polyacrylamide gels.

Immunoblot analysis

After SDS-PAGE, the proteins were transferred to nitrocellulose membranes and immunoblotted according to the method of Towbin et al., (1979) (Towbin et al., 1979), with modifications. Proteins were electrophoretically transferred to membranes for 1 hr at 100 V in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Once the optimal concentration of antigen and antisera was determined by titration analysis, these variables were kept constant. The membranes were treated with the following reagents: (1) blocking buffer (150 mM NaCl, 25 mM Tris-HCl [pH 7.5], 1 mM NaN_3, 1% nonfat powdered milk, 0.05% Tween 20); (2) two Tris-buffered saline (20 mM Tris, 500 mM NaCl, 0.05% Tween 20 [pH 7.5]; TTBS) washes; (3) elk serum (1:100); (4) two TTBS washes; (5) rabbit anti-elk IgG diluted 1:200 (Neumann et al., 1994); (6) three washes in TTBS followed by two TBS (20 mM Tris, 500 mM NaCl [pH 7.5] washes; (7) alkaline phosphatase-labeled goat anti-rabbit IgG (Bio-Rad Life Sciences Division); (8) p-nitroblue tetrazolium salt and 5-bromo 4-chloro-3-indolyl phosphate in aqueous dimethyl-formamide (Bio-Rad Life Sciences Division); and (9) tap water. Molecular weights (approximated within a three kDa range) were calculated from standard curves obtained from the separation of high and low molecular weight standards (Bio-Rad Life Sciences Division).

Statistics

Two-way analysis of variance and the matched pairs t-test were used to analyze the optical densities obtained from spectrophotometric analysis (SuperANOVA, Abacus Concepts Inc., Berkeley, California, USA). Probability values of ≤5% were considered to be significant.

RESULTS

Nematode recovery

Meningeal worm and *D. viviparus* were the only nematodes found in the elk. Only two of five elk given 15 infective larvae of *P. tenuis* had adult meningeal worms within the central nervous system (CNS), whereas numerous nematodes (mean = 39, SD = 14) were present in the CNS of elk exposed to 300 larvae of *P. tenuis* (Table 1). Elk in the low-dose group that had meningeal worms showed no noticeable neurological signs. Conversely, elk given 300 larvae showed clinical signs of depression, disorientation, torticollis, and with posterior paralysis, usually within a few days of death.

Four of the ten elk acquired natural infections of *D. viviparus* prior to being submitted to our study (Table 1). *Dictyocaulus viviparus* larvae were recovered from 164 of 167 fecal samples from these four elk. The absence of lungworm in the remaining elk was confirmed by fecal analysis (0 of 313 fecal samples infected), examination of fluids collected from macerated lung tissue, and at necropsy.

TABLE 1. Nematodes recovered from elk calves exposed to 15 or 300 infective larvae of *Parelaphostrongylus tenuis*.[a]

	Dose and elk identification number									
	15 larvae					300 larvae				
	18	111	3	113	116	17	115	114	5	117
Meningeal worms recovered	3	2	0	0	0	57	53	31	28	28
Lungworms recovered[b]	Yes	No	Yes	No	No	Yes	No	No	Yes	No
Death DPE[c]	120	104	147	154	143	71	60	94	102	111

[a] Unpublished data of W.M. Samuel and M.J. Pybus or Samuel et al. (1992).
[b] Naturally infected.
[c] Days post-exposure.

ELISA

When reactivity of antisera with molecules of infective *P. tenuis* larvae was compared over time, ELISA values were significantly higher ($P < 0.05$) on DPE 83 compared to day 0 for elk exposed to 15 or 300 parasites (Table 2). Similarly, ELISA values using protein of adult *P. tenuis* were significantly higher ($P < 0.05$) on DPE 83 compared to day 0 for elk exposed to 300 parasites. When differences between day 0 and 83 were analyzed, ELISA values using *P. tenuis* adult or larval protein were not significantly different from sera of elk infected or uninfected with *D. viviparus*. No significant differences were observed in the antibody responses directed against molecules of adult *D. viviparus* when sera from DPE 83 were compared to those from day 0.

