RESEARCH NOTES

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Uncinaria hamiltoni (Nematoda: Ancylostomatidae) in South American Sea Lions, *Otaria flavescens*, From Northern Patagonia, Argentina

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ABSTRACT: Thirty-one South American sea lion pups (*Otaria flavescens*) found dead in Punta León, Argentina, during the summer of 2002, were examined for hookworms (*Uncinaria hamiltoni*). Parasite parameters were analyzed in 2 locations of the rookery, i.e., a traditional, well-structured breeding area and an expanding area with juveniles and a lax social structure. Prevalence of hookworms was 50% in both localities, and no difference was observed in prevalence between pup sexes ($P > 0.05$). Hookworms were concentrated in the small intestine. Transmammary transmission is assumed because only adult hookworms were found in the pups. The mean intensity of hookworms per pup was 135; the mean intensity in females (92.78) was significantly different ($P < 0.05$) from that of males (230.25). No difference ($P > 0.05$) in intensity was found between the 2 breeding areas, although prevalence was higher in the traditional breeding area than in the other area. Location was the only factor affecting hookworm prevalence (*P* log-linear model: 0.9552; χ^2: 1.5629). No apparent trend between body condition and intensity of hookworms was observed.

South American sea lions, *Otaria flavescens*, occur in more than 85 rookeries and 'haul-outs' along the coast of Argentina in the southwestern Atlantic ocean, both on the midland and on islands, through the whole year (Reyes et al., 1999). The population was exploited for leather and oil during the first half of the 20th century. During this period, it was dramatically reduced, with no evident growth until 1990 when a positive increase in the number of pups was observed (Crespo and Pedraza, 1991). At present, the population in northern Patagonia is growing at an annual rate of 4–7%, although it still represents less than a quarter of the supposed original population size before the highest levels of exploitation (Dans et al., 2003). Besides population dynamics, their interactions with fisheries (Crespo et al., 1994, 1997) and the food habits of this species have been well reported (Koen-Alonso et al., 2000), although parasitological studies are scarce. However, *Uncinaria* spp. have been previously reported from the Falkland (Malvinas) Islands by Baylis (1933), from Uruguay by Botto and Mañé-Garzón (1975), and from Uruguay and Chile by George-Nascimento et al. (1992). These nematodes are found in the intestines of otariid pups (Lyons et al., 2001) and, unusually, phocid pups (George-Nascimento et al., 1992).

The taxonomic specificity of *Uncinaria* spp. remains uncertain (Lyons, De Long et al., 2000), and molecular-based analyses are underway elsewhere (Nadler et al., 2000). Originally, 2 species, *Uncinaria lucasi* Stiles, 1901, and *Uncinaria hamiltoni* Baylis, 1933, were described, although, subsequently, intermediate types were found by several authors (Lyons et al., 2001). The life cycle of *U. lucasi* is known and has been well described in northern fur seals (*Callorhinus ursinus*) from Alaska by Lyons (1963). Infections by *Uncinaria* spp. (uncinariosis) are regarded as one of the most important mortality factors in sea lion pups (Lucas, 1899; Baylis, 1933; Olsen and Lyons, 1965; Fowler, 1990).

The main objective of this study was to generate data on *U. hamiltoni* in northern Patagonia relative to the parasite's prevalence, mean intensity, range of intensity, and distribution in South American sea lion pups and to evaluate the presence of *U. hamiltoni* as a potential mortality factor.

Samples were taken in Punta León reserve (43°03′S, 64°47′W; Chubut province, Argentina), one of the largest rookeries in northern Patagonia, where approximately 2,000 pups are born every year (25% of total pup production). During the past 20 yr, together with an increase in the number of pups, there was an increase in the area occupied (from 4 to 7 km). The study was undertaken with the knowledge that the rookery now has 2 different locations, one with a traditional breeding structure (northern area) and another developed during the past 10 yr, with a less organized breeding structure (southern area). The only other pinniped species found in Punta León is the southern elephant seal (*Mirounga leonina*).

Hookworms were collected during the 2002 breeding season, between 11 January and 10 February. An additional and final survey was completed at the end of the breeding season, on 27 February. Thirty-one (13 males, 18 females) fresh, recently dead pups were collected. All animals were necropsied immediately in the field. Pups were measured and weighed, sex was determined, and thickness of subcutaneous fat (at the level of the throat, the sternum, and the belly button) was determined to assess the body condition of the pups. Intestines were removed, placed in plastic bags, and fixed in 70% ethanol. As soon as possible, i.e., within 24 and 48 hr, intestines were taken to the Marine Mammal Laboratory (LAMAMA-CENPAT/CONICET) where they were dissected according to the method of Aznar et al. (1997). Each fragment of an intestine was placed in a petri dish filled with 70% ethanol and dissected with scissors; all examinations were conducted using a stereomicroscope at ×6.3 magnification. All parasites were collected, counted, preserved in 70% ethanol, and cleared in lactophenol for identification. Terminology, i.e., prevalence, mean intensity, and range of hookworm infection is from Bush et al. (1997). Parasite distribution along the intestine was determined using variance-to-mean ratios (Krebs, 1989), and significance evaluated with a chi-square test. Student's *t*-test (Zar, 1996) was used to assess: (1) differences in mean intensity of hookworms between female and male pups, (2) differences in mean intensity between female and male hookworms, (3) differences in mean intensity of hookworms among female pups, (4) differences in mean intensity of hookworms in male pups, and (5) differences in mean intensity of hookworms between the northern and the southern breeding areas within Punta León. Fisher's exact test (Zar, 1996) was used to examine differences in prevalence between sexes. Finally, a log-linear model (Sokal and Rohlf, 1998), with 3 classification criteria, i.e., pup sex, location within rookery, and presence–absence of hookworm, was used to evaluate which factor influenced hookworm prevalence.

