

Shared Epitope for Monoclonal IR162 Between *Anisakis simplex* Larvae and *Clonorchis sinensis* and Cross-Reactivity Between Antigens

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Ocular Larva Migrans Caused by *Toxocara cati* in Mongolian Gerbils and a Comparison of Ophthalmologic Findings with Those Produced by *T. canis*

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ABSTRACT: To elucidate the pathogenic potential of *Toxocara cati*, we observed the ophthalmologic changes of the fundi in Mongolian gerbils, *Meriones unguiculatus*, after oral inoculation of 17 embryonated eggs/g body weight. Ophthalmic conditions in 8 *T. cati*-infected gerbils were monitored using an ophthalmoscope from day 0 to day 156 and were compared with those of 57 *T. canis*-infected gerbils. The results showed that *T. cati* larvae migrated into the eye of the gerbil and then elicited ophthalmic changes, including retinal (25%) and vitreous (50%) hemorrhaging, vasculitis (37.5%), and exudative lesions (25%). Lesions were less prevalent, however, in *T. cati*-infected than in *T. canis*-infected gerbils. Unlike in *T. canis*-infected gerbils, the hemorrhagic lesions did not reappear in *T. cati*-infected gerbils after they were absorbed. These findings suggested that *T. cati* larvae are a potentially hazardous pathogen for ocular toxocariasis and that Mongolian gerbils infected with *T. cati* may be a useful model for the study of human ocular toxocariasis caused by *T. cati*. This is the first study to report that *T. cati* larvae can induce ophthalmic lesions in the retina of gerbils.

Among the pet-associated zoonoses, toxocariasis is a common parasitic disease caused by the ingestion of the second-stage *Toxocara* larvae. The larvae often migrate to the retina, resulting in severe inflammation of the eye. A dog roundworm *T. canis*, is typically the pathogen involved with toxocariasis. However, a cat roundworm, *T. cati*, may also cause toxocariasis, although there has been no evidence for the involvement of *T. cati* in ocular invasion. Because the morphological characteristics of the second-stage larvae of both species are quite similar, it is difficult to distinguish between the pathology induced by *T. canis* and that induced by *T. cati* (Nichols, 1956). Eggs of *T. cati* have been found in the sandpits of public parks (Shimizu, 1993; Uga, 1993; Uga et al., 1996; Martinez et al., 1997). Therefore, some researchers believe that *T. cati*, rather than *T. canis*, more likely accounts for toxocariasis in humans (Val-kounova, 1982). In addition, ocular toxocariasis caused by *T. cati* has been serologically diagnosed in Japan (Sakai et al., 1998).

Despite epidemiological and serological evidence, the ophthalmologic characteristics of *T. canis* and *T. cati* infections have not been compared, mainly because no suitable animal models for ocular toxocariasis have been described. Recently, we reported that Mongolian gerbils, *Meriones unguiculatus*, showed a high prevalence of *T. canis* larvae in their eyes after oral inoculation with *T. canis* eggs (Takayanagi et al., 1999). Here, we describe the ophthalmoscopic differences between ocular toxocariasis due to *T. canis* and that due to *T. cati* in Mongolian gerbils.

Four- to 5-mo-old Mongolian gerbils were raised in our laboratory and maintained under pathogen-free conditions. Infected cats were identified by fecal examination, and adult worms of *T. cati* were collected from feces following the administration of anthelmintics. Female *T. cati* were isolated, and embryonated eggs were prepared as previously described (Oshima,

1961). Inoculation with the eggs was carried out as perviously described (Takayanagi et al., 1999). Each gerbil was inoculated with 17 eggs/g body weight by stomach tube under light anesthesia. Eight gerbils were infected with *T. cati* and 57 were infected with *T. canis*. Detailed data from 20 of the 57 gerbils infected with *T. canis* have been published (Takayanagi et al., 1999).

Ophthalmoscopic observation was carried out as previously described (Takayanagi et al., 1999). Under light anesthesia with pentobarbital sodium, ocular changes were monitored with an ophthalmoscope (Scalar VMS-170M, Abbe Science, Tokyo, Japan) after the pupils were dilated with tropicamide. In animals with highly sensitive corneas, surface anesthetics (oxypocaine hydrochloride, Santen Pharmaceutical Co. Ltd., Osaka, Japan) were applied. Ophthalmologic findings of the fundi were recorded on videotape from day 0 to day 156. We observed the fundi every day for the first 2 weeks, every 2 or 3 days between day 15 and day 30 of infection, and thereafter every 4–6 days until the end of experiment. One of the *T. canis*-infected gerbils and 1 of the *T. cati*-infected gerbils were killed on day 7 of infection for histopathologic and parasitologic investigation. The eyes were removed and fixed with 2.5% glutaraldehyde and 4% formaldehyde in phosphate buffer (pH 7.2). Thin sections embedded in JB-4 plastic resin were stained using the Giemsa method. Additionally, larvae that had migrated to the brain were counted by the Kondo method (Kondo, 1970).

Based on the data from the first 20 gerbils, we previously reported that *T. canis* larvae elicited severe ophthalmic changes in gerbils (Takayanagi et al., 1999). Thereafter, we continued to observe the fundi of 37 additional gerbils infected with *T. canis*. Abnormal findings observed in gerbils with *T. canis* and *T. cati* infections are summarized in Table I.

Hemorrhage, vasculitis, exudative changes, and motile larvae were less severe in *T. cati*-infected gerbils than in *T. canis*-infected gerbils, except for vitreous hemorrhage. In both *T. cati*-infected and *T. canis*-infected gerbils, the appearance of hemorrhages began on day 3 of infection. All hemorrhages, regardless of type, were gradually absorbed and subsequently disappeared within 10–14 days in both groups. Vitreous hemorrhages, however, did not absorb completely; brilliant yellowish granular lesions remained for some time (Fig. 1). Hemorrhagic lesions that appeared in the early stage of infection in *T. cati*-infected gerbils did not reappear once they were absorbed; however, such hemorrhages in *T. canis*-infected gerbils appeared repeatedly during the investigation. In 18 of 57 gerbils with *T. canis* infection, retinal hemorrhage also appeared at and after 14 days of infection. Furthermore, this type of hemorrhage was observed more than twice in the same fundus in 8 of the 57 gerbils.

TABLE 1. The incidence of abnormal findings in fundi of eyes of Mongolian gerbils infected with *Toxocara canis* or *T. cati*.

Findings	<i>T. canis</i> *		<i>T. cati</i> †	
	% Gerbils	% Eyes	% Gerbils	% Eyes
Hemorrhagic changes				
Chroidal hemorrhage	82.5	54.4	62.5	31.3
Retinal hemorrhage	36.8	26.3	25	18.8
Small retinal hemorrhage with white spot	43.9	27.2	6.25	12.5
Vitreous hemorrhage	21.1	12.3	50	25
Exudative lesion	77.2	64.9	25	12.5
Vasculitis	52.6	36	37.5	18.8
Larva(e)	82.5	67.5	50	31.3

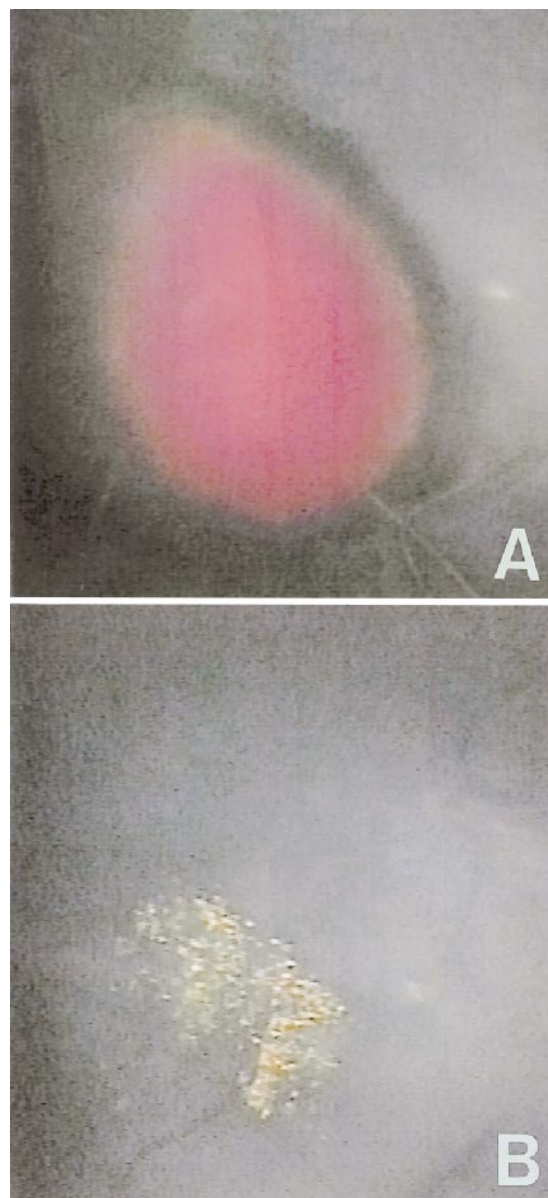
* 57 infected gerbils (114 eyes).

† 8 infected gerbils (16 eyes).

No significant difference in the incidence of hemorrhage, inflammatory lesions, and larvae was noticed in the right versus the left eye for either *T. cati*- or *T. canis*-infected gerbils. Migrating larvae in the retina were found in 47 (82.5%) of 57 *T. canis*-infected gerbils (77 of 114 eyes) and 4 (50%) of 8 *T. cati*-infected gerbils (5 of 16 eyes). In *T. cati*-infected gerbils, larvae were first noticed in 2 of 16 eyes on the fifth day of infection. From the first day of infection to day 10, 4 of 8 gerbils (4 of 16 eyes) had larvae in their retinas. Seven days after infection, 32 larvae were found in the brains of *T. cati*-infected gerbils and 185 in the brains of *T. canis*-infected gerbils. These results suggest that the migration pattern of *T. cati* larvae was different from that of *T. canis* larvae in gerbils.

We have previously reported that Mongolian gerbils are more suitable for use in studies of human ocular toxocariasis than are other animals that have been used, such as mice, guinea pigs, rabbits, and monkeys (Takayanagi et al., 1999). Although the migration profile of *T. canis* in gerbils is not fully understood, our preliminary study revealed that the migration route in gerbils is similar to that in mice. Thus, approximately half of the eggs inoculated successfully invaded the body and migrated in somatic muscle and brain of *T. canis*-infected gerbils. Prior studies, however, have indicated that *T. cati* larvae are seldom found in the mouse brain (Oda, 1976; Prokopic and Figallova, 1982). In the present investigation, larvae from approximately 3% of the eggs of *T. cati* administered were recovered from the brain, indicating that gerbils are more susceptible than mice to *T. cati* infection of the brain.

The present study provides evidence for the pathogenic potential of *T. cati* larvae in gerbils, a potential that was approximately equivalent to that of *T. canis*. This is the first study (1) to demonstrate that *T. cati* larvae are able to migrate into the eyes of Mongolian gerbils after the oral inoculation of eggs and then (2) to elucidate the ophthalmic changes in their eyes. Mongolian gerbils infected with *T. cati* could provide a useful model for human ocular toxocariasis caused by *T. cati*. The incidence of retinal lesions in *T. cati*-infected gerbils, however, was lower than that in *T. canis*-infected gerbils. Ophthalmic lesions did not reappear in the eyes of *T. cati*-infected gerbils, but they repeatedly appeared in *T. canis*-infected gerbils, retinal hemorrhages in particular. These differences might be attributed to

FIGURE 1. Vitreous hemorrhage in the peripheral retina of a *Toxocara cati*-infected gerbil 5 days after infection (A) and 34 days after infection (B).

the number of larvae migrating to the brain; the recovery rate of larvae from the brain of *T. cati*-infected gerbils was less than that in *T. canis*-infected gerbils. The present findings suggest that *Toxocara* larvae may migrate to the eye from the brain. Additionally, a preliminary study revealed that retinal lesions reappeared after steroid treatment (data not shown), further suggesting that *T. canis* larvae might move away from the brain and into the eye.

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Evidence of *Cowdria ruminantium* Infection (Heartwater) in *Amblyomma sparsum* Ticks Found on Tortoises Imported into Florida

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ABSTRACT: *Amblyomma marmoreum* and *A. sparsum* ticks were collected from tortoises imported into Florida from Africa and were tested for *Cowdria ruminantium* infection using a *C. ruminantium*-specific pCS20 polymerase chain reaction assay. In 1 shipment imported from Zambia, 15 of the 38 *A. sparsum* male ticks collected from the leopard tortoises (*Geochelone pardalis*) were found to be positive for infection with *C. ruminantium*. In contrast, all 148 *A. marmoreum* tested were negative for *C. ruminantium* infection. This is the first reported evidence of the introduction of heartwater-infected ticks into the United States, but there were no opportunities to confirm isolation of *C. ruminantium* from the ticks by either culture or transmission studies.

It has been known for several years that reptiles imported into the United States were on occasion infested with ticks (Becklund, 1968), but it was only recently that the extent and significance of these infestations became apparent. In 1997, the African tortoise tick *Amblyomma marmoreum* was discovered on imported leopard tortoises (*Geochelone pardalis*) outside importation facilities on a reptile-breeding premises in central Florida and found to have established a breeding colony (Allan et al., 1998). This discovery was followed by a survey of reptile facilities in Florida that found 8 exotic species of ticks on a variety of imported tortoises, snakes, and monitor lizards (Burrridge et al., 2000). Among those exotic ticks were 2 species, *A. marmoreum* and the large reptile tick *A. sparsum*, both of which are known to be vectors of heartwater (Norval and MacKenzie, 1981; Peter et al., 2000b). These findings were of considerable significance because heartwater is an acute tickborne disease of ruminants caused by the rickettsia *Cowdria ruminantium*, which poses a serious threat to the cattle, sheep, goat,

and deer populations of the United States (Burrridge, 1997). Consequently, all *A. marmoreum* and *A. sparsum* ticks identified in the survey were collected, and a sample of each collection was processed for testing for *C. ruminantium* infection. This research note reports the results of the testing of the tick samples.

Between July 1997 and May 1999, 148 *A. marmoreum* (57 males, 44 females, 32 nymphs, and 15 larvae) and 38 *A. sparsum* (all males) were collected for diagnostic testing from imported leopard tortoises and African spurred tortoises (*G. sulcata*) at 5 reptile facilities in central and southwest Florida. All ticks were preserved in 70% ethanol and shipped by air to our Heartwater Research Project laboratory in Harare, Zimbabwe.

In Zimbabwe, the ticks were dissected and processed for analysis by the pCS20 polymerase chain reaction (PCR) assay as described by Peter et al. (2000a). The pCS20 PCR assay uses the primers AB 128 (5'-ACTAGTAAATTGCACAATCTAT-3') and AB 129 (5'-TGATAACTTGGTGCGGGAAATCCTT-3'), which amplify a 279-base pair fragment of *C. ruminantium* DNA found in more than 37 *C. ruminantium* isolates throughout the geographical distribution of heartwater. This assay is more sensitive than all other assays available for detection of *C. ruminantium* infection in ticks and, in addition, it is also the most characterized assay (Mahan et al., 1998; Allsopp et al., 1999; Peter et al., 1999, 2000a). The pCS20 PCR assay has a sensitivity of detection of 100 organisms per tick. In addition to its high sensitivity, this PCR assay has a very high specificity, being unable to detect agents such as *Ehrlichia canis*

and *E. chaffeensis*, which are both phylogenetically and antigenically closely related to *C. ruminantium* (Peter et al., 1995, 2000a).

DNA from the 148 *A. marmoreum* and the 38 *A. sparsum* ticks was isolated as described by Peter et al. (2000a). At the same time, 18 known uninfected *A. hebraeum* ticks from our laboratory colony were processed similarly as negative control ticks. In addition, positive and negative control PCR samples were included in all assays, consisting of reagents with and without *C. ruminantium* genomic DNA, respectively. PCR assays were performed on all 204 ticks, with amplified products either dot-blotted directly onto nylon membranes or analyzed by agarose gel electrophoresis before Southern blotting onto nylon membranes. The DNA-bound membranes were hybridized to the ³²P-labeled pCS20 DNA probe to optimize detection of PCR products. Fifteen of the 38 *A. sparsum* ticks imported on tortoises from Zambia were found to be positive for *C. ruminantium* infection following hybridization with the pCS20 DNA probe. In contrast, none of the 148 *A. marmoreum* or of the 18 *A. hebraeum* negative control ticks was positive.

Previously it has been suggested that the introduction of tick vectors of heartwater on imported reptiles poses a potentially serious threat to the cattle, sheep, and goat industries, and the deer populations in the United States (BurrIDGE, 1997). Our finding of evidence of *C. ruminantium*-infected *A. sparsum* on leopard tortoises imported into Florida from Africa demonstrates that importation of infested tortoises poses a very real threat of introduction of heartwater into the United States and indicates an urgent need to take actions to control tick infestations on these reptiles.

The finding of positive reactions to the PCR assay for heartwater in *A. sparsum*, but not in *A. marmoreum*, is not altogether unexpected. Whereas *A. marmoreum* is primarily a tick of tortoises (Theiler and Salisbury, 1959), *A. sparsum* is both a reptilian and a mammalian tick, commonly infesting not only tortoises, but also large mammals such as the African buffalo (*Syncerus caffer*) (Norval and Mackenzie, 1981; Walker and Olwage, 1987), a wild ruminant that can be a carrier of heartwater (Andrew and Norval, 1989). It is possible that immature *A. sparsum* had fed as larvae or nymphs on an infected animal such as an African buffalo in Zambia, where heartwater is endemic (Provost and Bezuidenhout, 1987), had molted to adults, and had then attached to the leopard tortoises before their importation into Florida.

All ticks found to show evidence of *C. ruminantium* infection in this study were *A. sparsum* males that had been sent in 1 shipment of infested leopard tortoises from Zambia to a reptile importer in central Florida. All *A. sparsum* that were collected from that shipment were killed by immersion in 70% ethanol, precluding attempts to confirm heartwater. Consequently, although this study demonstrated evidence of *C. ruminantium* infection in imported African ticks, it was unable to confirm isolation of this organism from the ticks by either cell culture or

tick or blood transmission experiments, and no evidence of *C. ruminantium* infection was detected in any animal in Florida.

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Enzyme Activities in *Schistosoma mansoni* Soluble Egg Antigen

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ABSTRACT: *Schistosoma mansoni* egg antigens are mostly responsible for the granulomatous pathology in human intestinal schistosomiasis. Several previous studies have indicated that the induction of an immune response against some parasite enzymes may protect against pathology. The present work was designed to identify enzyme activities present in a standard soluble egg antigen (SEA) preparation. Simple colorimetric analyses were performed incubating SEA with 2-naphthyl, 2-naphthylamide (2NA), or *p*-nitrophenyl substrates at different pHs in the absence of added effectors. Results showed prominent acid phosphatase (pH 5.4), alkaline phosphatase (pH 8.5), and N-acetyl- β -glucosaminidase (pH 5.4) activities. Relevant peptidase activities were also detected at pH 6.5–7.5 against 2NA derivatives of (1) aliphatic (α -Ala > β -Ala > Leu > Met > S-benzyl-Cys), polar (Ser > Gln), basic (Arg > Lys > ornithine), and acidic (Glu) amino acids; (2) dipeptides: X-Ala (X = Gly > Leu > Lys > Asp), X-Arg (X = Ala > Arg > Phe > Gly > Pro > Asp), Ser-Met, and Phe-Pro; and (3) tripeptides (Ala-Phe-Pro > Phe-Pro-Ala). The data demonstrated that *S. mansoni* SEA contains a rich set of hydrolases with different specificities that might play a role in the egg physiology and possibly also in the host–parasite relationships.

In schistosomiasis, patient morbidity is caused by the granulomatous reaction and fibrosis initiated by disseminated eggs, occurring mainly in the host's liver in the case of *Schistosoma mansoni*. Schistosome eggs release soluble proteins and glycoproteins that are thought to be responsible for granuloma formation and other egg-induced reactions (Boros, 1989). The study of the nature and role of individual egg components may help us understand these phenomena. Some schistosome enzymes have relevant functions, i.e., leucine aminopeptidase in egg hatching (Xu and Dresden, 1986), egg pyruvate kinase (62-kDa antigen) as inducer of a potent CD4(+) T helper cell response in mice (Asahi et al., 1999), and glutathione S-transferase as a vaccinating antigen (Capron et al., 1995).

In the present study, we screened the ability of a standard soluble egg antigen (SEA) preparation (Boros and Warren, 1970) to hydrolyze a number of synthetic substrates to identify prominent egg enzymatic activities. Schistosome eggs were isolated from livers of hamsters infected with a Venezuelan strain (JL) of *S. mansoni* by a sieving and enzymatic methodology (Cesari, Ramos, and Alarcón de Noya, 1987). The livers were dissected and homogenized in sterile 0.01 M phosphate buffer, pH 7.4 (10 ml/liver), and the homogenate was digested with 40 μ g·ml⁻¹ trypsin (Sigma Chemical Co., St. Louis, Missouri) for 2 hr at 37 C. The suspension was sieved through consecutive screens of 420, 144, and 44 μ m. Eggs were recovered from the last screen and washed extensively with sterile 1.5% NaCl. The clean eggs (as seen under a light microscope) were stored at –20 C. After thawing, approximately 1×10^6 eggs were resuspended and homogenized in 4 ml sterile 0.01 M phosphate buffer, pH 7.0, and centrifuged at 100,000 *g* for 2 hr at 4 C. The supernatant (SEA) was filtered through a Minisart NML 0.2- μ m filter (Sartorius, Göttingen, Germany) and, if not used

immediately, frozen at –20 C. Livers from uninfected hamsters were also processed as above. Protein contents of the samples were determined by the method of Bradford (1976). For the enzyme assays, API ZYM (Bio-Mérieux, Marcy l'Etoile, France) kits were used. Substrates in the 2520 kit were naphthyl derivatives of phosphate, fatty acids, and carbohydrates (α - and β D-galactose, α - and β D-glucose, α D-mannose, β D-glucuronic acid, N-acetyl- β D-glucosamine, α L-fucose) and 2-naphthylamide (2NA) derivatives of L-Leu, L-Val, L-Cys, N-benzoyl-DL-Arg, and N-glutaryl-Phe. These substrates were tested at different pHs prestablished by the kit manufacturer. Substrates in the glycosidase kit were *p*-nitrophenyl (PNP) derivatives of the above carbohydrates and of α L-rhamnose, α L-arabinose, β D-galacturonic acid, α - and β -maltose, N-acetyl- α D-glucosamine, β L- and β D-fucose, β D-lactose, β D-mannose, and α - and β D-xylose. These substrates were tested at pH 5.5, 6.5, 7.2, and 8.5. Substrates in the AP LRA peptidase kits were 2NA and 4-methoxy-2NA derivatives of free or N-terminal blocked amino acids, di-, tri-, and oligopeptides and were also tested at pH 5.5, 6.5, 7.2, and 8.5. All the kits were used essentially according to the manufacturer's instructions, with some modifications to make the system more quantitative. SEA (65 μ l, 0.2 mg protein/ml) was added to all the wells of the kits, and the well strips were incubated aerobically in a humid chamber for 2 hr at 37 C. Diluent buffer (65 μ l) was placed in wells with and without substrate to obtain enzyme and substrate blank values, respectively. To stop reactions, 70 μ l 0.1 N NaOH was added to the wells of the glycosidase kit. Reactions in the 2520 and the peptidase kits were stopped by adding 35 μ l of reagent A (2.5 g TRIZMA base, 1.1 ml 37% [w/w] HCl, and 1.0 g of sodium dodecyl sulfate, diluted to 10 ml with distilled water) and 35 μ l of reagent B (35 mg fast blue salt in 10 ml 2-methoxyethanol); color was allowed to develop for 10 min at about 25 C (room temperature). A 100- μ l aliquot from each well was transferred into a corresponding well of a microtiter plate, and the released products were determined at different wavelengths appropriate to the colors according to products: *p*-nitrophenol (glycosidase kit) at 405 nm (yellow) and naphthol and naphthylamine derivatives coupled to fast blue (2520 and LRA peptidase kits) at 450 nm (brown), 492 nm (orange), 540 nm (violet), or 620 nm (blue). Absorbances were read in a Titertek Multiskan reader MCC/340 (Flow Laboratories, McLean, Virginia). Under the above conditions, absorbances were linear for up to 3 hr of incubation at 37 C.

