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Source: Journal of Wildlife Diseases, 38(4) : 796-803

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-38.4.796

Journal of Wildlife Diseases, 38(4), 2002, pp. 796–803

DETECTION OF ANTI-*PARELAPHOSTRONGYLUS TENUIS* ANTIBODIES IN EXPERIMENTALLY INFECTED AND FREE-RANGING MOOSE (*ALCES ALCES*)

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ABSTRACT: Confirming *Parelaphostrongylus tenuis* infection in moose (*Alces alces*) and other susceptible hosts is difficult. An enzyme-linked immunosorbent assay (ELISA) was developed using the excretory-secretory (ES) products of third-stage *P. tenuis* larvae (ES-ELISA) and the test applied to serum samples obtained from seven moose calves (5–9.5 mo old) given infective larvae (L3) in doses approximating those likely to be received in nature (3–30 L3). Anti-*P. tenuis* immunoglobulin G antibodies were detected in all seven inoculated moose during the course of infection until the termination of experiment 61–243 days post-inoculation (DPI). Five animals tested between 16–25 DPI had significant antibody levels, while a sixth animal did not test positive until 46 DPI. The seventh animal was not tested until 199 DPI. Antibody levels remained elevated in all five animals that harbored adult worms at the termination of the experiment. Whereas, antibody levels showed a gradual decline in the two remaining animals, presumably because of death of worms, and antibodies were undetected in one animal at the time of necropsy. The other animal displayed an anamnestic increase in antibody level following a challenge inoculation of infective larvae. Terminal and peak optical density (OD) values detected by ES-ELISA strongly correlated with inoculation dose ($r=0.98$, $P=0.02$ and $r=0.95$, $P=0.04$, respectively) among animals harboring adult worms ($n=4$) but not significantly with the number of worms recovered postmortem (peak OD, $r=0.82$, $P=0.18$; terminal OD, $r=0.93$, $P=0.07$). Unlike the ES products, use of somatic antigens of the adult worm in ELISA did not provide satisfactory results. Antibodies to *P. tenuis* were detectable by ES-ELISA in two of 21 free-ranging moose from an enzootic area but not from any of 23 animals from a non-enzootic area. The ES-ELISA appears to be a useful test for assessing exposure of moose to *P. tenuis.*

Key words: *Alces alces*, ELISA, moose sickness, *Parelaphostrongylus tenuis*, serodiagnosis.

INTRODUCTION

Parelaphostrongylosis in moose (*Alces alces*) is caused by the neurotropic meningeal worm *Parelaphostrongylus tenuis* (Anderson, 1964). The disease has been known for many years in eastern North America where moose at the southern limits of their range overlap with infected white-tailed deer (WTD; *Odocoileus virginianus*), the parasite's definitive host (Anderson, 1965). Clinical signs of "moose sickness" range from severe motor and sensory impairment (paresis, ataxia, stumbling, circling, blindness) to less specific signs including lameness, fearlessness, depression, or weight loss (Lankester and Samuel, 1997).

Despite rather conspicuous neurologic signs shown by some infected moose, definitive diagnosis of parelaphostrongylosis is often difficult. Presently, infection can only be confirmed by finding developing or adult *P. tenuis* in either the spinal cord, the brain, the subdural space, or the cranial venous blood sinuses. Removal and examination of the central nervous system (CNS) of a moose is extremely laborious, and even when the required resources and expertise are available, careful necropsy and histopathologic examination may fail to find evidence of worms (Lankester, 2001). As well, some moose receiving a low inoculation dose and known to have adult worms in the cranium may show only slight or transitory disease signs (Lankester, 2002). Clinically silent infections in free-ranging moose have been suspected for many years following reports of meningeal worm in apparently normal animals killed by hunters or by vehicles (Smith and

Archibald, 1967; Gilbert, 1974; Thomas and Dodds, 1988). Difficulties in identifying infected animals have hindered measuring the full impact of *P. tenuis* on moose populations and may explain, in part, the disparate historical records of the frequency of moose sickness (Whitlaw and Lankester, 1994a).