Voucher specimens of *U. hamiltoni* from the South American sea lion pups were deposited (U.S. National Parasite Collection [USNPC] 94041; 94042) in the USNPC Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland.

The hookworms found in the South American sea lion pups were identified as *U. hamiltoni* on the basis of comparison with described morphospecies only. Given the present uncertainty in the taxonomy of hookworms, we should say that our morphological identification is provisional and that the ongoing molecular studies may eventually result in the revision of the taxonomy of these *Uncinaria*. All the hookworms found were adults. Morphometric data corresponding to the specimens found are provided in Table I. Hookworms infected 50% of the pups examined. No difference was observed in prevalences between sexes (female pups, 56.25%; male pups, 38.46%; Fisher's exact $P = 0.4621$). Hookworms showed an overdispersed distribution in the small intestine ($I = 278.63$; $\chi^2 = 8080.25$, df = 29 [lower critical value = 16.05; upper critical value = 45.72]). Only 2 worms were found attached to the intestine wall; all the rest were free in the lumen and no hemorrhagic lesion was observed. The number of hookworms found in all infected pups ranged from 1 to 451: from 1 to 248 in female pups and from 17 to 451 in male pups (Table II). The mean intensity of hookworms per pup was 135 (±146.52). The mean intensity in females (92.78 ± 98.09) was significantly lower than in males (230.25 ± 206.8) ($t = 2.94$; $P = 0.013$). No difference was observed in the mean intensity of female and male hookworms (72.91 ± 69.69; 65.69 ± 69.94, respectively; $t = 0.79$,

TABLE I. Morphometric measurements on females and males of *Uncinaria hamiltoni* found in South American sea lions (*Otaria flavescens*) from Punta León, Patagonia (mean, ± SD, range) and from previous studies (range). All measurements are expressed in millimeters.*

	Uncinaria hamiltoni (this work)		*Uncinaria hamiltoni* (in *Otaria flavescens*) (Baylis, 1933)		*Uncinaria hamiltoni platensis* (in *Otaria flavescens*) (Botto and Mañé-Garzón, 1975)		*Uncinaria hamiltoni* (in *Eumetopias jubata*) (Baylis, 1933)	
	Females	Males	Females	Males	Females	Males	Females	Males
Sample size	30	29	—	—	30	30	3	1
Number of hosts examined	6	4						
BL	5.36–17.2 (11.37 ± 2.94)	4.64–10.32 (7.49 ± 1.53)	12.5–17.5	8.5–12.0†	8.3–19.4	6.4–11.4†	16.7–18.8	9.4†
BLB		4.88–10.64 (7.85 ± 1.52)		—				
OL	0.820–1.34 (1.15 ± 0.11)	0.79–1.83 (1.09 ± 0.19)	1.6–1.9	1.5–1.55	1.35–1.57	1.2–1.38	1.4–1.6	1.3
OW	0.11–0.22 (0.18 ± 0.02)	0.10–0.31 (0.16 ± 0.05)	—	—	—	—	—	—
LBC	0.21–0.34 (0.28 ± 0.03)	0.21–0.28 (0.24 ± 0.02)	0.32–0.38	0.28–0.3	0.24–0.29	0.21–0.26	—	—
WBC	0.2–0.31 (0.24 ± 0.02)	0.14–0.24 (0.19 ± 0.06)	—	—	—	—	—	—
VPE	2.13–6.62 (4.33 ± 1.34)		5.1–7.0		3.6–5.7		—	
EL	0.1–0.14 (0.12 ± 0.01)		0.13–0.14		0.1–0.13		—	
EW	0.04–0.10 (0.07 ± 0.01)		0.06–0.09		0.06–0.08		—	
SL		0.57–1.05 (0.89 ± 0.09)		1.0		0.75–0.99		0.67
DRL		0.01–0.08 (0.04 ± 0.02)		—		—		—
DRB		0.12–0.25 (0.15 ± 0.03)		—		—		—
Ratio DRB–DRL		1:3.65		1:2.4		1:3.0		—

* BL, body length; BLB, body length including bursa; OL, length of the esophagus; OW, width of the esophagus; LBC, length of buccal capsule; WBC, width of buccal capsule; VPE, distance from vulva to posterior end; EL, egg length; EW, egg width; APE, distance from anus to posterior end; SL, spicule length; DRL, dorsal ray length; DRB, dorsal ray bifurcation.
† Not specified if measured with or without the bursa.