SEA was highly positive for acid phosphatase (ACP), alkaline phosphatase (ALP), and β -N-D-acetyl-glucosaminidase (β NAG); it was negative with most glycosidase kit substrates at any pH tested, except for a consistently high β NAG activity seen at pH 5.5 and pH 6.5 with PNP-N-acetyl- β -D-glucosamin-

TABLE I. Activities* on naphthyl (N) and *p*-nitrophenyl (PNP) substrates by *Schistosoma mansoni* soluble egg antigen (SEA).†

Substrate	pH	A (nm)	Absorbance ($\bar{x} \pm SD\ddagger$)
Naphthol-AS-BI-phosphate	5.4	620	1.277 $\pm$ 0.135
1N-acetyl- β -D-glucosaminide	5.4	450	0.316 $\pm$ 0.150
PNP-acetyl- β -D-glucosaminide	5.5	405	0.545
PNP-acetyl- β -D-glucosaminide	6.5	405	0.764
PNP- α -D-mannopyranoside	6.5	405	0.103
2N-phosphate	8.5	540	1.391 $\pm$ 0.013

* Activities reported as absorbance readings at the different optimal wavelengths after 2 hr at 37 C; values corrected for absorbance in control wells without enzyme.

† SEA protein concentration = 13 μ g/well.

‡ Mean $\pm$ SD reported only for *n* = 3 (3 independent experiments with 3 different SEA preparations).

ide and a moderate α -mannosidase activity at pH 6.5 (Table I). To our knowledge, ACP, β NAG, and α -mannosidase activities, have not been previously reported in *S. mansoni* eggs, and their role is at present unknown. However, β NAG and α -mannosidase have been recently hypothesized by Khoo et al. (1997) in *Schistosoma* eggs as trimming enzymes for the tailoring of glycoproteins and glycolipids. ALP seems to play a role in embryo development. Previous histochemical work by Giboda and Zdarska (1994) revealed this enzyme inside immature *S. mansoni* eggs associated with the vitelline membrane and the body surface of embryonating miracidia; in mature eggs, it was associated with miracidial germ cells and sensory endings of the neural cells. In the eggs of the cestode *Taenia taeniaeformis*, ALP activity was found in the outer embryoforic and oncospherical membranes, where it seems to be involved in carbohydrate metabolism and transport of nutrients to the developing embryo (Ajayi et al., 1985). In adult *S. mansoni* worms, ALP is tightly bound to the tegumental membranes, is immunogenic in infected patients, and has been used as a marker of infection (Pujol et al., 1989). 2-Naphthyl derivatives of butyrate, caprylate, and myristate (esterase activities) showed hydrolysis values not higher than $A_{540nm} = 0.095$.

Several monoaminoacyl-peptidase (MAP) activities were present in SEA. These activities were optimal at pH 6.5–7.5 and were significant with several 2NAs, including aliphatic (α -Ala > β -Ala > Leu > Met > S-benzyl-Cys), polar (Ser > Gln), basic (Arg > Lys > ornithine), and acidic (Glu $\gg$ Asp) aminoacyl 2NAs (Table II). At present, we do not know whether the observed hydrolysis of a given aminoacyl derivative was performed by more than 1 MAP. Alpha- and β -alanyl aminopeptidases were prominent enzymes. Alpha-alanyl aminopeptidase was reported to have antigenic activity in other developmental forms of *S. mansoni* (Damonville et al., 1982). However, β -Ala MAP is not a common enzyme and it may liberate β -Ala, an important osmoprotectant (Dawson and Baltz, 1997), from natural substrates. The hydrolysis of β -Ala-2NA was probably induced by an enzyme different from that acting on α -Ala-2NA because α -Ala-2NA hydrolysis was inactivated upon prolonged storage at -20 C or after freezing and thawing. Leucine aminopeptidase (LAP) is particularly plentiful in eggs, where it is associated with the vitelline membrane (Bogitsh, 1983); it is also present in other developmental forms of *S.*

TABLE II. Arylamidase activities* on unblocked N-terminal amino acyl 2NA-substrates by *Schistosoma mansoni* soluble egg antigen (SEA).†

2NA substrate	pH			
	5.5	6.5 ($\bar{x} \pm SD\ddagger$)	7.2	8.5
L- α -Ala	0.479	0.859 $\pm$ 0.168	ND§	0.345
L- β -Ala	0.379	0.779 $\pm$ 0.188	0.733	0.315
L-Leu	ND	ND	0.707 $\pm$ 0.043	ND
DL-Met	0.323	0.585 $\pm$ 0.061	0.521	0.304
S-benzyl-L-Cys	0.480	0.443 $\pm$ 0.145	0.367	0.107
L-Ser	0.223	0.406 $\pm$ 0.020	0.338	0.087
L-Gln	0.212	0.332 $\pm$ 0.039	0.356	0.159
L-Lys	0.187	0.472 $\pm$ 0.067	0.353	0.130
L-Arg	0.637	0.819 $\pm$ 0.117	ND	0.218
L-Ornithine	0.220	0.243 $\pm$ 0.045	0.263	0.108
L- α -Asp	0.007	0.048 $\pm$ 0.036	0.017	0.017
L- α -Glu	0.085	0.183 $\pm$ 0.051	0.178	0.080

* Activities on the AP LRA substrate series reported as absorbance readings at 492 nm after 2 hr at 37 C; values corrected for absorbance readings in control wells without enzyme.

† SEA protein concentration = 13 μ g/well.

‡ Mean $\pm$ SD reported only at pH 6.5 and pH 7.5 for *n* = 3 (3 independent experiments with 3 different SEA preparations).

§ ND = not determined.

|| Activity seen on the API ZYM 2520 substrate series at pH 7.5.

mansoni (Damonville et al., 1982; Cesari et al., 1983; Doenhoff et al., 1988). This enzyme seems to play a role in egg hatching because bestatin (a specific aminopeptidase inhibitor) prevented this process (Xu and Dresden, 1986). Egg LAP was recognized as antigen by sera from mice infected with either *S. mansoni* or *S. japonicum* (Xu et al., 1988) or by IgG from *S. mansoni*-infected patients (Cesari, Pujol, et al., 1987). High methionine aminopeptidase activity was also observed (Table II). Methionine aminopeptidases are ubiquitous and cleave the N-terminal methionine from many newly translated polypeptides (Bradshaw et al., 1998). S-benzyl-L-Cys-2NA could have been hydrolyzed by this enzyme. Nonetheless, specific S-benzyl-L-cysteine aminopeptidases are known to act on the bioactive peptides oxytocin and vasopressin (Itoh and Nagamatsu, 1995). At present, it is difficult to suggest the role of the prominent activities observed with Ser-2NA and Gln-2NA (Table II). The strong arginyl aminopeptidase activity observed with Arg-2NA (Table II) was also sensitive to storage at -20 C; it was higher than that with ornithine-2NA (structurally related to arginine) and with Lys-2NA (Table II). At present, we do not know if this enzyme activity is related to the antigenic lysyl aminopeptidase activity hydrolysing L-Lys-naphthylamide reported by Doenhoff et al. (1988) in *S. mansoni* eggs. Although not shown in Table II, 2NAs of Gly, Pro, hydroxy-Pro, Thr, Cys, Val, Ile, His, pyro-Glu, Phe, Tyr, and Trp were hydrolyzed weakly or not at all.

SEA was also positive for dipeptidyl aminopeptidase (DAP) activities. Significant activities were seen with 2NA derivatives of X-Ala (X = Gly > Leu > Lys > Asp), X-Arg (X = Ala > Arg > Phe > Gly > Pro > Asp), Ser-Met, Phe-Pro, and Gly-Pro (Table III). DAP type I (Gly-Arg), II (Lys-Ala), III (Arg-Arg), or IV (Gly-Pro) activities might be responsible for some of the observed hydrolysis. It was difficult to assess if the dipeptide substrates were hydrolyzed exclusively by DAPs or by the consecutive action of different MAPs. However, Gly-Ala-

TABLE III. Arylamidase activities* on unblocked N-terminal 2NA-di-peptidyl and -tripeptidyl substrates by *Schistosoma mansoni* soluble egg antigen (SEA).†

2NA substrate	pH			
	5.5	6.5 ($\bar{x} \pm SD\ddagger$)	7.2	8.5
Gly-L-Ala	0.132	0.533 $\pm$ 0.051	0.505	0.107
L-Leu-L-Ala	0.119	0.421 $\pm$ 0.111	0.362	0.076
L-Lys-L-Ala	0.078	0.342 $\pm$ 0.028	0.238	0.030
L- α -Asp-L-Ala	0.038	0.134 $\pm$ 0.022	0.120	0.019
Gly-L-Pro	0.150	0.089	—§	0.243
L-Phe-L-Pro	0.193	0.247 $\pm$ 0.151	—	0.165
L-Ser-L-Met	0.181	0.462 $\pm$ 0.022	0.340	0.094
L-Ala-L-Arg	0.128	0.599 $\pm$ 0.154	0.562	0.085
L-Arg-L-Arg	0.163	0.493 $\pm$ 0.070	0.347	0.032
L-Phe-L-Arg	0.055	0.347 $\pm$ 0.001	0.364	0.039
Gly-L-Arg	0.054	0.218 $\pm$ 0.067	0.520	0.071
L-Pro-L-Arg	0.036	0.205 $\pm$ 0.020	0.457	0.046
L- α -Asp-L-Arg	0.031	0.105 $\pm$ 0.038	0.133	0.014
L-Ala-L-Phe-L-Pro	0.044	0.331 $\pm$ 0.076	0.543	0.046
L-Phe-L-Pro-L-Ala	0.017	0.233 $\pm$ 0.036	0.279	0.050

* Activities on the AP LRA substrate series reported as absorbance readings at 492 nm after 2 hr at 37 C; values corrected for absorbance readings in control wells without enzyme.

† SEA protein concentration = 13 μ g/well.

‡ Mean $\pm$ SD reported only at pH 6.5 and pH 7.5 for n = 3 (3 independent experiments with 3 different SEA preparations).

§ — = negative values.

|| Usually high blank values without enzyme, probably due to substrate instability.

2NA and Asp-Ala-2NA (Table III) were presumably hydrolyzed only by DAPs because no MAP activity was seen on Gly-2NA or Asp-2NA. Gly-Pro-2NA and Phe-Pro-2NA were probably hydrolyzed by a DAP IV (X-prolyl DAP), an activity that has been demonstrated in mammals, fungi, and bacteria and has been attributed to serine proteinases working at neutral pH (Walter et al., 1980). Little or no activity was seen with 2NA derivatives of Gly-Gly, His-Ser, Gly-Phe, Ser-Tyr, Gly-Trp, Glu-Glu, and Glu-His at any pH tested (data not shown). A DAP II activity, hydrolyzing Lys-Ala-4-methoxy-2NA at pH 6.3, was reported by Kramer and Bogitsh (1985) in adult *S. japonicum* worms. Also, a DAP I, hydrolyzing 7-amino-4-methyl coumarin (NHMeC) derivatives of Gly-Arg and Gly-Phe at pH 5.5, and a DAP III, hydrolyzing Arg-Arg-NHMeC at pH 8.5, were reported in adult *S. japonicum* and *S. mansoni* worms by Holo-Jamriska et al. (1999).

Tripeptidyl-aminopeptidase activities (Tomkinson, 1999) were observed at pH 7.2 with Ala-Phe-Pro-2NA and Phe-Pro-Ala-2NA (Table III). No activity was seen on 2NAs of His-Leu-His, Val-Tyr-Ser, Leu-Leu-Val-Tyr-Ser, and Ala-Phe-Pro-Ala (data not shown).

The endopeptidase substrates N-benzoyl-Ala-2NA, N-benzoyl-Leu-2NA, N-CBZ-Arg-2NA, and N-acetyl-Gly-Phe-2NA were not hydrolyzed at any pH tested. N-glutaryl-Phe-2NA (substrate for chymotrypsin, tested at pH 7.5) and N-benzoyl-DL-Arg-2NA (substrate for trypsin, tested at pH 8.5) were not hydrolyzed. However, N-CBZ-Gly-Gly-Arg-2NA (a substrate for plasminogen activator) was hydrolyzed at pH 5.5 ($A_{492nm} = 0.214$) and at pH 8.5 ($A_{492nm} = 0.296$).

No activity was seen in control experiments with heat-inactivated SEA (100 C for 5 min), indicating that positive reactions

were clearly due to enzyme action. The egg batches used to prepare SEA appeared microscopically free of host liver tissue, making gross contamination with host components improbable. This assumption was confirmed in an experiment with SEA prepared from eggs isolated by the sucrose-Percoll methodology of Dalton et al. (1997), which provides eggs free from contaminating host DNA. The activity profiles seen with this SEA preparation were identical to those shown in Tables I, II, and III. However, soluble host liver extracts processed and used with substrates at the same protein concentration as SEA generally resulted in activity not above background. Preliminary results using proteinase inhibitors indicated that various of the arylamidase activities observed herein were partially inhibited by bestatin and/or phenylmethyl sulfonyl fluoride (a serine proteinase inhibitor), suggesting that aminopeptidases and/or serine proteinases are in part responsible for those activities.

The egg hydrolases found here may have biological roles associated with processing of host proteins to fulfill nutritional requirements of the embryo or embryo differentiation. These hydrolases may also help eggs to pass through the wall of the intestine or bladder and help miracidia to penetrate the smooth muscle extracellular matrix of snail vectors, as suggested previously (Pino-Heiss et al., 1985; Yoshino et al., 1993), or may be responsible for the granulomatous pathology in schistosomiasis. From this perspective, the identification and availability of purified key egg enzyme antigens might enable the study and development of "antipathology" vaccines that could decrease the intensity of the host granulomatous inflammatory response in a way similar to that produced after host vaccination with the schistosome glutathione S-transferase vaccine, which has been shown to limit pathology and parasite transmission (Xu et al., 1991; Capron et al., 1995; Shuxian et al., 1997).

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Genetic Difference in Susceptibility and Fatality of Three Strains of Mice Experimentally Infected with *Neodiplostomum seoulense*

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ABSTRACT: The genetic influence on host susceptibility to *Neodiplostomum seoulense* infection and fatality of the host was studied in 3 inbred strains of mice (BALB/c [H-2d], C3H/He [H-2k], and C57BL/6 [H-2b]). The survival of the mice, worm expulsion kinetics, worm size, number of eggs produced per day (EPD), and number of uterine eggs were observed from day 1 to day 40 postinfection (PI) with 100 or 200 metacercariae per mouse. Infection with *N. seoulense* was highly lethal to all 3 strains of mice, but the lethality was dose-dependent and varied according to the genetic backgrounds of the mice. The C3H/He mice exhibited the highest mortality, the lowest worm burdens and EPD, and the quickest expulsion of worms. It is suggested that different genetic backgrounds of mice appear to affect the host's capacity to expel *N. seoulense* and the fatality of the hosts themselves.

Neodiplostomum seoulense (Seo et al., 1964) is an intestinal trematode of humans and rodents in the Republic of Korea

(Seo, 1990). Since human infection was first observed in a man who consumed the viscera of undercooked snakes and experienced severe gastrointestinal troubles and eosinophilia (Seo et al., 1982), 26 additional cases have been reported (Hong et al., 1984, 1986). The site of infection in the definitive host is the duodenum, but the parasites extend into the jejunum and ileum in heavy infections (Hong et al., 1983). In a study of the small intestines of mice and rats, severe degenerative changes of the villi and hyperplasia of the crypts were observed (Lee et al., 1985). Moreover, a heavy infection with *N. seoulense* in the ICR (Institute of Cancer Research) mice caused severe clinical disease and a fatal outcome (Huh et al., 1988). The pathogenicity of this fluke was studied in greater detail using 2 additional strains of mice and their F1 (BALB/cA × C3H/HeJ)

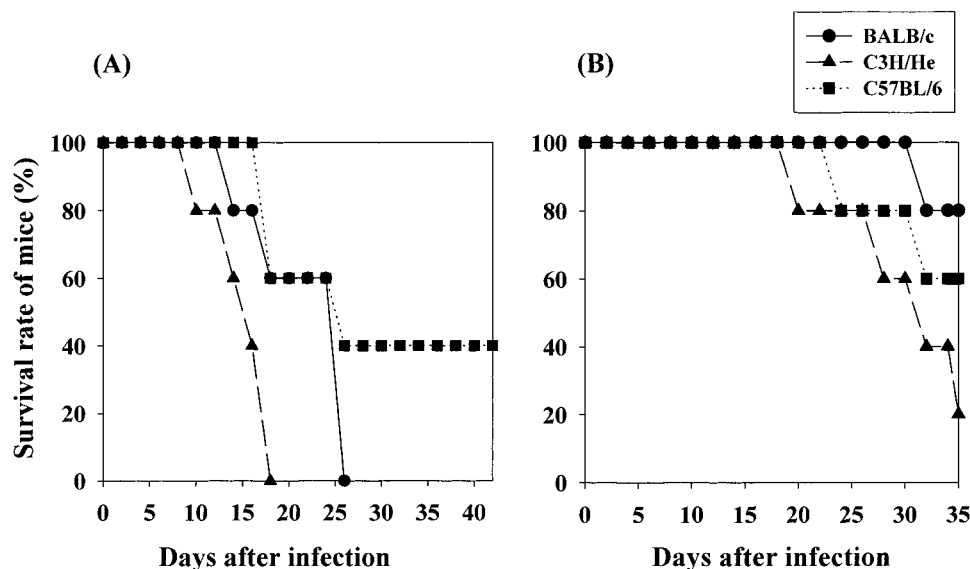


FIGURE 1. Survival rates of 3 inbred strains of mice, BALB/c, C3H/He, and C57BL/6, experimentally infected with (A) 200 or (B) 100 mc of *Neodiplostomum seoulense* ($n = 5$ for each value). The C3H/He mice had the highest fatalities with this trematode infection regardless of the infection dose. The C3H/He mice also had a significantly shorter survival period ($P < 0.05$) than other 2 strains of mice (see text).

progeny (Kook et al., 1998). The hybrid F1 mice showed an intermediate phenotype in terms of their survival and worm recovery, strongly suggesting a genetic influence on the host susceptibility (Kook et al., 1998).

There are many reports regarding the genetic influence of hosts in their susceptibility to parasite infections. For example, the differences in susceptibility to *Eimeria vermiformis* infection among different strains of mice were related to genetic differences in cellular and cytokine responses (Wakelin et al., 1993). Strains of mice with different genetic backgrounds exhibited varying susceptibility to *Metagonimus yokogawai* infection (Chai et al., 1984). These genetic effects were related to the phenotype of H-2 gene products and to different capabilities in eliciting immune responses that expel worms (Krcó et al., 1983; Else and Grecis, 1991; Wakelin et al., 1993). Although it was suggested that the H-2 gene possibly controls the differences in protection against *N. seoulense* infection (Kook et al., 1998), no further studies have been performed. The present investigation was undertaken to examine the genetic influence on susceptibility of mice to *N. seoulense* infection by observing the survival of mice, worm recovery rate, worm size, daily egg production, and number of uterine eggs in different strains of mice.

Three strains of male mice (BALB/c [H-2d], C3H/He [H-2k], and C57BL/6 [H-2b]), raised under specific pathogen free (SPF) conditions, were purchased from the Korean Animal Center (Jinju, Republic of Korea). They were 8 wk old at the beginning of the experiment and were maintained at the animal facility equipped with SPF conditions at Seoul National University College of Medicine, Seoul, Republic of Korea.

Snakes, *Rhabdophis tigrina*, naturally infected with the metacercariae (mc) of *N. seoulense* were purchased from Yongjongdo, Kyonggi-do, Republic of Korea, one of the local enzootic areas, and were maintained at 4 C until used. The mc were isolated by a digestion technique using artificial gastric juice containing 0.5% pepsin (1:10,000, Sigma Chemical Co., St.

Louis, Missouri) and 0.8% HCl, following the procedure described previously (Kook et al., 1998).

The mice were infected orally with either 100 or 200 mc in saline volumes of 0.2 ml using a gavage needle. Each dosage group consisted of 30 mice, 5 mice for observing the survival rate and 25 mice for other observations. The postinfection (PI) time of death of mice was recorded to calculate the survival rate. The dead mice were immediately examined at necropsy to recover worms. Briefly, their small intestines were opened longitudinally in petri dishes containing saline. Adult flukes attached at the mucosa were freed and counted using a stereomicroscope.

The degree of infection in each mouse was monitored every other day (200-mc infection group) or every 7 days (100-mc group) by examining the fecal egg counts (number of eggs produced per day [EPD]). Briefly, the mice were kept in individual cages and the feces were collected every 24 hr, then dissolved in 10 ml of tap water. Fifty microliters of the suspension were placed on a glass slide and the number of eggs was counted under a light microscope. The EPD was measured in duplicate.

After infection with 100 mc, 5 mice were killed every 7 days. Adult flukes were harvested from the small intestine; 20 were randomly selected from each group and fixed in 10% formalin under coverslip pressure. They were stained with Semichon's acetocarmine, their length and width were measured, and the number of eggs in the worm uteri was counted.

The survival rate of the mice and the worm burdens in each group of mice were expressed as the mean $\pm$ standard deviation (SD). The statistical significance among the groups was tested by the student's *t*-test. A value of $P < 0.05$ was considered significant.

