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A Novel *Giardia duodenalis* Assemblage A Subtype in Fallow Deer

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ABSTRACT: The molecular identification of species and genotypes of *Giardia* spp. infecting wild mammals represents the most reliable tool to understand the role played by these animals as reservoirs of cysts infectious for human and other animals. Of 139 fecal samples collected from fallow deer (*Dama dama* L.) hunted in a Natural Reserve of northern Italy, the prevalence of *Giardia* sp. was 11.5% (16 of 139 animals), and it was higher in fawns than in older animals. Fragments of the β-giardin and triose phosphate isomerase (tpi) genes were successfully polymerase chain reaction amplified and sequenced from 8 isolates. No sequence variation was observed between isolates at the 2 genetic loci. Sequence and phylogenetic analyses identified a *Giardia duodenalis* subtype that clusters with assemblage A isolates and that shows homologies of 98 and 97% at the β-giardin and tpi loci, respectively, compared with the A1 subtype. Because the *G. duodenalis* subtype found in fecal samples of fallow deer has never been detected previously, its role as a pathogen for humans and domestic animals is unknown, but, considering its genetic distinctiveness, it is likely to be low.

Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*) is the only species within the *Giardia* genus that is responsible for infection of humans and other mammals, including pets and livestock (Thompson and Monis, 2004). This protozoan produces robust cysts, which are voided in the feces and transmitted directly through fecal/oral contact, or indirectly by ingestion of contaminated water and food. Water has been increasingly recognized as an important vehicle, and many waterborne outbreaks have been reported in economically developed countries (Smith et al., 2006).

In spite of its invariant morphology, genetic analyses have demonstrated that *G. duodenalis* is a species complex composed of at least 7 distinct assemblages (Monis et al., 2003). Assemblages A and B have been detected in a wide range of mammalian hosts, including humans, whereas the remaining assemblages (C–G) are host-specific and have never been isolated from humans (Monis et al., 1999, 2003; Sulaiman et al., 2003). To understand the epidemiology of the infection, and in particular, the impact of the zoonotic transmission, the direct molecular characterization of *Giardia* spp. cysts in fecal samples represents the most reliable and informative approach (Cacciò et al., 2005).

In hoofed animals (Artiodactyla), molecular investigations have demonstrated the presence of the host-adapted assemblage E of *G. duodenalis* (Ey et al., 1997), but livestock animals also may be infected with potentially zoonotic assemblages A and B (Trout et al., 2004; Lalle et al., 2005; Ryan et al., 2005). Therefore, understanding the role of hoofed animals as potential sources of human infectious cysts is an area of epidemiologic interest (Appelbee et al., 2005).

In Italy, little is known about the prevalence of giardiasis in hoofed animals, and the data are limited to livestock (Berrilli et al., 2004; Giangaspero et al., 2005; Lalle et al., 2005). The aim of the present study was to examine the prevalence of *Giardia* spp. in a sample of fallow deer (*Dama dama* L.) and to determine the species/subtypes by using molecular methods.

Fecal samples were collected in the Boscone della Mesola (longitude 12°13′E; latitude 44°55′N; Emilia-Romagna Region, northeast of Italy), one of the largest Italian floodplain forest (1,058 ha) and a Natural Reserve since 1977. A population of 500/600 fallow deer live in this area. The extensive overgrazing by fallow deer in the forest has removed most of the ground flora and suppressed tree regeneration; therefore, a program of selective culling was initiated (Mattioli et al., 2003). Between September 2005 and February 2006, fecal samples were collected from the rectum of 139 killed animals (corresponding to an estimated 23% of the population), transferred to clean plastic bags, and brought immediately to the laboratory. The first screening for the presence of *Giardia* sp. cysts was made by flotation in a sodium nitrate solution (specific gravity 1.3 g/ml) and microscopic examination of unstained samples. Sixteen samples tested positive, and 13 samples were sent to the Istituto Superiore di Sanità of Rome for further parasitological analyses and for molecular identification. Samples were filtered through a mesh, washed with phosphate-buffered saline solution, and subjected to centrifugation on 1 M sucrose gradient for 10 min at 800 *g*. After the flotation step, the presence of *Giardia* spp. cysts was assessed by immunofluorescence (IF) microscopy using fluorescein isothiocyanate-conjugated cyst wall-specific antibodies (Merifluor, Meridian Bioscience, Cincinnati, Ohio).

DNA was extracted directly from fecal samples by using the Fast Prep (Qbiogene, Illkirch Cedex, France) procedure as described by da Silva et al. (1999). Briefly, an aliquot of fecal sample was homogenized using the FP120 Fast Prep Cell disruptor (Savant, Thermo Electro Corporation, Woburn, Massachusetts). The DNA released after the lysis step was purified using the Fast DNA extraction kit (Qbiogene).

A 511-base pair (bp) fragment of the β-giardin gene was amplified by nested polymerase chain reaction (PCR) by using the conditions described previously (Lalle et al., 2005). Similarly, a 530-bp fragment of the triose phosphate isomerase (tpi) was amplified by nested PCR by using the protocol described by Sulaiman et al. (2003). The primary PCR reaction consisted of 5 μl of 10× PCR buffer (Promega, Milan, Italy), 1.5 mM $MgCl_2$, 200 μM each of dNTP, 20 pmol of each primer, 0.2 μl (1 unit) of GoTaq DNA polymerase (Promega), and 3 μl of DNA in a total reaction volume of 50 μl. For the nested PCR, 2.5–5 μl of the first PCR was used as template. The primary PCR reaction was carried out for 35 cycles, each consisting of 94 C for 30 sec, 65 C (β-giardin) or 50 C (tpi) for 30 sec, and 72 C for 1 min in a T-personal thermocycler (Whatman-Biometra, Goettingen, Germany), with an initial hot start at 94 C for 2 min and a final extension at 72 C for 7 min. The conditions for the tpi nested PCR were identical to that of the primary reaction, whereas for the β-giardin, the annealing temperature was set at 53 C and the extension time at 30 sec. PCR products were separated by electrophoresis in 1% agarose gels stained with ethidium bromide.

PCR products were purified using the QIAquick purification kit (QIAGEN, Milan, Italy) and sequenced on both strands by using the ABI Prism BIGDYE Terminator Cycle Sequencing kit (Applied Biosystems, Milan, Italy) and the same sets of primers used for PCR. The sequencing reactions were analyzed using the ABI 3100 automatic sequencer (Applied Biosystems), and sequences were assembled using the software program SeqMan II (DNASTAR, Madison, Wisconsin). The tpi and β-giardin sequences from the fallow deer have been deposited in the GenBank database under the accession numbers DQ650648 and DQ650649, respectively.

The nucleotide sequences of tpi gene fragments from selected *Giardia* species and *G. duodenalis* assemblages (see legend to Fig. 1) were retrieved from the GenBank database and aligned with the fallow deer sequence by using the program Clustal X (Thompson et al., 1997). Phylogenetic analysis was performed using MEGA 3.1 (Kumar et al., 2004). Distance-based analyses were conducted using Kimura 2-parameter distance estimates, and trees were constructed using the neighbor-joining algorithm. Bootstrap proportions were calculated by the analysis of 1,000 replicates for neighbor-joining trees.

Of 139 fecal samples tested, 16 (11.5%) were positive for *Giardia* sp. cysts by using microscopic or IF assays. The prevalence was higher in fawns (average 14.9%) than in older animals (average 5.5%; Table I). The β-giardin and tpi gene fragments were successfully PCR amplified and sequenced from 8 of the 13 samples from which DNA extraction was attempted. No sequence variation was observed among fallow deer isolates at both loci. At the β-giardin locus, comparison of the fallow deer sequence with the reference subtype A1 (GenBank X85958) showed a homology of 98%, with 8 synonymous nucleotide substitutions (Table II). Importantly, the β-giardin sequence was found

TABLE I. Prevalence of *Giardia* spp. cysts in fecal samples of fallow deer as a function of age and sex.

Age group (yr)	No. of animals tested/ positive (%)	Male tested/ positive (%)	Female tested/ positive (%)
1–2	94/14 (14.9)	42/9 (21.4)	52/5 (9.6)
2–5	36/2 (5.5)	11/1 (9.1)	25/1 (4.0)
>5	9/0 (0)	9/0 (0)	0/0 (0)
Total	139/16 (11.5)	62/10 (16.1)	77/6 (7.8)

to be identical to that obtained from a wild moose (*Alces alces*) isolate from Norway (GenBank DQ648777), a result that enlarges the host range of the *G. duodenalis* subtype to different cervine species. At the tpi locus, the fallow deer sequence showed a homology of 97% compared with the reference subtype A1 (GenBank L02120), with 14 nucleotide substitutions, including 1 nonsynonymous substitution (Table II). The phylogenetic analysis of the tpi nucleotide sequences showed that the fallow deer subtype clusters with assemblage A subtypes, and bootstrap analysis indicated strong statistical support for this grouping (Fig. 1).

Little is known about the prevalence of *Giardia* spp. in cervine animals, and most surveys have been performed in North America. In northern California, only 3 (3.7%) samples of 82 collected from adult fallow deer, Columbian black-tailed deer (*Odocoileus hemionus columbianus*), and Tule elks (*Cervus elaphus nannodes*), contained *Giardia* sp. cysts (Deng and Cliver, 1999). Only 1 of 26 (3.8%) samples collected from hunter-killed white-tailed deer (*Odocoileus virginianus*) in a central county of Maryland, was positive for *Giardia* sp. cysts (Trout et al., 2003). A prevalence of 2.9 and 1.1% was observed in free-ranging white-tailed deer from Virginia and Mississippi, respectively (Rickard et al., 1999). These studies suggest an average prevalence close to 3% in the United States, albeit this figure may be underestimated due to sampling bias, the intermittent shedding of *Giardia* spp. cysts and the examination of a single fecal sample per animal.

The present study shows that molecular typing of *Giardia* cysts in fecal samples of Italian fallow deer identified a novel, unique subtype within the *G. duodenalis* assemblage A. As far as we know, this subtype has never been identified in humans or in other mammalian hosts, and this opens the question whether it is host-adapted or whether it can play a zoonotic role. The natural reserve under study is a popular spot for tourism, with an estimated 50,000 visitors/yr. Although the risk for direct zoonotic transmission of *Giardia* sp. cysts to humans is probably low, it is important to monitor *Giardia* sp. infections in wildlife, to assess the potential impact of the environmental contamination on human health. In this context, it should be pointed out that the area that surrounds the natural reserve is used for crop cultivation (mainly maize, carrot, asparagus, and melon) and that shellfish also are extensively produced in the near shore marine environment. Wildlife feces may, therefore, contaminate those foods if cysts are transported from the watershed into the canal system from which water is abstracted for irrigation or that drains into the Po River estuary.

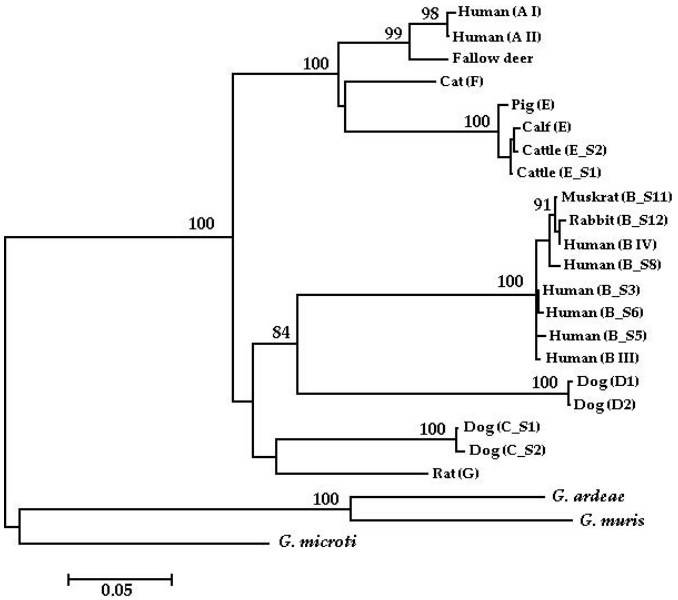

FIGURE 1. Phylogenetic relationships of *Giardia* inferred by the neighbor-joining analysis of the tpi nucleotide sequences. Only bootstrap values >70 are indicated. The GenBank accession numbers of the sequences used are A I (L02120), A II (U57897), fallow deer (DQ650649), Cat F (AF069558), Pig E (AF069559), Calf E (DQ157270), Cattle E S1 (AY228646), Cattle E S2 (AY228645), Muskrat B S11 (AY228638), Rabbit B S12 (AY228639), Human B IV (AF069560), Human B S8 (AY228635), Human B S3 (AY228630), Human B S6 (AY228633), Human B S5 (AY228632), Human B III (AF069561), Dog D1 (DQ220289), Dog D2 (DQ246216), Dog C S1 (AY228643), Dog C S2 (AY228642), Rat G (AF069562), *Giardia ardeae* (AF069565), *Giardia muris* (AF069564), and *Giardia microti* (AY228649).