P = 0.43), in the mean intensity of female and male hookworms in female pups (43.88 ± 36.88; 44.89 ± 44.35, respectively; t = 0.19, P = 0.85), and in the mean intensity of female and male hookworms in male pups (112.75 ± 115.25; 112.5 ± 96.49, respectively; t = 0.02, P = 0.98).

In the northern breeding area, 44.44% of the dead pups were infected, whereas 60% of the dead pups in the southern part of the rookery were infected. Among all the infected pups, 57.14% belonged to the traditional breeding areas and 42.86% to the new area. There was no interaction between the 3 factors in the log-linear model, and location was the only factor that accounted for the adjustment of the model (P of the log-linear model: 0.9552; χ^2: 1.5629, df: 6). This suggests that the breeding area influences the prevalence of infection. In addition, no difference was found between the mean intensity of hookworms in the northern (150.5 ± 123.5) and the southern areas (110.4 ± 191) (t = 1.025; P = 0.33).

Prevalence and mean intensity of *U. hamiltoni* in the South American sea lion were moderate compared with hookworm infections in other pinnipeds studied in other areas. For example, pups had lower levels of infection than the California sea lion (*Zalophus californianus*) and the northern fur seals from California (Lyons, Spraker et al., 2000), although they were more heavily infected than the northern fur seals from Alaska (Lyons, Spraker et al., 2000) and the Juan Fernández fur seal (*Arctocephalus philippii*) from Alejandro Selkirk Island, Chile (Sepúlveda, 1998). One reason that accounts for the moderate levels of infection preliminarily found at Punta León may be the kind of substratum. As it is known, the type of soil is related to the development and transmission of hookworms. On St. Paul Island, Alaska, for example, northern fur seal pups born on sandy rookeries have higher numbers of adult hookworms than pups born on less sandy areas (Lyons, Spraker et al., 2000) or as observed for Steller sea lions (*Eumetopias jubatus*) from a rocky terrain rookery in Oregon where no hookworm is found (Lyons et al., 2003). This matter will be intensively studied at northern Patagonia in the future, with more samples from different breeding seasons.

Hookworm burden did not differ significantly between the 2 locations of Punta León rookery. Also, it has to be noticed that pup mortality was 2 times higher in the southern than in the northern area, and this was attributed to the absence of a typical nursery structure in the new breeding area (E. A. Crespo, pers. obs.) rather than to hookworm infection.

A relationship between a host's sex and the number of hookworms present has not been previously reported. California sea lions, northern fur seals (from California), and Juan Fernández fur seals did not show differences in hookworm intensities in male and female pups (Lyons et al., 1997, 2001; Sepúlveda, 1998), contrary to that observed in this work.

Transmammary transmission of hookworms in South American sea lions has not been demonstrated, although it is assumed. Lyons (1994) and Lyons and De Long et al. (2000) found that adult hookworms in California sea lion and Northern fur seal pups were derived only from parasitic third stage larvae passed through mothers' milk, for a short time postpartum, and that there may be no other way of transmission (Lyons, 1994). The same pattern of transmission is presumed in the present case because only adult hookworms were found throughout the period studied, supporting the idea that pups would only be infected with colostrum. In addition, if the number of hookworms is directly related to the quantity of milk consumed by the pup, and assuming only transmammary transmission, it might be supposed that male South American sea lion pups acquired more milk than female pups. Our preliminary findings would support this prediction even though there is no evidence that female nursing differs according to the sex of the offspring (Cappozzo et al., 1991).

Hookworms appeared to be concentrated in the small intestine. This site is the same as that selected by hookworms in Juan Fernández fur seals (Sepúlveda, 1998) and California sea lions (Dailey and Hill, 1970; Lyons, De Long et al., 2000). However, it is different from the northern fur seal pups, where they appear concentrated in the cecum and proximal large intestine (Lyons et al., 1997).

Higher prevalences and mean intensities should be expected in the traditional nursing area than in a new expanding area. In the first site, reproductive females rather than juveniles (nonreproductive) are concentrated, facilitating hookworm transmission. Consistent with the expected pattern, differences in prevalence were observed in South Amer-

TABLE II. Mean intensity ± SD and range of intensity of *Uncinaria hamiltoni* found in South American sea lions (*Otaria flavescens*) from Punta León, Patagonia, Argentina.

Host	Total *Uncinaria hamiltoni* (n = 1,656)	Female *Uncinaria hamiltoni* (n = 800)	Male *Uncinaria hamiltoni* (n = 856)
All pups	135 ± 46.52 (1–451)	72.91 ± 69.69 (7–243)	65.69 ± 69.94 (1–208)
Female pups	92.78 ± 98.09 (1–248)	43.88 ± 36.88 (12–101)	44.89 ± 44.35 (1–136)
Male pups	230.25 ± 206.8 (17–451)	112.75 ± 115.25 (7–243)	112.50 ± 96.49 (9–208)

ican sea lions. However, contrary to the expected pattern, they exhibited no difference in the number of hookworms present in pups. The total number of individuals of the southern area of the rookery is now one-quarter that of the northern area. Therefore, density-dependent mechanisms could be still relaxed compared with a population size close to its carrying capacity *k.* This may be the more plausible explanation for the lack of differences observed between the 2 areas.