Infections of *P. tenuis* in WTD can be identified by detecting dorsal-spined larvae in feces followed by polymerase chain reaction (PCR) assay (Gajadhar et al., 2000). However, this method is of little value for detecting infected moose since few ever pass larvae. Immunodiagnosis appears to hold promise. Initial work done with antigens extracted from adult worms (Neumann et al., 1994) and infective third-stage larvae (L3; Bienek et al., 1998) suggest that infected wapiti (*Cervus elaphus*) could be detected by serologic testing. Nevertheless, not all infected animals could be identified and cross-reaction occurred with antibodies against *Dictyocaulus* sp. Also, as much as 83 days post-infection (DPI) were required to obtain a positive test in animals given a high inoculation dose of 300 L3. Even longer periods were required in animals given doses of 15 L3 (Bienek et al., 1998). Most of these problems were overcome by Ogunremi et al. (1999a, 2002) who used parasite excretory-secretory (ES) products as antigens to detect serum antibodies in WTD and elk. Anti-*P. tenuis* antibodies were detectable as early as 14 DPI in WTD, and between 14 and 28 DPI in elk.

In an effort to produce a more convenient and reliable diagnostic test for parelaphostrongylosis in moose, an enzyme-linked immunosorbent assay (ELISA) was developed similar to the one previously used to detect *P. tenuis* in WTD (Ogunremi et al., 1999a) but incorporating a moose specific antibody conjugate. In this study, preliminary assessment of test sensitivity and specificity was performed using sera from experimentally infected moose whose infection levels were confirmed by necropsy. Further assessment of test performance was done on samples obtained from a group of 21 hunter- or vehicle-killed moose and another group of 23 animals from an area free of WTD and *P. tenuis*.

MATERIALS AND METHODS

Source of *P. tenuis* and infection of animals

First-stage *P. tenuis* larvae used to infect laboratory-reared snails were obtained from a WTD killed near Grand Marais, Minnesota (USA; 47°45′N, 90°30′W) and the resulting L3 were given to seven moose calves in June 1992 and March 1993 as described by Lankester (2002). Doses of three, three, or 30 L3 were given to three 5 mo old calves (#1–3) and four calves (#4–7) of similar age were kept as uninoculated controls for 4.5 mo. At 9.5 mo of age, animals in the latter group were inoculated with five, five, 10, or 15 L3. Two animals (#6 and #7) that received five and 10 L3 (respectively), were each given a challenge dose of 15 L3, 199 days after the initial infection. Animals were bled for sera 1–4 times prior to infection and frequently thereafter in the case of #1–6, and were euthanized 61–243 DPI. Moose #7 was bled infrequently because of intractability, and only pre-infection and 199 DPI serum samples of this animal were analyzed. This animal was killed on 250 DPI but the terminal serum sample was not available. Sera were separated within 1.5 hr of blood collection and kept at −20 C until tested for antibodies.

Sera from free-ranging moose

Sera from 44 free-ranging moose in both enzootic (northeast Minnesota) and non-enzootic (northwest Ontario, Canada) areas were used to investigate the utility of the ES-ELISA for detecting natural infections of *P. tenuis*. Twenty-one wild moose serum samples came from animals killed by hunters or by vehicles in northeast Minnesota in the vicinity of Grand Marais. White-tailed deer in the area existed at summer densities of about 2/km^2 and 82% had *P. tenuis* in the cranium (Slomke et al., 1995). Another 23 serum samples were obtained from apparently normal moose handled for the purpose of radio-collaring in northwest Ontario approximately 50 km southeast of Dryden (49°35′N, 92°36′W). White-tailed deer were virtually absent in the area. Two serum samples used as positive controls were obtained from moose in northeast Minnesota from an 11 mo old wild animal that acted tame, walked in circles, and was found at postmortem to harbor one adult worm in the cranium, and from an 8

mo old animal that showed neurologic signs typical of parelaphostrongylosis such as torticollis and lack of fear of humans and was presumed to be infected with *P. tenuis* although the head was not examined for the parasite. Sera were separated from blood clots or from blood collected from the cavities of killed moose or from the jugular vein of collared moose handled without immobilizing drugs. Sera were frozen for 3–5 yr at −18 C before use.

