Transplacental Transmission of Cutaneous *Leishmania mexicana* Strain in BALB/c Mice

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Abstract. The vertical transmission of leishmaniasis has been reported in species that cause visceral leishmaniasis. However, this condition has scarcely been documented in species that cause cutaneous leishmaniasis. The aim of this study was to determine experimentally whether *L. mexicana* is transmitted vertically. A control group of BALB/c mice and a group infected with *L. mexicana* were mated, the gestation was monitored, and females were killed before delivery. Four resorptions ($P = 0.023$) and eight fetal deaths ($P = 0.010$) were observed in the infected female group; furthermore, the offspring body weight of the infected group was lower than the body weight of the healthy group ($P = 0.009$). DNA amplification by polymerase chain reaction (PCR) revealed that all placentas and maternal spleens as well as 39 of 110 fetal spleens obtained from the offspring of infected mothers tested positive for *Leishmania*. In conclusion, *L. mexicana* is transmitted transplacentally and causes fetal death, resorption, and reduction in offspring body weight.

INTRODUCTION

Leishmaniasis is a worldwide zoonotic disease. It is estimated that 14 million people are infected with the pathogen, with 2 million new cases per year and an at-risk population of 350 million distributed throughout 88 countries. Although they are considered endemic areas, these countries are not limited to migration related to tourism or labor activities. Clinically, leishmaniasis occurs in three different forms: cutaneous, mucocutaneous, and visceral. The forms of leishmaniasis are associated with the *Leishmania* species that parasitize the host. *L. donovani*, *L. aethiopica*, and *L. infantum* cause visceral leishmaniasis. *L. braziliensis*, *L. amazonensis*, and *L. major* cause cutaneous and mucocutaneous leishmaniasis. *L. amazonensis*, *L. braziliensis*, and *L. mexicana* cause localized and disseminated cutaneous leishmaniasis.

Under natural conditions, *Leishmania* parasites are acquired from infected hosts during feeding by the disease vector: females of the *Lutzomyia* or *Phlebotomus* genus. In recent years, cases in which individuals/animals have become infected by different routes, such as organ transplants, contaminated sharps, and sexual and vertical transmission,^{1,2} have become increasingly common.

The first reported case of vertical transmission occurred in 1926 in a pregnant woman who lived in India during the first trimester of her pregnancy. She was diagnosed and treated for leishmaniasis by *L. infantum* with a good response. She subsequently returned to her home country (England), and no complications were observed during delivery. However, during the first 1 year of life, her baby suddenly developed symptoms of leishmaniasis; a spleen biopsy was performed, and *Leishman-Donovan* bodies were observed. The child was treated, and the symptoms disappeared.³

Travel to endemic areas by women before and during pregnancy has been identified as a risk factor for vertical infection in children, even when the mothers do not develop symptoms.^{4–6} In many such cases, babies were delivered by caesarian section, ruling out infection by the birth canal.

Furthermore, because breastfeeding was not performed in these cases, parasite transmission seemed to be associated with the placental route. Among the changes that have been observed in the placentas of babies from mothers with visceral leishmaniasis, ischemic–thrombotic-type changes are particularly notable when babies have a low birth weight or fetal death occurs during pregnancy.^{7,8} Premature deliveries have been observed in cases of maternal infection with visceral leishmaniasis.⁹ Because of variability in the population and the lack of evidence linking leishmaniasis infection to pregnancy in these cases, several research groups have proposed infection models in which animals are infected with visceral strains.

