ANIMAL SCIENCE

Anti-*Brucella canis* antibodies in dogs naturally infected with *Leishmania infantum* and associated histological alterations in the genital tract

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Abstract: In canine visceral leishmaniasis, coinfections can aggravate the disease. Our aim was to investigate *Brucella canis* in dogs infected with *Leishmania infantum*. One hundred and six *L. infantum*-seropositive dogs were submitted to serology for *B. canis*, PCR for *B. canis* and *L. infantum*, and histopathological analysis of the genital tract. Anti-*B. canis* antibodies were detected in seven dogs whose clinical signs, *L. infantum* load and histological alterations were similar to those of seronegative animals. The circulation of anti-*B. canis* antibodies was low but demonstrates the exposure of dogs to this bacterium in a visceral leishmaniasis-endemic area.

Key words: canine brucellosis, canine visceral leishmaniasis, histopathology, PCR, serology.

INTRODUCTION

Brucella canis is the main causative agent of canine brucellosis, a worldwide zoonotic disease. Transmission occurs by the venereal route and through contact of broken skin or mucosae with or ingestion of contaminated tissue or body fluids such as aborted fetuses, placenta and reproductive secretions. This bacterium can cause late abortion, as well as endometritis, epididymitis, prostatitis, and orchitis in dogs (Hollett 2006, Rodrigues et al. 2016).

The protozoan *Leishmania infantum* is the causative agent of canine visceral leishmaniasis (CVL), a zoonosis with a worldwide distribution whose main reservoir in urban areas is the dog (Oliveira et al. 2017). Despite the classical transmission of the parasite by the bite of infected phlebotomine sand flies, venereal and vertical transmission between dogs has been reported (Turchetti et al. 2014). Infection with *L. infantum* can cause orchitis, epididymitis, prostatitis, vaginitis, and endometritis (Boechat et al. 2020).

Coinfections of *L. infantum* with other pathogens can induce progression of the disease and increase mortality due to CVL (Toepp et al. 2019). However, the circulation of *B. canis* in dogs infected with *L. infantum* and the effects of this coinfection on the aggravation of CVL and canine brucellosis are unknown. Therefore, the aims of this study were to investigate the presence of *B. canis* in dogs naturally infected with *L. infantum* and their clinical signs, as well as the histological alterations and *L. infantum* load in the genital tract.

MATERIALS AND METHODS

Between January 2013 and December 2016, 106 domiciled dogs (63 males and 43 females) that tested seropositive for *L. infantum* in periodic serological surveys performed by public health services participating in the CVL surveillance and control program were included. The animals were from the municipality of Barra Mansa (22°32'38" S and 44°10'15" W), state of Rio de Janeiro, Brazil, a CVL-endemic area. The included dogs tested seropositive for anti-*L. infantum* antibodies by the rapid dual-path platform (TR DPP®) assay and enzyme immunoassay (ELISA), both produced by BioManguinhos (Fiocruz, Rio de Janeiro, Brazil). The procedures of this study were approved by the Ethics Committee on Animal Use (CEUA/Fiocruz; Permit Nos. LW-54/13 and LW-24/17).

After mechanical restraint, the dogs were evaluated clinically by inspection of the skin and mucosae, and palpation of the superficial lymph nodes and abdominal organs. Blood was collected from the dogs by puncture of the cephalic vein and the serum obtained was stored at -20°C for the gel immunodiffusion (AGID) assay using cell wall antigens of *B. ovis* to detect anti-*B. canis* antibodies. The dogs were then sedated by intramuscular administration of ketamine hydrochloride (10 mg/kg) and acepromazine maleate (0.2 mg/kg) and euthanized by intravenous injection of an overdose of sodium thiopental and potassium chloride. During necropsy, tissue samples were collected from the male (right and left epididymis, right and left testes, and prostate) and female genital tract (vulva, vagina, and body of the uterus). Three fragments were collected per tissue: the first fragment was immersed in sterile saline and submitted to parasitological culture for detection of *Leishmania*; the second fragment was stored at -20°C for subsequent analysis by

quantitative polymerase chain reaction (qPCR) for the detection of *L. infantum* and *Brucella* spp. DNA; the third fragment was fixed in 10% buffered formalin and embedded in paraffin (FFPE) for the diagnosis of *Leishmania* spp. by immunohistochemical and histopathological techniques and for the detection of *L. infantum* and *Brucella* spp. DNA by qPCR only in *B. canis*-seropositive dogs from which frozen genital tract samples were not collected.

The AGID assay was carried out using as antigen lipopolysaccharide and soluble proteins extracted from the cell wall of *B. ovis* strain Reo 198 (TECPAR – Instituto de Tecnologia do Paraná, Curitiba, PR, Brazil) according to manufacturer specifications. The serum samples were double tested and the test was interpreted by an experienced professional to avoid false-positive results.