Antigens

Excretory-secretory (ES) products of L3 and the somatic adult worm antigens of *P. tenuis* were prepared following published protocols (Ogunremi et al., 1999a, 2002) with some modifications. Briefly, batches of ES products were prepared by culturing about 2,500 L3 in RPMI containing 5% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (1.25 μg/ml) at 37 C with 5% CO_2 for about 28 hr. Thereafter, the culture fluid was harvested and clarified by centrifugation at 16,000×G for 10 min and the supernatant containing the ES products was harvested and stored at −20 C until used. Two female and two male adult worms were rinsed three times in phosphate buffered saline (PBS), pH 7.4 and resuspended in 500 μl of ice-cold PBS, followed by sonication at 300 W (Sonic Dismembrator, Fisher Scientific, Pittsburg, Pennsylvania, USA), 1 min at a time, for a total of 3 min. The sonicated adult worm antigen suspension was clarified and stored as above. The protein concentration of the somatic adult antigen preparation was determined by the bicinchinonic acid (BCA) assay kit (Pierce, Rockford, Illinois, USA).

Mouse anti-moose immunoglobulin G conjugate

Moose serum (1 ml) was clarified at 16,000×G for 20 min and mixed with protein G sepharose (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) following suspension in 40 mM Tris (pH 7.8) containing 500 mM NaCl (0.5 ml). The mixture was allowed to rock on a shaker for 1 hr at room temperature and then centrifuged at 3,800XG for 2 min. The protein G sepharose with bound moose immunoglobulin G (IgG) was washed three times with 5 ml of 40 mM Tris containing 500 mM NaCl. Bound moose IgG was released by treatment of sepharose with 0.1 M glycine-HCl, pH 2.7, at room temperature for 30 min and neutralized with 75 μl of 1M Tris-HCl, pH 9.0. The purified moose IgG was dialyzed against PBS and the protein concentration determined by the BCA assay. Mice were inoculated subcutaneously with 15 μg of moose IgG mixed with an equal volume of Freund's complete adjuvant. A booster dose of moose IgG antibodies mixed with Freund's incomplete adjuvant was administered subcutaneously to the mice 2 wk later. Two weeks after the booster dose, blood was obtained from mice and allowed to clot for serum collection. Mouse serum containing anti-moose IgG antibodies was applied to a protein G sepharose column and the bound mouse IgG antibodies eluted with glycine-HCl as described above. Purified mouse IgG antibodies including molecules directed against moose IgG antibodies (i.e., mouse anti-moose IgG) were conjugated to alkaline phosphatase (AP; Sigma Biosciences, St. Louis, Missouri, USA) using 1% glutaraldehyde and following standard procedures (Harlow and Lane, 1988). After treatment with 1M ethanolamine, pH 7.4, the mouse anti-moose IgG-AP conjugate was dialyzed against PBS at 4 C and stored in aliquots at −20 C until tested. The conjugate reacted with the heavy and light chains of moose immunoglobulins when tested by western blot under reducing conditions (data not shown).

Enzyme-linked immunosorbent assay

Details of anti-*P. tenuis* ELISA using either ES products of L3 or somatic adult parasite antigens have been described previously (Ogunremi et al., 1999a, 2002). In this study, microtiter plates were coated with ES products diluted in PBS (1 in 3) or with 0.46 μg of somatic adult antigens. Serum samples were diluted 1:100 in PBS containing 0.1% Tween-20 (PBST). Mouse anti-moose IgG-AP conjugate was used at a dilution of 1:250 in PBST. Optical density (OD) values were read after 60 min of color development. Appropriate controls were included in each run by omitting one reagent at a time (i.e., antigen, serum, conjugate controls) to ensure proper test performance. The medium used to culture L3 for ES products preparation (i.e. RPMI containing fetal calf serum, penicillin, streptomycin, and amphoterin B) was also substituted in place of ES products as an ELISA antigen control. Each serum sample was tested three times by ES-ELISA, and 2–4 times by somatic adult-ELISA. Mean OD values are reported. A serum sample was scored positive if the mean OD value was greater than two times mean OD value of sera collected from all seven experimental moose prior to inoculation with *P. tenuis*. In moose sampled many times before inoculation, the highest pre-inoculation OD value was used for the calculation.