Longevity of hookworms in intestines of pinnipeds from North America is well known (Lyons, De Long et al., 2000, and references therein). For *O. flavescens* pups, this phenomenon is yet to be examined. Long-term examinations of pups dying during the nursing period should give an indication when hookworms are eliminated, assuming that they resemble California sea lion and northern fur seal pups (Lyons, De Long et al., 2000).

A clear trend between the body condition of the pup and the number of hookworms present was described in other pinnipeds (cf. Lyons et al., 2001, and references therein), although a similar relationship could not be observed in South American sea lion pups. Critical biological features (related to thickness of the subcutaneous fat or pup weight and health status) not yet known for the species are required to establish any possible pattern or trend (if there were any).

One additional fact to consider in the future is the presence of southern elephant seals in the same area as another potential host species for *U. hamiltoni*, although hookworm infection is believed to be unusual in phocids (George-Nascimento et al., 1992).

Population dynamics of pinniped hosts and hookworms are correlated (Lyons, Spraker et al., 2000). The *O. flavescens* population is increasing and, hence, a corresponding increase in its hookworm population may exist. Within the present conditions, a study during the future breeding seasons should reveal such trends.

Finally, uncinariosis is considered one of the most important mortality factors among pinniped pups in the Northern Hemisphere (e.g., Lucas, 1899; Baylis, 1933; Olsen and Lyons, 1965). However, it may not be an important cause of mortality for the species studied in the Southern Hemisphere based on the low intensity of infection and the absence of lesions in the small intestine (Sepúlveda, 1998). Consequently, the moderate infection levels found in South American sea lion pups may show that uncinariosis could become a potential mortality factor as the population increases toward its original size.

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First Isolation of *Neospora caninum* From an Aborted Bovine Fetus in Spain

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ABSTRACT: *Neospora caninum* was isolated from the brain of a 6-mo-old aborted bovine fetus from Galicia, Spain. The fetal brain homogenate was inoculated intraperitoneally into cortisonized mice. The peritoneal exudate from the infected mice, along with mouse sarcoma cells (Tg180), was inoculated into a second group of mice, and parasites were harvested from the peritoneal exudate. The parasites were adapted to in vitro growth in Vero monolayers. The tachyzoites from the peritoneal exudate reacted positively with anti–*N. caninum* antibodies and not with anti–*Toxoplasma gondii* antibodies on indirect fluorescent antibody test. The tachyzoites were lethal to interferon gamma gene knock out (KO) mice and could be identified immunohistochemically in the tissues. The identity of the parasite was also confirmed by polymerase chain reaction amplification of *N. caninum*–specific fragments. The sequences of the amplified gene 5 fragments (GenBank AY494944) were found to be identical to that of an Austrian isolate of *N. caninum* but not to that of NC-1. This is the first isolation of viable *N. caninum* from Spain.

Neospora caninum is a major cause of abortion in cattle worldwide (Dubey, 2003). In Spain, antibodies to *N. caninum* have been detected in bovine sera, and *N. caninum* antigen has been detected in aborted bovine fetuses (Fondevila et al., 1998; González et al., 1999; Mainar-Jaime et al., 1999; Quintanilla-Gozalo et al., 1999; Pereira-Bueno et al., 2003). We report the first isolation of viable *N. caninum* from an aborted bovine fetus in Spain.

A 6-mo-old aborted bovine fetus and a blood sample of the dam were collected from a farm located in La Coruña district, Galicia, Spain. The cow was Holstein–Friesian, barn housed, milked twice a day, and was fed total mixed rations. The entire fetus and serum from the dam were refrigerated and sent by overnight mail to the Center of Parasite Immunology and Biology Laboratory, Porto, Portugal.

The cow was found to have an antibody titer of 1:160 or more in the *Neospora* agglutination test as described by Romand et al. (1998). The fetal brain was used for isolation of *N. caninum* according to the procedure of Canada, Meireles, Rocha, Sousa et al. (2002). Briefly, the brain was trypsinized and the washed homogenate was inoculated intraperitoneally into cortisonized mice. The peritoneal exudate from these mice, along with mouse sarcoma cells (Tg180), was inoculated into a second group of mice, and parasites were harvested from the peritoneal exudate of this group. For in vitro propagation of the parasite, the final pellet obtained from the peritoneal wash was inoculated onto confluent monolayers of Vero cells as described (Canada, Meireles, Roch, Sousa et al., 2002).