In the study of Trout et al. (2003), a *G. duodenalis* isolate from a white-tailed deer was typed as assemblage A, subtype A1, which has a clear zoonotic potential, by sequence analysis of the tpi gene (GenBank AY302562). However, when the same isolate was typed at the β-giardin locus, a unique subtype was identified, which differed from A1 by 2 nucleotide substitutions (GenBank AY302561; Table II). In The Netherlands, 1 *G. duodenalis* isolate from a roe deer (*Capreolus capreolus*)

TABLE II. Nucleotide substitutions between the fallow deer sequence and the reference subtype A1 of *Giardia duodenalis* assemblage A. The white-tailed deer β-giardin sequence (GenBank AY302561) is shown for comparison. Positions are numbered with respect to the start codon of the complete coding sequence (GenBank X85958 for the β-giardin and L02120 for the tpi gene, respectively). Underlined positions indicate nonsynonymous substitutions.

	β-Giardin									
Position	133	255	354	378	429	444	462	534	564	582
A1	a	c	t	t	t	t	a	g	t	a
White-tailed deer	T	c	t	t	C	t	a	g	t	a
Fallow deer	a	T	C	C	C	C	G	A	C	G

	tpi													
Position	93	108	117	120	133	144	162	174	189	231	352	394	492	498
A1	t	t	c	c	g	g	a	a	a	c	c	t	g	c
Fallow deer	C	C	T	T	T	A	G	G	G	T	T	C	T	T

was typed as assemblage A by the 18S rDNA gene sequencing, but the analysis of the glutamate dehydrogenase gene identified a novel subtype (GenBank DQ100288), which differs from those found in humans and other animals (van der Giessen et al., 2006). These data suggest that cervids can harbor various subtypes of *G. duodenalis*, all belonging to the assemblage A, but only the A1 subtype identified in the feces of white-tailed deer has an established zoonotic potential. It is likely that the subtype identified in fallow deer and wild moose isolates represents a *G. duodenalis* subtype adapted to these animals. It is noteworthy that the hoofed-specific assemblage E (Ey et al., 1997), which is common in livestock, has not been identified in any of the cervine species tested to date. Further studies are needed to fully describe the nature of *G. duodenalis* assemblages/subtypes infecting these animals in different parts of the world.

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Immunomodulation of the Response to Excretory/Secretory Antigens of *Fasciola hepatica* by Anapsos® in Balb/C Mice and Rat Alveolar Macrophages

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ABSTRACT: It is known that excretory/secretory antigens of *Fasciola hepatica* (ESFh) trigger a Th2-like immune response. Anapsos® (A) is an aqueous hydrosoluble extract obtained from the rhizomes of the fern *Polypodium leucotomos* that has shown immunomodulator effects in some parasitic infections and immunological disorders. In this work we assess the effect of Anapsos® and ESFh and *Quillaja saponaria* extract (Qs) on BALB/c mice and rat alveolar macrophages. Anapsos® modulates the response of mice immunized with ESFh, decreasing IgG1 antibodies in A+ESFh− and A+Qs+ESFh−treated mice and triggering high levels of γIFN in spleen cell culture in comparison with ESFh−

TABLE I. Enzyme-linked immunosorbent assay (ELISA) showing IgG, IgG1, and IgG2a antibodies against *F. hepatica* excretory secretory antigen (ESFh) in BALB/c mice at the end of the experiment.

Group	IgG (Mean ± SEM)	IgG1 (Mean ± SEM)	IgG2a (Mean ± SEM)
G1b Ø	0.261 ± 0.061	0.179 ± 0.068	0.292 ± 0.054
G2b A	0.254 ± 0.078	0.166 ± 0.048	0.215 ± 0.045
G3b Qs	0.194 ± 0.090	0.206 ± 0.054	0.233 ± 0.071
G4b A + Qs	0.217 ± 0.076	0.161 ± 0.030	0.237 ± 0.061
G5b ESFh	0.601 ± 0.069*	0.590 ± 0.139*	0.263 ± 0.048
G6b A + ESFh	0.486 ± 0.099*	0.249 ± 0.098†	0.179 ± 0.047
G7b Qs + ESFh	0.610 ± 0.081*	0.824 ± 0.229*	0.280 ± 0.058
G8b A + Qs + ESFh	0.581 ± 0.103*	0.490 ± 0.277‡	0.221 ± 0.032

* $P < 0.05$ compared with G1b.
† $P < 0.05$ compared with G5b.
‡ $P < 0.05$ compared with G7b.

and Qs+ESFh−treated groups. Moreover, Anapsos® showed statistically significant inhibitory effects on the nitrite production by rat alveolar macrophages prestimulated with lipopolysaccharide (LPS) as well as ESFh antigen in comparison with macrophages stimulated only with LPS. The application of ESFh and Anapsos® combined avoids this inhibitory effect. Thus, Anapsos® modulates the immune response against ESFh in naive mice and on the nitrite production in prestimulated rat alveolar macrophages.

Fascioliasis is a widespread parasitic infection caused by the trematode *Fasciola hepatica*. The infection causes great economic losses, mainly in cattle and sheep (Boray, 1985), and represents an emerging pathogen for humans with up to 17 million people infected in the world (Mas-Coma et al., 1999). The parasite is acquired by ingestion of metacercariae, which excyst in the small intestine and penetrate the intestinal wall into the peritoneal cavity. The juvenile flukes then move through the peritoneal cavity and enter the liver parenchyma, eventually migrating into the bile ducts (Behn and Sangster et al., 1999). During migration through the liver, parasite trails are surrounded by an extensive local inflammatory reaction. A dominant immunoglobulin G1 isotype, massive eosinophilia, and increase in IL-4, IL-5, and IL-10 observed in murine fascioliasis indicate a Th2 immune response (Chauvin and Boulard, 1996; Tliba et al., 2002). Several studies have demonstrated that *F. hepatica* excretory/secretory antigens (ESFh) in rats, sheep, and humans inhibit the proliferative response of spleen mononuclear cells stimulated with mitogens in a dose-dependent manner (Moreau et al., 2002). A decrease in nitric oxide production by lipopolysaccharide (LPS)–stimulated peritoneal macrophages has also been observed with ESFh (Cervi et al., 1999).

Anapsos® is a hydroalcoholic extract of the rhizome of the fern *Polypodium leucotomos* that grows in the rain forests of Central and South America. It has been used in the treatment of autoimmune diseases and has demonstrated immunomodulatory effects on lymphocyte subsets and cytokines. It has also been used for the treatment of atopic dermatitis and psoriasis (Sempere-Ortells et al., 2002). Moreover, it immunomodulates the response of mice injected with crude antigens from the third-stage larvae of *Anisakis simplex* (Cuellar del Hoyo et al., 1997), crude antigens from the first-stage larvae of *Trichinella spiralis* (Dea-Ayuela et al., 1999), and *Trichomonas vaginalis* down-regulating the Th2 immune response (Nogal-Ruiz et al., 2003).

The objective of the present work is to assess whether Anapsos® can immunomodulate the response induced by excretory/secretory antigens from *F. hepatica*. We examine the production of IL-4/γIFN cytokines and IgG1/IgG2a antibodies an in vivo murine model, and the in vitro production of nitric oxide by rat alveolar macrophages.

Fasciola hepatica excretory/secretory antigens (FhES) were prepared as described by Casanueva et al. (2001), with some modifications. Adult *F. hepatica* worms were collected from naturally infected cows in a local abattoir and washed 4 times at room temperature for 1 hr with PBS, pH 7.2. The worms were incubated at 37 C for 3 hr, 1 worm/ml in PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM ethylenediaminetetracetic acid (EDTA), 1 mM N-ethylmaleimide (NEM), 0.1 mM pepstatin A, and 0.1 mM N-tosylamide-L-phenylalanine chloromethyl ketone (TPCK). After incubation, the medium was collected and centrifuged at 5,000 *g*. The obtained supernatant fraction obtained was concentrated through 5 k, ultrafree-15 filters from Millipore (Bedford, Massachusetts), and then centrifuged at 5,000 *g* at 4 C. Protein concentration was determined by the BCA technique (Pierce, Rockford, Illinois). Ninety-eight 7-wk-old female BALB/c mice were used. Mice were maintained in a controlled environment with a 12-hr light/dark cycle and free access to water and food in the Experimental Animal Service facility (University of Salamanca, Salamanca, Spain). All animal experiments were carried out according to European Council Guidelines 86/609 EC.

We performed an experiment with 5 groups of 10 mice: G1a, control (Ø); G2a, daily intraperitoneal (i.p.) injection of 600 μg of Anapsos® (A; ASAC Pharmaceutical International AIE, Alicante, Spain) from day 0 to 10 of the experiment; G3a, subcutaneous (s.c.) injection with 20 μg of *Quillaja saponaria* extract (Qs; Sigma, St. Louis, Missouri) at days 5 and 10 of the experiment; G4a, s.c. immunized with 50 μg of ESFh at days 5 and 10; G5a, daily i.p. injection of 600 μg of A and s.c. immunized with 20 μg of Qs and 50 μg of ESFh at days 5 and 10. In another experiment we used combinations of A+Qs, A+ESFh, and Qs+ESFh in 8 groups of 6 animals each: G1b, control (Ø); G2b, daily i.p. injection of 600 μg of A from day 0 to 10 of the experiment; G3b, s.c. injection with 20 μg of Qs at days 5 and 10 of the experiment; G4b, daily i.p. injection of 600 μg of A from day 0 to 10 and s.c. injected with 20 μg of Qs at days 5 and 10; G5b, s.c. immunized with 50 μg of ESFh at days 5 and 10; G6b, daily i.p. injection of 600 μg of A and s.c. immunized with 50 μg of ESFh at days 5 and 10; G7b, s.c. immunized with 20 μg of Qs and 50 μg of ESFh at days 5 and 10; G8b, daily i.p. injection of 600 μg of A and s.c. immunized with 20 μg of Qs and 50 μg of ESFh at days 5 and 10. All mice were killed 20 days after the beginning of the experiment. Spleens were removed to obtain splenocytes.

Serum samples obtained at the end of the experiment were individually analyzed by indirect enzyme-linked immunosorbent assay (ELISA) in accordance with Muro et al. (1997). Plates were coated with 4 μg/ml of FhES antigen. Sera, in triplicate, were diluted at 1/100, and polyclonal sheep anti-mouse IgG (Sigma), monoclonal anti-mouse IgG1, and anti-mouse IgG2a (Nordic Immunology, Tilburg, The Netherlands) diluted at 1/1,000 were used as a conjugate. Development was performed with ortho-phenylene diamidine (OPD) substrate, and absorbances were measured at 492 nm.

