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NON-SAND FLY TRANSMISSION OF A NORTH AMERICAN ISOLATE OF *LEISHMANIA INFANTUM* IN EXPERIMENTALLY INFECTED BALB/C MICE

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ABSTRACT: *Leishmania infantum*, an etiologic agent of zoonotic visceral leishmaniasis, is endemic in the foxhound population in the United States and Canada. Leishmaniasis is usually transmitted by blood-feeding sand flies; however, epidemiological data do not support a significant role for sand flies in the maintenance of foxhound infections in North America, and an alternate mode of transmission may exist. The present study was conducted to determine if transplacental or direct transmission occurs in pregnant BALB/c mice experimentally infected with *L. infantum* isolated from a naturally infected foxhound from Virginia as well as to determine if the parasite was directly transmitted to the males used to breed the mice. Female BALB/c mice were intravenously inoculated with 1×10^6 promastigotes of the LIVT-1 strain of *L. infantum*. Mice were bred to uninfected male BALB/c mice 2 mo postinoculation. Pregnant mice were killed between days 13 and 18 of gestation. Pups and placentas were collected at necropsy, divided, and used for parasite culture and polymerase chain reaction (PCR) analyses. Culture and PCR analyses were performed on spleens from the male mice to determine the possibility of sexual transmission. *Leishmania* sp. DNA was detected in 4 of 88 pups and 3 of 16 placentas from LIVT-1–inoculated mice. One male mouse used to breed infected females was PCR positive. This work provides evidence for a low level of nonvector transmission of North American *L. infantum* in a mouse model.

Leishmania spp. parasites are flagellated protozoans that are normally transmitted by blood-sucking phlebotomine sand flies. In North America, *Leishmania infantum*, a member of the *Leishmania donovani* complex and a causative agent of zoonotic visceral leishmaniasis, is endemic in the foxhound population (Gaskin et al., 2002; Rosypal et al., 2003). Sand flies are distributed throughout many parts of North America (Young and Perkins, 1984); however, to our knowledge, *L. donovani*–infected sand flies have not been described from the United States (Monti, 2000). Infections acquired by blood transfusions from infected U.S. foxhounds have been described infrequently (Owens et al., 2001; Giger et al., 2002). The transmission mode by which *L. infantum* infections are maintained in foxhounds in North America is currently unknown.

Rare cases of nonsand fly–vectored leishmaniasis by direct contact (Lainson and Bray, 1964; Nuwayri-Salti and Khansa, 1985) and sexual contact (Symmers, 1960; Catone et al., 2003) have been described. Additionally, occasional reports of congenital transmission of leishmaniasis have been reported in humans (Low and Cooke, 1926; Nyakundi et al., 1988; Yadav et al., 1989; Eltoum et al., 1992; Meinecke et al., 1999) and in dogs (Mancianti and Sozzi, 1995; Masucci et al., 2003). It has been suggested that maternal transmission plays a role in the spread of *L. infantum* among foxhounds in North America (Gaskin et al., 2002; Rosypal et al., 2003). We have previously shown that a North American isolate of *L. infantum* was transplacentally transmitted to puppies from an experimentally infected dog (Rosypal, Troy et al., 2005). In the present work, through the use of experimentally infected BALB/c mice, we report the results of a study of direct and maternal transmission of North American *L. infantum* isolated from a naturally infected foxhound.

MATERIALS AND METHODS

Parasites and mouse infections

Leishmania infantum promastigotes were isolated from popliteal lymph node and bone marrow tissues from a naturally infected foxhound from Virginia as previously described (Rosypal et al., 2003) (LIVT-1 strain, American Type Culture Collection, PRA-149, Manassas, Virginia). Promastigotes were subcutaneously inoculated into interferon-γ-gene knockout (BALB/c genetic background) mice. Chronically infected mice were killed, and their spleens were harvested, homogenized, and cultured in vitro at 25 C in *Leishmania* sp. culture media (30% v/v fetal bovine serum, 1% penicillin/streptomycin, and 2% human urine, in Grace Insect Media).

