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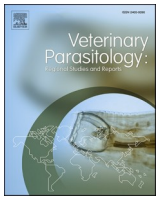

Regional report

Report of the presence of *Leishmania infantum* in the milk of a naturally infected female dog in Brazil

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ABSTRACT

Dogs are the most important reservoir of *Leishmania infantum*, the causal agent of visceral leishmaniasis in Brazil. Although lymphoid tissue is the most important biological tissue where amastigotes can be found, this paper describes the presence of *L. infantum* DNA in the milk of a lactating naturally infected female dog. This finding suggests the need for further studies to elucidate whether breastfeeding can be a route of infection.

1. Introduction

Canine leishmaniosis (CanL) is a serious and frequent disease worldwide. It is caused by *Leishmania infantum* transmitted during bloodmeal of sandflies of the genera *Lutzomyia* and *Phlebotomus*. It affects man and other animals and is considered to be an important zoonosis of which the dog is the main reservoir (Alvar et al., 2004; World Health Organization, 2017). CanL is manifested by a broad spectrum of clinical signs and degrees of severity, ranging from totally asymptomatic patients to those presented with different levels of dermopathy, lymphadenopathy, onycogryphosis, weight loss, abnormalities of the musculoskeletal system and ocular lesions (Alvar et al., 2004; Baneth et al., 2008; Ribeiro, 2020).

The sources of transmission of *Leishmania* protozoans among dogs, humans, and other animals, are matters of constant investigation. In dogs, several forms of transmission have been suggested besides the bite of infected sand flies. Blood transfusion, venereal and transplacental transmission have been proven (Boggiatto et al., 2011; da Silva et al., 2009; de Freitas et al., 2006; Pangrazio et al., 2009; Rosypal et al., 2005; Silva et al., 2008). As discussed before (Solano-Gallego et al., 2011), a suspected but unproven route includes direct dog-to-dog transmission through bites or wounds, which could explain the presence of autochthonous CanL clinical cases (Shaw et al., 2009) in non-endemic areas in the absence of apparent vectors, as described in foxhounds in the USA (Duprey et al., 2006) or in breeding kennels in Europe (Chamaillé et al., 2010). Moreover, transmission by other hematophagous arthropods such as ticks and fleas has been considered (Coutinho et al., 2005; Coutinho and Linardi, 2007; Dantas-Torres, 2011). Such parasite transmission sources require further investigation to elucidate their real role in the epidemiology of the disease (Solano-Gallego et al., 2011, Ribeiro, 2020).

Metzdorf et al. (2014) reported a case of a female dog infected by *L. infantum* in which 40 days postpartum and 20 days after weaning, the presence of amastigote forms in cytological and immunohistochemical examination was detected in mammary secretion and identified as *L. infantum* through PCR-RFLP of the ITS-1 region. Similarly, the possibility of elimination of parasitic forms of *L. infantum* via milk of lactating female dogs was suggested by Boechat et al. (2016), when the presence of amastigotes in mammary gland lumens of naturally infected female dogs was detected. Souza and Halverson (2019) reported the case of a female dog with a condition suggestive of Sticker tumor in the vulvar region and nodules in the inguinal breasts. Cytological examination of the content of these nodules revealed cellular atypia, inflammatory cells, and amastigote forms of *Leishmania* sp. Thus, this paper reports the

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finding of *L. infantum* DNA in the milk of a nursing female dog by molecular techniques.

2. Materials and methods

2.1. Sampling and previous laboratory tests results

In April 2021, a six-year-old female Keeshond dog was received in a veterinary hospital in Belo Horizonte, Brazil, which is an endemic region for CanL. The female had given birth to four puppies 20 days earlier. Three of them were stillborn and one was healthy and breastfeeding normally. This female dog had previous serological and molecular diagnosis of CanL as of April 2021 - optical density (OD) in ELISA 2220/0.620; 1:40 in indirect immunofluorescence reaction (IFAT), bone marrow *q*PCR with 822,739.44 parasite DNA copies/μL and negative PCR in skin samples of the inner surface of the ear tip. The patient was under medication with allopurinol (10 mg/kg twice a day) was clinically healthy and results of blood tests that included a complete blood count (CBC) and complete biochemistry panel were within normal limits except for the elevation of total globulins, 5.3 g/dL (reference values 2.5–4.5 g/dL) (Idexx Laboratories®). The milk was collected by finger milking of different teats, stored in sterile 1.5 mL microtubes and sent for molecular examination.