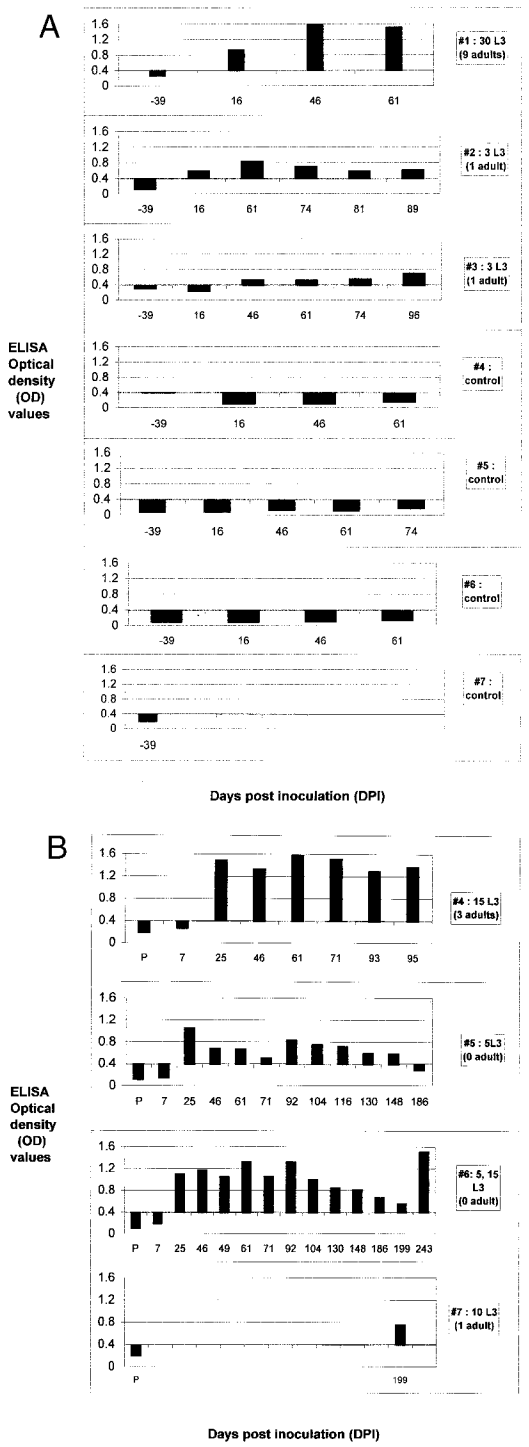

FIGURE 1. Serum antibodies against the excretory-secretory (ES) products of the third-stage larvae (L3) of *Parelaphostrongylus tenuis* in experimentally infected and control moose. A: Moose calves were inoculated with 30 (#1) or three (#2 and #3) third-stage larvae (L3), and an additional four calves (#4–7) kept as uninoculated control animals. Blood samples were collected from all animals 39 days before inoculation (−39) and at the indicated days thereafter. B: Moose #4–7 that previously served as control animals were bled (P) and inoculated with 15 (#4), five (#5 and #6), or 10 (#7) L3. A challenge inoculation of 15 L3 was given to moose #6, 199 days after the initial inoculation. Animals were bled at the indicated times post-inoculation. Mean optical density (OD) values from triplicate ELISA to detect serum immuglobulin G antibodies directed against the ES products of L3 are shown. All pre-inoculation samples from each animal are designated P. The number of adult worms recovered from the central nervous system of each moose at necropsy is indicated.

Statistical analyses

Correlation analyses were performed to investigate the effect of infective dose and adult worm recovery on antibody detection as measured by OD values. Infective dose, peak OD value, terminal OD value, and number of adult worms recovered were $\log_{10}$ transformed, tested for normality by the Kolmogorov-Smirnov test, and analyzed for correlation using the Pearson product moment correlation. One way analysis of variance was used to compare the group mean OD of negative free-ranging moose serum samples (i.e. hunter-killed, vehicle-killed, or collared moose) for statistically significant differences. All analyses were done with SigmaStat (SPSS Inc., Chicago, Illinois). Differences and correlations were considered significant if $P<0.05$.