Six-day-old LIVT-1 promastigotes cultured from murine spleen tissue were used for inoculum in the present study. Female 12-wk-old BALB/c mice (N = 20, Jackson Laboratories, Bar Harbor, Maine) were intravenously (IV) inoculated with 1×10^6 LIVT-1 promastigotes suspended in 0.05 ml of Hanks balanced salt solution (HBSS). Female BALB/c mice (N = 4) IV inoculated with 0.05 ml of HBSS served as controls and were housed separately. Prior to injection, mice were anesthetized by intraperitoneal injection of 60 mg/kg bodyweight (BW) ketamine and 5 mg/kg BW xylazine. Mice were warmed on a heating pad to induce vasodilation before IV injection into the lateral tail vein. *Leishmania infantum*–infected female mice were housed together for 8 wk to allow the mice to establish chronic infections before breeding.

Breeding

Beginning 8 wk postinoculation, female mice were bred to uninfected male BALB/c mice. Female mice were checked daily for the presence of sperm plugs, and, if present, the females were separated from the males after breeding. Pregnant mice were killed between days 13 and 18 of gestation to preclude potential transvaginal transmission of the parasite during natural birth. To verify infections in the mothers, spleens were harvested and divided at necropsy. Half of the spleen tissue was homogenized and cultured in vitro at 25 C in *Leishmania* spp. culture media. The remaining spleen halves were used for polymerase chain reaction (PCR) analysis.

Mouse pups and *Leishmania* spp. PCR analysis

At necropsy, the mouse pups were removed from the mother, and the placentas were divided and homogenized. Half of each pup was cultured in vitro in *Leishmania* spp. culture medium at 25 C. Cultures were examined for parasite growth with an inverted microscope every day for 4 wk. The remaining halves of the mouse pups were used for PCR analysis. Placental tissue was divided and used for culture and PCR analyses.

DNA was extracted from mouse pups, placentas, and maternal spleen tissue using a commercial kit (DNA Maxi Kit, Qiagen®, Valencia, California). For each 50-μl reaction, 1 μl of DNA was added to 45 μl of Platinum® PCR Supermix (Invitrogen™ Life Technologies, Carlsbad, California) in a 0.5-ml thin-walled microcentrifuge tube. To the reaction tube, 2 μl of primers 13A (5′-GTGGGGGAGGGGCGTTCT-3′) and 13B (5′-ATTTTACACCAACCCCCAGTT-3′) were added, which am-

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TABLE I. Results of North American *Leishmania infantum* inoculation in groups of BALB/c mice used for breeding.*

	LIVT-1 group (n = 16)	Control group (n = 4)
Treatment	1×10^6 LIVT-1 promastigotes	HBSS
Total number of pups from all females in group	88	30
Average litter size	5.5 pups	7.5 pups
Total number of culture-positive spleens from dams	5	0
Total number of PCR-positive spleens from dams	15	0
Total number of culture-positive placentas from dams	0	0
Total number of PCR-positive placentas from dams	3	0
Total number of culture-positive pups	0	0
Total number of PCR-positive pups	4	0

* HBSS, Hanks balanced salt solution; PCR, polymerase chain reaction.

plify a conserved minicircle region of kinetoplast DNA from all species of *Leishmania* (Rodgers et al., 1990).

Optimal PCR amplification conditions consisted of initial denaturation at 95 C for 2 min, 38 cycles consisting of denaturation at 94 C for 30 sec, annealing at 62 C for 30 sec, an extension at 68°C for 30 sec, and a final extension at 72°C for 10 min. PCR products were electrophoresed on a 2% agarose gel along with size markers to detect the 116-bp PCR product. DNA extracted from LIVT-1 promastigotes was used a positive control, and a negative control without DNA was included. Sensitivity of the PCR assay was less than 1 organism (data not shown).