After 11 months, the female dog was well and clinical pathology exams were normal. The serological and molecular tests for CanL were repeated and the results obtained were an OD in ELISA of 3707/0.659; 1:1280 in IFAT; positive and strong reaction in SNAP Leish Idexx® (Maine, USA) and 174,000 parasite DNA copies/μl in bone marrow through qPCR. Besides, the dog owner was strongly recommended to maintain treatment and protective measures against the vector. A follow-up treatment was prescribed, and the patient was transferred to another veterinary practice by owner decision. The surviving puppy remained healthy until 11 months old and, according to the owners, remained negative for *Leishmania* infection.

2.2. Genotyping and parasite load quantification

Bone marrow and milk samples of the female dog were submitted to PCR. For bone marrow, sample gDNA (genomic DNA) was extracted with Wizard kit (Promega, Madison, WI, USA) according to manufacturer's recommendations and the reaction was performed in a real time PCR platform by using a pair of primers that amplifies a 90 bp single copy DNA polymerase gene (Forward 5′-TGT CGC TTG CAG ACC AGA TG-3′ and reverse 5′-GCA TCG CAG GTG TGA GCA C-3′) as described in Bretagne et al. (2001) and Celeste et al. (2017). For the milk samples collected, gDNA was extracted by using the Invitrogen® PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's recommendations, and submitted to three molecular approaches - conventional PCR, qPCR and PCR-RFLP - in attempt to detect the presence of *Leishmania* spp. gDNA (genomic DNA), for species characterization, and to quantify parasite load.

For conventional PCR, kinetoplast DNA (kDNA) minicircle sequence was used as a molecular target since that gene is present in large amounts in *Leishmania*, making the technique more sensitive as it is easier to detect minimal amounts of DNA. The primers used were 150–5′ (C/G)(C/G)(G/C) CC(C/A) CTA T(T/A)T TAC ACC AAC CCC 3′ and 152–5 ‘ GGG GAG GGG CGT TCT GCG AA 3’ (Degrave et al., 1994), which amplifies a 120 bp fragment visualized in 1% agarose gel.

PCR-RFLP technique was used to characterize the species of *Leishmania* involved in the infection by targeting ITS1 (Internal Transcribed Spacer – 1) region, that constitutes a non-coding transcribed region located between the small and large rRNA genes subunits generating a fragment of approximately 350 bp with the primers L5.8S – 5′ TGA TAC CAC TTA TCG CAC IT 3′ and L5.8SR – 5′- AAG TGC GAT AAG TGG TA 3′ (El Tai et al., 2000). The former reaction generated PCR products that were digested with *Hae*III restriction enzyme and the results were visualized in a 2% agarose gel, allowing the identification of the species involved in the infection (Schönian et al., 2003). AS positive controls, *Leishmania* reference strains *L. infantum* (MHOM/BR/74/PP75), *L. braziliensis* (MHOM/BR/75/M2903), *L. amazonensis* (IFLA/BR/67/PH8) and *L. guyanensis* (MHOM/BR/75/M4147) were used and autoclaved Milli-Q® water was used as negative control.

The qPCR method for parasite load assessment (DNA copies/μL) in milk sample was performed employing probe-based qPCR (Real-Time PCR with TaqMan Probe) method with standard curve and targeting kDNA minicircle sequence of L. *infantum* genome (TECSA Laboratories®, Belo Horizonte, Minas Gerais, Brazil).

3. Results

3.1. Conventional PCR

In conventional PCR agarose gel, amplicons with an expected size for *Leishmania* spp. gDNA were visualized as demonstrated in Fig. 1.

3.2. RFLP PCR

The PCR-RFLP technique also confirmed the presence of *Leishmania* spp. DNA in the milk sample (Fig. 2). Besides, the *Hae*III digest reaction generated fragments corresponding to the expected amplicon size visualized for the L. *infantum* reference strain (MHOM/BR/74/PP75) used in the Leishmaniasis Studies Laboratory – René Rachou Institute – Fiocruz Minas (Fig. 3).

3.3. qPCR

The parasite DNA load of *L. infantum* obtained using qPCR in milk was 683,686.87 DNA copies/μL (TECSA Laboratories®) (data not shown – laboratory confidentiality).