In several trials conducted in canines using female dogs that were either naturally¹⁰ or experimentally¹¹ infected with *L. donovani* and *L. infantum*, the parasite has been detected by polymerase chain reaction (PCR) in the placenta and various organs of the progeny.¹² The presence or absence of symptoms in naturally infected females did not affect the likelihood of infection of the progeny.¹³ Furthermore, in murine models, vertical transmission after an inoculum of 1×10^6 parasites/mL *L. infantum* has been observed.¹⁴

Vertical transmission of cutaneous species has not been described in humans; however, some recent evidence suggests that it may occur. For example, in a study of human cutaneous leishmaniasis caused by *L. braziliensis*, 2 of 19 mothers experienced miscarriages, and 2 of 19 mothers experienced preterm deliveries.¹⁵ In an experimental model in which hamsters were infected using 10^6 parasites/mL *L. panamensis* during the first 1 week of pregnancy, producing an acute infection, 24 of 93 (25.8%) offspring from infected mothers were positive by PCR at 1 and 2 months after the birth.¹⁶ In mice that were infected with a *L. mexicana* strain that was considered to be cutaneous, the parasite was detected in internal organs by PCR.¹⁷ This finding leads us to believe that vertical transmission of cutaneous *Leishmania* species can occur, including possible transplacental transmission.

MATERIALS AND METHODS

Parasite cultivation. *L. mexicana* strain MHOM/BZ/61/M379 promastigotes were cultivated at 32°C in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). A parasite count was performed in a Neubauer chamber, and the concentration was

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adjusted to inoculate 10^6 parasites in 20 μ L. PBS was used to wash the parasites and as a vehicle for the inoculum.

Murine model. Four-week-old, pathogen-free BALB/c mice (*Mus musculus*), both female and male, were obtained from the vivarium of the National Institute of Perinatology in accordance with the international recommendations for the use of laboratory animals (World Medical Association in the Declaration of Helsinki). The animals were given *ad libitum* access to food and water, and the temperature of the vivarium was maintained between 21°C and 24°C. The female population was distributed randomly into two groups of 7–10 mice (experiment 1: 7 healthy and 7 infected; experiments 2 and 3: 10 healthy and 10 infected). On reaching 9 weeks of age, each of the female mice in the group selected for infection was subcutaneously injected in the right footpad 11 weeks before the mating period.

Reproduction and tissue procurement. The sizes of the inoculated footpads and body weights of all of the females were measured at 19 weeks. Each female was transferred to an individual box. The mating period was initiated when the mice were 20 weeks of age, at which point a healthy male mouse was placed into the box of each female. The females were checked daily over a 5-day period for the presence of a mucous plug, which is a positive sign of mating and an indicator of the possible onset of pregnancy. Day 1 was the detection of a mucous plug. The body weights and inoculated footpad sizes of the mice were recorded each week. Pregnancy was interrupted on the 20th day of gestation, when the females were killed. Tissue samples were obtained by an abdominal approach to avoid transmission by the birth canal or breastfeeding. The number of offspring was counted, and the litters were weighed. Using the parameters described above, we calculated the average body weight of the offspring in the litters. At necropsy, the maternal spleen and placenta and the fetal spleen were collected. All of the tissues were preserved at –70°C until PCR analysis was performed.

Purification of DNA and PCR analysis. Complete organs, spleens or placentas, were suspended in 500 μ L lysis buffer. To disrupt the tissue, we used tungsten beads (TissueLyser Stainless Steel Beads, 5 mm; QIAGEN, Hilden, Germany); homogenization was performed by blending the samples using a TissueLyser LT (QIAGEN, Hilden, Germany) for 1 minute. Saturated phenol (300 μ L; Sigma-Aldrich, St. Louis, MO) was then added to each of the samples, which were stirred and centrifuged at $1,000 \times g$ for 10 minutes. The aqueous phases of the samples were recovered and placed in microcentrifuge tubes. Subsequently, 150 μ L saturated phenol and 150 μ L chloroform (Sigma-Aldrich, St. Louis, MO) were added to the samples, which were stirred and centrifuged at $1,000 \times g$ for 10 minutes. The aqueous phases were then recovered, and 600 μ L chloroform were added before stirring the samples for 10 minutes. The samples were then centrifuged at $1,000 \times g$ for 10 minutes. The aqueous phases were recovered, and 30 μ L sodium chloride (pH 7.4) and 1 mL absolute ethanol (Sigma-Aldrich, CA) were added at 4°C. The samples were then mixed by inversion and centrifuged at $15,000 \times g$ for 20 minutes at 4°C. The supernatants were carefully decanted to avoid the loss of the precipitate; 800 μ L 70% ethanol were added to each of the samples, which were then centrifuged at $6,000 \times g$ for 5 minutes at 4°C. The supernatants were again carefully decanted to avoid the loss of the precipitate before being allowed to dry at room temperature.

