



Ciência Rural

ISSN: 0103-8478

cienciarural@mail.ufsm.br

Universidade Federal de Santa Maria  
Brasil

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Ciência Rural, vol. 47, núm. 1, 2017, pp. 1-4  
Universidade Federal de Santa Maria  
Santa Maria, Brasil

Available in: <http://www.redalyc.org/articulo.oa?id=33148021020>

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### Molecular evidence of early vertical transmission of *Leishmania (Leishmania) infantum* in a dog

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**ABSTRACT:** Visceral leishmaniasis (VL) is caused by the protozoan *Leishmania infantum*. Transmission of this parasite to hosts occurs mainly through the bite of infected sand flies. However, alternative infection routes have been hypothesized, especially in areas where the biological vector is absent. The exact time of infection and whether in utero transmission occurs have still not been fully elucidated. This report demonstrates molecular evidence of vertical transmission of *L. infantum* from a pregnant dog to the embryo. Samples (e.g. vulva, vagina, cervix, uterine body, uterine horn and ovaries) from a female naturally infected by *L. infantum* and from her embryo were molecularly analyzed by means of qPCR and cPCR followed by DNA sequencing. The gestational age was estimated to be  $23 \pm 1$  day. Through qPCR, the presence of *L. infantum* DNA was detected in all the samples analyzed ( $n=7$ ), including the embryo, conversely through cPCR, only four samples (vagina, cervix, uterine body and embryo) were positive. This study demonstrated that transmission of *L. infantum* from a pregnant dog to the embryo might occur in the early days of pregnancy. In conclusion, this is the first report showing *L. infantum* infecting a canine embryo.

**Key words:** leishmaniasis, dog, vertical infection, embryo.

### Evidência molecular da transmissão vertical precoce de *Leishmania (Leishmania) infantum* em um cão

**RESUMO:** Leishmaniose visceral (LV) é causada pelo protozoário *Leishmania infantum*. A transmissão deste parasito para hospedeiros é principalmente vetorial. No entanto, rotas alternativas de infecção têm sido investigadas, especialmente em áreas onde o vetor biológico está ausente. O período exato de infecção e se a transmissão in utero ocorre ainda não está totalmente elucidada. Este estudo demonstra uma evidência molecular da transmissão vertical de *L. infantum* de uma cadela prenhe para o embrião. As amostras (vulva, vagina, cérvix, corpo do útero, corno do útero e ovários) de uma fêmea naturalmente infectada por *L. infantum* e de seu embrião foram analisados molecularmente por meio de qPCR e cPCR, seguido de sequenciamento de DNA. A estimativa da idade gestacional foi de  $23 \pm 1$  dia. Na qPCR, a presença de DNA de *L. infantum* foi detectado em todas as amostras ( $n=7$ ) analisadas, incluindo o embrião. No entanto, na PCR convencional, apenas quatro amostras (vagina, cérvix, corpo do útero e embrião) foram positivas. Este estudo demonstrou que a transmissão de *L. infantum* de uma cadela prenhe para o embrião pode ocorrer nos primeiros dias de prenhez. Em conclusão, de acordo com o conhecimento dos autores, este é o primeiro relatório evidenciando infecção de embrião canino por *L. infantum*.

**Palavras-chave:** leishmaniose, cão, infecção vertical, embrião.

Visceral Leishmaniasis is an important parasitic disease that affect mammals and is primarily transmitted by insect vectors belonging to the sub-family *Phlebotominae*. Nonetheless, alternative transmission routes among dogs have been hypothesized in VL (OLIVEIRA et al., 2015). For instance, involvement of ticks in the transmission of *Leishmania infantum*, especially *Rhipicephalus*

*sanguineus* sensu lato, was first demonstrated in France in the 1930s, through observation of the ability of these ticks to mechanically transmit the protozoon to rodents (BLANC & CAMINOPETROS, 1930). Similarly, the potential role of fleas has already been assessed (FERREIRA et al., 2009), but the real role of these ectoparasites in the epidemiology of *L. infantum* remains a matter of controversy.

In addition, over the past few years, venereal and vertical transmissions of this protozoon have been demonstrated as possible infection routes, especially in areas where the biological vectors are absent (OLIVEIRA et al., 2015). This hypothesis is supported by the retrieval of viable forms of *Leishmania* spp. in semen of infected dogs (SILVA et al., 2009a). Indeed, it has been demonstrated that this type of infection reported above can occur between an infected male and a non-infected female (SILVA et al., 2009b).

Conversely, although vertical transmission in humans has been known for a long time (LOW & COOKE, 1926), this event has not been completely elucidated in dogs. In fact, so far, all cases of vertical transmission reported in the scientific literature have described transmission between the female and fetus or newborn puppies (NAUCKE & LORENTZ, 2012). The exact time of infection and whether *in utero* transmission occurs remain unknown. Therefore, the aim of this report was to provide molecular evidence of vertical transmission of *L. infantum* from a pregnant dog to the embryo in early stages of pregnancy.

A female mixed-breed dog naturally infected by *L. infantum* diagnosed by immunofluorescence antibody test (IFAT $\geq$ 40) and parasitological examination of the bone marrow (RAMOS et al., 2013) was used in this study. This animal came from the Zoonotic Disease Control Center of the city of Caruaru (8°16'59" S and 35°58'33" W), in the state of Pernambuco, Brazil.