Infection with *N. seoulense* was lethal to all 3 strains of mice. The survival rate of the mice was dose-dependent; high fatality was associated with a high infection dose, and varied according to genetic backgrounds of the host (Fig. 1A, B). When 200 mc were given to each mouse, all C3H/He and BALB/c mice died

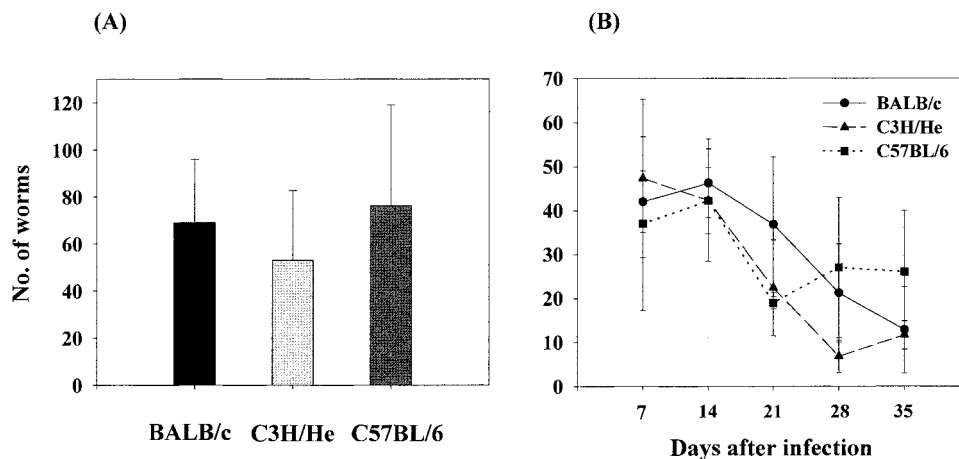


FIGURE 2. Worm recovery from 3 strains of mice, BALB/c, C3H/He, and C57BL/6, after infection with (A) 200 or (B) 100 mc of *Neodiplostomum seoulense*. The worm burden was measured (A) on the death day of the mice, except for 2 C57BL/6 mice that were sacrificed on day 42 PI ($n = 5$), or (B) every 7 days by killing mice until day 35 PI ($n = 5$). The vertical bars indicate mean $\pm$ SD.

by day 26 PI, whereas 40% of the C57BL/6 mice survived beyond day 40 PI, indicating partial resistance of the C57BL/6 mice against the fatal *N. seoulense* infection (Fig. 1A). The mean days of survival was significantly greater ($P < 0.05$) for BALB/c mice (22.0 ± 5.6 days) than C3H/He mice (14.2 ± 3.3 days). The genetic influence on the pathogenicity was reduced, with a decrease of the infection dose to 100 mc per mouse, although the C3H/He mice still showed an 80% mortality rate by day 35 PI (Fig. 1B). More than half of the BALB/c and C57BL/6 mice survived beyond day 35 PI at this dose (Fig. 1B).

The worm recovery rates were measured in 2 ways. First, the number of flukes remaining in the small intestine of each mouse ($n = 5$) directly after its death was calculated, except in 2 C57BL/6 mice that were killed at day 42 PI (Fig. 2A). Or, second, the weekly worm recovery rates ($n = 5$) were observed between day 7 and day 35 PI (Fig. 2B). Using the first method, the worm recovery rates were highest for C57BL/6 mice, followed by BALB/c, and then C3H/He mice (Fig. 2A). Very similar results were obtained by the second method, with C57BL/6 mice showing the highest worm recovery rates from day 28

to day 35 PI, and C3H/He mice the lowest rates during the same period (Fig. 2B). Worm expulsion started before day 21 PI in all 3 strains of mice, but a considerable proportion of worms persisted in the intestine until day 35 PI (Fig. 2B). The speed of worm expulsion was the fastest in the C3H/He mice and slowest in the BALB/c mice (Fig. 2B).

The average worm size at day 7 PI was 0.97–1.05 mm in length and 0.48–0.51 mm in width, without significant difference among the 3 strains of mice (Table I). The worms attained their full size by day 21 PI, but, after that time, they atrophied relative to the mouse strain up to day 35 PI (Table I). The decrease in the worm size was greatest in C3H/He mice, followed by BALB/c and C57BL/6 mice. Significant differences were present ($P < 0.05$) in the length and width of the worms recovered from the 3 strains of mice at day 28 or day 35 PI.

The reproductive output of *N. seoulense* (EPD) was observed in 2 ways, by checking the EPD every other day after infection with 200 mc (Fig. 3A) or every 7 days after infection with 100 mc (Fig. 3B). In the former method, the EPD began to increase from day 7 PI, and continued until day 16–18 PI in all 3 strains of mice (Fig. 3A). The peak EPD was significantly lower ($P <$

TABLE I. Measurements of the worms recovered from the 3 strains of mice experimentally infected with *Neodiplostomum seoulense*.

Mouse strain	Worm size by days postinfection (μm)				
	7 days	14 days	21 days	28 days	35 days
BALB/c mice					
Length	970.3 $\pm$ 162.8*	1257.4 $\pm$ 170.4	1273.4 $\pm$ 131.1	938.3 $\pm$ 96.7†	881.7 $\pm$ 119.6†
Width	510.9 $\pm$ 84.1	623.5 $\pm$ 74.2	628.1 $\pm$ 77.9	495.0 $\pm$ 75.1†	440.0 $\pm$ 63.2†
C3H/He mice					
Length	1051.9 $\pm$ 145.6	1146.4 $\pm$ 132.6	1230.4 $\pm$ 116.0	685.0 $\pm$ 134.4†	622.9 $\pm$ 164.6†
Width	509.6 $\pm$ 69.6	608.9 $\pm$ 37.5	605.4 $\pm$ 41.8	380.0 $\pm$ 36.8†	375.0 $\pm$ 104.9†
C57BL/6 mice					
Length	978.3 $\pm$ 112.1	1060.0 $\pm$ 121.3	1282.7 $\pm$ 130.4	1123.3 $\pm$ 129.3†	1011.7 $\pm$ 125.3†
Width	475.0 $\pm$ 39.0	538.3 $\pm$ 49.9	592.3 $\pm$ 72.4	578.3 $\pm$ 63.2†	526.7 $\pm$ 72.2†

* Mean $\pm$ SD of 20 worms.

† Significantly different between the C57BL/6 and C3H/He mice ($P < 0.05$) and between BALB/c and C3H/He mice ($P < 0.05$).

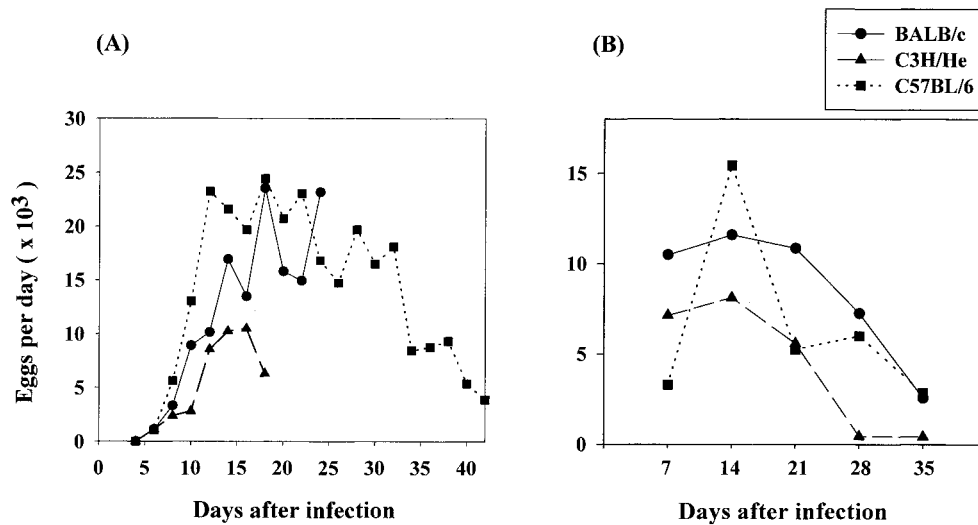


FIGURE 3. The chronological patterns of the EPD in the feces of C3H/He, BALB/c, and C57BL/6 mice ($n = 5$ for each value) infected with (A) 200 mc or (B) 100 mc of *N. seoulense*. The EPD was counted (A) every other day or (B) every 7 days PI. The peak EPD was significantly lower ($P < 0.05$) in C3H/He mice than in BALB/c or C57BL/6 mice in both cases.

0.05) in C3H/He mice than in the other 2 strains. After day 18 PI in C3H/He mice and day 24 PI in BALB/c mice, it was impossible to check the EPD because most of the mice had died. The C57BL/6 mice showed a plateau in the degree of the EPD from day 12 to day 32 PI, and a gradual decrease thereafter (Fig. 3A). In the latter method, the EPD peaked on day 14 PI and decreased gradually thereafter in all 3 strains of mice (Fig. 3B). The level of the peak EPD was, however, significantly different ($P < 0.05$) across the mouse strains, where the highest was in the C57BL/6 strain, followed by the BALB/c and C3H/He strains. Also, the decrease in EPD after the peak

was the most rapid and remarkable in the C3H/He mice compared with the other 2 strains.

The average number of eggs in the worm uteri increased progressively from day 7 to day 21 PI, but quickly diminished thereafter until day 35 PI in all 3 strains of mice (Fig. 4). However, the number of eggs was significantly smaller ($P < 0.05$) in the worms recovered from the C3H/He mice than in those from the other two strains.

In our previous study using C3H/HeJ, BALB/cA, and their hybrid F1 mice, it was strongly suggested that mice with different genetic backgrounds differ in their susceptibility to *N. seoulense* infection (Kook et al., 1998). The present study supports that suggestion and further suggests that the pathogenicity is also dependent upon H-2 gene differences in mice. A greater proportion of C3H/He (H-2k) mice succumbed to *N. seoulense* infection despite having fewer worms in their intestines and shorter duration of infection than BALB/c (H-2d) or C57BL/6 (H-2b) mice.

This kind of genetic influence on worm expulsion kinetics and host pathogenicity is likely to be related to differences in the properties of the host immune responses against the parasites. The genetic control of inbred strains of mice against *Trichinella spiralis* infection was, for example, primarily determined by the development of inflammatory responses that lead to worm expulsion (Wakelin and Donachie, 1981). The marked variations in the protective responses to parasite infections among the different strains of mice were due to T-cell-dependent immune responses (Krco et al., 1983; Wassom et al., 1984; Else and Grecnis, 1991). It was explained that such variations are dependent upon a genetic control influenced by the products of the H-2 genes in mice. Moreover, the proliferative responses of the cloned *T. spiralis*-specific T-cells were specifically inhibited by anti-I-E but not by anti-I-A subregion monoclonal antibodies, suggesting that I-A molecules play an important role in a mouse's immunity to *T. spiralis* (Krco et al., 1983).

In the present study, however, the discrepancy between the strong protective responses of C3H/He mice against *N. seoulense* infection and their severe clinical course (including di-

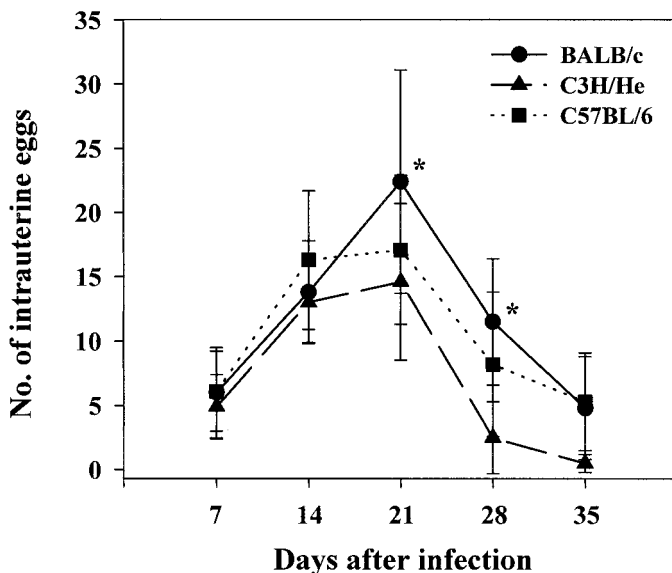


FIGURE 4. Number of intrauterine eggs in worms recovered from BALB/c, C3H/He, and C57BL/6 mice infected with 100 mc of *Neodiplostomum seoulense*. The data represent the mean $\pm$ SD of 20 worms. The difference was statistically significant ($P < 0.05$) at day 21 and day 28 PI between BALB/c and C3H/He mice.

arrhea, weakness, and mortality) raised many questions. In our previous study with *N. seoulense* (Kook et al., 1998), the entire small intestine of heavily infected C57BL/6 mice was severely contracted and shortened in length, suggesting intestinal paralysis, which was proposed as an important reason for the death of the mice (Kook et al., 1998). Structural and functional alterations of the intestine following enteric parasite infections are well known (Castro, 1989); they are caused by immunological responses of the host against the parasite and are associated with altered motility, smooth muscle hyperplasia, and decreased food transit times. The detailed pathogenesis leading to intestinal paralysis of mice infected with *N. seoulense*, however, is a subject for future research.

With regard to the effector cells responsible for the expulsion of *N. seoulense*, only a few studies have been conducted. Mucosal mast cells (MMC) were strongly suggested as the major effector in rats (Kho et al., 1990). Later, however, neither the MMCs nor the goblet cells were found to play important roles in the expulsion of *N. seoulense* from mice (Chai et al., 1998). In the expulsion of *N. seoulense*, little importance of the MMCs was observed again in mast-cell-deficient W/W^v and +/+ mice (Kook et al., 1998).

Therefore, further studies are required to better understand the expulsion mechanisms of *N. seoulense* from mice and its pathogenicity. Nevertheless, it could be speculated by the results obtained so far that the low worm burden and high mortality of C3H/He mice may have been caused by a rapid occurrence of hyperprotective responses such that the mice themselves were damaged by their own immune responses. The susceptibility as well as the fatality of mice may have been influenced by different levels of immune response determined by the genetic background of the host, e.g., H-2 gene products. It would be instructive to study the BALB/c mice in greater detail because they were susceptible to fatal infection at 200 mc but more or less resistant to a lower-dose infection. A detailed analysis of the immune responses induced in BALB/c mice would be helpful to understand their protective mechanisms against *N. seoulense* infection.

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Shared Epitope for Monoclonal IR162 Between *Anisakis simplex* Larvae and *Clonorchis sinensis* and Cross-Reactivity Between Antigens

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ABSTRACT: IR162 is a rat monoclonal IgE antibody (mAb). In the investigation of rat IgE production, the antigens recognized by IR162 (IR-Ags) were found to be expressed by a variety of helminthic parasites. By western blot analysis, IR162 detected bands in crude extracts of *Anisakis simplex* larvae, *Clonorchis sinensis*, *Paragonimus westermani*, plerocercoids of *Spirometra mansoni*, and *Toxocara canis*. Excretory–secretory material from *A. simplex* larvae also contained a protein recognized by IR162. IR162 mAb obtained from both Serotec and Zymed recognized identical bands of *A. simplex* larvae. Sandwich enzyme-linked immunosorbent assay analysis indicated that both IR-Ags of *A. simplex* larvae (IR-As) and *C. sinensis* (IR-Cs) were important antigens with respect to induction of specific IgG in human infections with these 2 organisms. Even though minimal cross-reaction between IR-As and IR-Cs was observed using sera from infected individuals, these results indicate that IR-Ags are found in the parasites examined, that IR-As and IR-Cs are the antigens that induce specific antibody in natural infection, and that the epitope that binds IR162 is shared by *A. simplex* larvae and *C. sinensis*.

IgE is one of the most important factors involved in immune responses to parasites. The production of specific IgE contributes to parasite removal (Hagan, 1993), and it has been found to enhance antigen presentation (Pirron et al., 1990; Squire et al., 1994). However, additional studies are required to further our understanding of the physiological role of IgE. Monoclonal anti-dinitrophenyl (anti-DNP) mouse IgE has been useful in understanding IgE responses in vivo (Yamaguchi et al., 1997). The observation that CD23 is related to proliferation of B cells was made using polymerized DNP and anti-DNP monoclonal mouse IgE (Campbell et al., 1992).

Bazin et al. (1974) cloned a number of cell lines from immunocytoma tumors arising spontaneously in ileocecal lymph nodes; these cell lines expressed several immunoglobulin isotypes. Among these lines was IR162, a monoclonal IgE that has been investigated as a prototype of rat IgE (Hellman et al., 1985). However, the antigen(s) recognized by this antibody were not known, reducing the usefulness of this reagent.

Clonorchis sinensis and *Anisakis simplex* larvae are important parasites of humans. The latter is a nematode responsible for mackerel allergy (Kasuya et al., 1990), as well as severe abdominal pain. The frequency of allergy development caused by *A. simplex* larvae is higher than other, well-known food-induced allergies, such as those caused by nuts or shrimp (Del Pozo et al., 1997). *C. sinensis* is a trematode that accelerates the development of cholangiocarcinoma (Lee et al., 1994).

We have identified antigens interacting with IR162 (IR-Ags) in several parasites, including *A. simplex* larvae, *C. sinensis*, *Paragonimus westermani*, plerocercoids of *Spirometra mansoni*, and *Toxocara canis*. We have also characterized the antigenicity of the IR-Ags from *A. simplex* larvae (IR-As) and from *C. sinensis* (IR-Cs) in natural human infections.

Rabbits and rats were purchased from Samyook Animal Center (Kyonggi-do, Korea). Dogs were purchased at a market in

Seoul. Animals were maintained at Korea University Medical College (KUMC) following the guidelines of the KUMC animal care committee for experimental animals. *A. simplex* third-stage larvae were collected from mackerels purchased at a fish market in Seoul. Adult *C. sinensis* were obtained from rabbit livers 3 mo after per os (p.o.) infection with metacercariae (Hong et al., 1997). Adult *P. westermani* were obtained from dog lungs 3 mo after p.o. infection with *P. westermani* metacercariae (Park et al., 1997). Plerocercoids of *S. mansoni* were collected from subcutaneous nodules of outpatients treated at Korea University Hospital in Ansan. *T. canis* worms were obtained from a naturally infected dog after anthelmintic treatment (Romaris et al., 1996).

Crude extracts of worms were prepared by homogenization with a Teflon pestle in a solution of phosphate-buffered saline pH 7.4 (PBS)/1% Igepal containing the proteinase inhibitors phenylmethylsulfonyl fluoride (10 µg/ml); leupeptin (0.5 µg/ml); aprotinin A (2.0 µg/ml); pepstatin A (1 µg/ml); iodoacetamide (3.7 mg/ml); and ethylenediaminetetraacetic acid (EDTA, 0.4 mg/ml) (Sigma, St. Louis, Missouri). Homogenized extracts were centrifuged at 10,000 *g* at 4 C and the supernatants used as the crude extracts (Iglesias et al., 1993). Excretory–secretory material of *A. simplex* larvae (ES-As) was prepared by culture of the larvae in RPMI-1640 supplemented with penicillin/streptomycin at 37 C in a humid incubator for 4 days (Iglesias et al., 1995). Culture supernatant was concentrated 10-fold by acetone precipitation and redissolved in PBS. Protein concentrations of all antigen preparations were estimated using the DC protein assay kit (Bio-Rad, Hercules, California) using bovine serum albumin as standard control.

Western blot analysis was performed as described by Gallagher et al. (1991) with some modifications. Proteins electrophoresed on 8–16% gradient Tris–glycine polyacrylamide gels (Novex, San Diego, California) were transferred to nitrocellulose membranes (Millipore, Bedford, Massachusetts) and blocked with 1% gelatin/0.1% Tween 20 Tris-buffered saline. The membrane was incubated with 2 µg/ml IR162 from Serotec (Oxford, U.K.) or Zymed (South San Francisco, California) overnight at room temperature (RT). After washing, the membrane was incubated with 4 µg/ml B5 (monoclonal mouse IgG anti-rat IgE) (Conrad et al., 1983) for 2 hr at RT, washed, and incubated for 2 hr with goat anti-mouse IgG that was conjugated to alkaline phosphatase and adsorbed to rat serum (AP-anti MoIg) (Sigma), diluted 1:1,000; 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium liquid substrate system (Sigma) was used to visualize antibody binding.

Indirect enzyme-linked immunosorbent assay (ELISA) was performed for detecting antigen-specific human IgG on the basis of the method described by McLaren et al. (1978), with some modifications. ELISA plates were coated with 100 µl of

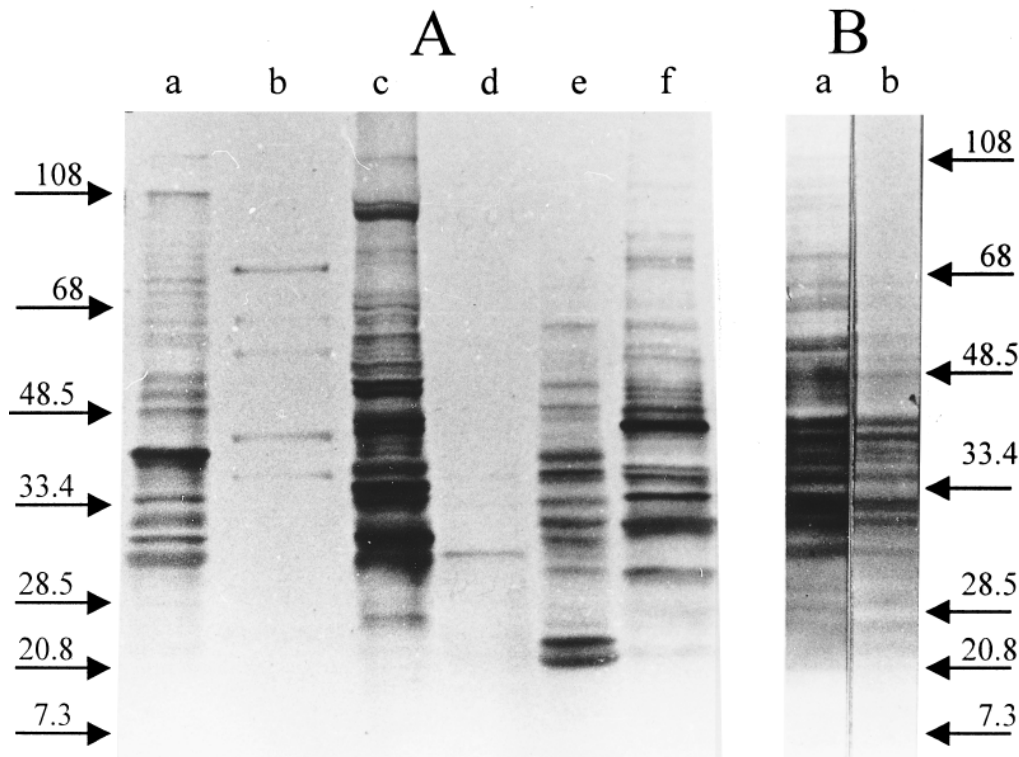


FIGURE 1. Western blot analysis of the parasite antigens recognized by rat monoclonal IgE, IR162. (A) Ten-well gradient gel loaded with 30 μ g of protein per well, blotted and probed with IR162 from Zymed. Antigens recognized by IR162 in parasite crude extracts, *Anisakis simplex* larvae (lane a), excretory-secretory material of *A. simplex* larvae (lane b), *Toxocara canis* (lane c), *Clonorchis sinensis* (lane d), *Paragonimus westermani* (lane e), plerocercoids of *Spirometra mansoni* (lane f). (B) Two-well gradient gel loaded with 250 μ g of protein per well of *A. simplex* larvae per well and probed with IR162 from Serotec (lane a) or IR162 from Zymed (lane b). Molecular masses (kDa) of standard markers are shown on both sides of blots.

antigen (4 μ g/ml). Plates were blocked with 1% bovine calf serum (BCS)/0.1% Tween 20-PBS (PBST) for 1 hr at 37 C, which was also the solution used for dilution of ELISA reagents. The plates were incubated with each serum diluted 1:200 for 2 hr at 37 C and peroxidase-conjugated goat anti-human IgG (ICN-Cappel, Costa Mesa, California), diluted 1:1,000 for 2 hr at 37 C. O-phenylene diamine was used as substrate. Optical density (OD) was read at dual wavelengths (450/650 nm) with a Spectramax ELISA reader (Molecular Devices, Menlo Park, California).