Immunoblot analyses

Molecules of third-stage larvae recognized by sera from elk infected with 15 or 300 nematodes were not unique to *P. tenuis*. All of the clearly recognized antigens were either cross-reactive with adult *D. viviparus* molecules (Fig. 1) or of the same molecular weight as those present in immunoblots incubated with serum collected on day 0 (Table 3). Sera collected at death from nine of 10 elk recognized the 34–36 kDa antigen of *P. tenuis* larvae. This antigen was also recognized by negative sera collected from four elk (3, 5, 115, and 117) (Table 3). However, the staining of these molecules was more intense on immunoblots reacted with sera collected 83 DPE and at death than those reacted with negative serum (Fig. 2).

Larval molecules of molecular weight

TABLE 2. ELISA values (optical densities) for serum collected from elk calves exposed to 15 or 300 infective larvae of *Parelaphostrongylus tenuis*.[a]

Antigen	DPE[b]	Dose	
		15 larvae	300 larvae
Larval *P. tenuis*	0	0.170 ± 0.055	0.141 ± 0.018
	83	0.213 ± 0.046[c]	0.248 ± 0.014[c]
Adult *P. tenuis*	0	0.173 ± 0.037	0.142 ± 0.027
	83	0.163 ± 0.023	0.163 ± 0.031[c]
Adult *Dictyocaulus viviparus*	0	0.116 ± 0.030	0.128 ± 0.024
	83	0.094 ± 0.012	0.134 ± 0.023

[a] Responses are expressed as the mean ELISA value ± standard deviation.
[b] Days post-exposure.
[c] $P < 0.05$ when values within the same dosage group are compared to day 0.

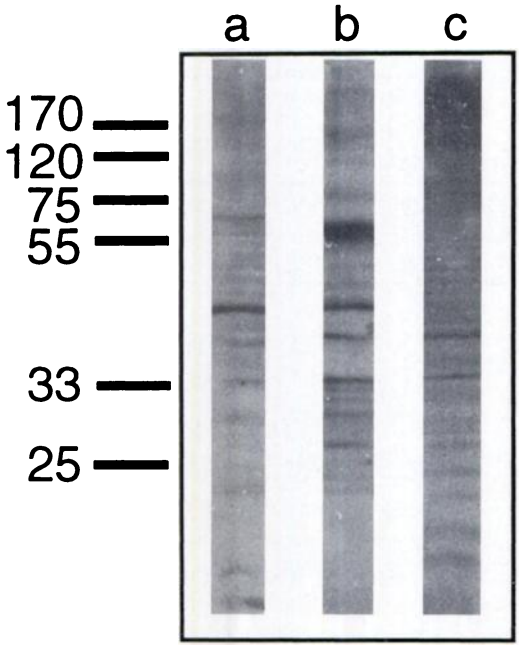

FIGURE 1. Immunoblot of *Parelaphostrongylus tenuis* third-stage larvae (lanes a and b) or *Dictyocaulus viviparus* adult antigens (lane c) probed with serum collected from elk 111. Serum in lane a was collected prior to exposure. Serum in lanes b and c were taken at the death of the animal.

25–27 kDa and 28–30 kDa were recognized by sera collected at death from seven of 10 elk. The samples that did not recognize these molecules were collected from elk (3, 113, and 116) that were exposed to 15 infective larvae but did not develop patent infections or had adults present in the CNS. Negative serum from elk 5 and 18 recognized the antigens with a molecular weight of 25–27 kDa and 28–30 kDa, respectively. Similarly, the staining intensity of these antigens was notably more intense on the immunoblots reacted with sera collected at death than those reacted with negative serum. In addition, when antigen recognition of sera (elk 18, 114, and 117) collected on day 0, 41, and 83 and at necropsy was analyzed by immunoblotting, it was evident that sera did not consistently recognize the 25–27, 28–30, and 34–36 kDa antigens until 83 DPE.

While many *D. viviparus* antigens were recognized by elk sera, the antibody reactivity with these antigens was inconsistent (Table 3). For example, serum collected from elk 3 (which only had lungworm at necropsy) did not recognize the 25–27, 28–30, and 34–36 kDa antigens of *D. viviparus*, while serum from elk (which only had *P. tenuis* at necropsy) recognized none, some, or all of these lungworm antigens. Interestingly, serum from elk 113 and 116 reacted with one or more of these antigens.