Spleen cell suspensions were prepared in accordance with López-Abán et al. (1999) and then pooled for every group. Spleen cells from every pool (1×10^6 cells/well) were incubated in triplicate alone and with 2.5 μg/ml of concanavalin A (Con A). Cells were incubated at 37 C in 5% CO_2 atmosphere for 48 hr. Cell suspension was centrifuged at 250 *g* for 10 min. Cell-free supernatants were recovered, aliquoted, and stored at −80 C until they were assayed for γIFN and IL–4. The assay employed in the first experiment was based on a quantitative ELISA, a kit using specific antibodies for mouse γIFN (EM-1001, Endogen, Inc. Woburn, Massachusetts) and IL-4 (EM-IL-4, Endogen). These determinations were performed in triplicate. In the second experiment, the assay employed was based on quantitative flow cytometry, a kit BD

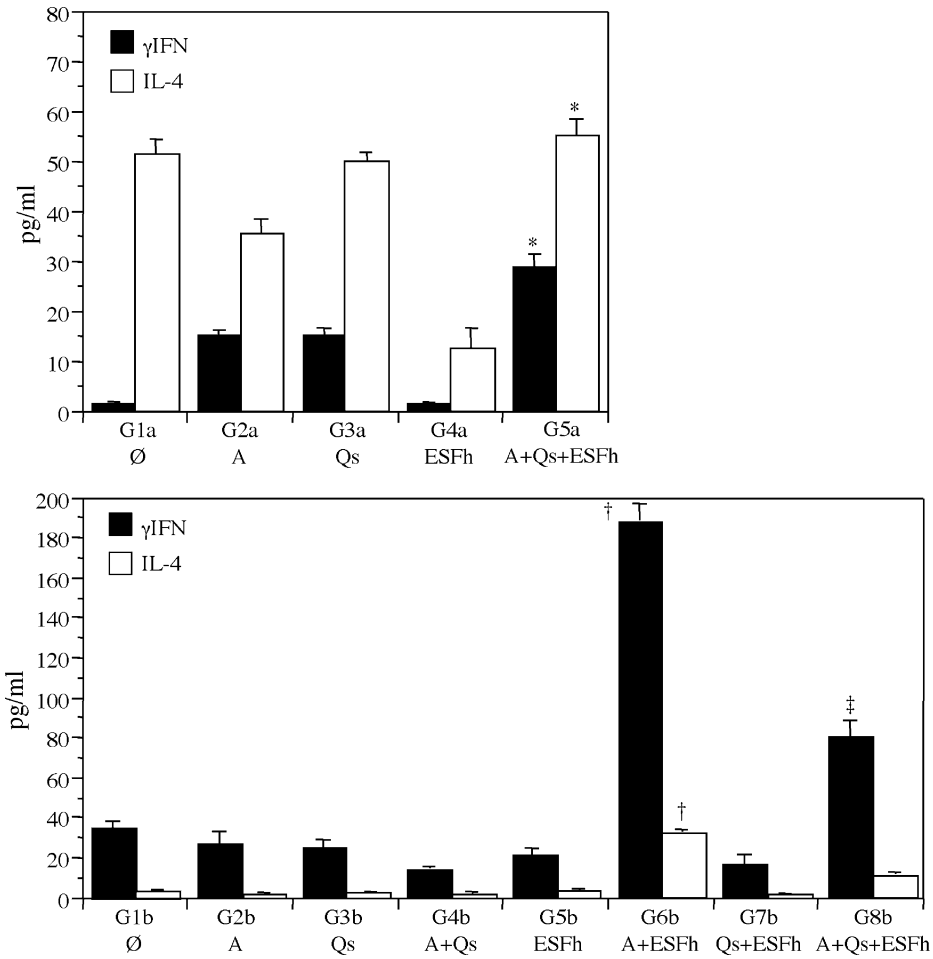

FIGURE 1. γIFN and IL-4 detection in culture supernatants from spleen mononuclear cell stimulated with 2.5 μg/ml of Con A: G1a, control (Ø); G2a, daily i.p. injection of Anapsos® (A) from day 0 to 10 of the experiment; G3a, s.c. injection of *Quillaja saponaria* extract (Qs) at days 5 and 10 of the experiment; G4a, s.c. immunized with ESFh at days 5 and 10; G5a, daily i.p. injection of A and s.c. immunized with Qs and ESFh at days 5 and 10; G1b, control (Ø); G2b, daily i.p. injection of Anapsos® (A) from day 0 to 10 of the experiment; G3b, s.c. injection of *Quillaja saponaria* extract (Qs) at days 5 and 10 of the experiment; G4b, daily i.p. injection of A from day 0 to 10 and s.c. injected with Qs at days 5 and 10; G5b, s.c. immunized with ESFh at days 5 and 10; G6b, daily i.p. injection of A and s.c. immunized with ESFh at days 5 and 10; G7b, s.c. immunized with Qs and 50 μg/dose of ESFh at days 5 and 10; G8b, daily i.p. injection of A and s.c. immunized with Qs and ESFh at days 5 and 10. (*$P < 0.05$ compared with G4a; †$P < 0.05$ compared with G5b; ‡$P < 0.05$ compared with G7b.)

mouse Th1/Th2 Cytokine Cytometric Bead Array (BD Biosciences, San Diego, California). Beads were coated with capture antibodies specific for γIFN and IL–4 cytokines samples, and standards were added to form sandwich complexes that were resolved in the FL3 channel of a BD FACScalibur flow cytometer. The concentration of cytokines was calculated from standard curves using known concentrations of mouse recombinant γIFN and IL-4.

For nitrite determination, alveolar macrophages were obtained from 250–300 g male Wistar rats (IFFA, Credo, Spain) by bronchoalveolar lavage as previously described (Espinoza et al., 2002). Cell viability, determined by the trypan blue vital exclusion stain, was higher than 90% in all cases. Adherent alveolar macrophages (10^6/well) were incubated in triplicate alone (negative control), with 10 μg/ml lipopolysaccharide (LPS; Sigma) positive control or with increasing concentrations of ESFh antigen (10, 30, and 50 μg/ml) or A (100, 500, and 1,000 μg/ml). To evaluate inhibitory effects, cells were preincubated with 10 μg/ml LPS for 1 hr at 37 C and then incubated with 10, 30, and 50 μg/ml of ESFh and 100, 500, and 1,000 μg/ml of A. Every condition was tested in 6 different cultures. After 18 hr at 37 C in 5% CO_2, the supernatant was collected and centrifuged at 500 *g* for 10 min and stored at −80 C until analysis. After removing the supernatant for nitrite determination, the cell viability was assessed by the MMT test described by Kiemer et al. (1997). Nitric oxide release was measured in triplicate indirectly using a quantitative, colorimetric assay based on the Griess reaction (Ding et al., 1988). The results were expressed in nmol nitrite per 10^6 macrophages. Results were expressed as mean and standard error of the mean (SEM). Differences among groups were determined by an ANOVA test, and Fishers Protected Least Significant Difference (PLSD) test was used when appropriate. A value of $P < 0.05$ was considered to be statistically significant.

Antibodies against ESFh detected by ELISA in BALB/c mice immunized with ESFh (G4a and G5b) and Qs+ESFh (G7b) showed high levels of IgG and IgG1 ($P < 0.05$) compared with control groups (G1a and G1b). Those immunized with A+ESFh (G6b) and A+Qs+ESFh (G5a and G8b) showed high levels of IgG ($P < 0.05$), but less levels of IgG1 in comparison with control groups (G1a and G1b). IgG2a-specific antibodies against ESFh were detected at low levels in all groups, and there were no differences between groups (Table I).

The release of γIFN by Con A–stimulated spleen mononuclear cells in the first and the second experiments was higher in mice immunized with A+ESFh (186.7 pg/ml) (G6b), A+Qs+ESFh (79.3 pg/ml) (G8b), and 28.5±2.5 pg/ml (G5a) in comparison with other groups (1.0±0.1–33.9 pg/ml). The release of IL-4 was high in mice immunized with A+ESFh (31.3 pg/ml) (G6b), A+Qs+ESFh (10.1 pg/ml) (G8b), and 54.5±3.5 pg/ml (G5a) compared with other groups (0.3–51.1±3.0 pg/ml) (Fig. 1).

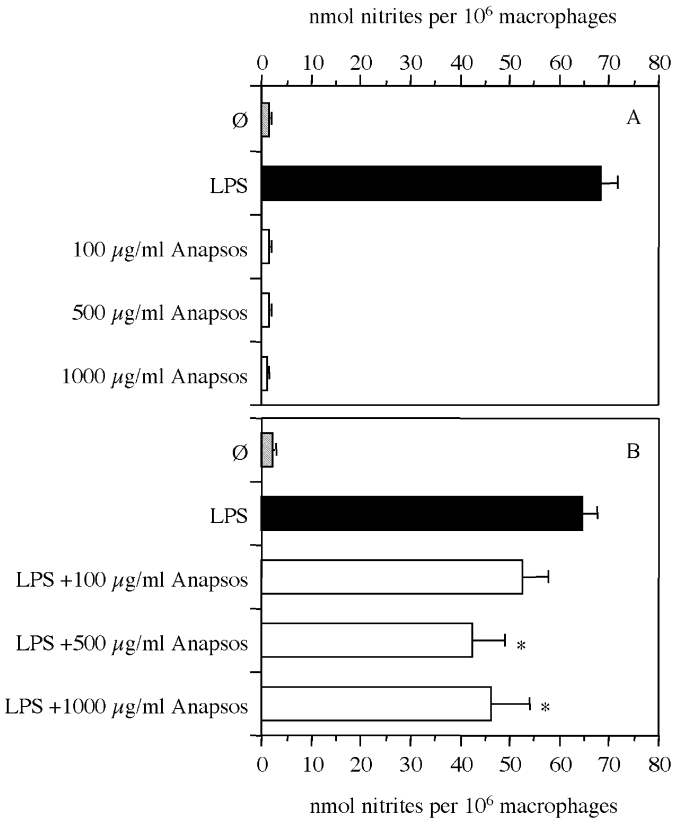

FIGURE 2. Effects of increasing concentrations of Anapsos® (**A**) on the nitrite production by alveolar macrophages and (**B**) LPS-prestimulated macrophages. $*P < 0.05$ compared with LPS treatment.

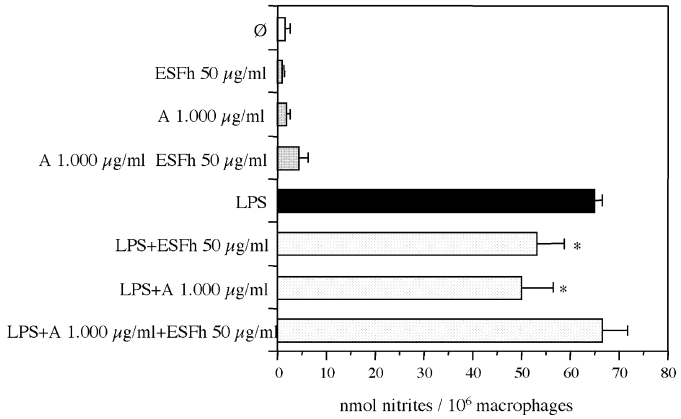

FIGURE 3. Effects of Anapsos® and ESFh antigens on the nitrite production by rat alveolar macrophages and LPS-prestimulated macrophages. $*P < 0.05$ compared with LPS treatment.

Rat alveolar macrophages did not release nitrites when stimulated with increasing concentrations of Anapsos®, but a significant inhibition of nitrite release was observed in LPS-prestimulated alveolar macrophages incubated with 500 μg/ml and 1,000 μg/ml of Anapsos® ($P < 0.05$) (Fig. 2). Similar results were found when we used the ESFh antigens to stimulate alveolar macrophages. Thus, ESFh did not cause naive alveolar macrophages to release nitrites, but it did cause inhibition of nitrate release by LPS-prestimulated macrophages at a dose of 50 μg/ml ($P < 0.05$) (Fig. 3). Moreover, we used 1,000 μg/ml of Anapsos® and 50 μg/ml of ESFh separately and together with normal and prestimulated alveolar macrophages. We did not find nitrite production in normal alveolar macrophage cultures when stimulated with Anapsos®, ESFh, or both substances together (Fig. 3). When we used LPS-prestimulated alveolar macrophages, we observed a reduction in nitrite production in cultures stimulated with Anapsos® ($P < 0.05$) as well as those with ESFh ($P < 0.05$) in comparison to the LPS control, but their effect was abrogated when used in combination (Fig. 3).