RESULTS

All pre-inoculation serum samples tested by ES-ELISA were below the cut-off OD value of 0.386. Serum samples collected from the first group of three moose (#1–3) inoculated with *P. tenuis* at 5 mo of age had significantly elevated OD values starting from 16, 16, or 46 DPI when compared to the samples from four uninoculated control moose (Fig. 1A). The control group of calves (#4–7) were eventually inoculated and by 25 DPI, #4–6 had significantly elevated OD values. The only post-inoculation sample available from moose #7 was similarly elevated (Fig 1B). Typically, OD values fluctuated during the course of infection but consistently re-

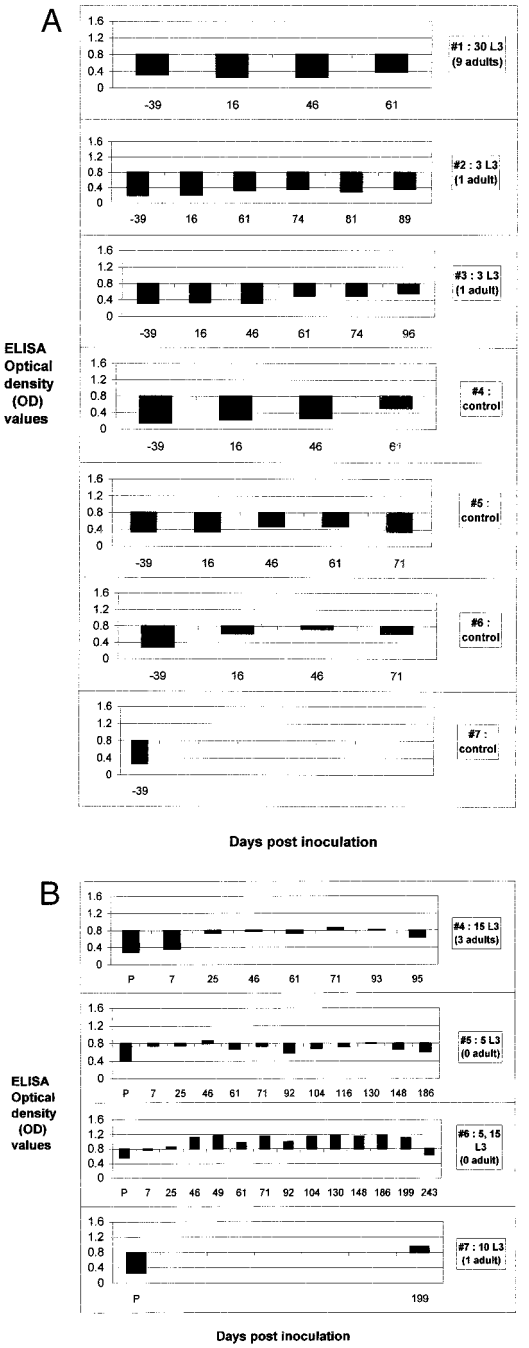

FIGURE 2. Serum antibodies against the somatic antigens of adult *Parelaphostrongylus tenuis* in experimentally infected and control moose. A: Moose calves were inoculated with 30 (#1) or three (#2 and #3) third-stage larvae (L3), and an additional four calves (#4–7) kept as uninoculated control animals. Blood samples were collected from all animals 39 days before inoculation (−39) and at the indicated days thereafter. B: Moose #4–7 that previously served as control animals were bled and inoculated with 15 (#4), five (#5 and #6) or 10 (#7) L3. A challenge inoculation of 15 L3 was given to #6, 199 days after the initial inoculation. Animals were bled at indicated times post-inoculation. The presence of serum immunoglobulin G antibodies directed against the adult worm antigens were tested 2–4 times by ELISA and mean optical density (OD) values are shown. All pre-inoculation samples from each animal are designated P. The number of adult worms recovered from the central nervous system of each moose at necropsy is indicated.

mained elevated in all animals until the end of the experiment with the exception of moose #5 from which no adult worm was recovered at postmortem (Lankester, 2002). Log transformed terminal OD values of non-challenged animals harbouring adult worm(s) correlated with the log transformed value of the infective larvae given (r=0.98; P=0.02, n=4). Log transformed peak OD values also showed significant correlation with the log transformed infective dose among non-challenged animals (r=0.95, P=0.01, n=5), and among only those harbouring adult worms (r=0.95, P=0.04, n=4). In moose #6 which did not have an adult worm at postmortem, the antibody levels waned as infection progressed but was still detectable by 199 DPI and became elevated after a challenge inoculation of 15 L3 was administered. A post-challenge serum sample was not available from the second challenged animal (#7).