Two months after the initiation of the cell culture, a flask infected with this isolate of *N. caninum* was sent to the Animal Parasitic Diseases Laboratory (APDL), U.S. Department of Agriculture, Beltsville, Maryland for further characterization. Tachyzoites (1×10^6) from culture were inoculated subcutaneously into 2 interferon gamma gene knock out (KO) mice, 2 Swiss Webster mice, and 2 gerbils (*Meriones ungulatus*) as described (Dubey et al., 1998; Dubey and Lindsay, 2000), and observed for infection. The surviving animals were killed after 3 mo, their sera tested for *Toxoplasma gondii* antibodies by modified agglutination test (MAT) (Dubey and Desmonts, 1987), and brain smears examined for protozoan tissue cysts. The tissues were examined microscopically after immunohistochemical staining using anti–*N. caninum* antibodies (Lindsay and Dubey, 1989).

The tachyzoites from the culture supernatant were used to extract DNA using DNAzol (MRC, Cincinnati, Ohio) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed with the *N. caninum*–specific primers Np6–Np21, as described (Yamage et al., 1996), including positive (NC-1) and negative controls. The PCR products were resolved on a 1.5% agarose gel and observed for the presence of the specific target fragment. The specific PCR product was extracted from the gel using MinElute gel extraction kit (Qiagen, Valencia, California) and directly sequenced in both directions using the Big Dye terminator system, version 3.1 (Applied Biosystems, Foster City, California) using an ABI 377 sequencer. The sequence chromatograms were edited using Sequencher 4.1 software (Genecodes Corp., Ann Arbor, Michigan). Searches were performed in BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to determine whether the sequences were similar to any of the previously published sequences of *N. caninum* available in the public database.

Neospora caninum–like tachyzoites were seen in the final pellet obtained from peritoneal washings on microscopical examination. These tachyzoites reacted with anti–*N. caninum* antibodies but not with anti–*T. gondii* antibodies using an indirect fluorescent antibody test as described (Canada, Meireles, Rocha, Sousa et al., 2002).

Tachyzoites were also observed in the Vero cell monolayer 10 days after infection with the mouse peritoneal wash. The new isolate of *N. caninum* (designated NC-Sp1) has been maintained in cell culture since April 2003.

The KO mice inoculated with the tachyzoites died of acute neosporosis 8 days later, with demonstrable tachyzoites in the immunohistochemical reaction to anti–*N. caninum* antibodies. The gerbils and Swiss Webster mice remained asymptomatic. Antibodies to *T. gondii* were not found in the sera of these mice and gerbils by MAT. Protozoan tissue cysts were not found in the brains of these animals killed 3 mo after inoculation.

The identity of the isolate was confirmed as *N. caninum* by PCR. Amplification of the 328-bp target fragment was observed with both NC-1 and the Spanish isolate of *N. caninum*. No amplicon was observed in the negative control. The gel-cleaned fragments were sequenced, and the sequences submitted to GenBank (AY494944). The BLAST search revealed that, whereas the 249-bp stretch of the sequence from the NC-Sp1 isolate was identical to that of an Austrian isolate (AF190701), it was polymorphic at 5 nucleotide positions from the sequences of NC-1 (X84238).

The parasite isolated in this study was identified as *N. caninum* on the basis of the morphology, infectivity to rodents, immunohistochemically and molecular characteristics. *Neospora caninum* and *T. gondii* are closely related parasites. Both are abortifacients, but *T. gondii* is rarely isolated from bovine fetuses (Canada, Meireles, Rocha, Costa et al., 2002). However, there is a need to fully characterize each *N. caninum* isolate from cattle because there are only a few isolates known from cattle, and this information was recently summarized (Dubey, 2003). To this list we add the first isolate of *N. caninum* from Spain.

There was absolute homology between the sequences of the Spanish isolate and that of an Austrian isolate, but not the NC-1 strain. It could be possible that the European isolates are distinct from the American isolates. However, this premise can only be confirmed by comparing sequence data from more isolates from these regions.

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First Report of *Neospora caninum* Infection in Adult Alpacas (*Vicugna pacos*) and Llamas (*Lama glama*)

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ABSTRACT: *Neospora caninum* is a cyst-forming coccidian that mainly affects bovines, although *Neospora* infection has also been described in other domestic and wild ruminant species. Serum samples from 78 alpacas (*Vicugna pacos*) and 73 llamas (*Lama glama*) at a unique dilution of 1:50 tested by indirect fluorescent antibody test (IFAT) were further analyzed serologically by IFAT and Western blot in both ruminant species to avoid cross-reactions with closely related coccidian parasites and to confirm the existence of *N. caninum*–specific antibodies. IFAT titers ranging between 1:50 and 1:800 were found. When using Western blot, *N. caninum* tachyzoite–specific immunodominant antigens with apparent molecular weights of 17–18, 34–35, 37, and 60–62 kDa were also recognized, although some sera with 1:50 IFAT titers proved not to have *N. caninum*–specific antibodies. As expected, higher IFAT titers were associated with higher anti–*N. caninum* reactivity in Western blot. This

TABLE I. Frequency distribution of IFAT titers to *Neospora caninum* species found in 78 alpacas and 73 llamas.

	Number of animals				
	Negative IFAT titers	Positive IFAT titers			
		1:50	1:100	1:200	1:800
Alpaca	50	20	0	0	8
Llama	50	9	4	4	6

report documents for the first time the presence of *N. caninum* infection in adult alpacas and llamas from Peru.