Previous studies of the immunological response against *F. hepatica* based on the detection of immunoglobulins and the production of cytokines indicate that a Th2-type response is produced. Moreover, vaccination trials against this parasite have shown the relationship between resistance to infection and Th1-type response (O'Neill et al., 2000; Mulcahy and Dalton, 2001). Thus, an effective vaccination against fascioliasis could be potentially achieved driving response avoiding Th2 response. In this study BALB/c mice immunized with ESFh (G4a and G5b) and Qs+ESFh (G7b) presented high levels of IgG and IgG1 and low levels of IgG2a antibodies. Moreover, IL-4 and γIFN were detected with low levels in supernatant from culture of spleen mononuclear cells in these groups. Thus, our data showed that ESFh is able to modulate the immune response of naive mice to a Th2-like response, as other authors have reported in immunizations with ESFh (O'Neill et al., 2000). In groups immunized with A+ESFh (G6b) or A+Qs+ESFh (G5a and G8b), we found important differences, i.e., the IgG1 response was much lower than in mice treated with ESFh (G4a and G5b) and Qs+ESFh (G7b) comparable to control groups, and higher levels of γIFN in supernatants from spleen cell cultures were detected in comparison with mice treated with ESFh (G4a and G5b) and Qs+ESFh (G7b). These data indicate that Anapsos® modulates the immune response induced by ESFh in mice similar to its effect in other host-parasite models (Cuellar et al., 1997; Dea-Ayuela et al., 1999; Nogal-Ruiz et al., 2003). We observed that ESFh antigen inhibits the nitric oxide production by rat LPS-prestimulated alveolar macrophages. This agrees with previous studies with ESFh (Cervi et al., 1999). In our study Anapsos® showed an inhibition of the nitric oxide production by rat alveolar macrophages prestimulated with LPS. In addition, the application of ESFh and Anapsos® combined on naive macrophages does not stimulate the production of nitrites. Nevertheless, when we assayed them with LPS-prestimulated macrophages we could not find any effect. Thus, Anapsos® is able to affect the immunological properties of the ESFh in vitro. In summary, we have shown that Anapsos® modulates the immune response against ESFh in naive mice and nitrite production in prestimulated rat alveolar macrophages. These immunomodulatory features could be interesting in the formulation of new vaccines against *F. hepatica*.

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Experimental Infection of *Dirofilaria immitis* in Raccoon Dogs

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ABSTRACT: Canine heartworm (*Dirofilaria immitis*) is a nematode that naturally parasitizes in the pulmonary arteries and the right ventricle of domestic dogs (*Canis familiaris*) as final hosts. Japanese raccoon dogs (*Nyctereutes procyonoides viverrinus*) also are known to be susceptible to infection by the parasite. However, prevalence of this infection among free-ranging raccoon dogs is low and so is the worm burden. To examine the susceptibility of the raccoon dog to *D. immitis* infection, 3 raccoon dogs and 2 beagles were inoculated 4 times with 25 third-stage larvae (L3s) of *D. immitis* at 3-wk intervals. Worms were recovered from 2 raccoon dogs and both domestic dogs. The average percentage of recovery (2.3%) of the raccoon dogs was almost 10 times lower (24.5%) than that of the domestic dogs, but there was no significant difference in the body length of worms recovered from 2 types of hosts. To examine microfilaremia, 2 raccoon dogs were infected with 100 L3s. Microfilaremia was observed for 180 days postinoculation (PI) but disappeared at about 300 days PI. The raccoon dog was mildly susceptible to infection with *D. immitis*, but surviving worms developed and matured normally.

Canine heartworm (*Dirofilaria immitis*) is a parasitic nematode harbored by domestic dogs (*Canis familiaris*) as definitive hosts in Japan, but the parasite also has been found in many animal species, including humans (Rodrigues-Silva et al., 1995). Canids are normal hosts to the parasite (Pappas and Lunzman, 1985; Fox et al., 1986; Starr and Mulley, 1988; Gortazar et al., 1994; Sacks, 1998), and the infection in other species seems to be aberrant. Infected domestic dogs are still considered reservoirs for canine heartworm infection in Japan. However, in 1938, the Japanese raccoon dog (*Nyctereutes procyonoides viverrinus*) also was reportedly infected with canine heartworm in Japan (Itagaki and Kume, 1938). The raccoon dog is a basal species with respect to the *Vulpes*/*Canis*/*Dusicyon* clade that includes domestic dogs, foxes, wolves, and coyotes (Wayne et al., 1997). The habitat of raccoon dogs has been affected by land development in Japan, resulting in a decline in their number. Nevertheless, owing to their omnivorous behavior, the animals have managed to adapt themselves to the residential areas of humans. Thus, with raccoon dogs and humans living closer together, the risk of infection with the parasite is actually greater than previously. Our previous survey indicated the low prevalence of infection and the light worm burden of canine heartworm among free-ranging raccoon dogs and discussed possible explanations (Nakagaki et al., 2000). To follow up on this effort, the next step was to determine experimentally the susceptibility of raccoon dogs to *D. immitis*. For the susceptibility assessment, we experimentally infected raccoon dogs and domestic dogs with third-stage larvae (L3s) of *D. immitis*, and we compared the susceptibility and microfilaria production data of the 2 experimental hosts.

Five raccoon dogs of both sexes, aged 6–12 mo and 2 female domestic dogs (12 mo old) were studied. To examine their susceptibility to *D. immitis*, the dogs were infected subcutaneously 4 times with 25 infective L3s over a period of 3 wk. These animals were killed by overdose of 100 mg/kg pentobarbital sodium and necropsied 116 days after the last inoculation. To examine the microfilaria production in raccoon dogs, 2 raccoon dogs were infected once with 100 L3s.

L3 were harvested by Boehrman's method from *Aedes togoi* that fed

TABLE I. Comparison of susceptibility to canine heartworm between raccoon dogs and domestic dogs.

Species	No. of animals (infected/total)	No. of recovered worms	Means of % recovery
Raccoon dogs	2/3	2, 5, 0	2.3
Domestic dogs	2/2	22, 29	25.5

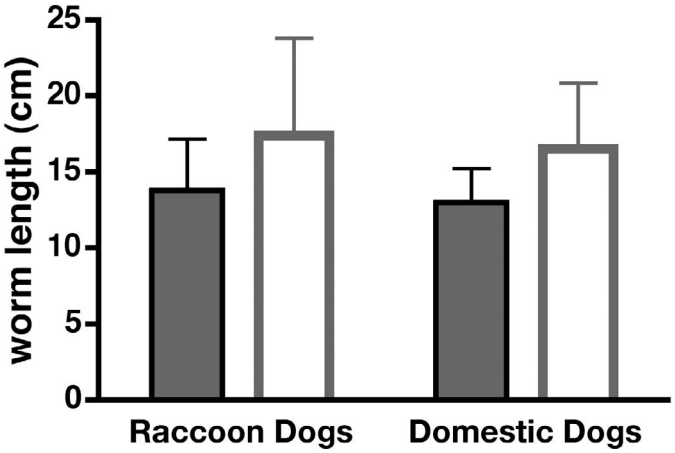

FIGURE 1. Body lengths of worms recovered from raccoon dogs and domestic dogs at 116 days postinfection with the third stage larvae. Solid and open bars are the body lengths of male and female worms, respectively. There were no significant differences in worm body length between raccoon dogs and domestic dogs of matched gender by Wilcoxon rank sum test.

on the blood from a highly microfilaremic dog at 10–14 days after the blood meal. L3s were isolated under a dissection microscope and washed several times in Hank's balanced salt solution containing penicillin and streptomycin. Subsequently, the animals were subcutaneously injected with L3s in 1 ml of sterile saline.

Microfilariae (mff) in peripheral blood were counted using the acetone microfilaria (mf) concentration method (Ohishi et al., 1959). Briefly, 2 ml of blood was added to 10 ml of acetone mf concentration solution in a 15-ml conical tube, which was then shaken vigorously for 1 min. The tube was subsequently left standing for at least 30 min at room temperature or in a refrigerator overnight. The mff were spun down at 2,000 rpm for 10 min and washed 3 times with distilled water. Finally, methylene blue-stained mff in packed sediments were counted in a hemocytometer under a light microscope.

Worms were recovered from 2 of 3 experimentally infected raccoon dogs; both domestic dogs had worms in the pulmonary arteries and the heart. The worm burden averages for the raccoon dogs and the domestic dogs were 2.3 and 25.5%, respectively, at 116 days after the last inoculation (Table I). There were no significant differences in body length of sex-matched worms recovered in the raccoon and the domestic dogs by Wilcoxon rank sum test (Fig. 1).

Microfilariae in the peripheral blood of 2 infected raccoon dogs were first detected at 201 and 188 days postinoculation (PI). The mf number increased and reached a peak at 220 to 250 days PI, but the raccoon dogs showed amicrofilaremic conditions at 348 and 282 days PI (Fig. 2). On the basis of seasonal periodicity, the peripheral blood was

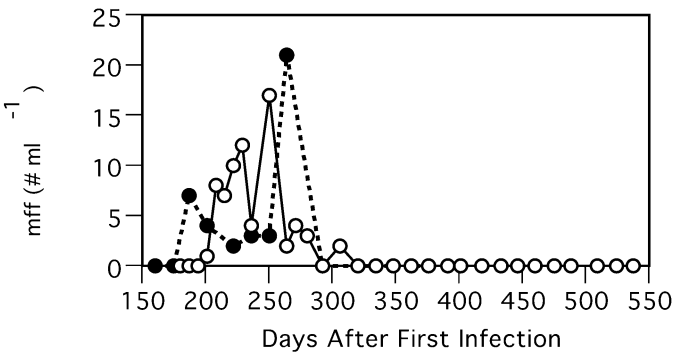

FIGURE 2. Microfilaremia in 2 experimentally infected raccoon dogs. The raccoon dogs were infected with 100 L3s.

TABLE II. Worm burden in the raccoon dogs experimentally infected with 100 L3 at 565 and 590 days PI.

Raccoon dog	No. of recovered worms		
	Male	Female*	Total
4	2	1	3
5	3	3	6

* Developed embryos and mff were observed in the uterus of worms.

checked until the end of the second wet season, but it was not examined after the blood had turned amicrofilaremic. Adult worms were recovered at 565 and 590 days after infection in 2 infected raccoon dogs (Table II); microfilariae were present in the uterus of the females.

Although the domestic dog is a definitive host for *D. immitis*, various other species have been found to harbor it. For example, California sea lions (*Zalophus californianus*) and ferrets (*Mustela putorius furo*) seem to be highly susceptible to the parasite (Forrester et al., 1973; Supakorndej et al., 1994). Our results seem to concur with the reported data on domestic cats (*Felis domestica*) (Ohishi et al., 1973; Nogami and Sato, 1997; Roncalli et al., 1998). The low prevalence of infection in domestic cats is obviously caused by the animal's low susceptibility to *D. immitis*. The domestic cat is located at a phylogenetically far distant position from the domestic dog, and the lower susceptibility may thus be dependent upon the specificity of the host species. Indeed, experimental infections with L3s of *D. immitis* have shown that a lesser number of worms were recovered in the heart and pulmonary arteries of the cat compared with the domestic dog (Kume, 1970; Donahoe, 1975). The low prevalence of infection and the light worm burden in the free-ranging raccoon dogs also might be induced by the animal's low susceptibility. The raccoon dog is phylogenetically closer to the domestic dog than to the domestic cat, but there is still significant distance between the domestic dog and the raccoon dog (Baranov et al., 1976; Wayne et al., 1997). Moreover, no report has so far confirmed the strict correlation between phylogenetic distance and susceptibility to canine heartworm infection. Our results indicate that the migrating worms into the pulmonary arteries and the heart were able to mature normally and to release microfilariae into the peripheral blood but that microfilaremia was transient. Ohishi (1997) observed that the immature fifth stage worms, which were recovered from infected dogs, remigrated equally into the pulmonary arteries and the heart of both dogs and cats, and they concluded that the larvae also developed normally and matured in cats after passing some barrier in the host defense of the parasite infection. The female worms recovered from the raccoon dogs that resulted in amicrofilaremic infection still produced microfilariae in the uterus at 565 and 590 days PI. A similar clearance of microfilariae has been observed in domestic cats infected experimentally (McCall et al., 1992). This information suggests that there is the possibility that the microfilariae in the peripheral blood of raccoon dogs are a source of transmission of *D. immitis*. However, the raccoon dog does not seem to be a reservoir for the parasite in nature, given that we are dealing mostly with single-sex infections in naturally infected raccoon dogs (Nakagaki et al., 2000), and also considering the recovery of worms in the pulmonary arteries and the heart and the low density of microfilaremia in this study.

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A Rapid Method for Producing Highly Purified *Cryptosporidium parvum* Oocysts

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ABSTRACT: Most procedures that have been described for purifying *Cryptosporidium parvum* oocysts are designed to either identify the parasites in clinical specimens or isolate oocysts from a small volume of feces from infected animals. The present study describes a rapid method for purifying high numbers of *C. parvum* oocysts from feces of infected calves that contains minimal contaminating fecal material and bacteria. The isolation method is based on differential flotation of *C. parvum* oocysts in NaCl, followed by ether extraction to solubilize lipids in calf feces. This procedure regularly yields $>10^9$ purified *C. parvum* oocysts within 1–2 days of feces collection.