During the necropsy, it was possible to observe that the animal was in an early stage of pregnancy. Therefore, the whole reproductive tract was carefully removed, put into a sterile plastic vial and sent to the laboratory. Subsequently, the uterus was opened and five embryos were found. Fragments from the vulva, vagina, cervix, uterine body, uterine horn and ovaries and from one embryo were collected and stored in sterile plastic tubes for molecular processing.

Genomic DNA (samples from vulva, vagina, cervix, uterine body, uterine horn, ovaries, and embryo) was extracted using the QIAamp DNA Blood and Tissue<sup>®</sup> kit (Qiagen<sup>®</sup>, Hilden, Germany), following the manufacturer's instructions. All the procedures were performed using micropipette barrier tips to prevent contamination.

The qPCR reactions were performed using the primers LEISH-1 (5'-AACTTTTCTGGTCCTCCGGGTAG-3') and LEISH-2 (5'-ACCCCCAGTTTCCCGCC-3') and the FAM probe (5'-AAAAATGGGTGCAGAAAT-3'-NFQ-MGB) that amplify the kinetoplast minicircle

DNA of *Leishmania* spp. (FRANCINO et al., 2006). The reaction mixture (12.5 $\mu$ L) contained 6.25 $\mu$ L of Taqman Universal PCR Master Mix, each primer at a concentration of 900nM, the probe at a concentration of 200nM and 100ng of template DNA. The amplification protocol consisted of initial denaturation at 95°C for 3 minutes, followed by 42 cycles of denaturation (at 95°C for 10 seconds) and annealing-extension (at 60°C for 30 seconds). All assays were carried out in triplicate, with each battery containing a negative control (bone marrow DNA from a dog that was negative in microscopic, molecular and serological examinations) and a positive control (DNA from an *in vitro* culture of *L. infantum*).

Positive qPCR samples were further tested by means of cPCR using the primers MC1 (5'-GTTAGCCGATGGTGGTCTTG3') and MC2 (5'-CACCCATTTTCCGATTTTG3') (CORTES et al., 2004). Amplicons (embryo and uterine body) were then purified using the Qiaex II kit (Qiagen<sup>®</sup>, Hilden, Germany) and sequenced in both directions by means of Sanger's method (SANGER et al., 1977) in an automated sequencer (ABI-3130; Applied Biosystems). Identity of the DNA sequences was determined by making comparisons with sequences available in Genbank<sup>®</sup> using the BLASTn search tool (ALTSCHUL et al., 1990).

Based on the macroscopic characteristics of the reproductive system (e.g. uterine edema) and embryo morphology (embryo in C shape), the canine gestational age was estimated as 23 $\pm$ 1 days. The embryo was 1cm in length and was particularly characterized by the presence of anterior and posterior limb buds.

Through qPCR, the presence of *L. infantum* DNA was detected in all the samples analyzed (n=7), including the embryo. Conversely, through cPCR, only four samples were reported to be positive (e.g. vagina, cervix, uterine body and embryo). The search for homology in the Genbank<sup>®</sup> database showed similarity of 98-99% (for both sequences) with DNA sequences of *L. infantum*. Sequences obtained in this study were deposited in Genbank<sup>®</sup> under accession numbers KT735370 and KT735371.

This report provides molecular evidence of vertical *in utero* transmission of *L. infantum* from a pregnant dog to the embryo in early days of pregnancy. Over the last years, the vectorial role of invertebrates in the transmission of *L. infantum* among mammals has been studied; however other potential routes of infection have been speculated. In dogs, it has been suggested that this transmission may occur through blood transfusion (OWENS et al., 2001), and through venereal and

vertical routes (OLIVEIRA et al., 2015). In fact, vertical transmission in these domestic carnivores was first demonstrated in Italy, where *L. infantum* parasites were isolated from the liver, spleen and lymph nodes of newborn puppies (MANCIANTI & SOZZI, 1995). However, this infection route remained controversial for a long time and several authors stated that this was an unusual transmission route. For instance, a previous study conducted in a Brazilian endemic area, in which 63 puppies from 18 naturally infected dogs were analyzed, concluded that *L. infantum* was not transmitted vertically, since at the end of the study, all the puppies were reported to be negative through molecular examination (ANDRADE et al., 2002). Conversely, more recently, this transmission route was demonstrated in Brazil (SILVA et al., 2009a), in the USA (BOGGIATTO et al., 2011) and in Germany (NAUCKE & LORENTZ, 2012). However, all cases of vertical transmission reported in the scientific literature so far have described transmission between a female and fetus or newborn puppies, but never in an embryo.

This is the first report of detection of *L. infantum* DNA in an embryo of estimated age  $23 \pm 1$  days. In this study, sampling of the embryo directly from the uterus prevented contact with the vaginal canal, thereby reducing the possibility of contamination. Indeed, *L. infantum* DNA has already been detected in the uterus of a pregnant female (ROSYPAL et al., 2005), and amastigote forms have been observed in the placental trophoblast (DUBEY et al., 2005). It has been demonstrated that circulation of parasites in the blood from the female to the placenta and annexes allows passage of *L. infantum* to the fetus (SILVA et al., 2009a).

The exact time of infection within vertical transmission is still unknown. Based on the findings of this report, the passage of *L. infantum* from a female to the embryo may occur in the early days of pregnancy. Therefore, this transmission route needs to be taken into consideration with regard to spreading of this infection among dogs. Finally, this is the earliest detection of *L. infantum* infecting a canine embryo.

## ACKNOWLEDGEMENTS

The authors thank the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for its financial support.

## BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The present study was approved by the Ethics Committee for Animal Experimentation of the Universidade

Federal Rural de Pernambuco (UFRPE) (protocol ECAE: 23082.015186/2012).

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