Neospora caninum is a cyst-forming coccidian parasite that has been recognized as a major cause of bovine abortion worldwide. This parasitic infection is also associated with neonatal mortality and encephalomyelitis in congenitally infected calves. It has been recorded in other domestic ruminants, such as sheep and goats, and wild ruminants, such as deer (Dubey, 1999) and antelope (Peters et al., 2001). However, to date, the presence of *N. caninum* infection in adult llamas and alpacas, ruminants with an important economic role in the Andean region, has not been described.

Two major veterinary problems affecting llamas and alpacas are high abortion and neonatal mortality rates mainly caused by parasitic and infectious agents (Fernandez-Baca, 1975; Leguia, 1991; Rivera et al., 1997). Infection by *N. caninum* is suspected to occur also in New World camelids because both compatible lesions and *N. caninum* DNA have been detected in brain tissue from llama and alpaca fetuses (Serrano-Martínez et al., in press).

Indirect fluorescent antibody test (IFAT) and Western blot were used in an attempt to provide preliminary data on the presence of *N. caninum* infection in adult llama (*Lama glama*) and alpaca (*Vicugna pacos*) from Peru.

Serum samples from adult camelids (alpacas and llamas) were collected in different regions of highland Peru and kept in a sera bank at the Veterinary Medicine Faculty of Lima. Alpaca sera (n = 78) and llama sera (n = 73) were screened for *N. caninum* antibodies using the IFAT at a unique dilution of 1:50. Positive sera were titrated by IFAT in 2-fold serial dilutions from 1:50 to 1:3,200 and tested on *N. caninum* tachyzoite–coated IFAT slides. To ensure that antibodies detected by IFAT were specific to *N. caninum* antibodies (NC-1 isolate), Western blot was carried out on all positive and 8 negative serum samples from both alpaca and llama species. A goat antibody anti-llama–conjugated IgG fluorescein isothiocyanate (VMRD, Pullman, Washington) (1:1 dilution) was used. Western blots were performed as described (Alvarez-Garcia et al., 2002) with slight modifications. After incubation with sera (1:25 dilution), the membranes were exposed to goat antibody anti-llama IgG (1:7 dilution) and then incubated again with anti-goat–conjugated IgG with peroxidase (Sigma Chemical Co., St. Louis, Missouri) at 1:1,000 dilution. Western blot results were based on the reactivities of the sera with tachyzoite immunodominant antigens (IDAs) of 17–18, 34–35, 37, and 60–62 kDa (Alvarez-Garcia et al., 2002). The cutoff required to consider a single serum as positive consisted in the recognition of at least IDAs of 17–18 or 37 kDa (or both).

The IFAT results showed that 28 alpacas and 23 llamas had detectable *N. caninum* antibody titers ranging from 1:50 to 1:800. As shown in Table I, the most predominant IFAT titer corresponded to 1:50. However, when sera with titers equal to 1:50 were tested by Western blot, 6 sera from alpacas (n = 20) and 3 sera from llamas (n = 9) recognized *N. caninum*–specific IDAs, whereas the other ones detected nonspecific bands. On the other hand, sera with titers equal to, or higher than, 1:100 proved to be positive by Western blot. The pattern of tachyzoite antigen recognition observed was similar in alpacas and llamas and was also similar to the one described for bovines (Alvarez-Garcia et al., 2002). Positive sera predominantly detected IDAs of 17–18 and 37 kDa, whereas IDAs of 34–35 and 60–62 kDa were recognized with less frequency (Fig. 1). Another important feature was intense recognition of the 17- to 18-kDa band by most *N. caninum*–reacting sera. Negative sera by IFAT did not bind to any *N. caninum* protein although nonspecific binding was observed in most seronegative animals. Moreover, an association between IFAT titers and protein band recognition was found (Fig. 1).

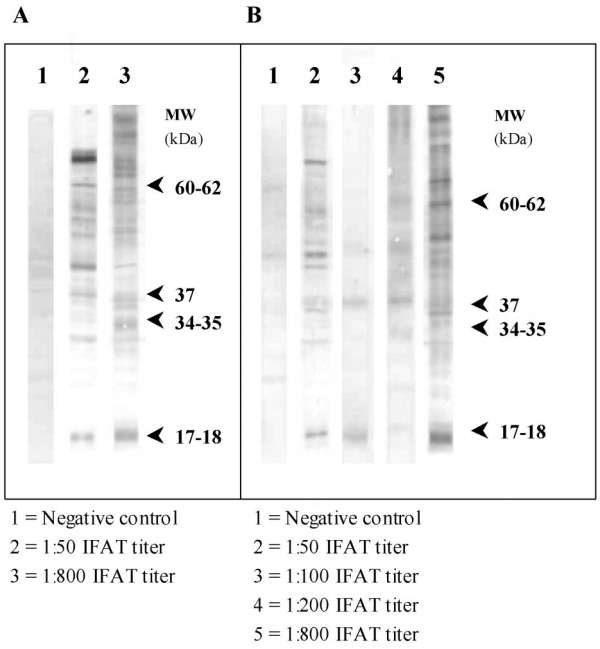

FIGURE 1. Relationship between IFAT titers and *Neospora caninum*–specific antigen recognition in camelids. (**A**) Alpacas. (**B**) Llamas.