4. Discussion

The presence of *Leishmania* spp. amastigotes is described in several organs of infected dogs. In the epidemiological point of view cutaneous parasitism, very common in dogs, propitiates its main source of transmission to vertebrate animals, including humans, through the bite of infected sandflies (Alvar et al., 2004, Ribeiro, 2020). In addition to skin (Cavalcanti et al., 2012), parasitic forms have already been recorded in blood, through blood transfusion transmission in dogs and humans (de Freitas et al., 2006; L. P. Silva et al., 2020); in the nervous system (Giannuzzi et al., 2017; Llanos-Cuentas et al., 2013); in the heart (Santos et al., 2015); in the lungs (Goncalves et al., 2018); in bones and joints (Silva et al., 2021a; Wallborn et al., 2016), in the pancreas, spleen, liver and bone marrow (Kost et al., 2021), in the gastrointestinal tract (Pinto et al., 2011); in lymph nodes (de Vasconcelos et al., 2016), in the oral cavity (Pinna Parpaglia et al., 2007), in the kidneys (Soares et al., 2005), in the eye balls (Brito et al., 2010), in the adrenal glands (Momo et al., 2014), and in thymus (Silva et al., 2020a, 2020b).

Also, in the male genital tract the infection has already been described in the external (glans, urethra, foreskin, scrotum, smegma) and internal (testis, epididymis, prostate) genitalia (Amara et al., 2009; Boechat et al., 2020, 2016; Diniz et al., 2005; Manna et al., 2012, Manna et al., 2009; Oliveira et al., 2016a; da Silva et al., 2021a; Silva et al., 2021b; Silva et al., 2014). The presence of *Leishmania* was also identified in seminal fluid and in semen (Boechat et al., 2016; Diniz et al., 2005; Manna et al., 2012; Oliveira et al., 2016a).

In the female genital tract, external (vulva, vagina and mammary glands) and internal (ovary, uterus, placenta, cervix) genitalia were found to be infected with *Leishmania* in bitches with or without clinical signs of visceral leishmaniasis (Boechat et al., 2020, Boechat et al., 2016; Dubey et al., 2005; Magro et al., 2017; Oliveira et al., 2016b; da Silva et al., 2021a; Silva et al., 2008).

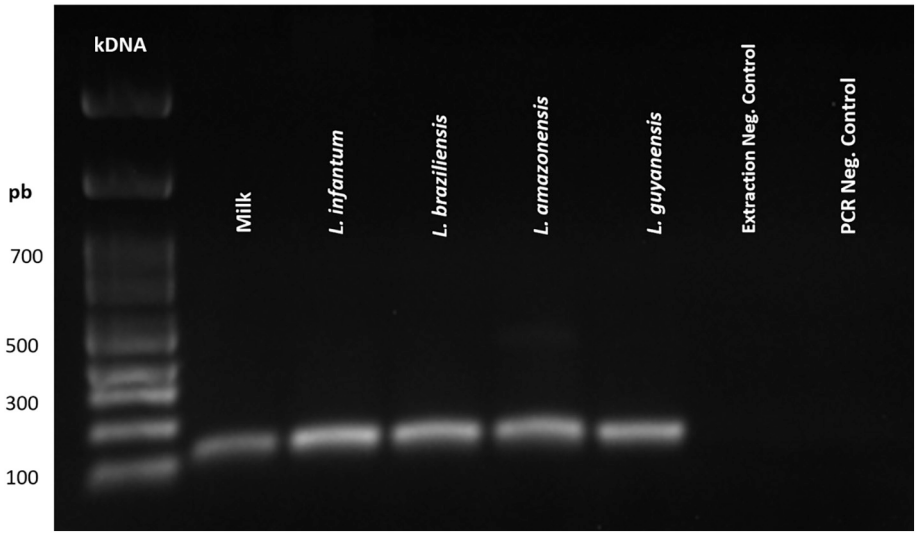

Fig. 1. Amplification profile of gDNA from milk sample from a female dog naturally infected with *Leishmania* spp. using kDNA genus specific primers. gDNA samples were amplified using a kDNA target pair of primers that amplified a 120 bp fragment. PCR products were visualized on 1% agarose gel. Milk corresponds to genomic DNA (gDNA) extracted from fraction of milk; *L. infantum*, *L. braziliensis*, L. *amazonensis* and *L. guyanensis*: gDNA from reference samples, respectively; Extraction Neg. Control and PCR Neg. Control: negative controls; pb: base pairs.