Finally, the precipitates were rehydrated with 100 μ L ultrapure water and stored at –20°C. The PCR mixture was prepared using the Taq PCR Master Mix from QIAGEN (1.5 mM $MgCl_2$, 0.5 U/ μ L Taq DNA polymerase, 200 μ M 2-deoxyribonucleoside 5-triphosphates (dNTPs) and 10 $\times$ PCR Buffer QIAGEN), 10 pM each JW11 and JW12 primers,^{18,19} and genomic DNA. Assay samples, at a final volume of 25 μ L/vial, were generated for the healthy and infected mice, the positive controls (i.e., *L. mexicana* cultures), and the negative controls (i.e., samples without DNA). Using a gradient thermal cycler (Mastercycler Gradient; Eppendorf, Westbury, NY), end-point PCR was performed under the following conditions: initial denaturation at 94°C for 4 minutes, denaturation at 94°C for 1 minute, alignment at 58°C for 30 seconds, and elongation at 72°C for 30 seconds. Forty cycles were performed followed by a final extension stage at 72°C for 10 minutes and finally, a cooling step at 4°C. Subsequently, the vials were stored at a temperature of –20°C. A 2.5% ultrapure agarose gel (Invitrogen, Sao Paulo, Brazil) was prepared and diluted in Tris-Acetate-EDTA (TAE) buffer 1 $\times$ (containing 40 mM Tris-acetate and 1 mM EDTA, pH 8.3), which was boiled until complete dissolution was achieved. The samples were mixed with 1 μ L bromophenol blue loading dye (10 $\times$ BlueJuice Gel Loading Buffer; Invitrogen, Sao Paulo, Brazil), and a molecular weight marker (50-bp DNA ladder; Invitrogen, Sao Paulo, Brazil) was loaded in the first well; 5- μ L aliquots were taken from each PCR sample and added to the wells, and the gel was run at 60 V for 2 hours. The samples were then stained with 0.5 μ g/mL ethidium bromide (Ultrapure Ethidium Bromide; Invitrogen, Sao Paulo, Brazil) for 20 minutes and washed with bidistilled water before observation using a gel imaging system (AlphaImager HP system, Cell Biosciences, Inc., Santa Clara, CA). An amplification product of 120 bp was expected.

Statistical analysis. For comparing two groups of continuous variables, data were analyzed by the Students *t* test and Mann-Whitney *U* test for comparisons of categorical variables. A *P* value < 0.05 was considered statistically significant. Analysis was performed with SPSS, version 20, for Windows (SPSS Inc., Chicago, IL).

RESULTS

General conditions of the mice. As anticipated, the healthy female mice exhibited no symptoms of leishmaniasis, whereas the inoculated female mice displayed a single, bright, non-ulcerated nodule at the site of inoculation. The average footpad size in the infected females was 4.5 ± 0.5 mm; in contrast, the average footpad size in the healthy females was 3.0 ± 0.0 mm (*t* test, *P* < 0.001). Increased lesion sizes were observed only in the infected animals and resulted from the PCR-confirmed presence of parasites.

Weight gain. Before mating, the average body weight of the 20-week-old healthy females was 22.5 ± 1.7 g, and the average body weight of infected females of the same age was 22.5 ± 2.3 g (*t* test, *P* = 0.970). The average body weight gain observed during pregnancy was similar for both groups, which is presented in Table 1 (*t* test, *P* > 0.05).