Similar to third-stage larval proteins, many adult-worm antigens of *P. tenuis* are shared with *D. viviparus* antigens. Although several antigens of *P. tenuis* adults were recognized by sera from the high dose group, only 32–34 kDa molecules were consistently recognized by the host immune response (data not shown). Sera collected day 0 and at necropsy from four of five elk (exposed to 15 larvae) recognized the 50–52, 53–55, and 56–58 kDa molecules of *P. tenuis* adults. However, the staining intensity of these antigens was darker only on the immunoblots reacted with serum collected at death from elk 111. Sera from elk in the low dose group, that were parasitized by the meningeal worm, strongly recognized the 29–31 kDa antigens of *P. tenuis* adults (Fig. 3).

DISCUSSION

In the natural environment, ungulates become infected with *P. tenuis* by ingesting terrestrial snails or slugs that contain infective third-stage larvae. As the gastropod is digested, larvae are released, penetrate the abomasum, begin to migrate to the spinal cord, and eventually locate as adults in the cranial cavity associated with the meninges (Anderson, 1963). It is reasonable to speculate that in the course of migration to the spinal cord, larval antigens are accessible to the immune system. Our results suggest that immunoassays using third-stage larvae protein have diagnostic potential, since a significant increase of ELISA values over time was observed in both groups. Moreover, sera collected from elk (parasitized by adult nematodes)

TABLE 3. Antigens recognized by ≥70% of the samples collected from elk exposed to 15 or 300 infective larvae of *Parelaphostrongylus tenuis*.

	Dose and elk identification number									
	15 larvae					300 larvae				
Molecular weight	3	18	111	113	116	5	17	114	115	117
25-27 kDa	-[a]	+[b]	+	-	-	+	+	+	+	+
28-30 kDa	-	+	+	-	-	+	+	+	+	+
34-36 kDa	+	+	+	+	-	+	+	+	+	+

a *P. tenuis* larval protein not recognized by elk serum.
b *P. tenuis* larval protein recognized by serum collected at death.
P. tenuis larval protein recognized by negative serum.
Dictyocaulus viviparus adult protein recognized by serum collected at death.
Recognition of both *P. tenuis* larval protein by negative serum and *D. viviparus* antigen by serum collected at death.

recognized the 25–27, 28–30, and 34–36 kDa molecules after 83 days. These observations are in agreement with Duffy et al. (1993) who showed that after 75 days there was seroconversion in deer exposed to 20 infective *P. tenuis* larvae (Duffy et al., 1993). These findings suggest that before wild ungulates are translocated, a quarantine period of at least 11 to 12 wk may be required to allow detection of disease present in the incubation stage.

When analyzed by one-dimensional SDS-PAGE, the 25–27, 28–30, and 34–36 kDa molecules of *P. tenuis* larvae also were recognized by negative serum. However, the staining intensity of these molecules was darker on those immunoblots reacted with sera collected 83 DPE and at death. These observations suggest that there is stronger recognition of these molecules as the infection progresses. Alternatively, there may be numerous molecules within these molecular weight ranges. Valuable data should emerge from future investigations that determine if these molecules have properties (e.g., isoelectric point) which make them antigenically unique to *P. tenuis*.

Within the literature there are contrasting reports of immunoglobulins produced against *P. tenuis* adults. For example, an ELISA using soluble antigen extract from adult worms demonstrated that meningeal worm infection is accompanied by a substantial increase of antibody in white-tailed deer fawns (>34-wk-old) (Duffy et al., 1993) and goats (Dew et al., 1992), but not in 9-wk-old white-tailed deer fawns (Dew et al., 1992). Our results suggest that elk infected with numerous worms evoke a humoral response against the adult meningeal worm. However, the value of adult-worm proteins for serodiagnosis is questionable, because no significant differences were observed in the low dose group (exposed to 15 larvae) when the ELISA values from DPE 83 were compared to those from day 0.