Studies on the molecular biology of *Cryptosporidium* spp. have been considerably advanced by the complete sequencing of the *Cryptosporidium parvum* and *Cryptosporidium hominis* genomes (Abrahamsen et al., 2004; Xu et al., 2004). The confirmation of gene expression in *Cryptosporidium* spp. sporozoities requires large numbers of highly purified oocysts that are devoid of contamination by microbes normally found in fecal material. A number of procedures have been described for concentrating and isolating *C. parvum* oocysts from feces. These include sucrose flotation (McNabb et al., 1985; Kuczynska and Shelton, 1999; Massanet-Nicolau, 2003; Truong and Ferrari, 2006), formalin-ethyl acetate sedimentation (McNabb et al., 1985; Weber et al., 1992; Kuczynska and Shelton, 1999), Percoll (Waldman et al., 1986; Kilani and Sekla, 1987; Kuczynska and Shelton, 1999), or Ficoll (Truong and Ferrari, 2006) gradient centrifugation, discontinuous sucrose gradient centrifugation in combination with Percoll gradient isolation (Arrowood and Sterling, 1987; Truong and Ferrari, 2006), and cesium chloride gradient centrifugation (Kilani and Sekla, 1987; Kuczynska and Shelton, 1999; Truong and Ferrari, 2006). In general, most procedures are designed to identify *C. parvum* oocysts in clinical specimens (<10 ml volume) (Garcia et al., 1983; McNabb et al., 1985; Heyman et al., 1986; Weber et al., 1992; Bukhari and Smith, 1995; Clavel et al., 1996). A few reports exist on successful isolation of *C. parvum* from diarrheic calf feces, but these involve processing multiple small volume samples (Waldman et al., 1986; Arrowood and Sterling, 1987; Kilani and Sekla, 1987; Kuczynska and Shelton, 1999; Massanet-Nicolau, 2003; Truong and Ferrari, 2006). In the present study, a technique for rapidly isolating nearly 100% pure *C. parvum* oocysts from large volumes of diarrheic calf feces is described, which does not require discontinuous sucrose or CsCl gradient centrifugation.

Cryptosporidium parvum (Iowa strain) oocysts were obtained by infecting 1-day-old calves with 10^6 oocysts. The calves were obtained at birth from the Beltsville Agricultural Research Center dairy and were handled according to animal care guidelines as approved by the BARC Animal Care Committee. The calves were housed in a 2.4 m × 1.2 m metabolic crate from days 3–10 postinfection for fecal collection; feces were analyzed daily for the presence of *C. parvum* oocysts using a commercial oocyst detection kit (Merifluor, Meridian Diagnostics, Cincinnati, Ohio). Fecal material from peak shedding days (generally days 5–7) were pooled and filtered through a stainless steel mesh strainer (0.5 mm) followed by passage through a 150-μm, followed by a 45-μm, stainless steel mesh sieves. The sieved fecal suspensions were centrifuged at 720 *g* for 15 min in 1-L polypropylene bottles in an IEC model K centrifuge with a swinging bucket rotor. After centrifugation, 750 ml of the supernatant was discarded, and the remaining supernatant-sediment was mixed, diluted 1:4 with NaCl (360 g/L, specific gravity, 1.21) in a 1-L polypropylene bottle, and centrifuged at 720 *g* for 20 min. After centrifugation, the bottles were placed on the bench top for 10 min, and then 200–300 ml of supernatant from each bottle was collected into a single container, diluted 1:4 with cold water, and centrifuged for 20 min at 720 *g*. The supernatants were discarded and pellets were washed twice with H_2O and centrifugation for 20 min at 720 *g*. The pellets were combined in a final volume of 240 ml cold H_2O, and 40-ml aliquots were distributed into 50-ml conical centrifuge

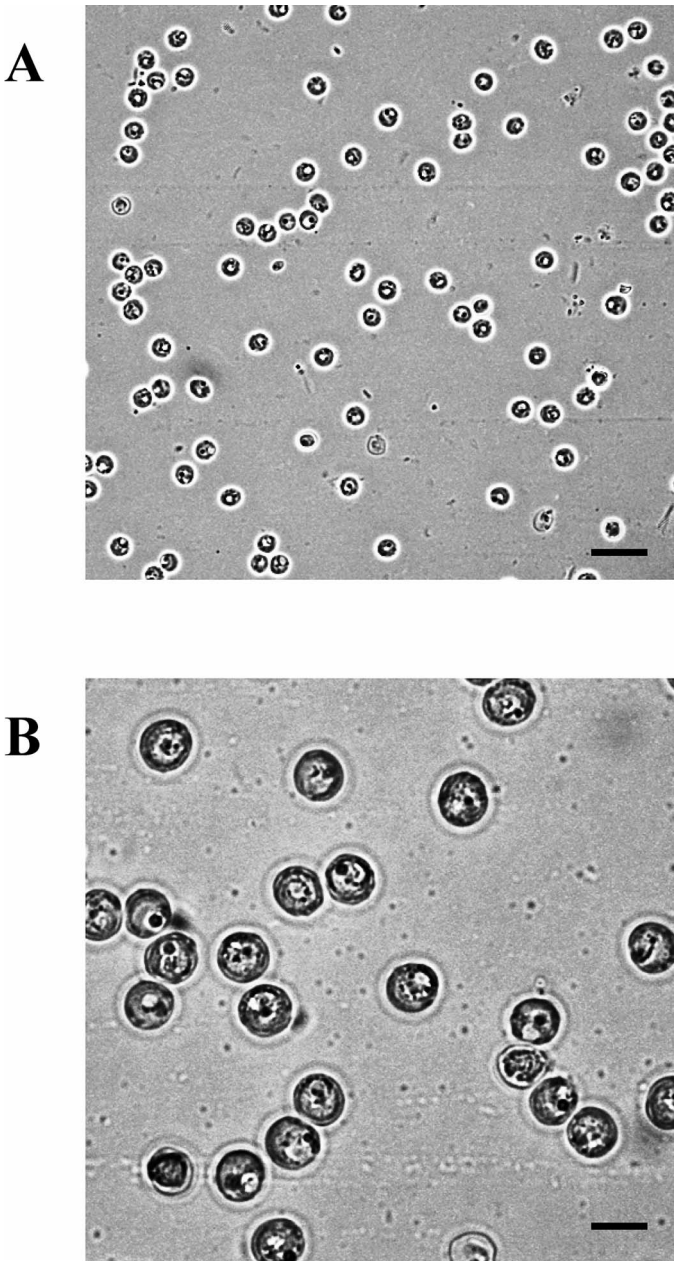

FIGURE 1. Light microscopic image of *Cryptosporidium parvum* oocysts purified from calf feces using rapid purification method. (**A**) ×400 magnification. Bar = 15 μm; (**B**) ×1,000 magnification. Bar = 5 μm.

tubes and then overlaid with 8-ml ether. The slurry was mixed for 20 sec to solubilize lipids and then centrifuged for 10 min at 1,200 *g* in a tabletop centrifuge with a swinging bucket rotor. After centrifugation, 3 layers overlying an oocyst pellet were observed, i.e., a top solvent layer, an interface layer consisting of fecal debris, and a lower aqueous layer. The 3 layers were removed using a 25-ml pipette, and the oocyst pellet was suspended in 50-ml deionized H_2O. The oocyst pellet was washed 3 times with deionized H_2O and centrifugation at 1,200 *g* for 10 min, followed by suspension in 10-ml deionized H_2O. To assess viability, the *C. parvum* oocysts were excysted using a standard technique (Gut and Nelson, 1999), which was modified by inclusion of a brief 1 min 1% sodium hypochlorite treatment, followed by repeated washing and centrifugation of the oocysts in water prior to excystation. The level of bacterial contamination was measured by spread-plating serial dilutions of the oocyst suspension onto Petri dishes containing LB agar (Sigma Chemical Co., St. Louis, Missouri) or blood agar (BBL, Cockeysville, Maryland) prior to, and after, bleach treatment. Also, the level of contaminating protein in the purified oocyst suspension was measured using a BCA assay test kit (Pierce Chemical Co., Rockford, Illinois) on supernatant after the oocysts were centrifuged for 5 min at 12,000 *g*.

The volume of diarrheic feces from a single calf infected at 1 day of age with 10^6 *C. parvum* oocysts is between 1–2 L per day during peak shedding (days 5–7). Applying the rapid isolation method, greater than 10^9 highly purified *C. parvum* oocysts were recovered from peak shedding days. In 3 propagation studies, an average of $1.9 \times 10^9 \pm 0.7 \times 10^9$ oocysts was obtained. The oocyst preparation had negligible protein contamination (data not shown). Prior to bleach treatment, 2.5×10^4 total bacteria/ml were present in the preparation, which is equivalent to 0.01% of the total number of *C. parvum* oocysts produced (Fig. 1). After bleach treatment, no bacterial colonies were observed. In addition, an excystation rate greater than 90% was observed using the modified rapid excystation method. The advantage of this technique is that a single NaCl flotation step, followed by ether extraction, can recover a large number of *C. parvum* oocysts with minimal contamination by fecal microorganisms. Other techniques that have been described require multistep procedures and discontinuous gradients of sucrose, Percoll, or CsCl to purify oocysts from small volume samples (Waldman et al. 1986; Arrowood and Sterling, 1987; Kilani and Sekla, 1987; Kuczynska and Shelton, 1999; Truong and Ferrari, 2006). The present technique was developed to recover high numbers of *C. parvum* oocysts from a large volume of fecal material without the cost of using reagents such as CsCl or Percoll. In our experience, employing sucrose gradients is not only time-consuming, but also produces fairly impure oocyst preparations, possibly stemming from the higher viscosity of sucrose. The high level of oocyst purity is due to the dual effect of NaCl and ether, i.e., the former dispersing particulate matter and preventing oocyst entrapment (Kuczynska and Shelton, 1999) and the latter solubilizing lipids commonly present in fecal material. *Cryptosporidium parvum* oocysts purified using the procedure described in the present study are stable for at least 3 mo when stored at refrigeration temperature, and can be used for excystation, cell culture, protein analysis, or infecting susceptible animals.

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Infection Dynamics of *Centrocestus armatus* Cercariae (Digenea: Heterophyidae) to Second Intermediate Fish Hosts

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ABSTRACT: The infection dynamics of *Centrocestus armatus* cercariae with respect to second intermediate fish hosts were investigated using cercariae collected from naturally infected *Semisulcospira libertina*. Cercariae survival and infectivity were recorded at 2-hr intervals. Survival remained constant but decreased abruptly at 30 hr of age. An age-dependent model presented the best-fit curve for the survival data (r^2 = 0.936), implying that cercariae tended to allocate resources equally among themselves and then died once those resources were depleted. Cercariae infectivity remained constant over the first 10 hr of life and then declined; an age-dependent model also provided a better fit (r^2 = 0.956). The transmission rate of *C. armatus* cercariae to the fish host was relatively low near the time of emergence from the snail host but peaked at 85% after 1 hr and then maintained a plateau period until 5 hr of exposure to fish. The pattern of transmission was also investigated initially at varying densities of cercariae and then by maintaining a constant cercariae density but varying the total number. Results revealed that the pattern of transmission was frequency-dependent.

Fish are important final or intermediate hosts in digenean life cycles. Many species of trematodes enter the fish hosts by active invasion of their cercariae and the behavior of these stages is crucial in determining the dynamics of parasite infection. In digenean parasites with complex life cycles, an understanding of infection dynamics involves the production and transmission of parasites through a series of intermediate hosts. To understand these processes, it is necessary to estimate both the survival of free-living cercariae stages and the rate and pattern of transmission. This study focused on the heterophyid digenean, *Centrocestus armatus* and the transmission process between the 2 intermediate hosts, the freshwater snail (*Semisulcospira libertina*) and the minnow (*Zacco temmincki*). Despite the importance of previous epidemiological studies on this particular system (Kimura and Uga, 2003, 2005), no data exist on infectivity and transmission of its cercariae between the snail and fish hosts. Detailed information is available on the infectivity and transmission of *Schistosoma mansoni* (Lawson and Wilson, 1983; Whitfield et al., 2003) and *Diplostomum spathaceum* (Whyte et al., 1991; Karvonen et al., 2003), but that is only of indirect value in predicting transmission patterns of fish infection, particularly in Asian systems.