To determine the antigenicity of IR-Ags in natural infections and the cross-reaction between IR-As and IR-Cs, a sandwich ELISA was performed on the basis of the method described by Iglesias et al. (1997), with some modification. ELISA plates were coated with 4 μ g/ml IR162 in coating buffer overnight at 4 C and blocked with BCS/PBST for 30 min at 37 C. Crude extracts of parasites were added to each well and incubated overnight at 4 C. Washed plates were incubated for 1 hr at 37 C with human sera diluted 1:200; detection was performed with peroxidase-conjugated goat anti-human IgG (2 hr at 37 C; ICN-Cappel) diluted 1:1,000. OD determinations utilized the Spectramax reader as described. The sera used were obtained from the Institute for Tropical and Endemic Diseases, Korea University. All ELISA experiments were run in duplicate and expressed as ± 1 standard error (SE).

Initially, we used IR162 antibody as a negative control in the study of specific rat IgE directed against *A. simplex* larvae and regarded the bands obtained as a nonspecific phenomenon. How-

ever, the repeated results showing specific bands in ES-As encouraged us to investigate further to see if ES-As has antigens that bind IR162, i.e., IR-Ags. When we searched for IR-Ags in the crude extracts of *A. simplex* larvae (cAs) with biotinylated MARE-1 (monoclonal mouse IgG anti-rat IgE; Zymed) and alkaline phosphatase-conjugated Extavidin (Sigma), we could not differentiate any specific bands by IR162, because there were avidin binding materials in cAs; a similar result was noted by Romaris et al. (1996). We therefore selected purified B5 and rat serum-adsorbed alkaline phosphatase-conjugated goat anti-mouse IgG and, with this system, we could differentiate the bands for IR-Ags even in the crude extracts of *A. simplex* larvae. To determine whether IR-Ags were unique in *A. simplex* larvae, we western blotted to nitrocellulose the crude extracts from several parasites, including *C. sinensis*, *P. westermani*, *S. mansoni*, and *T. canis*. The results indicated that all parasites probed had antigens recognized by IR162, i.e., were IR-Ags (Fig. 1A). Visible bands ranged from 10 to 120 kDa and the number of bands recognized from each species was different. To evaluate the consistency of the binding, we applied the IR162 obtained from either Serotec or Zymed and observed that the binding was identical regardless of the commercial source of the antibody (Fig. 1B; lanes a and b). However, the bands were not visualized by B5 when IR162 was omitted (data not shown).

The antigenicity of IR-As in human infection was analyzed with the sera specific for *A. simplex* larvae using sandwich ELISA. To verify the appropriateness of the ELISA preparation, the antigen dose relation of cAs was performed using human

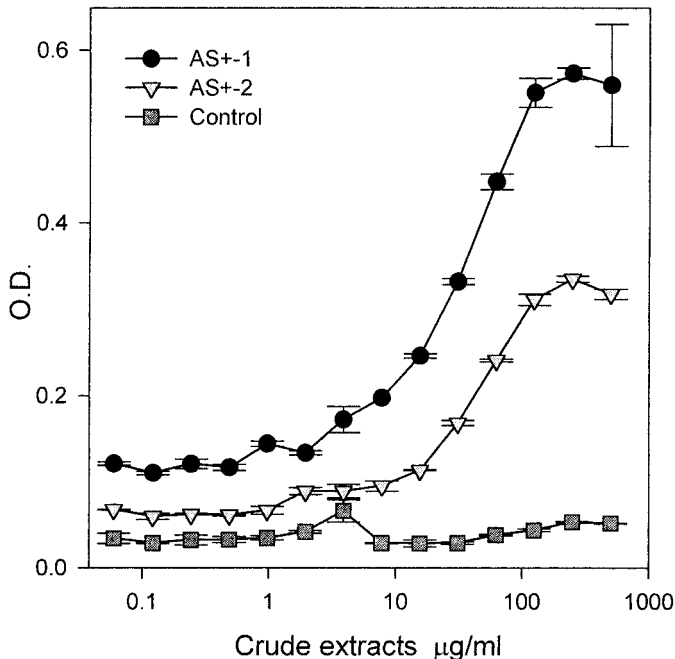


FIGURE 2. Sandwich ELISA with crude extracts of *Anisakis simplex* larvae. Analysis of appropriate human sera demonstrates that the parasite proteins recognized by IR162 are important antigens in natural human infections. ELISA plate was coated with IR162 from Zymed. Crude extracts of *A. simplex* larvae, ranging from 0.06 to 500 µg/ml, were applied to each well. As+ refers to 2 human serum samples containing specific antibody against *A. simplex* larvae. Human sera from noninfected individuals served as a negative control and was designated control.

serum-specific *A. simplex* larvae (As+ control) and a nonspecific control serum (As- control). A clear dose response was seen with dilutions of the As+ sera when applied to the cAs proteins bound by the basal coat of IR162. In contrast, no dose-related binding of IgG was seen when As- sera were used in lieu of the As+ sera. The OD at saturation (250 µg of cAs per well) using As+ sera was 0.57 and 0.34 as compared to 0.05 for As- sera. These results suggest that the binding was antigen-specific and that IR-As is, or are, antigenic in the human host.

To examine any cross-reactivity between IR-As and IR-Cs, we tested As+ and human serum specific for *C. sinensis* (Cs+) in the indirect ELISA and sandwich ELISA, respectively. The indirect ELISA results demonstrated low cross-reactivity between the 2 sera, as substrate development was much stronger when the appropriate antibody was added to the corresponding antigen(s) (Fig. 3A, B). Essentially similar results were obtained when these 2 sera were tested in the sandwich ELISA in which IR162-pretreated plates were coated with either cAs or crude extracts of *C. sinensis* (cCs) (Fig. 3C, D). In all, a total of 3 different As+ sera and 4 Cs+ sera was tested and all gave a similar result. These findings suggest that the cross-reaction between IR-As and IR-Cs was quite low. The results also suggest that both parasites have clinically important antigens that are recognized by the IR162 rat IgE antibody.

IR162 has become a most useful mAb and now is a cornerstone of IgE research. Before the description of this antibody by Bazin et al. (1974), only human IgE was available in any quantity. As the prototype of rat IgE, intensive research has been done (Hellman et al., 1985) and the value of these en-

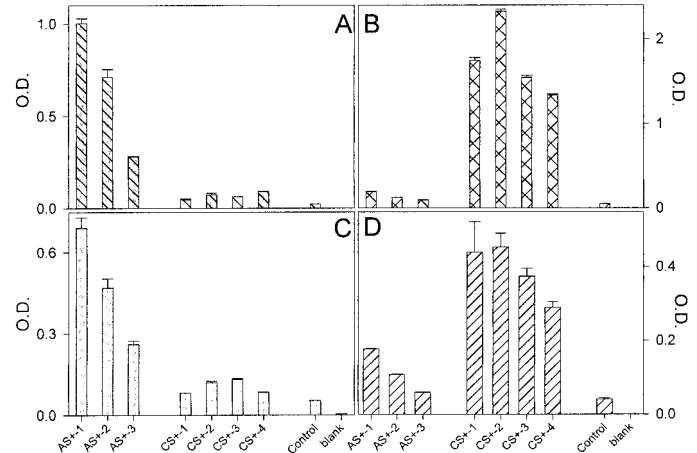


FIGURE 3. Cross-reactivity between antigens recognized by IR162 from crude extracts of *Anisakis simplex* larvae and those from crude extracts of *Clonorchis sinensis*. (A and B): Indirect ELISA was performed by coating wells with crude extracts of (A) *A. simplex* larvae or (B) *C. sinensis*. The indicated sera (1:200 dilution) or buffer was added to duplicate wells and IgG binding was determined. (C and D): Sandwich ELISA was performed on ELISA plates coated with IR162 from Zymed. Crude extracts (100 µg/ml) of (C) *A. simplex* larvae or (D) *C. sinensis* were added to each well. The same serum and control set was then added and specific human IgG binding determined. As+ indicates a serum containing anti-*A. simplex* antibodies and Cs+ indicates a serum containing anti-*C. sinensis* antibodies.

deavors will be amplified now that the antigens have been identified. The initial identification of the IR-Ags was performed using commercially obtained IR162 from Serotec to characterize antigens in ES-As. In addition, we have tested different lots of IR162 from Serotec and from another vendor (Zymed) and, in each case, the antibodies bound to the same ES-As bands on western blots (data not shown).

Hahn et al. (1996) reported that the plasma of *Biomphalaria glabrata*, the intermediate snail host of *Schistosoma mansoni*, contains a protein that binds immunoglobulins from a variety of vertebrates. When IR162 was omitted from the sandwich ELISA for cAs, a few nonspecific bands were labeled by alkaline phosphatase-conjugated anti-mouse Ig; however, these bands were not seen when a peroxidase-conjugated goat anti-rat IgG whole molecule (Serotec) was used (data not shown). The most likely explanation for this nonspecific labeling is that the alkaline phosphatase reagent had some anti-parasite activity. This nonspecific staining can be characterized by fewer bands and ones of lesser intensity than are seen with specific antisera or IR162 mAb. When blots were performed with peroxidase-conjugated goat anti-rat IgG whole molecule, the antigen bands of IR-Ags were recognized with IR162, but not with rat monoclonal antibodies 11B11 (rat IgG1 mAb; Pharmingen, San Diego, California) or JS6-1A12 (rat IgG2a mAb; Pharmingen) (data not shown). The staining intensity of cAs with IR162 from Serotec (Fig. 1B, lane a) was stronger than that observed using IR162 mAb from Zymed (Fig. 1B, lane b). Currently, it is not known whether the difference in staining intensity reflects a variance in IR162 from the different vendors or, alternatively, it could reflect differences in quality of the antibody preparations. In any case, the difference in staining intensity may be a

minor problem in the identification of the antigens recognized by IR162.

Konishi (1991, 1997) described naturally occurring IgM antibodies to *Toxoplasma gondii* in humans and mouse IgM monoclonal antibodies multireactive with parasite antigens from *T. gondii* and *T. canis*. He noted that naturally occurring antibodies are the cause of such "nonspecific reactions." Although it is very interesting that all the parasites tested expressed IR-Ags, we wanted to determine whether IR-Ags induced specific antibody production in human infections. Furthermore, whether IR-Ags cause cross-reaction between parasite antigens in routine laboratory tests for parasite-specific antibody remains to be determined. To characterize antigenicity in natural human infection as well as cross-reactivity, we prepared sandwich ELISA, reasoning that the principles underlying the technique were similar to those used in the diagnosis of *A. simplex* larvae infection with a monoclonal anti-*A. simplex* antibody as the base coating (Iglesias et al., 1997). We selected *A. simplex* larvae and *C. sinensis* to evaluate cross-reactivity between species because there are fewer bands in the latter (Fig. 1A). Antigen dose dependency is clearly demonstrated in Figure 2, which shows that the IR-As induced a specific human IgG response. The similarities between the patterns observed in the comparison of cross-reactivity using either the direct parasite antigen-coated plates compared with IR162 rat IgE-coated plates (Fig. 3) was striking, and essentially all of the parasite antigens of both parasites were bound to the IR162 rat IgE. These conditions indicate that the binding of human IgG is restricted to IR-Ags and not other antigens. The results further suggest that the cross-reaction between IR-As and IR-Cs are not sufficient to significantly influence the identification of antigen specificity (Fig. 3). Rather, IR-As are antigenically distinct from IR-Cs. The results suggest that whereas the epitope bound by IR162 is shared among many parasites investigated, the antigens are not identical, as was shown using *A. simplex* and *C. sinensis*. Perhaps the epitope interacting with IR162 is either a carbohydrate structure common to parasite antigens or a repeat motif common to parasite antigens.

Konishi (1997) noted that the multireactive monoclonal IgM antibodies are not efficient to defend mice from infection with *T. gondii*. Although it is not known whether the naturally occurring IgM antibodies described by Konishi and IR162 bind to the identical epitope, it would be of interest to see if passive transfer of IR162 can influence infection outcome because IgE is thought to be so important in parasite rejection (Hagan, 1993).

We believe that these findings will contribute to further study of IgE and parasite antigens and that the finding that many parasites have antigens recognized by IR162 will help accelerate the purification and identification of the IR-Ags in each parasite species. Utilization of IR162 should contribute greatly to unlocking the physiological role of IgE in the rat.

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Frenkelia microti Infection in a Chinchilla (*Chinchilla laniger*) in the United States

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ABSTRACT: A *Frenkelia microti* tissue cyst was found in the brain of a chinchilla (*Chinchilla laniger*) used for biomedical research. This is the first report of *Frenkelia* infection in this animal in the United States.

Frenkelia microti (Findley and Middleton, 1934) Biocca, 1968 is a coccidian with many species of small mammals as intermediate hosts, and buzzards (*Buteo buteo*) and hawks (*B. borealis* and *B. jamaicensis*) as definitive hosts (Rommel and Krampitz, 1975; Dubey et al., 1989; Lindsay et al., 1992; Upton and Mckown, 1992). Intermediate hosts become infected by ingesting sporocysts or oocysts shed in the feces of definitive hosts. Merogony occurs mostly in hepatocytes, and tissue cysts are found in the brain (Rommel and Krampitz, 1975; Gobel et al., 1978). Tissue cysts of *Frenkelia* are lobulate and can be macroscopic. The life cycle of *Frenkelia* is similar to *Sarcocystis* and some authors regard *Frenkelia* as a synonym of *Sarcocystis* (Votýpka et al., 1998; Mugridge et al., 1999).

In North America, tissue cysts of *Frenkelia* have been reported from wild voles (*Microtus modesticus*; Frenkel, 1953, 1956), a rat (*Rattus rattus*; Hayden et al., 1976), a muskrat

(*Ondatra zibethica*), and a porcupine (*Erithizon dorsatum*; Karstad, 1963; Kennedy and Frelie, 1986). The only report of a *Frenkelia* infection in chinchillas (*Chinchilla laniger*) is from Germany (Meingassner and Burtscher, 1977). We herein report a *F. microti* infection in a chinchilla in the United States.

The animal was a retired breeder female obtained from a supplier in Rochester, Minnesota. It was from a group of chinchillas used for biomedical research on noise-induced hearing loss. The chinchillas were housed individually and fed commercial chinchilla chow, supplemented with raisins, carrots, and apples.

No clinical signs were noted. The animal was killed in good health and necropsied. Specimens from all major organs were fixed in 10% buffered neutral formalin. Paraffin-embedded sections were cut at 5 μm thickness and examined after staining with hematoxylin and eosin.

One large lobulated tissue cyst was seen in the cerebrum near the meninges (Fig. 1A). The tissue cyst was 330 μm long and 220 μm wide. The cyst wall was thin (<0.5 μm thick) and enclosed hundreds of bradyzoites (Fig. 1B). Septa were thin; there was no host reaction.

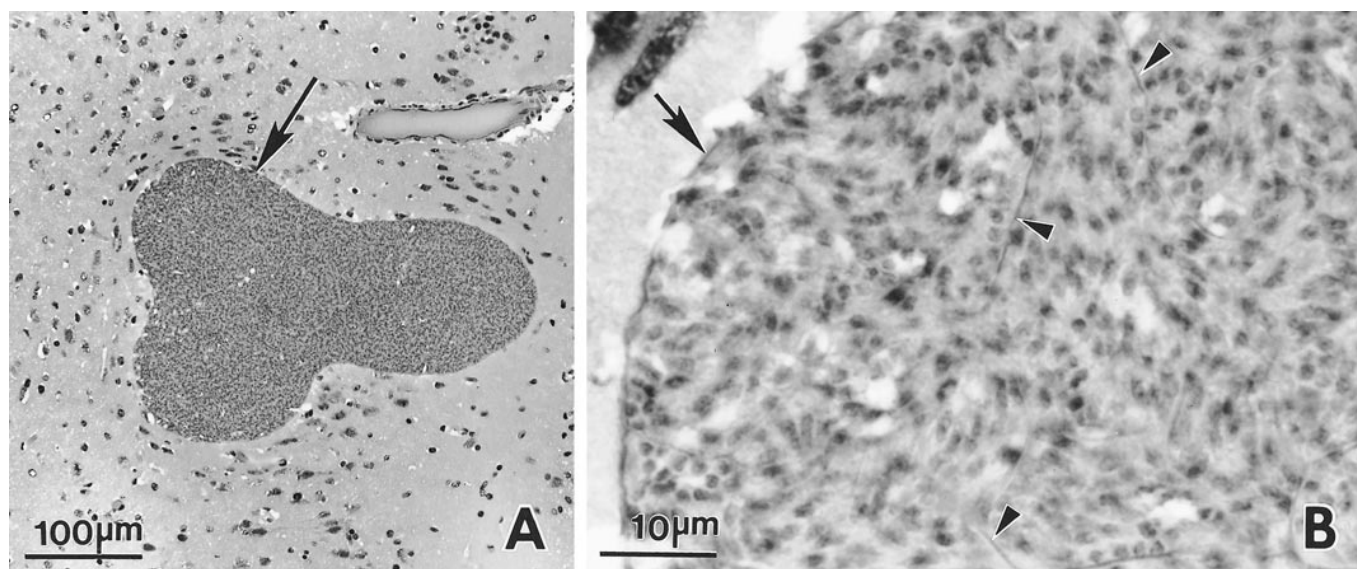


Figure 1. *Frenkelia microti* tissue cyst in the cerebrum of chinchilla. **A.** A low magnification showing a lobulated tissue cyst (arrow). **B.** Tissue cyst with cyst wall (arrow) and thin septa. Hematoxylin and eosin stain.

The tissue cyst in this chinchilla was probably an incidental finding. The chinchilla probably became infected by ingesting food contaminated with feces of an infected hawk. The tissue cyst phase of *Frenkelia* generally does not cause clinical signs in animals (Dubey et al., 1989). Although the merogonic phase of *Frenkelia* spp. in naturally infected animals is unknown, merogony occurs in hepatocytes in experimentally infected animals, and can be pathogenic (Rommel and Krampitz, 1975; Gobel et al., 1978). *Frenkelia* meronts are structurally similar to those of *Sarcocystis* spp that develop in parenchymal cells, e.g., *Sarcocystis muris* with cat-mouse cycle. Acute hepatic sarcocystosis was reported in 2 chinchillas by Rakich et al. (1992), and has been seen in 3 other chinchillas in the United States (J. Dubey, unpublished observation). Whether acute hepatic sarcocystosis in chinchillas represents a phase of *F. microti* needs investigation. In this respect, of the 22 chinchillas necropsied over the past year at the Naval Medical Center, San Diego, 14 had gross hepatic lesions with an undetermined etiology.

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Immunohistochemical Confirmation of *Sarcocystis neurona* Infections in Raccoons, Mink, Cat, Skunk, and Pony

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ABSTRACT: In the central nervous system of 2 raccoons, 1 cat, 1 pony, 2 mink, and 1 skunk, protozoa previously thought to be *Sarcocystis*-like reacted positively to *Sarcocystis neurona*-specific antibodies in an immunohistochemical test. In addition, *S. neurona* was identified in the brain of another skunk. These observations indicate that *S. neurona* is not confined to opossums and horses.

Sarcocystis neurona is an etiologic agent for equine protozoal myeloencephalitis (EPM) in horses, and EPM is the most common cause of neurologic disorders in horses in the Americas (Dubey et al., 1991; Hamir et al., 1992; MacKay, 1997). The life cycle of *S. neurona* is not fully known. The opossum (*Didelphis virginiana*) is a definite host, and horses are considered aberrant intermediate hosts. Horses are thought to become

infected by ingesting *S. neurona* sporocysts excreted in the feces of opossums (Fenger et al., 1997; Dubey and Lindsay, 1998). How opossums become infected with *S. neurona* is not known because intermediate hosts harboring *S. neurona* sporocysts are not known. Only schizonts and merozoites are found in tissues of horses, and these stages are confined to the brain and the spinal cord. Live *S. neurona* has been isolated only from the central nervous system (CNS) of horses and the intestines of opossums (Dubey et al., 1991; Dubey and Lindsay, 1998). Recently, *S. neurona*-like infections were found in sea lions from California and a sea otter from Oregon; the protozoa in the CNS of these animals reacted with *S. neurona* antibody (Lapointe et al., 1998; Rosonke et al., 1999). More recently, *S.*

TABLE I. Detection of *Sarcocystis neurona* by immunohistochemistry in brains of naturally infected animals in the United States.

Species	Location	Reference
Pony	Maryland	Dubey and Miller (1986)
Mink	Oregon	Dubey and Hedstrom (1993)
Cat	California	Dubey et al. (1994)
Skunk	Massachusetts	Dubey et al. (1996)
	Oregon	this study
Raccoon	Ohio	Dubey et al. (1990)
	New York	Stoffregen and Dubey (1991)

neurona was cultivated in vitro from the brain of a naturally infected sea otter (Lindsay et al., 2000). Previous to that, *S. neurona*-like infections were reported in raccoons (Dubey et al., 1990; Stoffregen and Dubey, 1991; Thulin et al., 1992), skunks (Dubey et al., 1996), mink (Dubey and Hedstrom, 1993), a cat (Dubey et al., 1994), a monkey (Klump et al., 1994), and a pony (Dubey and Miller, 1986).

Although antibodies to *S. neurona* have been reported in ponies and other equids, clinical EPM has been confirmed only in horses (Saville et al., 1997). The only case of EPM in a pony is that reported by Dubey and Miller (1986) before the discovery of *S. neurona*. Recently, a high-titer *S. neurona*-specific serum was produced in a rabbit using cultured merozoites (Dubey et al., 1999). Here, we report specificity of this serum and confirm *S. neurona* infections in various species of wild and domestic animals.

Tissues of 2 raccoons, 2 mink, a skunk, a cat, and a pony with *S. neurona*-like infections previously reported (Table I), tissues of a monkey (Klumpp et al., 1994), and tissues of an additional skunk from Corvallis, Oregon, with multifocal non-suppurative encephalitis were used for immunohistochemical (IHC) demonstration of the parasites.

The *S. neurona* antibody was obtained from the serum of a rabbit injected with cultured merozoites of an *S. neurona* isolate from opossum no. 95 (Dubey et al., 1999). The positive control tissues were obtained from gamma-interferon knockout mice injected with *S. neurona* cultured merozoites and mice fed sporocysts from opossum feces (Dubey and Lindsay, 1998). The positive equine tissues were from the spinal cord of the horse that was used to describe the morphological characteristics of *S. neurona* (Dubey et al., 1991) and an EPM horse in which infection was verified by isolation of the SN6 isolate of *S. neurona* (Dubey et al., 1999). The negative control tissues used for the demonstration of specificity of the test serum were sarcocysts and second generation schizonts of *Sarcocystis cruzi* from cattle, schizonts from the liver of a horse infected with an undetermined species of *Sarcocystis* (Davis et al., 1999), tissues from a *Sarcocystis canis*-like infection in bears (Zeman et al., 1993; Garner et al., 1997), tissues from a chinchilla with hepatic sarcocystosis (Rakich et al., 1992), schizonts and sarcocysts of *Sarcocystis speeri* from mice (Dubey and Lindsay, 1999), tachyzoites and tissue cysts of *Neospora caninum* from mice, dogs, and cattle, *Neospora*-infected spinal cord from a naturally infected horse (Hamir et al., 1998), tachyzoites and tissue cysts of *Toxoplasma gondii* from cats and mice, and sarcocysts of *Sarcocystis kirkpatrickii* from raccoons (Snyder et al., 1990). For the IHC technique, the sections were deparaffinized, di-

gested in 0.4% pepsin, and reacted with a 1:10,000 dilution of serum from the rabbit using the avidin-biotin peroxidase complex method (Dubey et al., 1999). The rabbit serum had a ≥ 1 :16,000 titer in an indirect fluorescent antibody test using *S. neurona* merozoites from cell culture as antigen.