Characteristics of gestation. The number of pregnant females was similar in both groups: 15 healthy and 13 infected mice (χ^2 , *P* = 0.785). The number of offspring produced in both groups was also similar: 120 pups were harvested from

TABLE 1

Maternal and fetal characteristics in experimental cutaneous leishmaniasis

Variable	Healthy (N = 27)	Infected (N = 27)	P value
General conditions of the population			
Size of the footpad at 20 weeks of age (mm)	3.0 ± 0.0	4.5 ± 0.5	< 0.001*
Weight of females at 20 weeks of age (g)	22.5 ± 1.7	22.5 ± 2.3	0.970*
Weight gain of pregnant mice after mating (g)			
Week 0	23.2 ± 1.9	22.9 ± 2.4	0.714*
Week 1	25.5 ± 2.5	24.3 ± 2.5	0.220*
Week 2	33.9 ± 7.0	32.5 ± 6.7	0.583*
Week 3 (day 20)	38.3 ± 6.7	35.0 ± 6.6	0.203*
Behavior of gestation			
Non-pregnant	12	14	0.785†
Pregnant	15	13	
Live offspring (n)	120	110	0.870‡
Live offspring median (range)	9 (3–11)	9 (6–11)	
Total deaths (n)	0	12	0.004‡
Fetal deaths	0	8	0.010‡
Resorptions	0	4	0.023‡
Offspring weight (average)	1.6 ± 0.4	1.3 ± 0.3	0.009*
PCR in tissues			
Maternal spleens	0/15	13/13	< 0.001‡
Placentas	0/120	110/110	< 0.001‡
Fetal spleens	0/120	39/110	< 0.001‡

*t test.

† χ^2 test.

‡Mann-Whitney U test.

healthy females, and 110 pups were harvested from infected females (Mann-Whitney U test, $P = 0.870$). However, eight dead offspring (Mann-Whitney U test, $P = 0.010$) and four resorptions (Mann-Whitney U test, $P = 0.023$) were produced in the infected group, whereas no events of this type were observed in the healthy group. The average body weight of the offspring from healthy females was 1.6 ± 0.4 g, whereas the average body weight of the offspring from infected females was 1.3 ± 0.3 g (t test, $P = 0.009$), which is presented in Table 1. Electrophoresis of the PCR products revealed that 100% of the maternal spleens and placentas collected from infected mothers tested positive for the presence of *Leishmania* species. As for the offspring, 39 of 110 fetal spleens from the infected female group tested positive for *L. mexicana*, exhibiting a 120-bp amplification product (Figure 1). All of the tissues from the healthy females and their offspring tested negative for *L. mexicana*.

DISCUSSION

The vertical transmission of leishmaniasis has been reported in humans and animals infected with the visceral species *L. donovani* and *L. infantum*.^{3,5,7,20,21} The presence of *Leishmania* parasites has previously been identified in the human placenta, revealing parasites both inside and outside macrophages, necrotic areas in the placental tissue, and compromised vascular tissue.⁷ In a canine animal model using Beagles, a canine breed that exhibits low levels of genetic variability, the presence of parasites in the maternal bone marrow, liver, spleen, and placenta of naturally infected females was shown by PCR.¹¹ However, the amount of the inoculum was not stated in the paper, and mating was conducted with a chronically infected male. Therefore, the

FIGURE 1. The presence of *L. mexicana* in maternal and fetal tissues. Measurements were performed by PCR in 2.5% agarose gel. Lane 1, 50-bp molecular weight marker; lane 2, infected maternal spleen; lane 3, healthy maternal spleen; lane 4, infected fetal spleen; lane 5, healthy fetal spleen; lanes 6 and 7, infected placenta; lane 8, healthy placenta; lane 9, negative control; lane 10, M379 axenic culture positive control. The size of the expected product is 120 bp.

contribution of unknown pre-disposing factors, such as the concentration of the inoculum and factors related to sexual transmission (a situation that has been described based on the appearance of genital lesions in humans^{22,23} and animals² harboring the visceral species), could not be excluded.