Patterns of survival and infectivity were examined in relation to the age of the free-living stages. Because cercariae have limited energy reserves (Anderson and Whitfield, 1975; Lawson and Wilson, 1980), there must be a trade-off between resource depletion and rate of host encounter, which may shape the optimal infection strategy of this particular system. The parasites could use one of the several alternative strategies. First, the parasite could allocate resources at random between the infective stages, so they live for variable periods of time; in which case, mortality of cercariae will be constant, and numbers and infectivity will decline exponentially. Second, the cercariae could carry similar quantities of resources; in this case, mortality will be highly age-dependent, and number and infectivity will remain high and then dramatically drop at a specific age. Thus, one aim of this study was to investigate survival and infectivity in relation to age and examine the particular strategy employed by *C. armatus*.

Another critical factor influencing infection dynamics is the transmission of the parasite to the next host. In this particular host–parasite system, it is assumed that fish hosts move randomly with respect to the infective stages and can be modeled in terms of density or pesudo–mass action (McCallum et al., 2001). An alternative view is that hosts do not move randomly but remain within a set home range such that infection rate is dependent, not on density alone, but on the frequency that the host encounters infective stages within their home range; this could be modeled as frequency-dependent or true mass action (Karvonen et al., 2003). Thus, the pattern of transmission can be evaluated by examining the relationship between transmission rate and cercariae density.

We tested these hypotheses by performing replicated infection trials under laboratory conditions. Cercariae were obtained by exposing naturally infected snails to bright light conditions at 24 C for 3 hr. Suspensions of cercariae from different snails were then pooled. To examine cercariae survival, a suspension was divided into 6 containers, each containing 500 cercariae. Division was done by directly counting cercariae using a stereomicroscope and then pipetting into 60-mm petri dishes. Containers were maintained on a 12:12-hr light/dark photoperiod at room temperature (20 C). Survival of cercariae was determined by counting the number alive in individual containers every 2 hr. The procedure for cercariae infectivity was done by exposing individual, uninfected *Z. temmincki* (mean fork length = 8.85 ± 1.39 cm; mean weight = 4.50 ± 1.87 g) to 200 cercariae in a glass container with 400 ml of dechlorinated tap water of 5-cm depth at room temperature. The length of exposure to cercariae was 1 hr. The procedure was then repeated every 2 hr using a new fish until infectivity reached zero. The

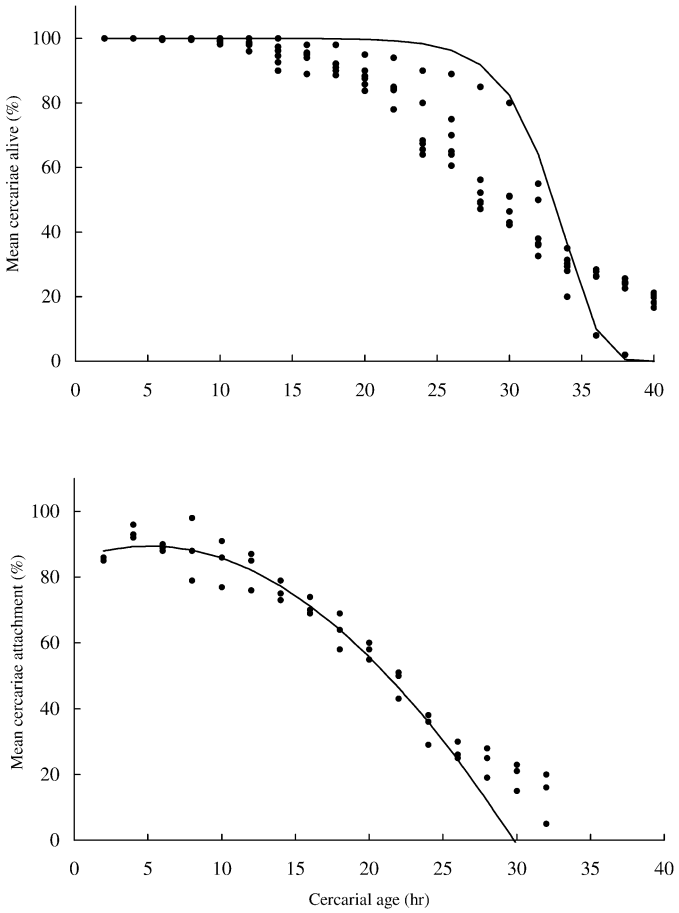

FIGURE 1. Age-dependent survival and infectivity of *Centrocestus armatus* cercariae at 20 C. Cercariae survival (**A**). Data points represent raw survival rate values, whereas the best-fit curve was modeled according to an exponential equation of the form $P_t = \exp(a/b[1 - \exp[bt]])$, where $a = 3.4 \times 10^{-7}$, $b = 0.42296$, and P_t is the proportion of cercariae alive at time t. Cercariae infectivity (**B**). Data points represent raw infectivity-rate values, whereas the fitted curve was modeled according to nonlinear regression with the use of a polynomial equation $I_t = at^3 + bt^2 + 1.720t + 85.19$, where $a = 0.0006$, $b = -0.1715$, and I_t is the cercarial infectivity rate at time t.

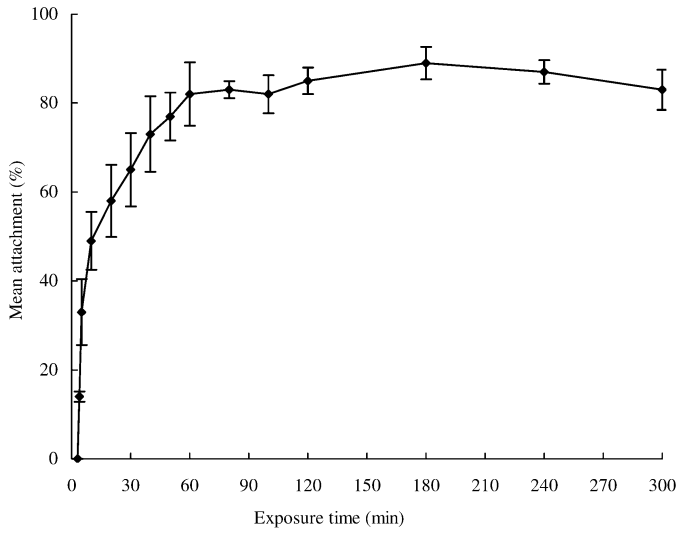

FIGURE 2. Mean transmission rate of *Centrocestus armatus* to *Zacco temmincki* exposed to 200 cercariae under standardized conditions. n = 5 fish/interval. Bars = SD.

percentage of infectivity was determined by direct counting of unattached cercariae using a stereomicroscope after 1 hr of exposure.

Transmission rate was assessed by exposing individual juvenile *Z. temmincki* of similar size to 200 cercariae at the same time. In this case, the transmission of the parasite to the fish host was determined at different time intervals over a 5-hr period. Infection procedure was the same as above. Transmission was determined by directly counting the number of unattached cercariae per given time interval. The relationship between transmission and cercariae density was also assessed by replicated infection trials at varying densities and at constant density but manipulating the total number of cercariae. Densities in the glass container of 400 ml dechlorinated water included 50, 100, 200, 350, and 500 cercariae. The infection time was 1 hr; after which, the fish were removed, and the number of unattached cercariae were counted using a stereomicroscope. To determine whether transmission differed with the total number of cercariae, water volume in the tank was manipulated so that total cercariae numbers varied but the density remained constant at 500 cercariae/L of water. Individual fish were introduced, and the same infection procedure used.

In general, survival of *C. armatus* cercariae remained relatively constant at 98% until 10 hr after emergence and gradually decreased thereafter. A sharp decline in survival was observed from 30 hr of age, when the number immobile and dead rapidly increased. This pattern did not vary greatly between containers (GLM, $F = 1.245$, $P > 0.05$). The number of cercariae decreased to almost zero at 38 hr of age, based from the age-dependent model that described the data. (Fig. 1A, $r^2 = 0.936$). Cercariae infectivity was also relatively constant at 87% for the first 10 hr but dropped to 50% by 22 hr and to values approaching 0% at 30 hr. Although an exponential model fitted the infectivity data ($r^2 = 0.855$), an age-dependent model provided a better fit (Fig. 1B, $r^2 = 0.956$).

Figure 2 shows that the transmission rate rose from an initial value of 14% (mean percentage of attached cercariae) at 4 min after exposure and progressively increased thereafter, attaining a maximum of 85% at 1 hr. That value was then maintained for a long plateau period of 5 hr. A linear regression model was used to evaluate the effect of density and number of cercariae on the transmission pattern of *C. armatus*. The total number of cercariae attached to fish increased in both cases. However, transmission did not vary with density of cercariae but did vary significantly with the number of cercariae in the tank (Fig. 3).

Overall, the results have implications for the mode of infection of *C. armatus* cercariae to second intermediate fish hosts. Significant data from this investigation relate to the age-dependent infection capabilities of *C. armatus* cercariae. The model showed that the time of abrupt decrease in survival rate generally coincided with the time when infectivity was at 0%. The age-dependent dynamics of cercariae survival and infectivity are driven by the rate of the use of their resources because cercariae possess limited energy levels. These resources were not measured directly, but it was assumed they were glycogens, based on the studies of Anderson and Whitfield (1975) and Lawson and Wilson (1980). Previous studies on other digenean parasites found that the mortality of cercariae is age-dependent rather than constant (Karvonen et al., 2003; Whitfield et al., 2003). Nothing in these results invalidates the conclusion of those authors that survival and infection capacity of a live cercaria remain constant throughout its life. However, the age at which mortality increased was shown to vary among parasite species recorded at the same temperature (Karvonen et al., 2003; Whitfield et al., 2003). That could imply that different parasite species employ different traits and may have different responses to temperature.

In addition, quantitative information on the age-dependence of cercariae survival and infectivity has contributed to an understanding of the infection dynamics of *C. armatus* to second intermediate fish hosts in the natural environment. If the pattern of infectivity described in this study reflects what occurs in the field, there is a midday peak in the diurnal emergence rhythm of cercariae, and larval infectivity will be maintained at a maximum level for the remainder of daylight. Because these fish hosts are active diurnally, this temporal patterning of maximal cercariae infectivity could be considered an important component of an ''optimal transmission strategy.''

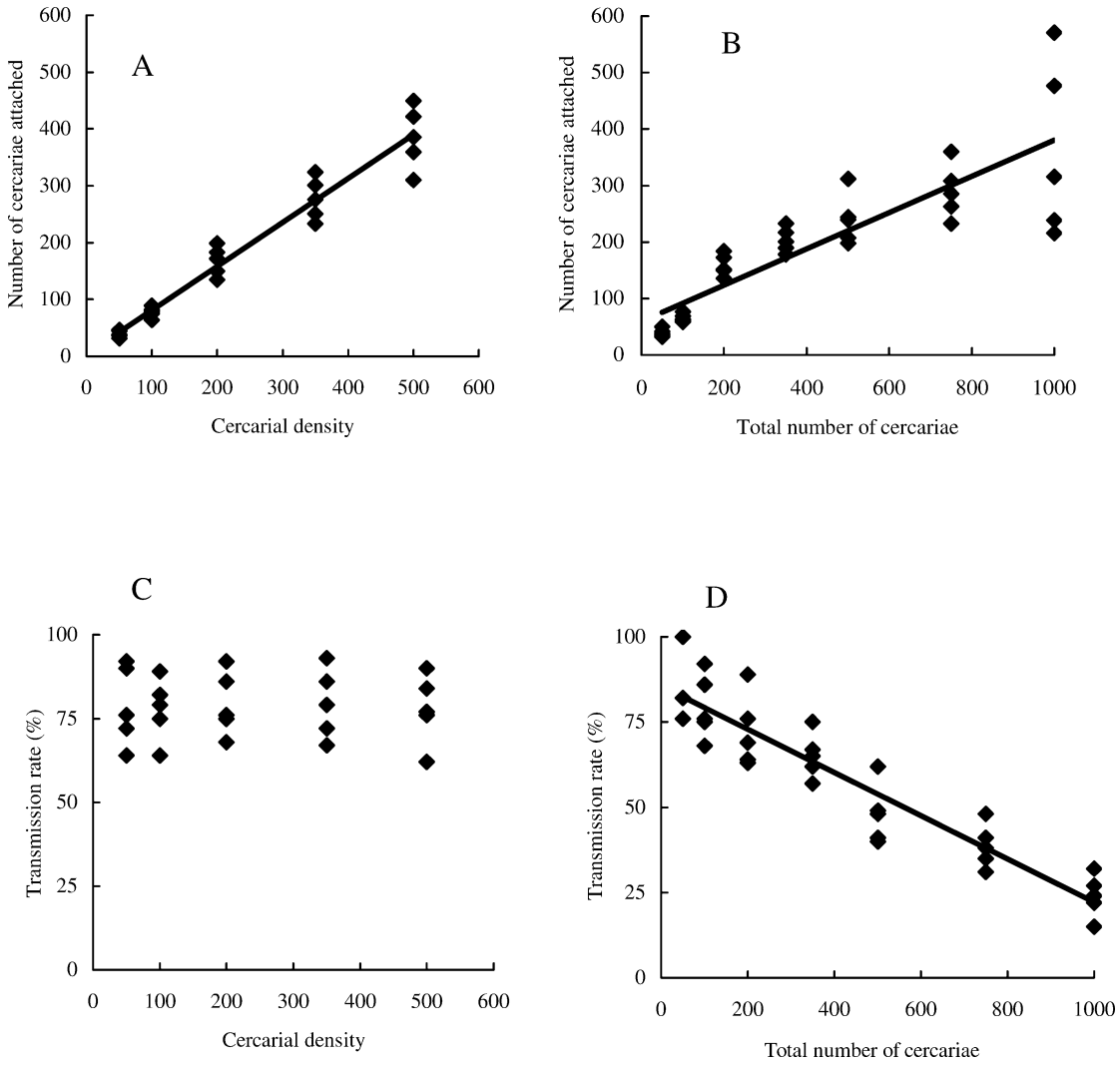

FIGURE 3. Total number of *Centrocestus armatus* cercariae attached per fish at various cercariae densities (**A**). Constant cercariae density but with an increasing number of cercariae and volume of water in the tank (**B**). Transmission rate of the parasite at various cercariae densities (**C**). Transmission rate at constant cercariae density but increasing number of cercariae (**D**). Fitted lines calculated based on the linear regression model.