Organisms from all brains of animals in Table I (including 1 skunk from Oregon) reacted with the *S. neurona* antibody. Organisms in the spinal cord of the monkey previously considered to be *S. neurona* (Klumpp et al., 1994) did not react with *S. neurona* antibody in the present study. These findings suggest that another *S. neurona*-like organism might have been associated with encephalomyelitis in the monkey.

Tissues infected with other organisms did not react with the *S. neurona* antibody. Results of this study confirm that *S. neurona* infections occur in animals other than horses and opossums. The role of these animals in the life cycle of *S. neurona* remains to be determined.

Until recently, there was no immunocompetent animal model to study pathogenesis of *S. neurona*. Horses fed *S. neurona* sporocysts developed clinical signs and lesions consistent with EPM and had antibodies to *S. neurona*, but the parasite was not demonstrable in equine tissues (Fenger et al., 1997). Thus, Koch's postulates were not fulfilled. Although clinical EPM has been induced in immunodeficient mice (Marsh et al., 1997; Dubey and Lindsay, 1998), the small size of mice precludes evaluation of many clinical parameters. Findings of *S. neurona* in association with lesions in CNS tissues of nonequid animals indicates that these animals may also serve as natural aberrant intermediate hosts for *S. neurona*.

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***Gyrodactylus anguillae* (Monogenea: Gyrodactylidae) From Anguillid Eels (*Anguilla australis* and *Anguilla reinhardtii*) in Australia: A Native or an Exotic?**

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ABSTRACT: A species of *Gyrodactylus* collected from 2 species of anguillid eels (*Anguilla australis* Richardson, 1841 and *Anguilla reinhardtii* Steindachner, 1867) from Australia is identified as *Gyrodactylus anguillae* Ergens, 1960. The morphology of sclerites of *G. anguillae* specimens from Australia is in accordance with previous descriptions of specimens collected from *A. anguilla* (Linnaeus, 1758) from Europe and *A. anguilla* imported into Japan. *Gyrodactylus anguillae* was previously thought to be a parasite specific to *A. anguilla*, an eel that is native to freshwater catchments throughout Europe. Information on eel translocations and host and parasite biology is reviewed and it is hypothesized that *G. anguillae* is a naturally occurring parasite in Australia and not an introduction.

Despite increasing interest in the culture of anguillid eels in Australia, little attention has been given to their parasite fauna on this continent. Kennedy (1995) provides the only thorough investigation of the parasite fauna of a species of eel (*Anguilla reinhardtii* Steindachner, 1867) in Australia. Kennedy (1995) identified 2 species of Monogenea, namely *Pseudodactylogyrus anguillae* (Yin and Sproston, 1948) and *Pseudodactylogyrus*

bini (Kikuchi, 1929), but not another well known parasite of eels, *Gyrodactylus anguillae* Ergens, 1960. We have found a species of *Gyrodactylus* on 2 species of anguillid eels from several localities in Australia. In the present paper, we provide a description of this species of *Gyrodactylus* and discuss whether it is an exotic or native parasite in Australia.

Infected *Anguilla australis* Richardson, 1841 and *A. reinhardtii* were collected from sites in Queensland, New South Wales, and Victoria. Specific localities are given below. Before being examined for parasites, eels collected from the Albert River in southeast Queensland were cultured for almost 1 yr at either the Queensland Department of Primary Industries (QDPI) Freshwater Fisheries and Aquaculture Centre (FFAC), Walkamin, north Queensland or the QDPI Bribie Island Aquaculture Research Centre (BIARC), southeast Queensland. Eels from other localities were dissected soon after capture. Fish were identified using Allen (1989). For the study of sclerites, parasites were mounted in ammonium picrate glycerin (Malmberg, 1970).

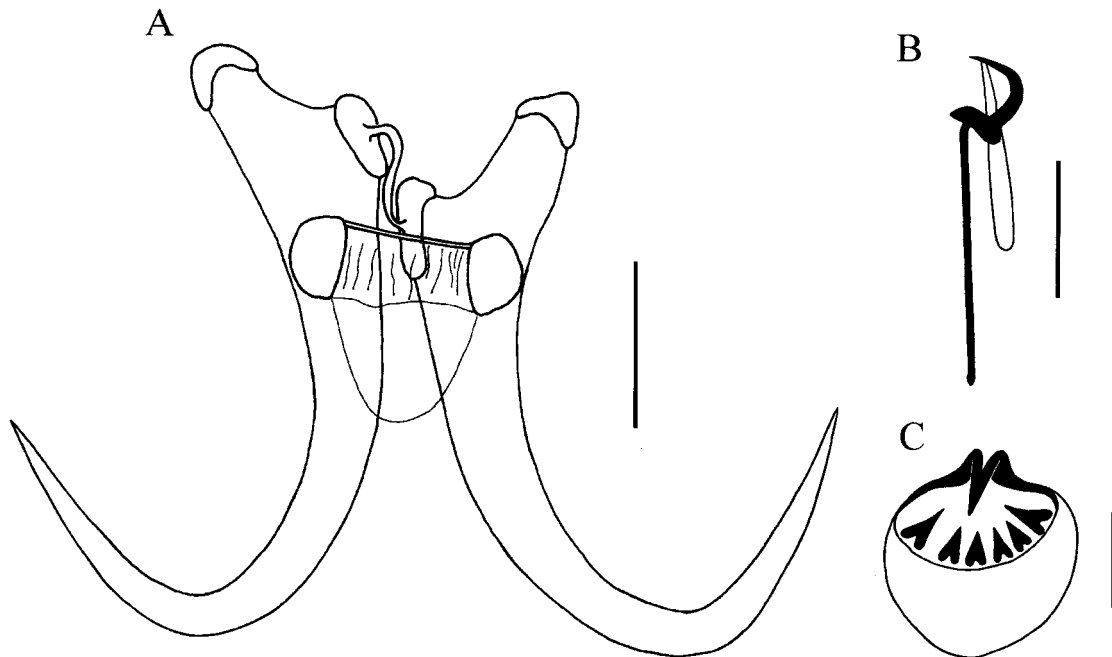


FIGURE 1. Sclerites of *Gyrodactylus anguillae* from *Anguilla australis* and *Anguilla reinhardtii* in Australia. **A.** Hamuli, ventral and dorsal bars. **B.** Marginal hook. **C.** Male copulatory organ. Scale bars 10 μm (A, B) and 5 μm (C).

Fourteen sclerite characters were measured for each worm. Measurements reported here generally follow the method of Malmberg (1970). Measurement of the marginal hook follows Ogawa and Egusa (1978). Measurements were made using a computerized digitizing system similar to that described by Roff and Hopcroft (1986) and were rounded to the nearest 0.5 μm . Unless otherwise stated, all measurements are given in micrometers as the mean with the range in parentheses. Measurements of marginal hooks are the average of 4 per individual specimen. Hamuli measurements were also averaged for each specimen.

Malmberg's (1970) voucher specimens of *G. anguillae* were borrowed from the Swedish Natural History Museum (SNHM), P.O. Box 50007, S-104 05 Stockholm, Sweden. Voucher specimens (QM G217347-8) of the present material are deposited in the Queensland Museum (QM), P.O. Box 3300, Brisbane, Queensland 4101, Australia.

Specimens of *G. anguillae* were collected from the following hosts and locations in Australia: *Anguilla australis* and *A. reinhardtii* from the Albert River, southeast Queensland (cultured at BIARC, length 49–57 mm, $n = 3$, prevalence 100%; cultured at FFAC, length 73–122 mm, $n = 11$, prevalence 64%); *A. reinhardtii* from the Fitzroy River, central Queensland (length 180 mm, $n = 1$); *A. reinhardtii*, Repton, NSW (length 427–541 mm, $n = 8$, prevalence 25%); *A. australis*, Skipton, Victoria (length 635–706 mm, $n = 5$, prevalence 100%). All specimens of *G. anguillae* were collected from the gills of their hosts.

The following diagnosis is based on 14 worms from FFAC mounted in ammonium picrate glycerin. Observations on soft body organs were taken from 11 Mayer's hematoxylin-stained whole mounts from BIARC. Total length 254 (220–290); maximum width 68 (58–79). Hamulus length 36 (34–37.5); hamulus point length 17.5 (16–19); hamulus shaft length 32.5 (30.5–33.5); hamulus root length 12.5 (11.5–13.5) (Fig. 1A). Marginal

hook total length 24.5 (23.5–25); length of marginal hook sickle 5.5 (5–6.5); length of marginal hook handle 19 (18.5–20) (Fig. 1B). Distal width of marginal hook sickle 5 (4.5–6); proximal width 4 (4–4.5). Marginal hook filament length 13.5 (12.5–14.5). Ventral bar medial length 4 (3.5–5); ventral bar lateral length 5 (4.5–5.5); ventral bar width 13 (12–14.5). Ventral bar membrane rarely visible; length 8 ($n = 2$). Total length of ventral bar and membrane 13.5 (13–13.5). Dorsal bar medial length 1 (0.5–1.5); width 12 (8.5–15.5). Male copulatory organ with 1 large, 2 moderate, and 4 small spines (1 + 2 + 4) (Fig. 1C). Embryo normally folded dorsoventrally in uterus. Of 11 unflattened whole mounts with large embryos, 5 were folded with embryo haptor dorsal, 5 folded with haptor ventral, and 1 was folded laterally.

The shape and size of the haptoral sclerites of specimens described here are very similar to descriptions of *G. anguillae* by Ergens (1960), Malmberg (1970), and Ogawa and Egusa (1978, 1980) (Table I). Some small discrepancies do, however, occur. *Gyrodactylus* specimens from Australia have smaller sickle distal width than reported by Malmberg (1970), although sickle distal width reported by Ogawa and Egusa (1978, 1980) is comparable. Marginal hook filament length is larger than reported by Malmberg (1970); however, he used a procedure for measuring this character different to that used by Ogawa and Egusa (1978) and us. Embryos of *G. anguillae* from Australia are folded dorsoventrally in the uterus rather than the normal situation in *Gyrodactylus* where worms are folded laterally. The significance of this is unclear because mature embryos are previously undescribed in this species and the usual orientation of embryos could not be determined in the flattened voucher specimens from SNHM. Measurements of specimens from BIARC, Fitzroy River, Repton, and Skipton are in accordance with those from FFAC. We conclude that all the *Gyrodactylus* specimens

TABLE I. Sclerite measurements of *Gyrodactylus anguillae* from *Anguilla* spp.

Author	Ergens, 1970	Malmberg, 1970	Ogawa and Egusa, 1978	Ogawa and Egusa, 1980	This study
Host(s)	<i>A. anguilla</i>	<i>A. anguilla</i>	<i>A. anguilla</i>	<i>A. anguilla</i>	<i>A. australis</i> , <i>A. reinhardtii</i>
Locality	Albania	Sweden	England	Japan	Australia
No. of specimens	1	10	17	14	14
HL	32.3	36.1 (35.2–37.0)	31 (30–34)	(34–37)	36 (34–37.5)
HPL	15.2	16.7 (15.8–17.2)	14 (13–15)	(15–16)	17.5 (16–19)
HSL	28.5	31.4 (30.8–32.6)	27 (26–29)	(29–32)	32.5 (30.5–33.5)
HRL	9.5	11.7 (9.7–13.2)	8 (7–9)	(9–11)	12.5 (11.5–13.5)
LMH		24.0 (22.9–25.1)†	(22–25)†	(22–24)	24.5 (23.5–25)
LSI	5.7	5.8 (4.8–6.2)†	(5–6)†	(5–6)	5.5 (5–6.5)
LH		18.7 (18.0–19.4)†	(17–19)†	(17–18)	19 (18.5–20)
SDW		5.9 (5.7–6.2)†	(5–6)†	(5–6)	5 (4.5–6.0)
SPW		4.0 (3.5–4.0)†	(3–4)†	(3.5–4)	4 (4–4.5)
MHFL		9.8 (8.8–11)†	(10–12)†		13.5 (12.5–14.5)
VBL	3.8	4.5 (4.0–5.3)	(2.5–3)	(2–3.5)	4 (3.5–5)
VBLL		4.7 (4.0–5.3)			5 (4.5–5.5)
VBW	9.5	14.1 (13.6–14.5)	13 (12–14)	(11–13)	13 (12–14.5)
VBML		7.8 (6.2–9.7)	(5–7)		8
VBTL		13.6 (11.0–15.4)			13.5 (13–13.5)
DBL	1	1.4 (1.3–1.8)	1		1 (0.5–1.5)
DBW	9.5	14.8 (14.1–15.8)	(12–14)		12 (8.5–15.5)
MCO		1+2+4 (1+4–7)			1+2+4

* Means are given with the range in parentheses. Abbreviations: HL, hamulus length; HPL, hamulus point length; HSL, hamulus shaft length; HRL, hamulus root length; LMH, total length of marginal hook; LSI, length of sickle; LH, length of handle; SDW, sickle distal width; SPW, sickle proximal width; MHFL, marginal hook filament length; VBL, ventral bar medial length; VBLL, ventral bar lateral length; VBW, ventral bar width; VBML, ventral bar membrane length; VBTL, ventral bar total length; DBL, dorsal bar length; DBW, dorsal bar width; MCO, male copulatory organ.

† Original measurements combined to form a single mean or range, or both.

we have found on *A. australis* and *A. reinhardtii* are *G. anguillae*.

Several species of *Gyrodactylus* have been reported from anguillid eels including: *G. anguillae* from *Anguilla anguilla* in Europe (see Malmberg, 1970) and Japan (see Ogawa and Egusa, 1980); *Gyrodactylus egusai* Ogawa and Hioki, 1986, *Gyrodactylus joi* Ogawa and Hioki, 1986 and *Gyrodactylus nipponensis* Ogawa and Egusa, 1980 from *Anguilla japonica* Temminck and Schlegel, 1846 in Japan (see Ogawa and Egusa, 1978; Ogawa and Hioki, 1986). *Gyrodactylus anguillae* is distinguished easily from *G. egusai* and *G. joi* because, unlike these species, it does not have ventral bar processes. *Gyrodactylus anguillae* has similar sclerite morphology to *G. nipponensis*, indicating that both of these species may belong to the *G. anguillae*-species group (see Malmberg, 1970). *Gyrodactylus anguillae* can be distinguished from *G. nipponensis* because it has smaller hamuli and larger marginal hooks.

Previously, the natural distribution of *G. anguillae* was thought to include populations of *A. anguilla* throughout the freshwater catchments of Europe (see Malmberg, 1970) and possibly also *Anguilla rostrata* (LeSeur, 1817) in North America (Crane and Eversole, 1980). The latter record, however, requires verification because no descriptions or figures were provided, and morphologically similar species are known to occur on other anguillid eels, e.g., *G. nipponensis* on *A. japonica*. Records of *G. anguillae* from some other areas, e.g., Japan (Ogawa and Egusa, 1978), do not represent endemic parasite populations but are introductions resulting from importation of *A. anguilla* for aquaculture.

It is intriguing that *G. anguillae* is present on *A. australis* and *A. reinhardtii* in Australia. Eels have been transported widely around the world (McCosker, 1989), and it is tempting to explain the presence of *G. anguillae* in Australia through the introduction of an eel host. This explanation, however, seems unlikely because there has been no known imports of live anguillid eels into Australia.

Our work has revealed that the distribution of *G. anguillae* is much wider than previously thought. Despite many parasitological investigations of anguillid eels, several factors could conceal the true distribution of *G. anguillae*. The parasite is small, and at low prevalence and intensity it can be overlooked easily, especially if host tissues are fixed or frozen. *Gyrodactylus anguillae* appears to be more prevalent on small eels (elvers or glass eels) (Malmberg, 1970; see also Orecchia et al., 1987), so an accurate survey for this parasite should encompass broad host size ranges. Furthermore, the higher prevalence of *G. anguillae* on juvenile eels may be evidence that it is actually a marine or brackish water parasite that is carried into freshwater catchments with recruiting glass eels or elvers.

If the current distribution of *G. anguillae* is not the result of an anthropogenic introduction, then this parasite must have a natural, cosmopolitan distribution. It is difficult to explain how such a cosmopolitan distribution may have been maintained on anguillid eels that are believed to have evolved slowly from ancestral species in the Indo-Pacific (Aoyama and Tsukamoto, 1997; Tsukamoto and Aoyama, 1998). If we assume that the current distribution of *G. anguillae* is natural, 2 contrasting hypotheses can be proposed: (1) gene flow has occurred between

apparently isolated populations of *G. anguillae* and has prevented parasite speciation, or (2) isolated populations of this species have simply remained unchanged for long periods of time. For the first hypothesis, gene flow needs to have occurred between parasite populations from different continents. Such mixing could only be achieved by transfer of parasites between species of eels during their marine phase. *Gyrodactylus anguillae* can tolerate marine environments (Malmberg, 1970; Kennedy et al., 1997), and its direct life cycle is suited to infection of larval eel stages. However, this seemingly incongruous hypothesis can be excluded because Indo-Pacific and Atlantic eels have completely separate marine phases (Usui, 1991). Perhaps more plausible is the hypothesis that *G. anguillae* has remained unchanged on disjunct fish populations since the speciation events of their hosts. Marcogliese and Cone (1993) considered that the similarity between the parasite faunas of *A. anguilla* and *A. rostrata* to be evidence for allopatric speciation of the hosts without concomitant parasite speciation. Similarly, *G. anguillae* may have remained unchanged since Indo-Pacific and Atlantic eels became isolated when the Tethys Sea closed (see Aoyama and Tsukamoto, 1997). Marcogliese and Cone (1993) suggested that isolated populations of parasites occurring on both *A. anguilla* and *A. rostrata* may be undergoing speciation events that are not apparent morphologically. We also predict incipient speciation between populations of *G. anguillae* from eels in the Indo-Pacific and Atlantic regions.

It appears most likely that the *G. anguillae* group followed the evolutionary course of their anguillid hosts by evolving in the Indo-Pacific (specifically in the area of present day Indonesia; see Aoyama and Tsukamoto, 1997) and then dispersed and speciated elsewhere. There are probably 2 species of *Gyrodactylus* from eels that belong to the *G. anguillae* species group: *G. anguillae* reported from *A. anguilla*, *A. australis*, *A. reinhardtii*, and *A. rostrata*; and *G. nipponensis* reported only from *A. japonica*. It is worth noting that 3 eel species (*A. anguilla*, *A. australis*, and *A. rostrata*) reported as hosts for *G. anguillae* belong to a clade that arose from a westward dispersion into the Indian Ocean and later into the Atlantic (Aoyama and Tsukamoto, 1997). In contrast, *A. japonica* belongs to a separate clade originating from a northward dispersion to Japan, and it is infected with *G. nipponensis* that is not known from any other eel species. It follows that parasite speciation would have matched these early divergences of the host fauna. Support for this hypothesis and a western Indian Ocean origin of *G. anguillae* could be provided if a fourth member of that host clade, *Anguilla mossambica* (Peters, 1852), is also found to host *G. anguillae*.

The presence of *G. anguillae* on *A. reinhardtii*, however, does not fit with this evolutionary scheme. *Anguilla reinhardtii* dispersed southward to the Coral and Tasman seas (Aoyama and Tsukamoto, 1997) and was not part of the radiation to the Indian Ocean. It is possible that *A. reinhardtii* was infected secondarily by *G. anguillae* when this parasite arrived in Australia on *A. australis*. Alternatively, *A. reinhardtii* may be only a temporary host for *G. anguillae* in regions where these hosts are sympatric.

Origins of the *G. anguillae* group remain unknown, but a species of *Gyrodactylus* reported from apogonid fishes on the Great Barrier Reef may provide a clue (Ernst, 1999). This spe-

cies appears to have a *Gyrodactylus* (*Neonephrotus*)-type excretory system that is presently known only from *G. anguillae* in the *G. anguillae* species group (see Malmberg, 1970). It also has similar sclerite morphology to species in the *G. anguillae* species group but occurs on the more recently evolved apogonid fishes from the Great Barrier Reef. The excretory system of this *Gyrodactylus* sp. requires complete characterization, however, before it can be placed in *Gyrodactylus* (*Neonephrotus*) with certainty.

Pseudodactylogyrus anguillae is another monogenean parasite of eels that has been reported from several species of anguillid eels around the world. This parasite was first described from *A. japonica* from Asia and contrary views exist regarding its status as an exotic or an endemic parasite of *A. anguilla* in Europe (Nie and Kennedy, 1991; Škoriková et al., 1996). Our hypothesis that *G. anguillae* is a naturally occurring parasite on eels in Australia follows a similar conclusion by Cone and Marcogliese (1995) for their report of *P. anguillae* from *A. rostrata* from North America. For both of these parasite species, molecular data could provide definitive information to allow identification of endemic and introduced populations.

Kennedy and Di Cave (1998) recently reported what they suspected was the extinction of *G. anguillae*. This may be the case in Europe, but in Australia *G. anguillae* persists as a parasite of *A. australis* and *A. reinhardtii*.

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Host Associations and Seasonal Activity of *Amblyomma americanum* (Acari: Ixodidae) in Missouri

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ABSTRACT: From June 1993 through June 1996, 2,260 adult, 4,426 nymphal, and 2,178 larval lone star ticks *Amblyomma americanum* (L.) were collected in Missouri from vertebrate hosts and by dragging a cloth over vegetation. Prevalence, mean intensity, and relative abundance of each stage varied among hosts. The relative abundance of adult lone star ticks was highest on white-tailed deer, but this stage was also collected from raccoons, opossum, red fox, coyotes, and wild turkey. Nymphs were collected from gray squirrels, eastern cottontail rabbits, opossums, red fox, Carolina wren, and bobwhite quail, but the highest relative abundance occurred on wild turkey, white-tailed deer, and raccoons. Eastern cottontail rabbits, white-tailed deer, raccoons, and squirrels had the highest relative abundance of larval lone star ticks, but they were also found on opossums and wild turkey. The activity of adult lone star ticks was greatest from May through July. The activity for nymphs was highest from May through August, and for larvae, July through September.

The lone star tick *Amblyomma americanum* (L.) is among the most important pests of humans and livestock in the southern United States. This tick has been implicated as a vector of *Rickettsia rickettsii* Wolbach, the etiologic agent of Rocky Mountain spotted fever, and of *Francisella tularensis* McCoy and Chapin, the etiologic agent of tularemia (Bishopp and Trembley, 1945). It is also a probable vector of *Ehrlichia chaffeensis* Anderson, Dawson, Jones and Wilson, the etiologic agent of human monocytic ehrlichiosis (Ewing et al., 1995). There is also much debate over the association and vector competence of this tick species for *Borrelia burgdorferi* Johnson, Schmid, Hyde, Steigerwalt and Brenner, the etiologic agent of Lyme borreliosis. Gross infestations of lone star ticks can cause injury or death in deer (Bolte et al., 1970) and wild turkeys (Bishopp and Trembley, 1945), and reduce weight gain in cattle (Barnard, 1985; Ervin et al., 1987). Investigating tick–host interactions and seasonal activity throughout the geographic range of the lone star tick is necessary because of the importance of

this tick species as a biting pest and as a vector of human and animal pathogens.