Pre-infection serum samples from all moose tested in the somatic adult-ELISA had OD levels below the cut-off value of 0.799 (two times mean OD of pre-inoculation serum samples). However, the OD values were consistently below the cut-off value in all samples obtained from moose #1, #2, and #3 including those collected terminally (Fig 2A), even though the animals had nine, one, and one adult worms, respectively. Many of the samples obtained post-inoculation from moose #4 and #5 tested negative (Fig. 2B). The only excep-

TABLE 1. Serum antibodies against larval excretory-secretory products of *Parelaphostrongylus tenuis* in wild moose from enzootic and non-enzootic areas compared to positive controls, as measured by the enzyme-linked immunosorbent assay.

Sample source	Number	Optical density values from animals considered negative	Number considered positive	Optical density values from animals considered positive
Positive controls	2[a]	—	2	1.610, 1.158
Enzootic area				
—hunter-killed	11	0.216 ± 0.017 (0.150–0.296)	1	1.093
—vehicle-killed	8	0.241 ± 0.016 (0.200–0.318)	1	0.973
Non-enzootic area	23	0.192 ± 0.014 (0.110–0.379)	0	—

[a] Both animals showed abnormal locomotor signs typical of parelaphostrongylosis; the head of only one animal was available for examination and contained one adult *P. tenuis*.
[b] Mean±SEM (range).

tions were 71 and 93 DPI samples of #4 which tested positive. Similarly, only 46 and 130 DPI samples from #5 tested positive. In moose #6, samples obtained on 25 DPI and later tested positive, as did the only sample obtained after the inoculation of #7 (Fig. 2B). Log transformed terminal somatic-adult ELISA OD values did not correlate with log transformed infective dose among the five non-challenged animals ($r=-0.18$, $P=0.78$), nor was correlation observed if data used were only from non-challenged animals harboring adult worms ($r=0.19$, $P=0.82$, $n=4$).

Total numbers of worms found at necropsy totalled nine, one, one, three, zero, zero and one in moose #1–7, respectively (Lankester, 2002). Despite the absence of worms in moose #5 and #6, neurologic signs and positive serology by ES-ELISA indicated that developing worms had been present in both. Although statistical significance was not attained, the log transformed number of adult worms recovered at postmortem in non-challenged animals showed a better correlation with log transformed terminal OD values from ES-ELISA ($r=0.93$, $P=0.07$) than with the somatic adult-ELISA values ($r=-0.19$, $P=0.82$).

Serum samples from the 11 mo old moose with *P. tenuis* in the cranium, and the 8 mo old with overt neurologic signs attributed to *P. tenuis* were strongly positive in ES-ELISA (Table 1) and served as positive controls for samples from free-ranging moose. One of 12 hunter-killed moose from a *P. tenuis*-enzootic area in northeastern Minnesota had an OD value greater than the cut-off point (OD=1.093) and was considered positive. The mean±standard error (SEM) ELISA OD values for the remaining 11 animals were 0.216±0.017 (0.150–0.296).

Among nine vehicle-killed moose from the same general area, one had a positive ELISA value (OD=0.973), while the mean±SEM OD values for the remaining eight animals were 0.241±0.016 (0.200–0.318). Among 23 samples from the area in northwestern Ontario without WTD, the mean ELISA OD value was 0.192±0.014 (0.110–0.379). None of the animals in this group was considered positive. The mean OD values were similar among the negative animals in the three groups ($F=2.08$; $P=0.14$).