For cutaneous *Leishmania* species, such as *L. mexicana*, and *L. tropica*, the possibility of vertical transmission has not been considered, because it is typically thought that the parasite is limited to skin infections. However, recent data obtained using animal models of infection have revealed that dermal species may cause internal infections and colonize internal organs.^{24–26}

This study is one of the first experimental murine trials in which a cutaneous species, such as *L. mexicana*, was used to investigate the occurrence of vertical transmission through a transplacental route. To exclude confounding factors, our study used genetically identical animals that were infected with a reference strain of *L. mexicana*, the number of parasites inoculated into the females was controlled, the inoculated site was standardized across the groups, the post-inoculation period was defined, and matings were performed with healthy males. Furthermore, sacrificing pregnant females before delivery allowed us to evaluate the transplacental transmission of *Leishmania* parasites, ensuring that there was no contribution to vertical transmission of the birth channel during delivery or lactation in the post-natal days. Vertical transmission might explain the prevalence of this disease in pediatric patients who, at an early age, have exhibited visceral leishmaniasis caused by *L. mexicana*.²⁷

It has been shown that other protozoan parasites, such as *Plasmodium malariae*,²⁸ *Toxoplasma gondii*,^{29–31} and *Trypanosoma cruzi*,³² can cross the placental wall and affect both the mother and the neonate. The consequences of such infections by vertical transmission include abortion, perinatal death, maternal and fetal death, pre-term delivery, and intrauterine growth retardation. These effects have been reported in pregnant women infected with *L. donovani* and *L. infantum*.^{3–5,7,33} In our *L. mexicana* model of infection, no

maternal deaths occurred during pregnancy; however, eight fetal deaths and four resorptions were observed in the infected animals compared with none in the control group. The impact that *in utero* infection has on offspring development and survival needs to be further evaluated.

Maternal–fetal death has been reported in pregnant women with visceral leishmaniasis caused by *L. donovani*.³³ Spontaneous abortions with considerable damage to the placenta have been reported in patients exhibiting symptoms of leishmaniasis during the first trimester of pregnancy.⁷ In Beagles, perinatal death was observed in one of four pups from a chronically infected female.¹¹

In cases of cutaneous leishmaniasis in *L. braziliensis*-infected humans, a 10% miscarriage rate and a 10% pre-term delivery rate have been reported.¹⁵ In our study, using a murine animal model, we observed no decrease in the duration of pregnancy in infected mice compared with uninfected control mice; however, the infected females experienced fetal death and resorptions, whereas these symptoms were not observed in the healthy group. The number of live offspring was similar in both groups.

Birth weight is another factor that is affected during visceral infections by *Leishmania*; for example, in humans, babies with low birth weights from mothers infected with *L. donovani* have been reported.^{3,7} *Plasmodium* is another protozoan that causes intrauterine growth retardation²⁸; however, in our murine model, we observed that the average body weight per progeny was lower, and higher body weight variability was exhibited in the infected group compared with the healthy group. This result suggests that not all offspring become infected. This possibility was confirmed by PCR analysis, which revealed that the infection was present in 39 of 110 offspring from infected mothers. The weight loss observed in mothers with visceral leishmaniasis was not observed in the murine model of *L. mexicana*, and it was not observed before the initiation of the mating period with healthy males or during the gestation period.

L. mexicana infections did not affect the fertility of the females, and there are no publications on other species of *Leishmania* in which fertility has been reported to be affected. These results differ from other protozoan diseases, such as infection by *T. cruzi*, in which a decreased blood parasite load in response to benznidazole treatment increases the likelihood of pregnancy in infected mice.³⁴ Women living in malaria-endemic areas have reduced pregnancy rates relative to women who live in non-endemic areas.³⁵

Possible mechanisms for fetal damage include chronic infarction and ischemic alterations in the placental villi⁸; additionally, hematological and inflammation components, such as complement, cytokines, and prostaglandins, should be studied.

The results of the present study highlight the importance of considering cutaneous leishmaniasis to be a disease that affects the reproductive and maternal–fetal health; these findings should be further investigated in humans.

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