Adult lone star ticks infest medium- and large-sized mammals throughout their range (Bishopp and Trembley, 1945). Immature stages infest birds, medium- and large-sized mammals, and rarely small mammals. Host associations vary throughout the geographic range of the tick. For example, in central Tennessee, the eastern cottontail rabbit was an important host for immature lone star ticks (Zimmerman et al., 1988), whereas in Virginia the rabbit was not an important host (Sonenshine et al., 1965). Nevertheless, in a different region of Virginia, several lone star ticks were collected from rabbits (Jacobson et al., 1978). Variation in seasonal activity can also occur depending on geographic location. Generally, the lone star tick can be active throughout the year in the southern United States, but it is less abundant in midwinter (Bishopp and Trembley, 1945).

The study area was composed of 9 sites located in 5 counties in southeastern Missouri: Bollinger, Cape Girardeau, Scott, Stoddard, and Wayne. The collection period for trapping and dragging was from June 1993 through June 1996. Ticks were kept alive or placed in 70% isopropyl alcohol. Mammals were captured using 14-inch Sherman® live traps, and Tomahawk® squirrel-sized or Tomahawk raccoon-sized live traps. Traps were placed in a grid or transect with 40 Sherman live traps 10 m apart and 10 squirrel and 4 raccoon Tomahawk live traps 20 to 50 m apart, depending on habitat. Bait for rodent traps was peanut butter mixed with rolled oats with an additional sliced apple placed in squirrel traps. Raccoon traps were baited with fish-flavored cat food. Traps were placed at 1 site for 3 nights and checked every morning. Medium-sized wild mammals were anesthetized with a 1:1 mixture of ketamine hydrochloride (Ketaset®; Bristol Laboratories, Syracuse, New York) and acepromazine maleate (PromAce®; Ayerst Laboratories, New

TABLE I. Comparison of prevalence (χ^2), mean intensity (Kruskal-Wallis ANOVA), and relative abundance (Mann-Whitney test with Bonferroni adjustment) of adult *Amblyomma americanum* on different hosts in southeastern Missouri.

Variable	Raccoon	Opossum	Red fox	Coyote	Deer	Turkey
Number	27	43	1	1	9	38
Prevalence (%)	7	2	100	100	100	3
Mean intensity	1.0	2.0	12.0	8.0	13.5	47.0
Standard deviation	0	0	0	0	43.5	0
Relative abundance	0.07	0.09	12.0	8.0	13.5	1.2

York, New York), on the basis of estimated body weight. Opossums were only anesthetized if they posed a danger while handling. Rodents were killed using acepromazine maleate or by separation of neck vertebrae. Passerine birds accidentally captured in live traps were gently restrained while ticks were collected. Quail and wild turkey were shot or collected from hunter check stations. Ticks were collected from 10 deer shot from June 1995 through June 1996 and from hunter-killed deer from check stations during the months of November and December from 1993 to 1995. To identify periods of questing activity of *A. americanum*, attempts to collect them were made at each site once per month by dragging a 1-m² white loose-weave denim cloth for 100 m, with ticks being removed every 10 m. The number of ticks per square meter was determined by dividing the number of ticks collected by dragging by 100.

A total of 8,864 *A. americanum* was collected from vertebrates and by dragging during the 3-yr study. This included 2,260 adults, 4,426 nymphs, and 2,178 larvae. Adults were collected in all months except September, December, and January. Nymphs were collected from March through November and larvae were collected from April through October.

Differences in seasonal activity occur for all stages of the lone star tick, depending on the geographic location of the population. Single and dual peak activities have been found for different years and may be dependent on yearly differences in humidity (Patrick and Hair, 1977). In Missouri, adult lone star tick questing activity was highest in May, with a gradual decline to September (Fig. 1). In Virginia, peak adult activity was in May or June (Sonenshine and Levy, 1971; Sonenshine, 1979), and an additional activity peak occurred in July after the spring peak in wild mammals (Sonenshine and Stout, 1971). Dual spring and midsummer peaks were also found in eastern Oklahoma (Patrick and Hair, 1977) and central Tennessee (Bloemer and Zimmerman, 1988; Bloemer et al., 1988). In Missouri, questing nymphal lone star ticks were pri-

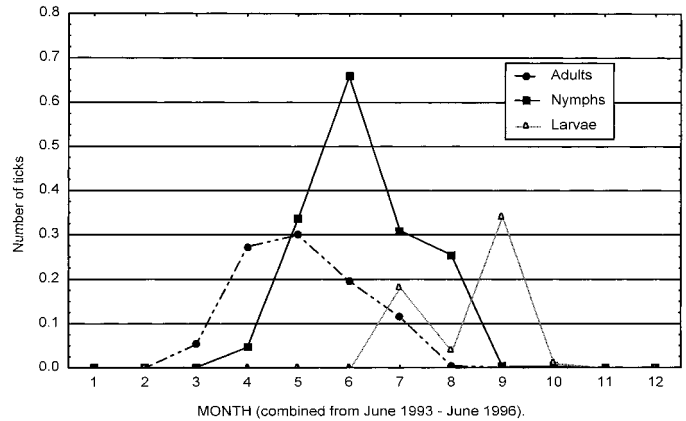


FIGURE 1. Monthly mean number of *A. americanum* per square meter collected by dragging.

marily active from April through September, with peak populations in June (Fig. 1). In Virginia, nymphal lone star tick populations were highest in May and June, followed by a rapid decrease in numbers in July (Sonenshine and Stout, 1971). In Oklahoma, nymphal activity was unimodal during 1 yr, with peak activity in June, and bimodal in another year, with peak activity in May and July (Patrick and Hair, 1977). In Tennessee, nymphal activity was unimodal, peaking in June (Bloemer and Zimmerman, 1988; Bloemer et al., 1988). In Missouri, larval lone star tick populations were active from July through October, with peak questing activity recorded in September (Fig. 1). In Oklahoma, larval populations were either unimodal, peaking in July, or bimodal, with dual peaks in August and October (Patrick and Hair, 1977). In Tennessee, larval populations peaked in August (Bloemer et al., 1988) or September (Bloemer and Zimmerman, 1988).

Differences among hosts and their interactions with larval, nymphal, and adult *A. americanum* were compared using prevalence, mean intensity, and relative abundance following Bush et al. (1997). Prevalences were compared using chi-square analysis with Bonferroni adjustment. Mean intensity and relative abundance were compared using Kruskal-Wallis ANOVA and Mann-Whitney *U*-test, with Bonferroni adjustment. STATISTICA for Windows (1996) by StatSoft, Tulsa, Oklahoma was used to analyze the data.

Statistical comparisons among hosts were made from combined yearly data, collected during seasonal periods of activity as evidenced by dragging and trapping. For example, 16 deer were collected during the seasonal activity of *A. americanum*, 6 in 1994, 8 in 1995, 2 in 1996, but none in 1993. Over 500 deer were checked for ticks during December, but no *A. amer-*

TABLE II. Comparison of prevalence (χ^2), mean intensity (Kruskal-Wallis ANOVA), and relative abundance (Mann-Whitney test with Bonferroni adjustment) of nymphal *Amblyomma americanum* on different hosts in southeastern Missouri.

Variable	Gray squirrel	Cottontail rabbit	Raccoon	Opossum	Red fox	Deer	Carolina wren	Bobwhite quail	Turkey
Number	10	96	33	63	1	14	6	7	38
Prevalence (%)	21	24	70	5	100	79	17	71	100
Mean intensity	2.0	9.2	11.1	9.7	8.0	121.1	3.0	4.8	6.0
Standard deviation	0	19.7	12.7	6.8	0	176.0	0	0.8	5.1
Relative abundance	0.43	2.2	8.0	0.46	8.0	95.1	0.5	3.4	6.0

TABLE III. Comparison of prevalence (χ^2), mean intensity (Kruskal-Wallis ANOVA), and relative abundance (Mann-Whitney test with Bonferroni adjustment) of larval *Amblyomma americanum* on different hosts in southeastern Missouri.

Variable	White-footed mouse	Fox squirrel	Gray squirrel	Cottontail rabbit	Raccoon	Opossum	Deer
Number	143	7	11	45	27	50	10
Prevalence (%)	0.7	57	91	18	52	2	70
Mean intensity	2.0	7.0	9.9	147.8	8.8	1.0	49.1
Standard deviation	0	7.0	5.6	333.6	7.3	0	59.9
Relative abundance	0.01	4.0	9.0	26.3	4.6	0.02	34.4

icanum were collected; therefore, these deer were excluded from statistical analysis.

No *A. americanum* was collected from the following mammals: northern short-tailed shrew *Blarina brevicauda* (Say), $n = 12$; deer mouse *Peromyscus maniculatus* (Wagner), $n = 3$; golden mouse *Ochrotomys nuttalli* (Harlan), $n = 21$; marsh rice rat *Oryzomys palustris* (Harlan), $n = 6$; western harvest mouse *Reithrodontomys megalotis* (Baird), $n = 13$; house mouse *Mus musculus* (L.), $n = 35$; prairie vole *Microtus ochrogaster* (Wagner), $n = 35$; pine vole *M. pinetorum* (LeConte), $n = 23$; eastern chipmunk *Tamias striatus* (L.), $n = 8$. No *A. americanum* was collected from a dark-eyed Junco *Junco hyemalis* (L.), 8 mourning doves *Zenaidra macroura* (L.), 1 brown thrasher *Toxostoma rufum* (L.), 1 red-winged blackbird *Agelaius phoeniceus* (L.), and 2 whippoorwills *Caprimulgus vociferus* (Wilson). Vertebrate species having no individuals parasitized by *A. americanum* were excluded from statistical analyses.

Significant differences in prevalence and relative abundance of adult lone star ticks occurred among hosts ($P \leq 0.05$) (Table I). Mean intensity of adult lone star ticks did not differ significantly among hosts. Significant differences in prevalence ($P \leq 0.05$), mean intensity ($H [8, N = 100] = 19.3, P \leq 0.05$), and relative abundance ($H [8, N = 264] = 119.1, P \leq 0.05$) of nymphal lone star ticks occurred among hosts (Table II). Significant differences in prevalence ($P \leq 0.05$) and relative abundance ($H [6, N = 293] = 139.4, P \leq 0.05$) of larval lone star ticks occurred among hosts (Table III). Mean intensity of larval lone star ticks was not significantly different among hosts. The total number of hosts analyzed differs between tables because only hosts captured during the time each stage was active were compared.

The white-tailed deer was the most important host for adult lone star ticks, with a higher prevalence and relative abundance of ticks than the raccoon, opossum, and wild turkey. Low captures of red foxes and coyotes prevented analysis of their importance as hosts for adult lone star ticks. In central Tennessee/southern Kentucky, coyotes and gray foxes both were parasitized by large numbers of adult lone star ticks and may be important hosts in areas of deer exclusion (Bloemer and Zimmerman, 1988). In Virginia, red foxes had the highest relative abundance of adults of all medium-sized mammals collected (Sonenshine and Stout, 1971). Raccoons, opossums, and wild turkeys were not important hosts of adult lone star ticks in Missouri or in previous studies in several southern states (Sonenshine and Stout, 1971; Bloemer and Zimmerman, 1988; Zimmerman et al., 1988; Kollars, 1993; Lavender and Oliver, 1996).

White-tailed deer, wild turkeys, and raccoons were the most important hosts of nymphal lone star ticks. Deer can be infested by large numbers of nymphs throughout the range of the lone

star tick. In South Carolina, most of the 4,800 ticks collected from 1 ear of a deer were nymphs (Bishopp and Trembley, 1945). In central Tennessee, raccoons had the highest relative abundance of nymphs of medium-sized mammals (Zimmerman et al., 1988) and in Virginia, raccoons were also important hosts of nymphs (Sonenshine and Stout, 1971). Rabbits, Carolina wrens, and bobwhite quail were also hosts for nymphs in Missouri. Immature stages are often found on ground-feeding birds (Sonenshine, 1979), particularly quail and wild turkey (Bequaert, 1945).

There were appreciable differences in the importance of mammals as hosts for larval lone star ticks as measured by prevalence and relative abundance. The white-tailed deer was the most important host of larval lone star ticks. Raccoons and gray squirrels were more important as hosts of larvae than rabbits were, as measured by prevalence, but rabbits were more important as measured by relative abundance of larvae. This may be explained by the high number of larvae found on 1 rabbit (1,100). Therefore, it is important to note that rabbits are capable of supporting massive infestations of larvae. This is particularly important in determining the epidemiology of Lyme borreliosis in Missouri if *A. americanum* is shown to be a vector of a *Borrelia* species.

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Sarcocystis campestris From Naturally Infected 13-Lined Ground Squirrels, *Spermophilus tridecemlineatus tridecemlineatus*, From Nebraska

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ABSTRACT: Grossly visible sarcocysts were seen in the skeletal muscles of 1 of 12 13-lined ground squirrels, *Spermophilus tridecemlineatus tridecemlineatus*, collected in Nebraska. The tissue cyst wall was up to 5.0 µm thick and contained spikelike projections. Transmission electron microscopy of tissue cysts revealed they were similar to *Sarcocystis campestris* Cawthorn, Wobeser, and Gajadhar, 1983, previously known only from experimental infections in Richardson's ground squirrel *Spermophilus richardsonii*. Prominent electron-dense bodies were observed lining the microfilaments present in the spikelike projections of the sarcocyst wall. This is the first report of *S. campestris* in a natural intermediate host and the first report of this parasite outside of Saskatoon, Canada.

Cawthorn et al. (1983) described *Sarcocystis campestris* from Richardson's ground squirrels *Spermophilus richardsonii*, fed sporocysts collected from naturally infected badgers, *Taxidea taxus*. They reported that sarcocysts became grossly visible by day 258 postinoculation. Sporocysts were found in badgers that were fed muscle from the experimentally infected ground squirrels, demonstrating that the life cycle consisted of a badger definitive host and a ground squirrel intermediate host. Wobeser et al. (1983) described the pathogenicity of *S. campestris* sporocysts for Richardson's ground squirrels. The natural intermediate host for *S. campestris* is not known.

We observed grossly visible sarcocysts in 13-lined ground squirrels, *Spermophilus tridecemlineatus tridecemlineatus*

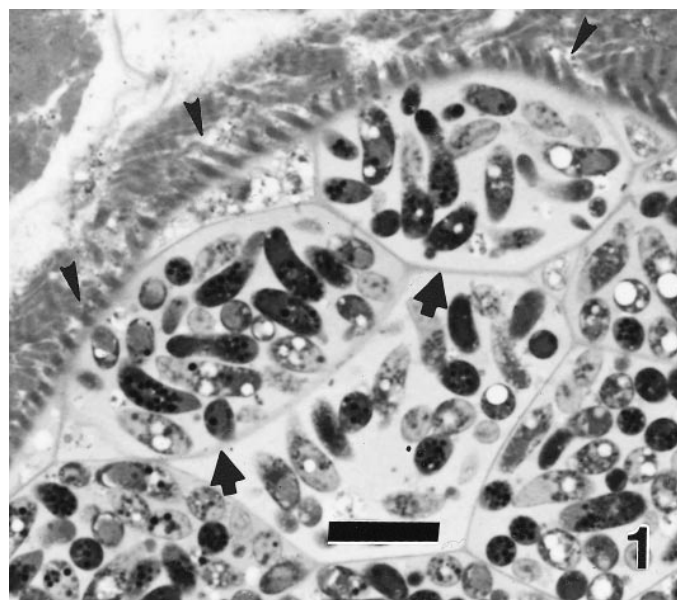
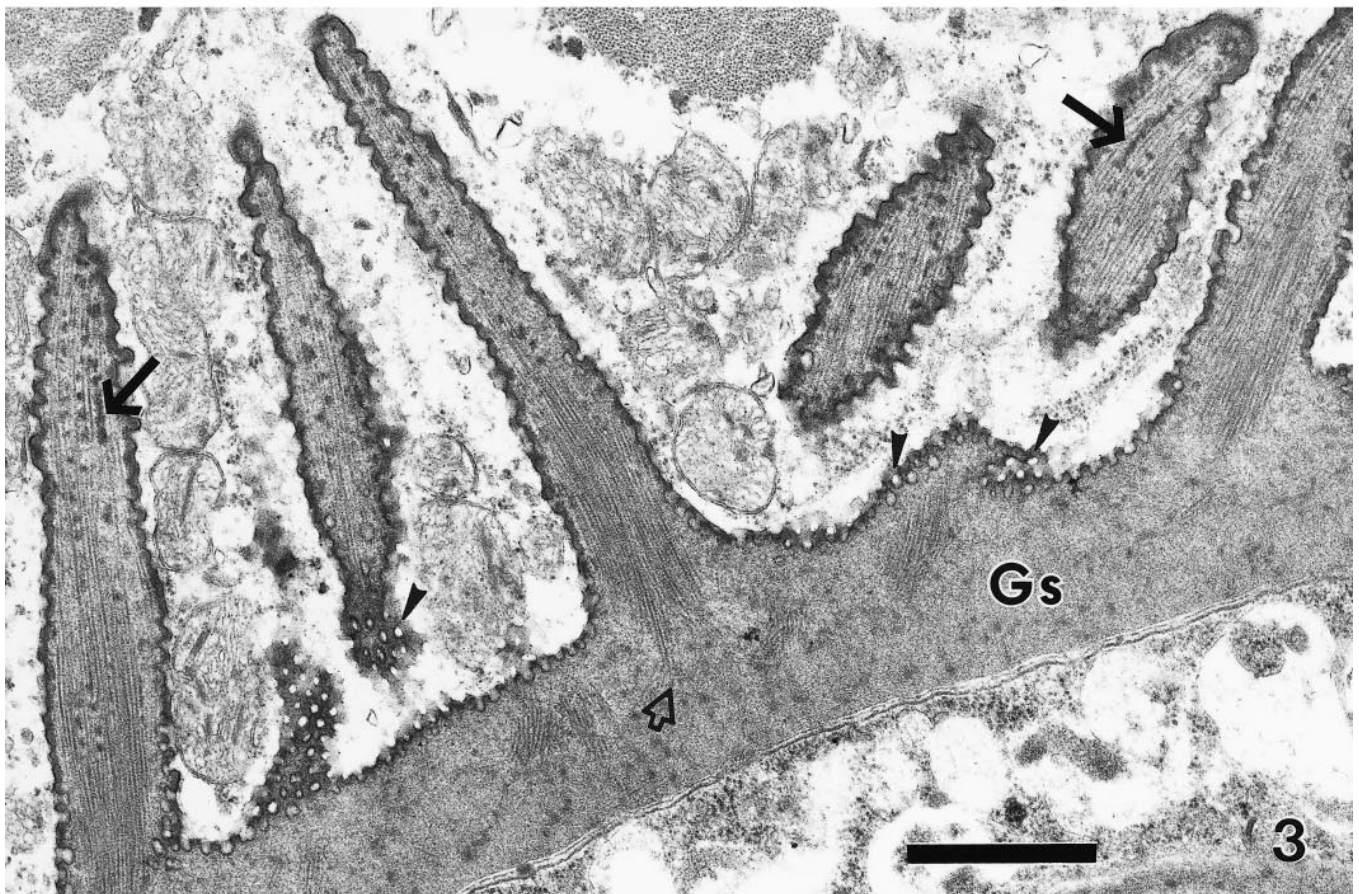
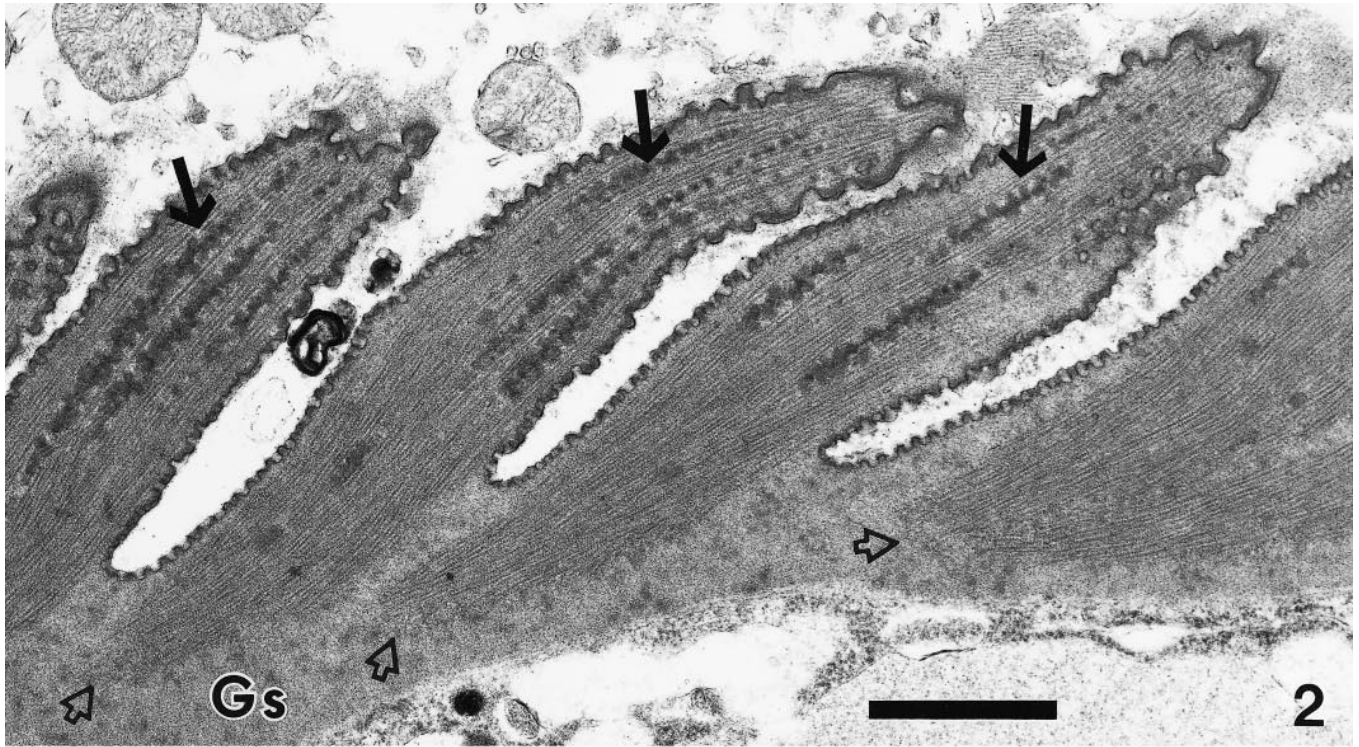


FIGURE 1. Photomicrograph of a sarcocyst of *Sarcocystis campestris* in a 13-lined ground squirrel. Note the spikelike projections (arrowheads) on the sarcocyst wall. The septa (arrows) divide the sarcocyst into compartments. Toluidine blue stain. Bar = 10 µm.



FIGURES 2, 3. Transmission electron photomicrographs of sarcocysts of *Sarcocystis campestris* in a 13-lined ground squirrel. 2. Spikelike projections containing rows of electron-dense bodies (arrows) along the microfilaments. Note that the microfilaments (open arrows) extend into the ground substance (GS). Bar = 1 μ m. 3. Note the ornamentations on the primary cyst wall and that holes (arrowheads) in the primary cyst wall are due to the plane of sectioning. Spikelike projections containing rows of electron-dense bodies (arrows) along the microfilaments are present and microfilaments (open arrows) extend into the ground substance (GS). Bar = 1 μ m.

(Mitchill), from Nebraska. We did not examine tissues for microscopic sarcocysts. The present report documents the occurrence of *S. campestris* in 13-lined ground squirrels that serve as a natural intermediate host and provides additional information on the structure of the sarcocyst of this species.