DISCUSSION

Excretory-secretory products of *P. tenuis* L3 proved useful as ELISA antigen in identifying experimentally infected moose and in monitoring the course of infections. Anti-*P. tenuis* antibodies were first detectable between 16–46 DPI and persisted until the end of the experiment in all infected moose. The magnitude of the anti-ES an-

tibody titers as measured by the peak and terminal OD values depended on the inoculation dose and the test was sensitive enough to detect infection in animals that had received the lowest infective dose (three L3) and which harbored as few as one adult worm. In contrast to ES products of L3 (ES-L3), somatic antigens of the adult worm performed poorly in ELISA. For instance, antibodies could not be detected by the test at any time in three animals given three to 30 L3 and from which one to nine adult worms were recovered postmortem. The relative effectiveness of the two antigen preparations in detecting *P. tenuis*-infected moose is similar to observations reported for WTD in which antibodies against the somatic antigens of the adult worm were not detected in two of six deer inoculated with six and 20 worms (Ogunremi et al., 1999a). The relative sensitivity of antigen preparations in an indirect ELISA used for experimentally infected deer was ES-L3>somatic-L3>somatic adult antigen. Thus, larval ES products appear satisfactory and superior to somatic antigen preparations for detecting and monitoring experimental *P. tenuis* infections in cervids.

Meningeal worm is known to complete its development to the adult stage in WTD at about 40 days (Anderson, 2000). Approximately the same period is required in moose (Lankester, 2002). The presence of antibodies against larval ES products beyond this time may be due in part to their persistence and to the presence of identical antigens in the adults that might continue to stimulate antibody production. The longevity of such induced antibodies contributes to the usefulness of larval ES antigens in serologic diagnosis, as demonstrated in *P. tenuis*-infected WTD (Ogunremi et al., 1999a, b). It is likely that these antigens may be similarly suited for the diagnosis of *P. tenuis* infections in other susceptible ruminant species such as sheep and llama.

Our attempt to determine the usefulness of the ELISA using sera from wild moose was encouraging. All 23 samples from normal moose in an area of northwestern Ontario where WTD were not present had ELISA OD values below the cut-off limit. This indicates a lack of significant cross-reactivity between *P. tenuis* and other helminth antibodies in this area of the moose range. Sera from two of 21 apparently normal moose from an enzootic area in northeastern Minnesota were positive. One of these was killed by a hunter and the other in a vehicle collision. Although heads were not available to confirm the presence of worms in these two animals, it is reasonable to conclude that they were infected with *P. tenuis* when the serologic data reported in this study is evaluated against published prevalence values of *P. tenuis* in moose: Smith and Archibald (1967) found *P. tenuis* in the heads of 5% of 115 clinically normal moose shot by hunters or dying accidentally in Nova Scotia and New Brunswick, Canada. Gilbert (1974) found *P. tenuis* in the cranium of 15% of 66 moose killed by vehicles and 25% of 44 shot illegally in Maine, while Thomas and Dodds (1988) found adult worms in the heads of 6.5% of 92 vehicle and hunter-killed moose in Nova Scotia.

The ability of the ES-ELISA to accurately determine exposure to *P. tenuis* in all experimentally inoculated moose including two animals given as few as three larvae, shows that the test is quite sensitive. Similarly, the test appears very specific since animals from an area free of *P. tenuis* all tested negative. Taken together, the ES-ELISA shows promise as a tool for the evaluation of exposure of moose to *P. tenuis* in the field.

This test may also be useful for more precisely evaluating the impact of this WTD parasite on populations of cohabiting susceptible cervids and other hosts. It should provide corroborating evidence of infection in moose that show clinical neurologic signs but in which no nematodes can be found. We believe that apparently normal moose with a subclinical infection

or a history of exposure to the parasite can now be identified serologically and their mortality rate and reproductive fitness compared to uninfected animals. As well, mean anti-*P. tenuis* antibody titers in moose sharing range with WTD at different densities and under varying conditions for parasite transmission should provide a basis for better understanding the inverse relationship seen between the densities of these two cervids in eastern North America (Whitlaw and Lankester 1994a, b).

ACKNOWLEDGMENTS

Financial support for this work was provided by Canada-Saskatchewan Agri-Food Innovation Fund and the Matching Investment Initiative (MII) program of the Canadian Food Inspection Agency and by a National Sciences and Engineering Research Council of Canada (NSERC) grant to MWL. Authors acknowledge the help of S. Dudzinski in animal care and bleeding of moose, and the excellent technical support of T. Steeves-Gurnsey, L. Pura, and R. Peterson, as well as other members of the Centre for Animal Parasitology. Published for the Canadian Food Inspection Agency, Minister of Public Works and Government Services, Canada.

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Received for publication 30 March 2001.