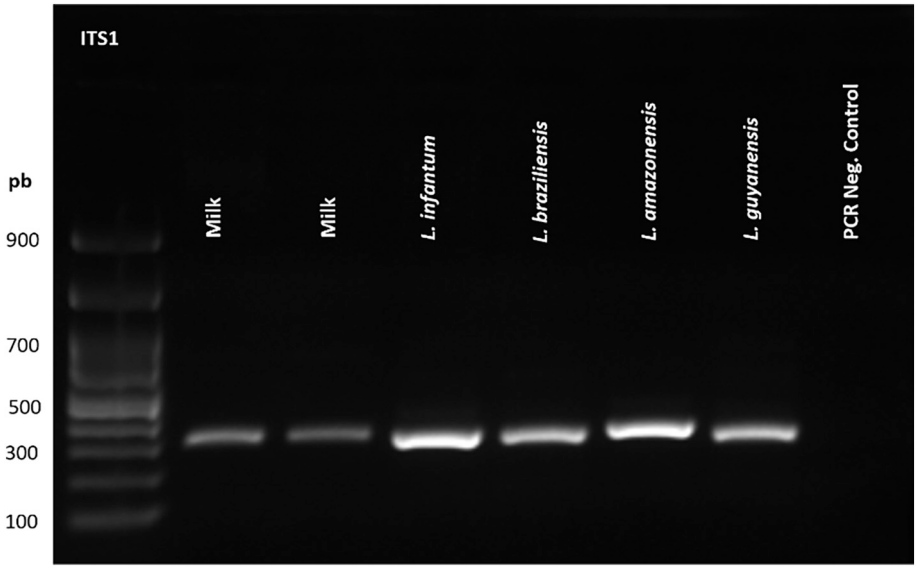

Fig. 2. RFLP-PCR amplification profile of gDNA from milk sample from a female dog naturally infected with *Leishmania* spp. using ITS-1 specific primers. gDNA samples were amplified using ITS-primers that amplified a 350 bp fragment. PCR products were visualized on 1% agarose gel. Milk corresponds to genomic DNA (gDNA) extracted from fraction of milk; *L. infantum*, *L. braziliensis*, *L. amazonensis* and *L. guyanensis*: gDNA from reference samples, respectively; PCR Neg. Control: negative control; pb: base pairs.

The frequency of parasitism in the genital tract of male and female dogs and the possibility of transmission of L. *infantum* through coitus and by the transplacental or vertical route have been adequately proven and should be considered in regions where the transmission through sandflies has not yet been recorded (Boechat et al., 2020; Boggiatto et al., 2011; Toepp et al., 2019).

Other ways of transmission, such as direct transmission from dog to dog by wounds and by other hematophagous arthropods, such as ticks and fleas have been suspected but not yet proven (Solano-Gallego et al., 2011). In humans, iatrogenic transmission through syringe sharing during injecting drug practice, especially in cases of HIV/*Leishmania* co-infection, is also a well described source of infection (Alvar et al., 1997).

No records of *Leishmania* parasitic forms in milk have so far been reported in humans, but in female dogs there are reports of amastigote forms found in cytological exams and of L. *infantum* identification in molecular tests of both mammary parenchyma and secretions, but not during the nursing period (Metzdorf et al., 2014; Boechat et al., 2016; Souza and Halverson, 2019).

On the other hand, Andrade et al. (2002) studying pregnant dogs naturally infected with L. *infantum* and their subsequent offspring, evaluated through culture in NNN/LIT medium, smears, and PCR samples on maternal milk from six lactating female dogs and had negative results.

Another publication studied 20 unneutered female dogs naturally infected with *L. infantum* that were euthanized and submitted to necropsy, and no macroscopic changes were observed in their mammary glands. Furthermore, histological analyses showed different levels of granulomatous inflammatory infiltrates that were associated or not to the presence of amastigotes, which varied in their intensity in the examined tissues (Boechat et al., 2016). These authors also observed that the detection of amastigotes in the lumen of the mammary glands of the studied female dogs indicated a possible elimination of this parasite by milk, but this was not proven.

The oral transmission of *L. infantum* was demonstrated in hamsters (*Mesocricetus auratus*) in two studies that used macerates of *Rhipicephalus sanguineus* and *Ctenocephalides felis felis*, respectively, taken from dogs naturally infected with *L. infantum* (Coutinho et al., 2005; Coutinho and Linardi, 2007). Recently, Reimann et al. (2022) demonstrated the occurrence of oral and intragastric *L. braziliensis* and *L. infantum* infection in hamsters (*M. auratus*) following the ingestion of promastigote or amastigote forms. These reports increase the importance of studies that evaluate the potential of the oral route in *L. infantum* transmission.