Portions of internal and external abdominal oblique muscles and internal intercostal muscles with grossly visible sarcocysts were fixed in 2% (v/v) glutaraldehyde in phosphate buffer or 0.4% glutaraldehyde/4% formalin and mailed to the Center for Molecular Medicine and Infectious Diseases, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia for processing and ultrastructural examinations. Tissues were postfixed in 1% (w/v) osmium tetroxide, dehydrated in a series of ethanols, passed through 2 changes of propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, Pennsylvania). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL-100 CX II transmission electron microscope (TEM) operating at 80 kV. Additionally, 1- μ m-thick sections were placed on glass microscope slides and stained with toluidine blue and examined by light microscopy. Portions of muscles were also fixed in 10% neutral buffered formalin solution and processed for light microscopic examination after staining with hematoxylin and eosin (H&E).

One of 12 13-lined ground squirrels collected from Adams (n = 2), Clay (n = 8), and Fillmore (n = 2) counties had grossly visible sarcocysts. Nine of the animals were male and 3 were females. The ages were not determined.

Examination of grossly visible sarcocysts in histological sections stained with toluidine blue or H&E were similar (Fig. 1). The cyst wall was 1–2 μ m thick and contained spikelike projections that were 0.5–5.0 μ m long depending on the plane of section. Bradyzoites were 10.0–12.0 by 2.0–3.0 μ m in size. Toluidine blue- and H&E-stained tissue sections have been deposited in the United States National Parasite Collection (USNPC), Beltsville, Maryland, USNPC no. 089747.00.

Transmission electron microscopic examination of 3 sarcocysts from the naturally infected 13-lined ground squirrel were similar (Figs. 2, 3). The primary cyst wall was composed of the parasitophorous vacuole (PV) membrane and underlying electron-dense material. The PV membrane was ornamented with bumplike or knoblike structures that were electron dense in the upper portions but composed only of a small portion of the PV membrane at the lower portion. When sectioned appropriately, the thinner portions of the electron-dense ornamentations appeared as holes in the primary sarcocyst wall (Fig. 3). There was a granular layer immediately beneath the primary sarcocyst wall, and it gave rise to the ground substance and septa that divided the sarcocyst into compartments (Fig. 1).

Metrocytes were visible at the periphery of the sarcocyst. The sarcocyst wall contained numerous spikelike projections. The core of the spikelike projections contained bundles of microfilaments that extended from the anterior portion to $\frac{1}{2}$ to $\frac{3}{4}$ the depth of the ground substance. Electron-dense bodies were observed in rows along groups of these microfilaments (Figs. 2, 3). These rows of electron-dense bodies were up to $\frac{3}{4}$ the length of a spikelike projection (Fig. 2) or in small clusters in other spikelike projections (Fig. 3) in sarcocysts that were appropriately sectioned. Bradyzoites contained all the organelles typical of this stage (Dubey et al., 1989).

None of 5 badgers collected from Adams (2), Clay (2), or Fillmore (1) counties had detectable sporocysts in their fecal samples after flotation in Sheather's sugar solution.

Cawthorn et al. (1983) only briefly described the ultrastructure of sarcocysts of *S. campestris* and their TEM photomicrograph is of a 113-day-old sarcocyst. They mentioned that a central core containing longitudinal striations was present in the projections of the sarcocyst wall. We interpret these to be the microfilaments observed in the spikelike projections in the present study. They did not mention the occurrence of rows of electron-dense bodies in the projections of the sarcocyst wall in their study. It is not clear if Cawthorn et al. (1983) examined sarcocysts that were older than 113 days. Our sarcocysts were grossly visible, and it may be that they were older than the sarcocysts studied by Cawthorn et al. (1983). The differences in ultrastructure may be due to age of the sarcocyst (Dubey et al., 1989). However, several TEM photomicrographs of sarcocysts of *S. campestris* illustrated by Dubey et al. (1989) contain electron-dense bodies that are associated with microfilaments present in the spikelike projections of the sarcocyst wall. The source of the Dubey et al. (1989) material was the study of Cawthorn et al. (1983). It appears that the electron-dense bodies were present in the sarcocysts of *S. campestris* existing in the experimentally infected Richardson's ground squirrels but were not mentioned in the original species description by Cawthorn et al. (1983).

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Neutral Lipids in Cercariae, Encysted Metacercariae, and Rediae of *Zygocotyle lunata*

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ABSTRACT: High-performance thin-layer chromatography was used to analyze the neutral lipids in the rediae, cercariae, and encysted metacercariae of the paramphistomid trematode *Zygocotyle lunata*. Visual observations of the chromatograms showed that the most abundant lipid fractions were free sterols and free fatty acids in all larval stages and triacylglycerols in the metacercariae and rediae. The weight of free sterols ($\bar{x} \pm \text{SE}$) was 120 ± 20 ng/cercaria, 56 ± 3.8 ng/redia, and 5.9 ± 1.5 ng/encysted metacercaria; the weight of triacylglycerols was 13 ± 0.88 ng/encysted metacercaria, 6.3 ± 0.063 ng/redia, and was not detectable in the cercaria; the weight of free fatty acids was 160 ± 17 ng/cercaria, 76 ± 9.1 ng/redia, and 4.2 ± 0.46 ng/encysted metacercaria. Oil red O staining of whole larvae showed the presence of neutral lipids in the rediae but not in the cercariae or encysted metacercariae. A dramatic reduction was seen in the quantity of free sterols and free fatty acids in the encysted metacercariae as compared with the cercariae, suggesting that these neutral lipids are used in some way during the transformation from cercaria to metacercaria.

Zygocotyle lunata is a paramphistomid trematode that cycles between *Helisoma* snails and various avian and mammalian hosts (Willey, 1941). Considerable information is available on the biology and life cycle of this digenean (Willey, 1936, 1941), but it is an understudied species in terms of the physiology and biochemistry of paramphistomid trematodes.

Recent studies have been concerned with neutral lipid analyses of adults of *Z. lunata* raised in experimentally infected vertebrate hosts (Lee et al., 1998, 1999) and with the pathobiological effects of *Z. lunata* infection on the neutral lipid content of the digestive gland gonad complex (DGG) in *Helisoma trivolvis* snails (Fried et al., 1998). Quantitative analysis of the neutral lipids in the various larval stages, i.e., cercaria, encysted metacercaria, and redia, have not been studied, and little is known about the content and function of neutral lipids in digenean larvae in general. In this study, we used high-performance thin-layer chromatography (HPTLC) to examine the neutral lipid content of cercariae, encysted metacercariae, and rediae of *Z. lunata*.

Helisoma trivolvis snails naturally infected with *Z. lunata* were collected from a pond in Northampton County, Pennsylvania, and isolated in artificial spring water to obtain cercariae (Schmidt and Fried, 1997). To obtain metacercariae, cercariae were allowed to encyst on the surface of glass containers. Rediae were obtained by dissection from the DGG of the snails.

Each larval stage was prepared separately, counted, and transferred to 1.5-ml microcentrifuge tubes half-filled with Locke's solution. Samples were prepared as follows: 3 samples of 31, 30, and 29 rediae per tube; 3 samples of 39, 50, and 40 encysted metacercariae per tube; 3 samples of 21, 17, and 25 cercariae per tube. The samples were maintained in the tubes overnight at 4 C to inhibit mobility of the cercariae and allow the organisms to aggregate at the bottom of the tube.

To extract the lipids, the Locke's solution above the larval pellet was removed with a Pasteur pipet and replaced with 1.0 ml of chloroform-methanol (2:1) in each tube. Because of the

microscopic size of these larvae, lipids were extracted from the larval stages without the need for additional treatment. The sample was evaporated to dryness under nitrogen gas, and the lipid residue was reconstituted with a 100- μ l digital microdispenser (Drummond, Broomall, Pennsylvania) containing 50.0 μ l of chloroform-methanol (2:1) for the cercariae and metacercariae and 40.0 μ l for the rediae.

HPTLC analysis of neutral lipids was performed on 10- $\times$ 20-cm LHPKDF silica gel plates (Whatman, Clifton, New Jersey) containing 19 lanes and a preadsorbent zone. The plates were precleaned by development to the top with dichloromethane-methanol (1:1) and air-dried under a fume hood. For qualitative and quantitative analysis, the neutral lipid standard 184A (Nu-Check Prep, Elysian, Minnesota) was used, which was diluted in chloroform-methanol (2:1) to contain 0.254 μ g/ μ l each of cholesteryl oleate, methyl oleate, triolein, oleic acid, and cholesterol. Volumes of 1.0, 2.0, 4.0, and 8.0 μ l of the standard and 16.0 and 20.0 μ l of the reconstituted sample were spotted on a plate using a 25- μ l digital microdispenser (Drummond). The standard was diluted 1:1 and 1:3 with chloroform-methanol (2:1), and a 1.0- μ l aliquot of each of the dilutions was also applied to the plates so that the lowest standard weight would bracket the lowest weight of the samples. Plates were developed in a paper-lined, twin trough HPTLC chamber (Camag, Wilmington, North Carolina) lined with the Mangold solvent system (petroleum ether:diethyl ether:acetic acid, 80:20:1) to a distance of 6.5 cm past the preadsorbent-silica gel interface. After development, the plate was air-dried under a fume hood, sprayed with 5% ethanolic phosphomolybdic acid, and heated at 110 C for 15 min. Neutral lipid zones appeared as dark blue spots against a yellow background.

Densitometry of the lipid zones was performed using a TLC Scanner II (Camag) with a tungsten light source set at 700 nm, a slit width setting of 4, a slit length setting of 4, and a scanning rate of 4 mm/sec. The scanner was controlled by a CATS-3 software package, which produces a calibration curve relating the weights of the standard zones and their peak areas. The weights of lipids in the sample zones were determined automatically from their areas by interpolation from the calibration curve. The weight of lipid per larval stage was calculated as described by Muller et al. (1999).

Qualitative analysis of neutral lipids showed the major lipid fractions to be free sterols ($R_f = 0.23$) and free fatty acids ($R_f = 0.31$) in all larval stages and triacylglycerols ($R_f = 0.62$) in the metacercariae and rediae. Quantitatively, the cercariae contained the greatest amount of free sterols ($\bar{x} \pm \text{SE}$) with 120 ± 20 ng/cercaria, followed by 56 ± 3.8 ng/redia, and 5.9 ± 1.5 ng/encysted metacercaria ($n = 3$). The encysted metacercariae had a significantly greater (Student's *t*-test, $P < 0.05$) amount of triacylglycerols, 13 ± 0.88 ng/metacercaria, compared with 6.3 ± 0.063 ng/redia, but the cercariae did not show this frac-

tion. For free fatty acids, the cercariae contained the greatest weight, 160 ± 17 ng/cercaria, followed by the rediae, with 76 ± 9.1 ng/redia, and the metacercariae, with 4.2 ± 0.46 ng/metacercaria.

Histochemical studies were done on at least 20 whole organisms of each stage using Oil red O (ORO). The material was fixed in cold 10% neutral buffered formalin and stained in ORO as described by Fried et al. (1998). No ORO-positive material was seen in cercariae or the encysted metacercariae. The rediae showed ORO-positive droplets in the bodies of developing cercariae and in the redial space between the cercarial bodies.

This is the second study of absolute weight of neutral lipids in larval trematodes as determined by HPTLC. Muller et al. (1999) reported that cercariae of *Echinoparyphium* sp. had a mean weight of free sterol per cercaria of 0.022 μ g. The present study confirms a previous histochemical observation of negative ORO staining of *Z. lunata* cercariae but positive ORO staining of the rediae (Fried et al., 1998). The negative ORO reaction in the *Z. lunata* encysted metacercariae is reported here for the first time.

Because of the microscopic nature of these organisms, accurate weight measurements by the usual gravimetric methods were not possible. We assumed that there were no major weight changes during larval transformation from the cercarial to the metacercarial stage. *Zygocotyle lunata* is a more robust species than is *Echinoparyphium* sp., and we assumed that both cercarial and metacercarial weights of *Z. lunata* are considerably greater than those of *Echinoparyphium* sp.

There is no consensus as to what is stained by ORO in larval trematodes, presumably true fat (triacylglycerols) and free fatty acids. The only *Z. lunata* stage that was ORO positive and demonstrated neutral lipid classes by HPTLC was the redia. Melanin is very prominent in the cercarial body and the encysted metacercaria of *Z. lunata* (see Willey, 1941), and that pigment may have interfered with ORO staining.

The encysted metacercaria of *Z. lunata* is formed on a substratum in the absence of a second intermediate host. Encystment occurs following release and hardening of cystogenous material from the cercarial body and the loss of the cercarial

tail. This reaction does not involve the contribution of additional chemicals from another host. Hence, the dramatic reduction in both free sterols and free fatty acids in the encysted metacercaria compared with the cercaria suggests that these neutral lipids are used in some way during the transformation from cercaria to metacercaria. Ginetsinkaya (1988) stated that the quantity of fat deposited in digenean tissues (specific examples of the species studied were not given) increased during successive phases of the life cycle and reached a maximum in the mature adult. Our observation of the reduction of free sterols and free fatty acids from the cercarial to the metacercarial stage of *Z. lunata* does not support Ginetsinkaya's (1988) statement.

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Thubunaea dactyluris Sensus Fabio and Rolas, a Synonym of *Physalopteroides venancioi* (Spirurida, Physalopteridae)

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ABSTRACT: Specimens of *Thubunaea dactyluris* Karve, 1938 sensu Fabio and Rolas, 1974 from the lizard *Ameiva ameiva* of Brazil and types of *Physalopteroides venancioi* (Lent, Freitas, and Proença, 1946) from the toad *Bufo paracnemis* of Paraguay were re-examined. The male caudal papillae and the tooth arrangement demonstrated that these specimens were conspecific and are all assigned to *P. venancioi*. The occurrence of *Physalopteroides dactyluris* (Karve, 1938), a nematode parasite of lizards of India and Turkmenistan, is invalidated for Brazil.

Physalopteroides Wu and Liu, 1940 (Physalopteridae; Thubunaeinae) currently contains 16 recognized species (Bursey and Goldberg, 1994), of which 2 occur in the Neotropical Realm: *Physalopteroides valdesi* Coy-Otero and Barus, 1980 of Cuba and *Physalopteroides venancioi* (Lent, Freitas, and Proença, 1946) of Paraguay. Baker (1987) erroneously cited the type

TABLE I. Measurements in millimeters of *Thubunaea dactyluris* sensu Fabio and Rolas (Brazil) and *Physalopteroides venancioi* (Paraguay) compared to measurements of *Physalopteroides dactyluris* (India).

Character	<i>T. dactyluris</i> (Brazil)		<i>P. venancioi</i> (Paraguay)		<i>P. dactyluris</i> (India)
	Fabio and Rolas, 1974	This study	Lent et al., 1946	This study	Karve, 1938
Male					
Total length	7.60–13.36	6.63–9.66	10.20–12.72	8.92	6.00–8.70
Maximum width	0.23–0.52	0.14–0.31	0.27–0.37	0.25	0.19
Muscular esophagus	0.17–0.30	0.19–0.26	0.23–0.29	0.22	0.26–0.35
Glandular esophagus	1.28–1.83	1.12–1.75	1.70–2.00	1.40	1.80–3.20
Nerve ring (from anterior end)	0.20–0.23	0.18–0.21	0.19–0.26	0.19	0.19–0.23
Excretory pore (from anterior end)	0.22–0.24	0.22–0.24	0.28–0.29	0.28	0.26–0.30
Tail length	0.10–0.17	0.10–0.13	0.13–0.18	0.19	0.22
Right spicule length	0.06–0.07	0.06–0.07	0.60–0.10	0.08	0.05–0.08
Left spicule length	0.08–0.89	0.08–0.09	0.80–0.11	0.09	0.07–0.11
Precloacal papillae	4 pairs	4 pairs	4 pairs	4 pairs	4 pairs
Adcloacal papillae	1 pair	1 pair	1 pair	1 pair	0
Postcloacal papillae	4 pairs	4 pairs	4 pairs	4 pairs	6 pairs
Female					
Total length	14.85–22.29	13.94–20.57	14.82–16.49	12.24–14.28	11.50–19.40
Maximum width	0.32–0.65	0.34–0.42	0.34–0.41	0.35–0.42	0.31
Muscular esophagus	0.24–0.37	0.23–0.29	0.27–0.31	0.28–0.30	0.26–0.35
Glandular esophagus	2.00–2.85	2.04–2.69	2.10–2.40	2.04–2.10	1.80–3.20
Nerve ring (from anterior end)	0.17–0.29	0.21–0.23	0.26–0.29	0.25–0.28	0.19–0.23
Excretory pore (from anterior end)	0.19–0.29	0.19–0.29	0.34–0.35	0.34–0.35	0.26–0.30
Tail length	0.07–0.10	0.08–0.10	0.11–0.13	0.12–0.13	0.19–0.20
Vulva (from anterior end)	1.14–2.09	1.75–1.82	1.80–1.90	1.82–1.89	3.00–3.90
Egg length (µm)	31–40	32–40	33–37	36–39	30–32
Egg width (µm)	20–26	18–22	20–25	24–25	18–22

locality of *P. venancioi* as Uruguay. A third species, *Physalopteroides dactyluris* (Karve, 1938), was reported from a lizard, *Ameiva ameiva* (Teiidae), collected in Rio de Janeiro State, Brazil by Fabio and Rolas (1974). However, Baker (1987) considered this to be a doubtful record because this species was originally described from lizards of India where gekkonid and agamid lizards as well as viperid snakes are definitive hosts; the species also occurs in skinks of Turkmenistan (Baker, 1987). The purpose of this note is to report on the re-examination of the specimens of Fabio and Rolas (1974) and the cotypes of *P. venancioi* and to correct the nomenclature of these specimens.

Twenty-seven specimens in the Helminthological Collection of the Instituto Oswaldo Cruz were borrowed and examined: CHIOC 31062a–r (specimens mounted on slides accessioned for Fabio and Rolas, 1974) and CHIOC 18553–18560 and 18713 (cotypes of *P. venancioi*). The latter specimens had been deposited in vials of alcohol and were transferred to glycerol mounts for examination with a compound microscope. Because of damage to some specimens, all measurements could not be made on each specimen.

Measurements of selected morphological characters for specimens from Fabio and Rolas (1974) and the cotypes of *P. venancioi* were within respective ranges reported in the original papers (Table I).

All specimens are white, cylindrical nematodes, each with a transversely striated cuticle, anteriorly rounded and posteriorly tapered ends. There is an asymmetrical oral opening with a cephalic collarete surrounding 2 well developed lateral lips.

The right lip is armed with 5 teeth, of which the middle tooth is largest; the lateral teeth are small, sometimes truncate rather than pointed (Figs. 1, 2). The caudal alae do not meet anteriorly and extend to just below the tip of the tail. They are unsupported by papillae. There are 9 pairs of papillae: 4 pairs precloacal, 1 pair adcloacal, and 4 pairs postcloacal (Fig. 3). The tip of the tail of the male ends in a blunt point; that of the female is rounded.

We conclude that the specimens reported by Fabio and Rolas (1974) are indistinguishable from *P. venancioi* but do differ from specimens of *P. dactyluris*. Based on this fact as well as geographic distribution, we consider *Thubunaea dactyluris* Karve, 1938 sensu Fabio and Rolas (1974) nec Karve, 1938 a junior synonym of *P. venancioi* (Lent, Freitas, and Proença, 1946).

The measurements of specimens from Brazil agree well with those from Paraguay (Table I). Fabio and Rolas (1974) reported 5 pairs of postcloacal papillae in their Brazilian specimens; however, it is evident from their figures 7 and 8 that the first of those pairs is better described as paracloacal. Fabio and Rolas (1974) reported their specimens to have 4 teeth and did not specify which lip; our examination suggests it is probable that they overlooked the fifth tooth because the lateral teeth are sometimes chisel-like rather than pointed.

Thubunaea dactyluris was originally described by Karve (1938) from specimens taken from the lizards *Calotes versicolor* and *Hemidactylus flaviviridis* collected in Nagpur and Poona, India. The species was transferred to *Physalopteroides*

Molecular Characterization of a *Cryptosporidium* Isolate From a Black Bear

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ABSTRACT: To further validate the observation of the existence of host-adapted strains of *Cryptosporidium parvum*, we genetically characterized an isolate of *Cryptosporidium* parasite from a black bear. Sequence analysis of the ribosomal RNA small subunit and the 70-kDa heat shock protein (HSP70) showed that this parasite represents a new genotype of *C. parvum* and is related to the *C. parvum* dog genotype. This finding is helpful for clarifying *Cryptosporidium* taxonomy.

Cryptosporidium spp. are common parasites of vertebrates, responsible for diarrheal and respiratory diseases in infected animals. A total of 22 *Cryptosporidium* species have been described, largely based on the host occurrence. Most researchers, however, accept only 6–8 species and consider that *C. parvum* is the species responsible for infection in most mammals (O'Donoghue, 1995; Fayer et al., 1997).

Results of recent molecular taxonomic studies have provided useful data to help clarify *Cryptosporidium* taxonomy. Sequence and phylogenetic analyses of the ribosomal RNA (rRNA) small subunit (SSU) have validated the species status of certain *Cryptosporidium* parasites, such as *C. muris*, *C. serpentis*, *C. baileyi*, and *C. parvum* (Morgan, Monis, et al. 1999; Morgan, Xiao, et al., 1999; Xiao, Escalante, et al., 1999; Xiao, Morgan, et al., 1999). The validity of other species such as *C. wrairi*, *C. meleagridis*, and *C. felis*, however, remains less certain because of their similarity to certain parasites traditionally classified as *C. parvum* (Morgan, Xiao, et al., 1999; Xiao, Morgan, et al., 1999). The genetic distances among some *C. parvum* parasites are frequently larger than those between *C. parvum* and *C. wrairi* or *C. meleagridis*.

It is now becoming clear that there are host-adapted strains of *C. parvum*. Thus far, 7 genotypes of *C. parvum* have been described: the bovine, mouse, human, ferret, marsupial, pig, and dog genotypes. With the exception of the bovine genotype, most strains are associated with a specific group of animals. Thus, the pig genotype has been found only in pigs and the mouse genotype only in mice and related small rodents. In this report, we genetically characterized a *C. parvum* parasite recently found in a black bear (*Ursus americanus*) in Virginia (Duncan et al., 1999). Results of the analysis support the presence of host-adapted strains of *C. parvum*.

A paraffin-embedded tissue block from the small intestine of a male black bear cub with cryptosporidiosis found dead in Virginia was the source of parasite material used in this study. The diagnosis of *C. parvum* infection was based on the finding of developmental stages of *Cryptosporidium* parasites in the microvillous border of the small intestine and reactivity to anti-*C. parvum* rabbit sera (Duncan et al., 1999). DNA was extracted from the tissue block as described previously (Xiao, Escalante, et al., 1999; Xiao, Morgan, et al., 1999), except that small pieces of the tissue block were heated in DNA extract solution to remove paraffin before they were used in DNA extraction.