Results also indicate a relatively slow period of parasite recruitment by the fish host at a time close to cercariae emergence from the intermediate snail host. As glycogen reserves must be at their highest level at that time, those initial, lower levels of infection rate seem somewhat paradoxical. However, cercariae might improve their overall transmission efficiency by having a below-optimal infectivity for a short period, thus facilitating their spatial dispersion. Whitfield et al. (2003) examined the infectivity of *Schistosoma mansoni* cercariae toward differentiated keratinocytes and also found an early period of low infection rate. A delay in attaining maximum infection may signify an adaptive mechanism, allowing time for cercariae dissemination and, thus, reducing super-infection and subsequent parasite-associated mortality of the fish hosts. On reaching maximum recruitment at 1 hr, there was a plateau period of maximum infection (87%) until 5 hr of age; there was no subsequent increase in cercariae transmission to the fish host thereafter.

Finally, data showed that transmission of *C. armatus* cercariae to fish increased with both density and total population size. However, when transmission rate was considered, it remained constant with density, but decreased with total number of cercariae. Based on the abovementioned hypotheses, the transmission pattern of this particular host–parasite system is frequency-dependent. It may also indicate that *C. armatus* cercariae have a passive nature in locating its host. This is an important contribution of this study, particularly as most work, so far, has been done with microparasite systems. In addition to this paper, a study by Karvonen et al. (2003) also examined the transmission pattern of *Diplostomum spathaceum* cercariae to secondary fish host, but at varying scales. They also revealed that the transmission pattern was frequency-dependent. However, fish in the natural environment are exposed in larger volumes of water and the transmission pattern could be density-dependent at a higher scale. Further experiments using a greater variation in scale and host density are necessary to demonstrate this phenomenon.

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Giardia sp. and *Cryptosporidium* sp. Infections in Primates in Fragmented and Undisturbed Forest in Western Uganda

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ABSTRACT: In June 2005, we collected 115 fecal samples from wild primates in western Uganda and examined them for *Cryptosporidium* sp. and *Giardia* sp. with the use of immunofluorescent antibody (IFA) detection. We sampled primates from an undisturbed forest in Kibale National Park and from 3 highly disturbed forest fragments outside the park. Of disturbed forest samples, red colobus (*Pilocolobus tephrosceles*) and red-tailed guenons (*Cercopithecus ascanius*) harbored species of *Cryptosporidium* or *Giardia*, but black-and-white colobus (*Colobus guereza*) did not. All primate samples from undisturbed forest were negative for both parasites. Seven of 35 (20%) red colobus and 1 of 20 red-tailed guenons (5%) from forest fragments were infected with either *Cryptosporidium* sp. or *Giardia* sp. The presence of *Cryptosporidium* and *Giardia* species in primates living in forest fragments, but not in primates in undisturbed forest, suggests that habitat disturbance may play a role in transmission or persistence of these pathogens.

Species of *Cryptosporidium* and *Giardia* have been found in wild apes, and their presence has been argued to indicate enhanced contact with humans and/or domestic livestock (Nizeyi et al., 1999, 2002). Few studies, however, have examined other species of wild nonhuman primates for these pathogens.

We examined *Cryptosporidium* sp. and *Giardia* sp. prevalence and intensity in 3 nonhuman primate species (red colobus, *Pilocolobus tephrosceles*; black-and-white colobus, *Colobus guereza*; and red-tailed guenons, *Cercopithecus ascanius*) living in undisturbed forest in Kibale National Park, Uganda, and from 3 highly disturbed forest fragments outside the park. The fragments range in size from 1.2 to 8.7 ha and occur in areas largely unsuitable for agriculture, i.e., swampy valley bottoms and steep rims of lake craters (Gillespie and Chapman, 2006). These fragments are used by local villagers to varying degrees and are surrounded by tea plantations and small-scale agriculture, including pasture for domestic livestock (Gillespie and Chapman, 2006).

Fecal samples were collected from habituated and semihabituated adult and subadult male and female primates and preserved in 10% neutral buffered formalin (Gillespie, 2006). A Merifluor® *Cryptosporidium*/*Giardia* Direct Immunofluorescent Detection Kit (Meridian Bioscience, Inc, Cincinnati, Ohio) was used to detect both parasites (Johnston et al., 2003). Fecal samples were scored both for presence or absence of the pathogens as well as quantification of oocysts and cysts in feces. Counts were calculated by analyzing 10 μl of a 10-ml solution of 0.1 g of feces from each sample. Oocysts or cysts were then quantified by counting total numbers in 150 microscope fields (×400 magnification) and extrapolating results to the entire sample. This method was validated by spiking negative fecal samples with known numbers of *Cryptosporidium* sp. oocysts.

Results were analyzed statistically with the use of the computer program EpiInfo, Version 3.3.2 (Centers for Disease Control, Atlanta, Georgia). Trends were considered statistically significant at an $\alpha = 0.05$ level.

In total, 115 nonhuman primate samples were collected and analyzed. The overall prevalences of *Cryptosporidium* and *Giardia* spp. in nonhuman primates within forest fragments (n = 80) were 6.3 and 3.8%, respectively. None of the primates within the undisturbed forest (n = 35) was positive for *Giardia* or *Cryptosporidium* spp. infections. No animals were found to be simultaneously infected with both parasites.

Prevalence and mean intensities of infection for both parasites are presented in Table I. Red colobus (*Pilocolobus tephrosceles*) and red-tailed guenons (*Cercopithecus ascanius*) harbored *Cryptosporidium* sp. or *Giardia* sp., but black-and-white colobus (*Colobus guereza*) did not. All nonhuman primate samples from the undisturbed forest were negative for both protozoan parasites. Seven of 35 (20%) red colobus and 1 of 20 red-tailed guenons (5%) sampled from forest fragments were infected with either *Cryptosporidium* sp. or *Giardia* sp. For the primates sampled, *Cryptosporidium* sp. was present only in the red colobus inhabiting the forest fragments (prevalence of 14.3%; Table I). Red colobus inhabiting forest fragments were at a significantly higher risk of infection than were red-tailed guenons and black-and-white colobus within the same fragments (Fisher's exact test; $P = 0.014$). Prevalence of *Giardia* sp. was universally low among species, and no significant differences in *Giardia* sp. prevalence among species were noted (Table I).

Location of sampling was significantly related to the prevalence of infection with both parasites in nonhuman primates. No primates sampled from the undisturbed forest harbored species of *Cryptosporidium* or *Giardia*, whereas prevalence of *Cryptosporidium* and *Giardia* spp. among primate species within the forest fragments ranged from 0 to 14.2% (Table I). This difference was marginally statistically significant (Fisher's exact test; $P = 0.049$).

The results of this study suggest that wild nonhuman primates living in disturbed habitats are at greater risk for infection of *Cryptosporidium* and *Giardia* spp. than are primates living in undisturbed habitats. These results further support previous findings that primates in the Kibale region living in disturbed habitats are at higher risk of infection with directly transmitted gastrointestinal parasites than are primates living in less disturbed habitats (Gillespie et al., 2005; Gillespie and Chapman, 2006).

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TABLE I. Prevalence and intensity of *Cryptosporidium* and *Giardia* spp. in wild primates sampled from 3 forest fragments near Kibale National Park, Uganda.*

Species	No. sampled	*Cryptosporidium*		*Giardia*	
		Prevalence (%)	Mean intensity†	Prevalence (%)	Mean intensity†
Red colobus	35	14.3	13.9 ± 9.1	5.7	3.3 ± 2.6
Black-and-white colobus	25	0	—	0	—
Red-tailed guenon	20	0	—	5.0	3.5 ± 0.0

* Differences in prevalence among the 3 forest fragments were not statistically significant for either parasite or for any host species, so results from all fragments were collapsed for analysis.
† Mean intensities are expressed as 10^4 oocysts (*Cryptosporidium*) or cysts (*Giardia*) per gram, ± standard error of the mean; negative samples are not included in this calculation.

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Human Pseudoterranovosis, an Emerging Infection in Chile

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ABSTRACT: Fifteen cases of human pseudoterranovosis are reported for Chile, representing an emerging parasitic infection in this country caused by larvae of the nematode *Pseudoterranova* sp. Our observations also included an outbreak of pseudoterranovosis in 3 of 4 individuals who shared the same raw fish dish (cebiche). Most of the cases occurred in adult patients. The main source of infection was from consumption raw or fried marine fish, including hakes (*Merluccius australis* or *Merluccius gayi*), pomfret (*Brama australis*), Inca scad (*Trachurus murphyi*), and corvina (*Cilus gilberti*). Seasonal distribution showed most of the cases to occur in fall and spring. Parasite larvae were isolated from the mouths of most of the patients after they reported a pharyngeal tickling sensation, coughing, vomiting, or a foreign body in the mouth or throat.

Human anisakidosis produced by anisakid nematodes is most frequently caused by larvae of *Anisakis* (anisakiosis) and *Pseudoterranova* (pseudoterranovosis) (Takahashi et al., 1998; Audicana et al., 2003). A few cases have been attributed to *Contracaecum osculatum* (Takahashi et al., 1998) and a single case to an immature adult of *Hysterothylacium aduncum* (Yagi et al., 1996).

About 97% of the recorded cases of human anisakidosis have occurred in Japan, with the remaining percentages distributed among 26 countries (Takahashi et al., 1998). In the Western Hemisphere, anisakidosis has been reported in Canada, the United States., Mexico, Brazil, Chile (Takahashi et al., 1998), and Peru (Cabrera et al., 2003). Generally, anisakiosis is more common than human pseudoterranovosis in Japan and Europe (Takahashi et al., 1998; Audicana et al., 2003), although in North America, the greatest prevalence occurs with larvae of *Pseudoterranova* spp. (Amin et al., 2000). In Chile, most of the cases reported also were due to pseudoterranovosis, representing 10 of 13 cases of anisakid worms (Sapunar et al., 1976; Apt et al., 1980; Mercado et al., 1997, 2001; Verhamme and Ramboer, 1988; Canese, 1995; Torres, Canales et al., 2000). Two of the anisakid cases acquired in Chile after consumption of raw (cebiche) or smoked fish were diagnosed in Belgium and Paraguay, respectively; the first patient presented symptoms while traveling from Chile to his country of origin, and the second patient while temporarily occupied professionally in Paraguay (Verhamme and Ramboer, 1988; Canese, 1995).