Direct transmission studies

To determine if the LIVT-1 strain of *L. infantum* was transmitted by direct contact, male mice were maintained to allow the mice to establish infections following sexual contact. After mating, male mice that bred LIVT-1–infected female mice were housed together for 12 wk. Male mice that bred control mice were housed together after breeding. Mice were bled from the retro-orbital plexus and killed 12 wk postbreeding. At necropsy, spleens were harvested and divided. Half of the spleen was used for parasite culture in *Leishmania* spp. culture media. The remaining spleen halves were subjected to PCR analysis as described above. Mouse sera were tested for anti–*L. infantum* antibodies by the indirect fluorescent antibody test (IFAT) as previously described (Rosypal, Zajac et al., 2005).

RESULTS

The results of culture and PCR analyses from the dams and offspring are summarized in Table I. None of the LIVT-1–inoculated mice or the HBSS-inoculated mice developed clinical signs of leishmaniasis. Of the 16 female mice inoculated with LIVT-1 promastigotes, infections were confirmed by culture and/or PCR analysis of splenic tissue in 15 mice. *Leishmania* sp. DNA was detected in placental tissue from 3 LIVT-1–inoculated mice. Promastigotes were not cultured from placentas from any of the mice. Cultures of mouse pups yielded all negative results, and 4 pups from infected mice were positive for PCR analysis. Spleens, placentas, and mouse pups from HBSS-inoculated mice were all negative by culture and PCR analyses.

Promastigotes were not cultured from the spleen tissue of male mice. *Leishmania* sp. DNA was demonstrable by PCR analysis in 1 of 8 male mice bred to LIVT-1–inoculated female mice. Spleens from males that mated HBSS-inoculated mice were negative by PCR analysis. The male mouse with the PCR-positive spleen tissue had an IFAT titer of 1:50. None of the other mice that were bred to either LIVT-1–inoculated female mice or HBSS-inoculated female mice developed antibody titers to *L. infantum*.

DISCUSSION

The results from this study suggest that BALB/c mice experimentally infected with a U.S. isolate of *L. infantum* are able to transmit the organisms by vertical and sexual transmission at a low level. *Leishmania* sp. PCR products were present in 4 of 88 (5%) mouse pups from LIVT-1–inoculated mice. The pups were most likely infected in utero. This is supported by the finding of *Leishmania* sp. DNA in 3 of 16 (19%) placentas from inoculated mice. In addition, the pups were removed from the dam before natural delivery, thereby eliminating the chance of infection during parturition. The possibility of placental tissues being present on the mouse pups cannot be eliminated, because the pups were not washed before attempting to isolate the parasites from the fetuses. *Leishmania* spp. DNA was detected in 1 of 8 (13%) male mice used to breed the infected mice. This evidence supports the possibility of direct or sexual transmission of *L. infantum* parasites.

There are 3 basic diagnostic methods used to detect *Leishmania* spp. infections: (1) parasitological diagnosis by microscopy or parasite culture, (2) anti-*Leishmania* spp. serological tests, and (3) molecular assays to detect *Leishmania* spp. DNA (Ferrer, 1999). Although in vitro cultivation provides definitive proof of the presence of parasites, this method suffers from low sensitivity (Piarroux et al., 1994), but the diagnostic dilemma can be overcome by the use of PCR analysis (Smyth et al., 1992; Ashford et al., 1995). Additionally, *Leishmania* spp. may not grow in culture if few parasites are present (Reale et al., 1999). No parasites were cultured from mouse pups, placentas, or spleens from male mice. This may be due to a low parasite load transmitted by the transplacental or sexual routes or to the inherent low sensitivity of the diagnostic test.

It is important for both human and veterinary medicine to understand the transmission dynamics of *L. infantum* in North America so that strategic control measures can be implemented. This work provides evidence for the nonvector transmission of a North American isolate of *L. infantum* in experimentally infected BALB/c mice. It is possible that a higher rate of parasite transmission occurs late in gestation, during natural delivery, or by nursing. Future studies should examine potential transmission during the perinatal period.

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