Neospora caninum infection was investigated in South American adult camelids because these free-ranging species are of great economic importance in the Andean region as they are bred to produce both fiber and meat. Antibodies developed against *N. caninum* were detected in 28 alpacas and 23 llamas among 78 and 73 sera tested, respectively. This constitutes the first confirmed record of this parasite infection in adult South American camelids. Diagnosis of neosporosis is difficult in adult cattle because of the absence of clinical signs in chronically infected animals. Thus, confirmation of suspected *N. caninum* infection is based on the detection of parasite-specific antibodies. In this study, specific antibodies were positively identified using 2 serological diagnostic tests, IFAT and Western blot. There is still some uncertainty concerning cutoff values in the IFAT, which reduces its diagnostic value, whereas Western blot is more specific and allows unequivocal serological diagnosis even in cases that are problematic for IFAT (Söndgen et al., 2001). In the present study, the detection of high IFAT titers, the recognition of *N. caninum* IDAs, and the association between IFAT and Western blot results provided conclusive proof for the existence of *N. caninum*–specific antibodies in adult alpacas and llamas. On the other hand, nonspecific binding observed in negative sera and 9 sera with IFAT titers equal to 1:50 from both species analyzed by Western blot could be due to cross-reactions with other closely related apicomplexan parasites reported in camelids such as *Sarcocystis* spp. and *Toxoplasma gondii*. The majority of alpacas and llamas from South America older than 2 yr are infected with *Sarcocystis* spp. (Leguia, 1991; Tenter, 1995). On the other hand, *T. gondii*–specific antibodies have also been detected in 33.5% of llamas (Dubey et al., 1992) and 16.3% of alpacas (Gorman et al., 1999). Because 1:50 IFAT titers are clearly more predominant, the use of an IFAT cutoff point of 1:50 could lead to an overestimation of the prevalence of the infection and, consequently, in such doubtful cases Western blot could be required.

The present study strongly suggests the need for further epidemiological studies in llamas, alpacas, and other South American camelids in the short term to determine the prevalence of the infection. Moreover, the detection of compatible lesions together with *N. caninum* DNA in alpaca and llama fetuses (Serrano-Martínez et al., 2004) support the idea of detecting the infection in adult animals. However, determination of the association between seropositivity and abortion, previously observed in cattle (Schares et al., 1999), and the direct detection of *N.*

caninum and other apicomplexan parasites in aborted fetuses are also required to understand the importance of this parasitic infection.

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Additional Observations on the Sporozoite Transmission of *Plasmodium knowlesi* to Monkeys

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ABSTRACT: *Saimiri boliviensis* monkeys were infected by the intravenous injection of 50 sporozoites of the H strain of *Plasmodium knowlesi* dissected from the salivary glands of *Anopheles dirus* mosquitoes; prepatent periods were 11, 12, 13, 13, 13, and 16 days. Sporozoites of *P. knowlesi* stored frozen for 7 days, 53 days, 20 mo, 7 yr and 7 mo, and 11 yr and 5 mo induced infections in *Macaca mulatta* monkeys with prepatent periods of 7, 6, 8, 10, and 7 days, respectively. After frozen storage for 11 yr and 5 mo, infections were induced in *S. boliviensis* with prepatent periods of 10–13 days.

Plasmodium knowlesi has been widely used for biologic and immunologic studies on malaria in *Macaca mulatta* monkeys. In recent years, this host has been difficult to obtain. In addition, its size and aggressiveness make it a difficult host to house and manage. As a consequence, investigations have switched to the use of New World *Aotus* sp. and *Saimiri* sp. monkeys infected with human-infecting malarial parasites as models for immunologic studies of human malaria. Nonetheless, the large background of information on *P. knowlesi* in the *M. mulatta* monkey can be useful, if this parasite were to be shown to behave similarly in New World primates.

On completion of initial vaccine and drug trials in New World monkeys, *Saimiri* sp. and *Aotus* sp. are available to us for additional study. Previously, we have reported the susceptibility of these hosts to *P. fragile* (Collins et al., 1974, 1990), *P. cynomolgi* (Collins et al., 1975, 1985, 1999), *P. fieldi* (Sullivan et al., 1998), *P. inui* (Collins et al., 1981; Sullivan et al., 2003), and *P. knowlesi* (Collins et al., 1978; Sullivan et al., 1996). Of particular interest has been the ready transmission of *P. knowlesi* via sporozoites to *Saimiri* sp. monkeys. Further examinations were made of the usefulness of the *Saimiri boliviensis* monkey for sporozoite-induced studies with *P. knowlesi*, the results of which are reported here.

The object of the study was (1) to determine whether *S. boliviensis* monkeys could be infected by the injection of <100 sporozoites of *P. knowlesi* dissected from the salivary glands of an infected mosquito and (2) could sporozoites maintained in the frozen state still be infectious after many years of storage.