The definitive hosts of *Anisakis* and *Pseudoterranova* species are marine mammals. McClelland (2002) reviewed the life cycle of *Pseudoterranova decipiens*, the eggs of which are released with the feces of the definitive host (seals). The larvae develop to the third stage (L3), and they are then consumed by benthic and epibenthic copepods. They also may be also transmitted to invertebrates such as mature amphipods, decapods, isopods, nudibranchs, cumaceans, mysids, and polychaetes. Bottom fishes are infected when they consume infected crustaceans; larvae are then localized in the musculature. Seals become infected through consumption of crustaceans or fishes with L3s.

The source of human pseudoterranovosis is the consumption of crustaceans or raw fish (as cebiche, sushi, or sashimi), smoked fish, pickled fish, salad, or other poorly cooked fish dishes containing L3s. The L3s are capable of penetrating the stomach or intestinal wall (Ishikura et al., 1993), and they can migrate from the stomach to the esophagus or pharynx, provoking the tingling throat syndrome, which leads to ex-

TABLE I. Data from 15 cases of infection by *Pseudoterranova* L4s in Chile.

Patient	Yr/Season	Age/Sex*	Place of occurrence of infection	Suspected source of infection (fish)	No. of larvae	Symptoms
1	2001/Spring	40/M	Santiago	Cebiche (pomfred)	1	Sensation of a foreign body between the teeth
2	2002/Autumn	54/F	Santiago	Cebiche (not known)	1	Elimination of larva, after general anesthesia, tickling in pharynx and cough
3	2002/Autumn	19/F	Santiago	Cebiche (hake)	1	Elimination of the larva through mouth
4	2002/Autumn	43/F	Santiago	Fried fish (not known)	2	Pharyngeal tickling associated with elimination of each larva
5	2002/Winter	50/M	Santiago	Cebiche (hake)	1	Cough, pharyngeal tickling
6	2002/Spring	33/F	Santiago	Cebiche (hake)	1	Passive elimination through the mouth
7	2003/Summer	15/M	Santiago	Cebiche (Inca scad)	1	Elimination of the larva from the mouth after heavy coughing
8	2003/Autumn	26/F	Santiago	Fried fish (hake)	1	Gastritis, elimination of the larva with vomit
9	2003/Autumn	47/F	Santiago	Fried fish (pomfred)	1	Sensation of asphyxia, cough and secretions upon awakening after eliminating the larva
10	2003/Spring	6/M	Santiago	Cebiche (hake)	1	Pharyngeal tickling and elimination of the larva through mouth
11	2004/Spring	24/F	Cartagena	Cebiche (hake)	1	Heavy coughing and elimination of the larva through the mouth
12	2004/Spring	Adult/M	Cartagena	Cebiche (hake)	1	Elimination of the larva through the mouth
13	2004/Spring	Adult/F	Cartagena	Cebiche (hake)	2	Elimination of the larva through the mouth
14	2005/Spring	1/F	Santiago	No data	1	Intense diarrhea, loss of appetite, elimination of larva through anus
15	2005/Spring	Adult/M	Osorno	Cebiche (corvina)	1	Sensation of a foreign body, pharyngeal tickling and heavy cough pre-elimination of the larva

* M = male; F = female.

pulsion by coughing up (Audicana et al., 2003). In general, invasive capacity in larvae of *Pseudoterranova* sp. is extremely rare when contrasted with that of *Anisakis* sp. larvae (Amin et al., 2000). *Pseudoterranova decipiens* are capable of molting to the fourth stage (L4) within the human digestive tract (Ishii et al., 1989).

The objective of the present study was to analyze 15 cases of human pseudoterranovosis in Chile in relation to age and sex of the patients and their symptoms, as well as sources of the infections and their seasonality. Sixteen nematode larvae were obtained between 2001 and 2005 from various clinical laboratories and gastroenterology services in Chile. Most of the specimens had been collected from oral cavity of the patients. The larvae were fixed in 70% ethanol and cleared in lactophenol for morphological study. The range of each measurement in millimeters is given first, followed in parentheses by the mean value and its standard deviation. Voucher specimens were deposited in the U.S. National parasite Collection, Beltsville, Maryland. (USNPC 98916-98918) and the collection of the Instituto de Parasitología, Universidad Austral de Chile (IPUAT 0271-0282).

All 16 larvae had 3 lips, 1 dorsal bearing 2 double-papillae, and 2 subventral, each bearing a single double-papilla. A boring tooth and interlabia were absent. An excretory pore was present at the base of the subventral lip. The lengths of larvae were 19.5-38.5 (27.7 ± 5.3); maximum width 0.4-0.9 (0.8 ± 0.2); nerve ring 0.4–0.7 (0.5 ± 0.08) from anterior end. Length of esophagus 1.6–2.4 (2.0 ± 0.3); ventriculus 0.6–1.2 (0.8 ± 0.2); intestinal caecum 0.7–1.4 (0.9 ± 0.2), tail 0.1–0.2 (0.2 ± 0.03) with or without evident knoblike processes. Ratios included body length to esophagus, 1:10.7–16.1; body length to intestinal caecum, 1:21.1–37.2; body length to ventriculus, 1:22.7–45.6; ventriculus to intestinal caecum, 1:0.6–1.3; and body length to tail, 1:117–205. All the worms were identified as L4 of *Pseudoterranova* sp.

Most of the cases represented adult patients of both sexes (Table I), with the exception of 1 case registered in the city of Osorno (40°28′S, 70°38′W) in southern Chile; the remaining cases were treated in Santiago (33°28′S, 70°38′W). Three patients had acquired the infection while visiting coastal area of Cartagena (33°32′S, 71°36′W) (Table I). Two patients eliminated 2 larvae. Eleven of the patients had consumed cebiche, whereas 3 patients affirmed to have consumed only fried fish in the 10 days before eliminating larvae. Twelve patients identified the fish consumed as pomfret (*Brama australis*), hake (*Merluccius australis* or *Merluccius gayi*), Inca scad (*Trachurus murphyi*), or corvina (*Cilus gilberti*) (Table I). In most of the cases in the present study, the larvae were expelled from the mouth of the patient. Two patients noted the larvae while brushing their teeth, or felt a foreign body between the teeth. In a single case, a larva issued from the anus of the patient. Seasonal distribution showed most of the cases to occur in either fall or spring. Three of the patients (11–13) from 2 married couples had shared the same cebiche in a restaurant.

No association between sex and human pseudoterranovosis has previously been noted for cases in Canada, the United States., or Japan, and most cases occurred in adults (Margolis, 1977; Ishikura et al., 1996). Previous published reports of *Pseudoterranova* spp. acquired in Chile were correlated with the consumption of raw (cebiche and sushi), smoked, or fried fish such as hake, Inca scad, conger-eel, and salmon (Verhamme and Ramboer, 1988; Canese, 1995; Mercado et al., 1997, 2001). Association of cases of pseudoterranovosis with the consumption of fried fish indicates that this method of cooking does not always imply efficient cooking of the fish. Elimination of nematodes from the mouth in most cases was closely correlated with results of previously reported cases in Chile (Sapunar et al., 1976; Mercado et al., 1997, 2001) and in North America (Margolis, 1977; Deardorff and Overstreet, 1990; Amin et al., 2000).

The seasonal occurrence of cases in the present study can be attributed to a greater consumption of host fishes or to a higher prevalence of infection of the fishes in spring and fall. Anisakiosis has been observed seasonally in herring in the Baltic Sea (Lang et al., 1990). In Japan, infection of humans with *Pseudoterranova* spp. seems to occur mainly in the winter on the Island of Hokkaido, at a time when host fishes are caught, and probably have acquired the infection due to migrations of the definitive host *Callorhinus ursinus* (Takahashi et al., 1998). Hauksson (2002) associated the decrease of pseudoterranovosis in the fish *Myoxocephalus scorpius scorpius* in Iceland to the decrease of definitive hosts *Halichoerus grypus* and *Phoca vitulina*. Global climatic change may influence the distribution of anisakid larvae in their

hosts, which also could affect human infection (Ishikura et al., 1996; Takahashi et al., 1998; Marcogliese, 2001).

Based on our results and previous data (Sapunar et al., 1976; Apt et al., 1980; Verhamme and Ramboer, 1988; Canese, 1995; Mercado et al., 1997, 2001; Torres, Canales et al., 2000), most of the 28 cases of anisakid worms recorded to date in Chile have been caused by *Pseudoterranova* sp. (89.3%) compared with 10.7% of cases by *Anisakis* sp. Three of the cases were diagnosed between 1976 and 1990, whereas the remainder (89.3%) were diagnosed over the past 15 yr. Of the 25 cases of pseudoterranovosis published in Chile, only 2 cases reported penetration of the stomach wall by larvae (Apt et al., 1980; Mercado et al., 1997). This finding suggested a similar behavior of parasites of human pseudoterranovosis in Chile and the United States, in the latter of which most recorded cases indicated larvae were eliminated from the mouth (Amin et al., 2000; Mercado et al., 2001). There have been reports, however, of invasive pseudoterranovosis in the United States, for example, in the abdominal cavity of a man (Little and MacPhail, 1972), and, in another case, an L3 of *P. decipiens* emerged from an ulcer on the neck of an adult, which had effected a transesophageal penetration (Amin et al., 2000). It is probable that the occurrence of pseudoterranovosis in Chile represents only the tip of the iceberg, as suggested for Japan (Takashi et al., 1998), and it may be the case in other countries.

The increase in the recognition of this emerging infection in Chile may be related to 1) a better knowledge of this infection among health care professionals; 2) greater popularity in consumption of raw fish; 3) elimination of fisheries waste from factory ship and artisanal fishing boats, which increase the abundance in the infection of marine mammals and fishes that feed on these wastes; and 4) fresh fish in commerce may not be well frozen, allowing for the survival of larvae of *Anisakis* and *Pseudoterranova* spp. in the musculature of fishes commercialized in Chile for human consumption (Torres, Moya et al., 2000). The greater recognition of pseudoterranovosis with respect to the anisakiosis in Chile could be in relation to the greater frequency and intensity of infection by larvae of *Pseudoterranova* spp. in fish flesh as observed in 5 fish species commercialized and the high rate of their consumption in the city of Valdivia (Torres, Moya et al., 2000).

At present, 8 species of *Pseudoterranova* have been recognized in the world, including *P. decipiens*, *Pseudoterranova krabei*, *Pseudoterranova bulbosa*, *Pseudoterranova azarasi*, *Pseudoterranova decipiens* E, *Pseudoterranova cattani*, *Pseudoterranova ceticola*, and *Pseudoterranova kogiae*, among which *P. cattani* has been recorded from 4 (McClelland, 2002; Zhu et al., 2002) of the 16 host fish species for pseudoterranovosis in Chile (Torres, Moya et al., 2000). The definitive host of *P. cattani* is the sea lion, *Otaria byronia* (George-Nascimento and Urrutia, 2000). L3s and L4s of *Pseudoterranova* sp. also have been recorded from the digestive tracts of the cetaceans *Phocoena spinipinnis* and *Cephalorhynchus eutropia* on the southern Chilean coast (Torres et al., 1992). It is possible that fishes from the Chilean coast carry more than 1 species of *Pseudoterranova*, which is potentially pathogenic for humans, due to the extensive migration of some of the marine mammals that are definitive hosts of these parasites. The Weddell seal, *Leptonichotes weddelli*, for example, is the only known host of *P. decipiens* E described from Antarctica (McClelland, 2002), and it is capable of migrating to the Chilean coast of Patagonia and as far north as the Juan Fernandez Islands (Sielfeld, 1983). In addition, the dwarf sperm whale, *Kogia simus*, which hosts *P. kogiae* on the Australian coast (Johnston and Mawson, 1939), has been observed on the central Chilean coast (Sielfeld, 1983). It has been determined that about 10% of the *Anisakis* spp. larvae isolated from the Patagonian grenadier, *Macruronus magellanicus*, in Chile were capable of surviving up to 90 hr in aqueous 0.15 M NaCl at −15 to −18 C (Torres et al., 1982). Health education of the public can contribute to the control of anisakid infection through the dissemination of information promoting awareness of the problem and application of good sanitary practice in the consumption of fish, such as cooking at 65 C for 10 min, or in a microwave oven at 77 C (Adams et al., 1999); or freezing at −20 C for 7 days or −35 C for 15 hr in a blast freezer, in accordance with the U.S. Food and Drug Administration Food Code. Improved recognition of the *Pseudoterranova* spp. distributed on the Chilean coast will be obtained through better knowledge of the occurrence of anisakid nematodes in marine mammals on this coast. Also of importance will be the application of molecular methods in studies of larvae isolated from fishes and from human infections occurring in this area.

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