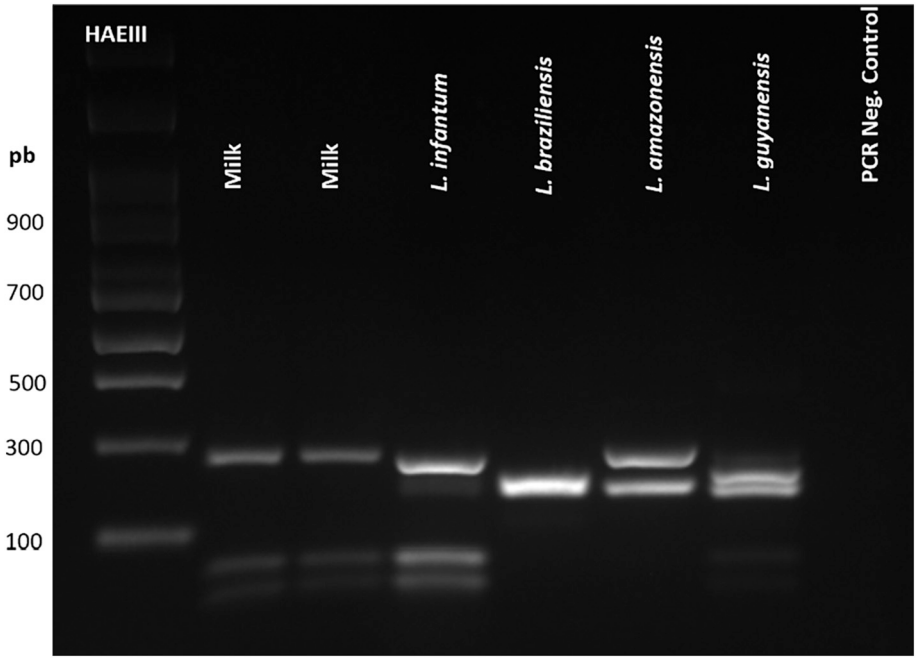

Fig. 3. RFLP-PCR internal amplification profile using *Hae*III restriction enzyme. gDNA samples were previously amplified using ITS-1 genus specific pair of primers and the product digested with HaeIII. Restriction fragments were visualized on 2% agarose gel. Milk corresponds to genomic DNA (gDNA) extracted from fraction of milk; *L. infantum*, *L. braziliensis*, *L. amazonensis* and *L. guyanensis*: gDNA from reference samples, respectively; PCR Neg. Control: negative control; pb: base pairs. The pattern observed for milk sample is the same observed in L. *infantum* positive control.

This paper reports the finding of *L. infantum* in the milk of a nursing female dog using molecular techniques. It is important to notice that this female dog did not show signs of mammary inflammation and no blood was observed in its milk. When compared to the study carried out by Martínez et al. (2011) in the bone marrow, the parasite load detected in milk in this study can be considered high, since those authors considered as high a load above 10,000 copies of *L. infantum* per milliliter of bone marrow content. In our report, the dog presented 683,686.87 copies of pathogen DNA/μL of DNA isolated from milk. Despite this high load, the female dog was in good health.

The occurrence of oral infection in hamsters (Coutinho et al., 2005; Coutinho and Linardi, 2007; Reimann et al., 2022), has opened a field of study in the same conditions with puppies during breastfeeding period.

As limitations of this report, we can mention the absence of a positive culture for *Leishmania* in milk, where the ability of the parasite to multiply and infect another animal could have been evaluated. Also, skin and bone marrow samples were not submitted simultaneously to the same molecular tests of milk samples which could have demonstrated differences in parasite load in the same scenario. The limited follow-up of the animal to repeat the exams was due to two main factors - the costs arising from examinations and procedures and the attachment of the owner to the animal, which limited the number of sample collections allowed.

On the other hand, the strong points of this unprecedented natural case were to report the importance of daily veterinary routine care in supplying scientific literature with relevant field data providing conditions to better understand the scenario of visceral leishmaniasis epidemiology and to contribute to its effective control in the future. It is important to emphasize that the partnership between a private institution (Veterinary Hospital) and a reference public laboratory (René Rachou Institute – Fiocruz Minas) supporting this type of study was of great relevance in the conduction of this report; this type of collaboration is of huge importance to raise the level of scientific publications.

Further studies are still needed to investigate the possibility of transmammary transmission of *L. infantum* amastigotes to lactating puppies and to elucidate its epidemiological role in canine disease.

Ethical statement

This study was carried out from a clinical service, in a private Veterinary Hospital (Santo Agostinho Hospital Veterinário) registered in Belo Horizonte, Brazil, in collaboration with René Rachou Institute (FIOCRUZ-MG) in the same city. Clinical examinations and treatments that animals were submitted was previously approved by their owners. Confidentiality and identity of patients and their owners may be preserved. A similar situation is present in other publications referenced in this study, such as da Silva et al. (2009) doi:https://doi.org/10.1016/j.vetpar.2009.08.011, Metzdorf et al. (2014) (don't have doi) and Souza et al. (2019) doi.org/10.31533/pubvet.v13n7a368.1-6, where no approvals from ethics committees were showed.

Declaration of Competing Interest

The authors declare no conflicts of interest related to the study.

Acknowledgments

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