Saimiri boliviensis and *M. mulatta* monkeys were colony-born animals. On arrival at the facility, all animals were quarantined for a 2-mo conditioning period, weighed, and tested for tuberculosis. Parasitologic and serologic examination indicated that the animals were free of infection with malarial parasites before primary inoculation. All *S. boliviensis* monkeys had been previously infected with human malarial parasites but were parasite free at the time of exposure to infection with *P. knowlesi*. All animals were fed a diet that has been proven to provide adequate nutrition and calories in captive *S. boliviensis* and macaque monkeys used in malaria-related research. Feed was free of contaminants and freshly prepared. Daily observations of the animals' behavior, appetite, stool, and condition were recorded. An attending veterinarian treated all animals as medical conditions arose.

Anopheles dirus (originally from Thailand) were laboratory reared and maintained at the CDC/DPD insectaries. Mosquito infection was obtained by allowing the caged anophelines to feed directly on a tranquilized *M. mulatta* monkey. For sporozoite challenge, sporozoites were dissected from the salivary glands of infected mosquitoes into 20% fetal bovine serum (FBS) in phosphate-buffered saline (PBS; pH 7.2). The glands were crushed under a coverslip; the released sporozoites were washed from the slide into a vial, and an aliquot was transferred to a Neubauer cell counting chamber for quantification. The sporozoites were then diluted in FBS-PBS and injected intravenously into the femoral vein of the monkey.

Blood-stage parasitemia was monitored by the daily examination of thick- and thin blood films by the method of Earle and Perez (1932). Infections were terminated by treatment with chloroquine (30 mg base over 3 days). All drugs were administered by oral intubation.

TABLE I. Infection of *Macaca mulatta* and *Saimiri boliviensis* monkeys with sporozoites of the H strain of *Plasmodium knowlesi* that had been stored frozen.

Monkey no.	Monkey species	Period frozen	Number of sporozoites	Prepatent period
C-224	*M. mulatta*	15 days	52,000	7 days
R-8811	*M. mulatta*	53 days	54,000	6 days
R-8936	*M. mulatta*	20 mo	125,000	8 days
R-8920	*M. mulatta*	7 yr, 7 mo	125,000	10 days
RH-0005	*M. mulatta*	11 yr, 5 mo	45,000	7 days
SI-2581	*S. boliviensis*	11 yr, 5 mo	45,000	12 days
SI-2187	*S. boliviensis*	11 yr, 5 mo	4,500	10 days
SI-2209	*S. boliviensis*	11 yr, 5 mo	450	13 days
SI-2046	*S. boliviensis*	11 yr, 5 mo	45	NI*

* No infection.

Anopheles dirus mosquitoes were infected with the H strain of *P. knowlesi* (Chin et al., 1965), by allowing them to feed on a sedated infected *M. mulatta* monkey. Salivary glands were harvested from infected *An. dirus* mosquitoes into FBS-PBS and dilutions made so that each of 6 *S. boliviensis* monkeys was injected intravenously with 50 sporozoites of the H strain. Daily blood films were made beginning 7 days after injection. All animals developed infection, with prepatent periods of 11, 12, 13, 13, 13, and 16 days.

Experimental evidence suggests that mosquitoes rarely inject over 100 sporozoites during feeding (Beier, Davis et al., 1991; Beier, Onyango, Koros et al., 1991; Beier, Onyango, Ramadhan et al., 1991). When 5–7 mosquitoes were fed on *S. boliviensis* to induce infection, prepatent periods were 8, 8, 8, 8, 10, 12, and 16 days (Sullivan et al., 1996). Whether or not the somewhat longer prepatent periods of 11–16 days after injection of 50 sporozoites would suggest that *An. dirus* infected with the H strain of *P. knowlesi* injects greater numbers of sporozoites during feeding resulting in shorter prepatent periods is open to further study. In contrast to squirrel monkeys, intravenous injection of 50 *P. knowlesi* H strain sporozoites resulted in prepatent periods of 6–7 days (median 6 days) in rhesus monkeys (M. Galinski, pers. comm.). This would suggest a greater innate immunity to *P. knowlesi* sporozoites in squirrel monkeys than in rhesus monkeys.

Blood-stage malarial parasites have long been maintained in the frozen state. Occasionally, sporozoites are stored frozen to initiate sporozoite-induced infections, particularly the relapsing-type malarial parasites. In 1992, sporozoites of the H strain of *P. knowlesi* were harvested from the salivary glands of *An. dirus* mosquitoes into 50% FBS-PBS, and aliquots containing different numbers of sporozoites were stored frozen in the vapor phase over N_2. Periodically, sample vials were thawed and injected into monkeys to determine the viability and infectivity of the sporozoites (Table I). Eleven years and 5 mo after freezing, a vial containing 145,000 sporozoites was thawed, and dilutions were made in an attempt to determine the percentage survival of the sporozoites. These results suggested that survival of infective sporozoites could have been less than 10%. However, the prepatent period of only 13 days after injection of 450 preserved sporozoites suggests survival high enough that frozen sporozoites may have a potential for challenge of animals where viability at this level may be sufficient to induce an infection.

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