Primary characterization of the bear *Cryptosporidium* parasites was conducted at the SSU locus. An 834-bp segment of the *Cryptosporidium* SSU rRNA gene was amplified by nested polymerase chain reaction (PCR). Primers and amplification conditions used in this study were as previously described (Xiao, Morgan, et al., 1999), except that the reverse primer used in the primary PCR was 5'-CCCATTTCCTTCGAAACAGGA-3'. For genotyping analysis, restriction enzyme length polymorphism (RFLP) was assessed by the digestion of the secondary PCR product with *SspI* and *VspI*. The secondary PCR product was further sequenced in both directions with an ABI377 autosequencer (Applied Biosystems, Foster City, California), with additional confirmation by sequencing a new PCR product.

A nested PCR protocol was used to amplify the HSP70 gene from genomic DNA of the bear *Cryptosporidium* isolate for nucleotide sequencing. For the primary PCR, a PCR product of ~2,015 bp was amplified using forward (5'-ATGCTCTGAGGTCCAGCTATTGGTATTGA-3') and reverse (5'-TTAGTCGACCTCTTCAACAGTTGG-3') primers. A total of 35 PCR cycles (94 C for 45 sec, 55 C for 45 sec, 72 C for 60 sec) were carried out, with an initial hot start (94 C for 5 min) and a final extension (72 C for 10 min). For the secondary PCR, a 1,939-bp fragment was amplified using 2.5 µl of primary PCR product and nested forward (5'-TA/CTTCATG/CTGTTGGTGTATGGAGAAA-3') and reverse (5'-CAACAGTTGGACCATTAGATCC-3') primers. The PCR conditions for the secondary PCR were identical to those of the primary PCR, except that the annealing temperature was 45 C. The

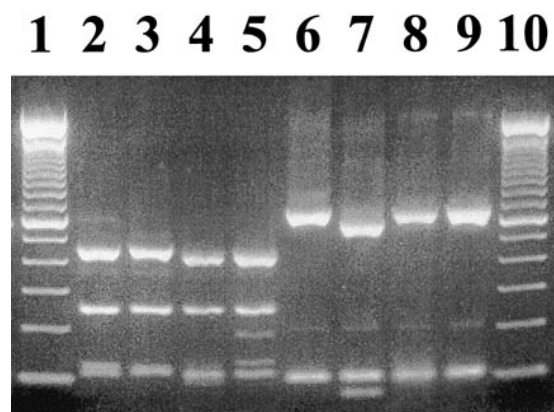


FIGURE 1. PCR RFLP analysis of an 834-bp region of the SSU rRNA gene of the bear *Cryptosporidium* isolate. Lanes 1, 5: 100-bp molecular ladders; lanes 2, 6: *C. parvum* bovine genotype; lanes 3, 7: *C. parvum* human genotype; lanes 4, 8: *C. parvum* dog genotype; lanes 5, 9: bear *Cryptosporidium* isolate. Lanes 2–5: *SspI* digestion; lanes 6–9: *VspI* digestion.

Bovine	GGAAGGGTTGTATTTATTAGATAAAGAACCAATATAATTGGTGACTCATAATAACTTTAC
Human	
Dog	TT.....T.....
Bear	TT.....T.....
Bovine	GGATCACATTAAAT--GTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTA
Human	A.T...--.....
Dog	TT...--.....
Bear	TT..AT.....
Bovine	GGGTATTGGCCTACCGTGGCAATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAG
Human	
Dog	
Bear	
Bovine	GGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC
Human	
Dog	
Bear	
Bovine	CTAATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTT-TGGTTTTGTAATTG
Human	-
Dog	AACA.....
Bear	AATA.....
Bovine	GAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGC
Human	
Dog	G.....
Bear	
Bovine	AGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGT
Human	
Dog	
Bear	
Bovine	AGTTGGATTTCTGTTAATAATTTATATAAAATATTTTGATGAATATTTATATAATATTAA
Human	
Dog	-A.C--.....
Bear	A.--.....
Bovine	CATAATTCATATTACTATATATTTT--AGTATATGAAATTTTACTTTGAGAAAATTAGA
Human	T.T...TTT.....
Dog	T...-----C.....
Bear	AC...-----.....
Bovine	GTGCTTAAAGCAGGCATATGCCTTGAATACTCCAGCATGGAATAATATTAAAGATTTTTA
Human	
Dog	T.T.....AG.....
Bear	AG.....
Bovine	TCTTT-CTTATTGGTTCTAAGATAAGAATAATGATTAAATAGGGACAGTTGGGGGCATTTG
Human	-T.....
Dog	-.....GA.....
Bear	CT.....GA.....
Bovine	TATTTAACAGTCAGAGGTGAAATTCTTAGATTTGTAAAGACAACTAATGCGAAAGCAT
Human	
Dog	T.....
Bear	T.....

FIGURE 2. Comparison of nucleotide sequence of the bear *Cryptosporidium* isolate with sequences from the *C. parvum* bovine, human, and dog genotypes in the 834-bp region of the SSU rRNA gene. Dots indicate nucleotide bases are identical to the *C. parvum* bovine genotype sequence, and dashes depict nucleotide base deletions. A portion of the conserved sequences in the 3' region is not shown.

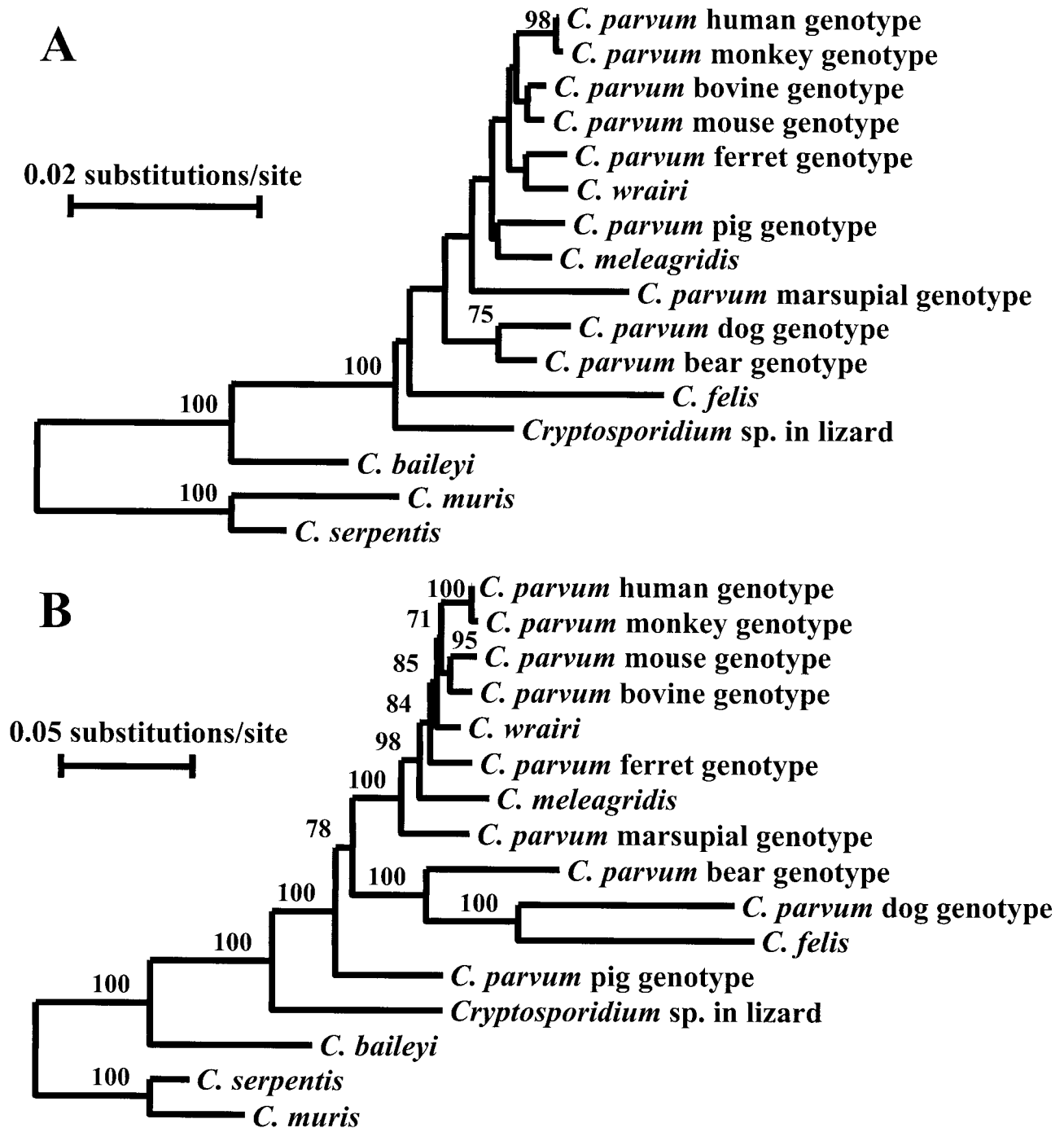


FIGURE 3. Relationship of the bear *Cryptosporidium* isolate to other *Cryptosporidium* parasites as inferred by neighbor-joining analysis of nucleotide sequences of the SSU rRNA (A) and HSP70 (B) genes. Numbers on branches are bootstrap values in percentage (only those greater than 70% are shown) based on 1,000 pseudoreplicates. The SSU rRNA (834 bp) and HSP70 (1,939 bp) of the bear *Cryptosporidium* isolate have been deposited in the GenBank database under accession nos. AF247535 and AF247536.

secondary PCR products were sequenced in both directions, and sequence accuracy was further confirmed by sequencing a second PCR product.

Sequences from the bear *Cryptosporidium* isolate were aligned with available nucleotide sequences from other *Cryptosporidium* species and *C. parvum* genotypes using the Wis-

consin package 9.0 (Genetics Computer Group, Madison, Wisconsin). Phylogenetic analysis of nucleotide sequences was conducted as previously described (Xiao, Escalante, et al., 1999; Xiao, Morgan, et al., 1999). Evolutionary distance between different isolates was calculated using the Kimura 2-parameter model, and phylogenetic trees were constructed using

the neighbor-joining algorithm (Saitou and Nei, 1987). Branch reliability was assessed using bootstrap analyses (1,000 replicates).

Restriction enzyme digestion of the secondary PCR product of the SSU rRNA gene showed that the bear *Cryptosporidium* isolate had *SspI* and *VspI* restriction patterns more similar to those of the *C. parvum* dog genotype than to those of any other *Cryptosporidium* species and *C. parvum* genotypes (Fig. 1; data not shown). Two additional *SspI* bands were present underneath the 254-bp band in the bear isolate that were not present in the *C. parvum* dog genotype. Sequence variation is known to occur among copies of the SSU rRNA in *Cryptosporidium* parasites (Xiao, Limor, et al., 1999). Resulting restriction site variation in the black bear isolate may account for these 2 additional bands.

Sequence analysis of the amplified SSU product confirmed the similarity between the dog genotype and the bear isolate; differences of 13 bp were seen between these 2 genotypes in the 834-bp region analyzed (Fig. 2). The close relatedness of the bear isolate to the *C. parvum* dog genotype is also reflected in a neighbor-joining tree constructed based on these sequences. Among 8 *Cryptosporidium* and 8 *C. parvum* genotypes used in the phylogenetic analysis, the bear isolate and the dog genotype clustered together (Fig. 3a).

Analysis of the 1939-bp fragment of the HSP70 gene also revealed that the bear isolate was most closely related to the *C. parvum* dog genotype. A difference of 235 bp was seen between the dog genotype and the bear isolate in the 1939-bp region of the HSP70 gene (data not shown). Phylogenetic analysis of the HSP70 sequences placed the bear isolate in the clade containing the *C. parvum* dog genotype and *C. felis* (Fig. 3b).

Results of the sequence and phylogenetic analyses in this study at both the SSU rRNA and HSP70 genes indicate that the bear isolate represents a new genotype of *Cryptosporidium* parasite that is closely related to the previously described *C. parvum* dog genotype and perhaps to *C. felis* (Xiao, Morgan, et al., 1999; Morgan et al., 2000). Thus to date, the following 8 distinct genotypes have been identified within what is currently recognized as *C. parvum*: human, bovine, pig, marsupial, mouse, ferret, dog, and bear genotypes (Morgan, Monis, et al., 1999; Morgan, Xiao, et al., 1999; Xiao, Escalante, et al., 1999; Xiao, Morgan, et al., 1999). Most of these genotypes have been found in a small group of related animals. The limited populations studied makes it difficult to generalize, but it appears that the parasites may have evolved to adapt to restricted hosts. The characterization of more host-adapted strains of *Cryptosporidium* parasites must continue so that host-parasite coevolution can be studied in detail.

The significance of these host-adapted genotypes in *Cryptosporidium* taxonomy is not fully clear. The genetic difference between some of the novel *C. parvum* genotypes and the classic *C. parvum* (mouse, bovine, and human) genotypes is much greater than what exists between some well-established species, such as *C. parvum* and *C. meleagridis* or *C. muris* and *C. serpentis*. It has been suggested recently that the *C. parvum* dog genotype should be considered a distinct species (Morgan, Xiao, et al., 1999; Xiao, Morgan, et al., 1999; Morgan et al., 2000; Sulaiman et al., 2000). Although the bear genotype is genetically related to the *Cryptosporidium* dog genotype, these 2 genotypes nevertheless differ as much as do

some of pairs of *Cryptosporidium* species, e.g., *C. parvum* and *C. meleagridis*.

The close relatedness of the bear genotype to that of the dog and *C. felis* can be a public health concern. Both the *Cryptosporidium* dog genotype and *C. felis* have been found recently in AIDS patients (Pieniazek et al., 1999) and children (L. Xiao, pers. obs.). Although direct evidence is still lacking, wildlife has been frequently considered a source of human *Cryptosporidium* infection (Laakkonen et al., 1994; Webster and MacDonald, 1995; Webster, 1996; Bajer et al., 1997; Chalmers et al., 1997; Sinski et al., 1998; Sturdee et al., 1999). If the bear *Cryptosporidium* is indeed infectious to humans, oocysts excreted by infected bears could end up in water systems, posing a public health threat.

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Molecular Differentiation of Three Species of *Metagonimus* by Simple Sequence Repeat Anchored Polymerase Chain Reaction (SSR-PCR) Amplification

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ABSTRACT: Genomic DNAs from 3 species of *Metagonimus* in the Republic of Korea, *M. yokogawai*, *M. miyatai*, and *M. takahashii*, were amplified by simple sequence repeat anchored polymerase chain reaction. Primers of 3' or 5' termini of the (CA)_n repeats were used; 3 kinds of 3' anchored primers (CA)₈RG, (CA)₄RG, and (CA)₈RY, and 2 kinds of 5' anchored primers BDB(CA)₇C and DBDA(CA)₇. The results showed distinguishable banding patterns among the 3 species of *Metagonimus*, suggesting that they have different genotypes.

Human infection with *Metagonimus yokogawai* (Digenea: Heterophyidae), which is contracted by eating raw sweetfish *Plecoglossus altivelis*, has been regarded as a parasitic disease of public health importance in the Republic of Korea (Chai and Lee, 1990). Many scattered endemic foci have been discovered along the riversides of the southern and eastern coasts (Chai et al., 1977; Seo et al., 1981; Chai and Lee, 1990; Chai et al., 2000). On the other hand, in inland areas, it has been reported that the metagonimiasis is not due to *M. yokogawai*, but to either *Metagonimus* Miyata type of Saito, 1984 or to *M. takahashii* Suzuki, 1930 (Chai et al., 1993; Yu et al., 1994). The fish hosts of the latter 2 species are the pale chub *Zacco platypus*, and the carp *Carassius carassius*, respectively. Although controversial, the Miyata type was proposed as a new species, *M. miyatai*, on the basis of its characteristic morphology and life cycle (Saito et al., 1997). On the genomic level, however, only a few studies have been performed on the species of *Metagonimus* using random amplification of polymorphic DNA (RAPD) (Yu et al., 1997a) or polymerase chain reaction-based restricted fragment length polymorphism (PCR-RFLP) (Yu et al., 1997b).

Simple sequence repeat (SSR) DNA loci can be identified in eukaryotic genomes. Interrepeat regions can be amplified by PCR and their products can be compared according to banding patterns. Thus, SSR-anchored PCR methods (SSR-PCR) have been applied to differentiate various animal and plant species (Ishibashi et al., 1995; Godwin et al., 1997). In the present

study, we attempted to apply this protocol to differentiate the 3 species of *Metagonimus* distributed in the Republic of Korea.

Metacercariae of *M. yokogawai*, *M. miyatai*, and *M. takahashii* were collected from *P. altivelis*, *Z. platypus*, and *C. carassius*, respectively, caught at different localities, and then isolated using an artificial digestion technique. Adult flukes were obtained after killing experimentally infected Sprague–Dawley rats at day 14 postinfection. Collected worms were preserved at –70 °C until genomic DNA extraction, using the method of Sambrook et al. (1989). Adult flukes were placed in a 1.5-ml tube containing the lysis buffer (10 mM Tris-Cl, pH 8.0, 100 mM EDTA, 0.5% sodium dodecyl sulfate). After mixing and incubation overnight at 37 °C, samples were treated with proteinase K (100 µg/ml) and RNase (20 µg/ml). Subsequently DNA was extracted using an equal volume of phenol:chloroform:isoamyl alcohol. The total genomic DNA was finally precipitated overnight after addition of 1/20 volume of 5 M NaCl and 2 volumes of 99% ethanol at –70 °C. The genomic DNA pellet was obtained by centrifugation at 10,000 *g* for 10 min and resuspended in 50 µl of TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) buffer. RNA in the sample was digested by addition of 0.1 volume of RNase A (10 mg/ml) and incubated for 1 hr at 37 °C. Extraction was done as described above. Residual phenol was removed by chloroform extraction. The genomic DNA extracted was resuspended in 100 µl of TE buffer and stored at 4 °C.

Primers of 3' or 5' termini of the (CA)_n repeats (Zietkiewicz et al., 1994) were used: 3 kinds of 3' anchored primers, (CA)₈RG, (CA)₄RG, and (CA)₈RY, and 2 kinds of 5' anchored primers, BDB(CA)₇C and DBDA(CA)₇. After determination of the annealing temperature (52 °C), template concentration (20 ng/ml), and primer concentration (0.5 µM/µl), SSR-PCR was done as follows. A total of 20 µl of solution containing sample DNA, 2.5 mM MgCl₂, 100 µM of each dNTP (Takara Shuzo, Shiga, Japan), primers, and 2.5 units of Taq polymerase was

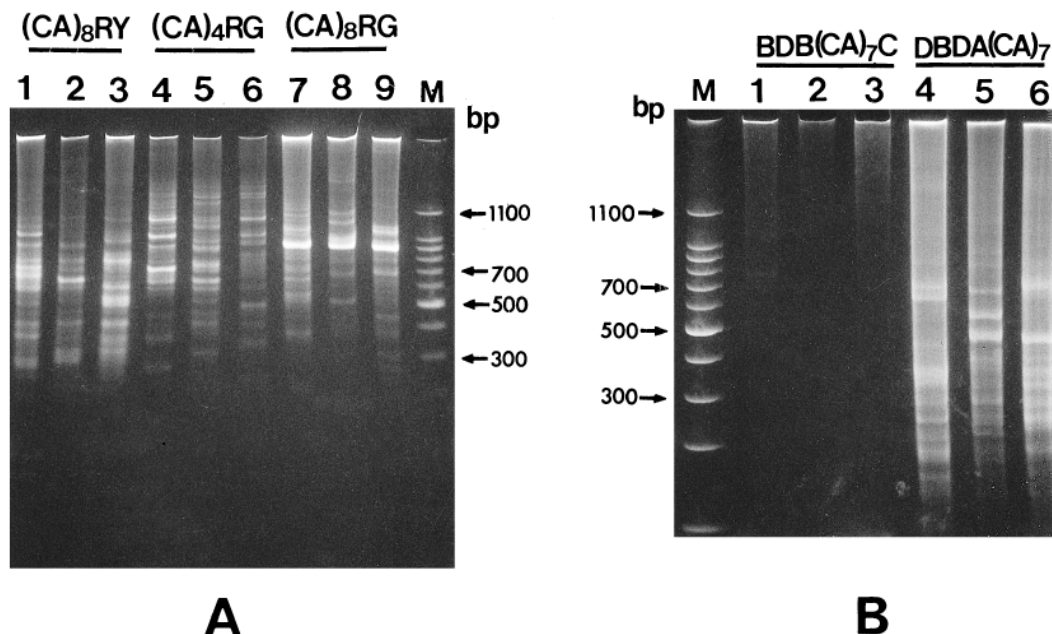


FIGURE 1. SSR-PCR patterns obtained from 20 ng/ml genomic DNAs of 3 species of *Metagonimus* and 0.5 μ M/ μ l primers at 52 C of annealing temperature. (A) The patterns in lanes 1–3 (lane 1, *M. takahashii*; lane 2, *M. miyatai*; lane 3, *M. yokogawai*), lanes 4–6 (lane 4, *M. takahashii*; lane 5, *M. miyatai*; lane 6, *M. yokogawai*), and lanes 7–9 (lane 7, *M. takahashii*; lane 8, *M. miyatai*; lane 9, *M. yokogawai*), respectively, were obtained using $(CA)_8RY$, $(CA)_4RG$, and $(CA)_8RG$ primers. (B) The patterns of lanes 1–3 (lane 1, *M. yokogawai*; lane 2, *M. miyatai*; lane 3, *M. takahashii*) and lanes 4–6 (lane 4, *M. yokogawai*; lane 5, *M. miyatai*; lane 6, *M. takahashii*) were obtained using $BDB(CA)_7C$ and $DBDA(CA)_7$ primers. Amplification was performed in Perkin-Elmer 9600 programmed for 30 cycles of 30 sec at 94 C, 45 sec at 52 C, and 1 min at 72 C. SSR-PCR fragments were separated by electrophoresis using 1% agarose gel and stained with ethidium bromide. M, marker.

amplified by PCR thermocycler (Perkin-Elmer 9600, Foster, California). The PCR reaction was done at 94 C for 30 sec, 52 C for 45 sec, and 72 C for 2 min, and repeated for 30 cycles. The PCR product was visualized by electrophoresis in 1% agarose gel staining with ethidium bromide.

All of the primers except the 5' anchored primer of $BDB(CA)_7C$ produced clearly distinguishable banding patterns among the 3 species of *Metagonimus* (Fig. 1A, B). The $BDB(CA)_7C$ primer did not anneal the template DNAs (Fig. 1B). The PCR products using the short oligonucleotide primer $(CA)_4RG$ showed more complex banding patterns than did other primers (Fig. 1A).

The results suggest that the 3 species of *Metagonimus* have different genotypes and support the validity of *M. miyatai* (Saito et al., 1997). All of the primers used were useful for differentiating *Metagonimus* species except for the 5' anchored primer $BDB(CA)_7C$. The present results support our previous report using RAPD (Yu et al., 1997a) or PCR-RFLP (Yu et al., 1997b), both of which demonstrated different banding patterns of polymorphic DNA or rRNA of the 3 species of *Metagonimus*.

It has been suggested that the SSR-PCR fingerprinting method might be applicable for taxonomic comparisons of animal and plant species (Ishibashi et al., 1995; Godwin et al., 1997), although the SSR-PCR method usually shows a higher level of polymorphism than PCR-RFLP or RAPD analyses. On the other hand, it has been shown that the SSR-mediated variations have important implications in epidemiological studies of bacteria (Belkum et al., 1998). The results of the present study suggest that the differences in SSR-PCR banding patterns among the 3 species of *Metagonimus* might be related to their

biological/ecological characteristics, e.g., differences in morphology and the requirement for different fish intermediate hosts.

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