Both rabbit (Neumann et al., 1994) and elk sera reacted with 50–55, 32–34, and 29–31 kDa molecules of *P. tenuis* adults. However, these antigens were not consistently recognized by all elk sera. Recognition of these antigens by the immune system of elk may be associated with an antigenic threshold, since the 32–34 kDa antigens were only recognized by serum from the elk infected with 300 larvae. Because of inherent experimental variation, it is possible that the 32–34 kDa antigen,

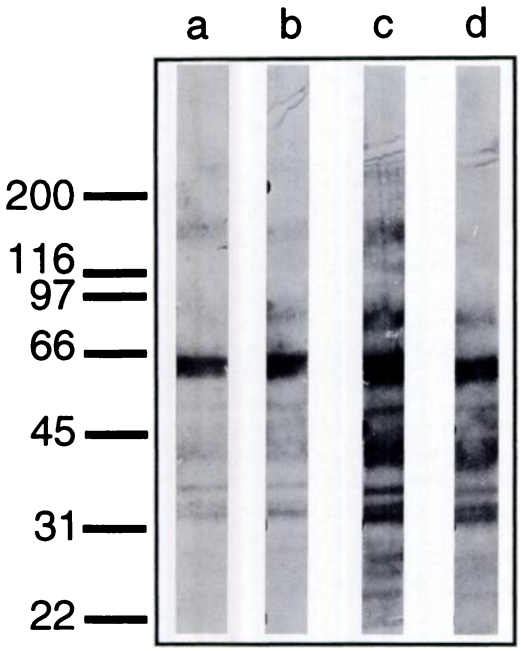

FIGURE 2. Immunoblot of *Parelaphostrongylus tenuis* third-stage larvae antigens probed with serum from elk 117. 'Lane a' serum collected prior to exposure. Lanes b and c sera collected 41 and 83 days after exposure, respectively. Lane d serum taken at the death of the animal.

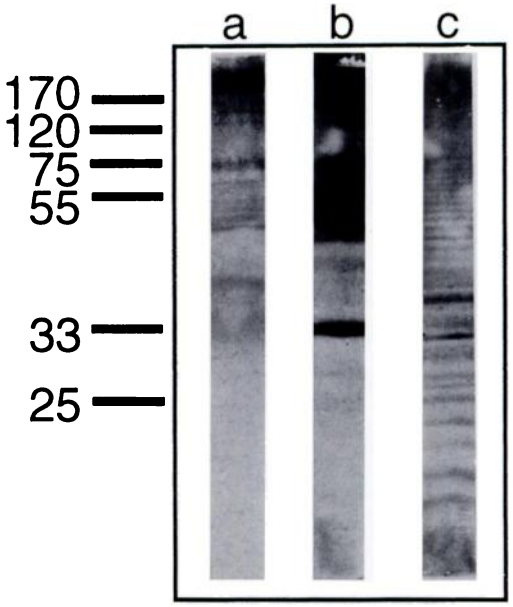

FIGURE 3. Immunoblot of *Parelaphostrongylus tenuis* (lanes a and b) or *Dictyocaulus viviparus* (lane c) adult antigens probed with serum collected from elk 111. Lane a serum collected prior to exposure. Lanes b and c serum taken at the death of the animal.

recognized by elk in the high dose group, is the same as the 29–31 kDa molecules recognized by sera from two elk that were exposed to 15 nematodes. These two elk differ from the others in the low dose group in that adult worms were found in the CNS at necropsy. Under these circumstances, it is difficult to conclude if these molecules may be of diagnostic value.

Serologic assays showed that several *D. viviparus* molecules are recognized by antibodies directed against *P. tenuis*. These observations are similar to that reported in rabbits immunized with *P. tenuis* adult worm or larval soluble extract (Neumann et al., 1994). In like manner, de Leeuw and Cornelissen (1991) reported that many antigens of *D. viviparus* adult worms are shared by parasites such as *Fasciola hepatica, Ostertagia ostertagi, Ascaris suum,* and other helminths (de Leeuw and Cornelissen, 1991). The later authors identified a 17 kDa antigen of *D. viviparus* that can be used to diagnose lungworm infections in cattle. Unfortunately, sera from only one of the four elk with *D. viviparus* in the respiratory tract in our study reacted with a 17 kDa molecule. Collectively, these findings emphasize that the full potential of a diagnostic test cannot be achieved until the inherent problems with cross-reactivity are fully defined. We believe that further studies are warranted to determine if antigens of third-stage larvae (25–27, 28–30, and 34–36 kDa) and adult meningeal worms (29–31 and 32–34 kDa) have properties that make them antigenically unique to *P. tenuis*.

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