Parasitological culture, histopathology and immunohistochemistry for the diagnosis of *Leishmania* spp., as well as qPCR for detection of *Brucella* spp. and *L. infantum* DNA and quantification of *L. infantum* load, were carried out following previously described protocols (Keid et al. 2007, Campos et al. 2017, Oliveira et al. 2017). The *L. infantum* load was expressed as the natural logarithm of the number of parasite genome equivalents (gEq)/ng.

RESULTS

Anti-*B. canis* antibodies were detected in seven (6.6%) of the 106 dogs examined (Table I). Six of these seven dogs (86%) exhibited clinical signs compatible with CVL, including lymphadenomegaly (71%), cutaneous ulcers (71%), splenomegaly (57%), alopecia (43%), onychogryphosis (43%), skin desquamation (43%), and thinness (43%) (Table I). Six of these seven dogs (86%) tested positive for *Leishmania*

Table I. Clinical data, histological alterations and *Leishmania infantum* positivity and load in the genital tract of seven dogs seropositive for anti-*B. canis* antibodies between January 2013 and December 2016 (Barra Mansa, Rio de Janeiro, Brazil).

Dog	Sex	Breed	Age (years)	Clinical signs	Histological alterations	*Leishmania* in genital tract (PC, IHC, HP and qPCR)	*L. infantum* load[a]
1	M	Mongrel	4	Thinness, alopecia, desquamation, cutaneous ulcers, lymphadenomegaly, splenomegaly, onychogryphosis	Moderate to intense bilateral granulomatous orchitis, seminiferous tubule atrophy and degeneration; moderate to intense, bilateral granulomatous epididymitis	T (+); E (+); P (-)	T=6.87; E=6.30
2	M	Mongrel	3	Thinness, alopecia, desquamation, cutaneous ulcers, lymphadenomegaly, splenomegaly, onychogryphosis	Moderate to intense bilateral granulomatous orchitis, seminiferous tubule atrophy and degeneration; moderate to intense unilateral granulomatous epididymitis; mild lymphoplasmacytic prostatitis with glandular fibrosis and atrophy	T (+); E (+); P (+)	T=13.93; E=10.53; P=13.27
3	M	Mongrel	2	Lymphadenomegaly, splenomegaly	Moderate to intense bilateral granulomatous epididymitis	T (-); E (+); P (-)	NQ
4	M	Pitbull	9	Cutaneous ulcer, lymphadenomegaly	Mild bilateral granulomatous orchitis; moderate to intense bilateral granulomatous epididymitis; moderate to intense lymphoplasmacytic prostatitis with cysts and glandular fibrosis and atrophy	T (+); E (+); P (+)	T= -1.91; E=2.64; P= -1.04
5	M	Mongrel	2	Cutaneous ulcer	Absent	T (-); E (-); P (-)	NQ
6	F	Mongrel	2	Thinness, alopecia, desquamation, cutaneous ulcers, lymphadenomegaly, splenomegaly, onychogryphosis	Moderate to intense granulomatous vulvitis; mild non granulomatous vaginitis; Endometrial hyperplasia	Vu (+); Va (+); U (+)	Vu=4.73; Va=10.73; U=12.34
7	F	Mongrel	3	Absent	Moderate to intense granulomatous vulvitis; mild non granulomatous vaginitis	Vu (+); Va (+);U (-)	NQ

a= *L. infantum* load expressed as the natural logarithm of the number of parasite genome equivalents (gEq)/ng; M= male; F= female; PC= parasitological culture; IHC= immunohistochemistry; HP= histopathology; qPCR= quantitative polymerase chain reaction; T= testis; E= epididymis; P= prostate; Vu= vulva; Va= vagina; U= uterus; (+)= positive for *Leishmania* in at least one test; (-)= negative in all *Leishmania* tests; NQ= not quantified.

in the genital tract by at least one of the diagnostic methods used (Table I). The main histological alterations observed in the seven dogs with anti-*B. canis* antibodies were granulomatous epididymitis (100%), vulvitis (100%), orchitis (75%), lymphoplasmacytic vaginitis (100%), and lymphoplasmacytic prostatitis with glandular fibrosis and atrophy (50%) (Table I and Figure 1).

Quantitative PCR for detection of *L. infantum* and *Brucella* spp. DNA was performed using genital tract samples from 74 of the 106 dogs included in the study; frozen samples were examined in 70 and FFPE samples in 4. These four dogs were seropositive for *B. canis* and only FFPE samples of their genital tract were available. In

32 dogs, qPCR was not performed because only FFPE samples of the genital tract were available and the animals were seronegative for *B. canis*.

Among the 74 dogs examined by qPCR, seven were positive (Table I) and 67 (43 males and 24 females) were negative for anti-*B. canis* antibodies and all tested negative for *Brucella* spp. DNA by PCR. In the seven dogs positive for anti-*B. canis* antibodies, the *L. infantum* load ranged from -1.91 to 13.93 gEq/ng in the genital organs of four dogs (Table I). In the 67 animals negative for anti-*B. canis* antibodies, the *L. infantum* load in the genital organs ranged from -2.04 to 18.81 gEq/ng in testes, from -1.16 to 15.34 gEq/ng in the epididymis, from 1.10 to 8.76 gEq/
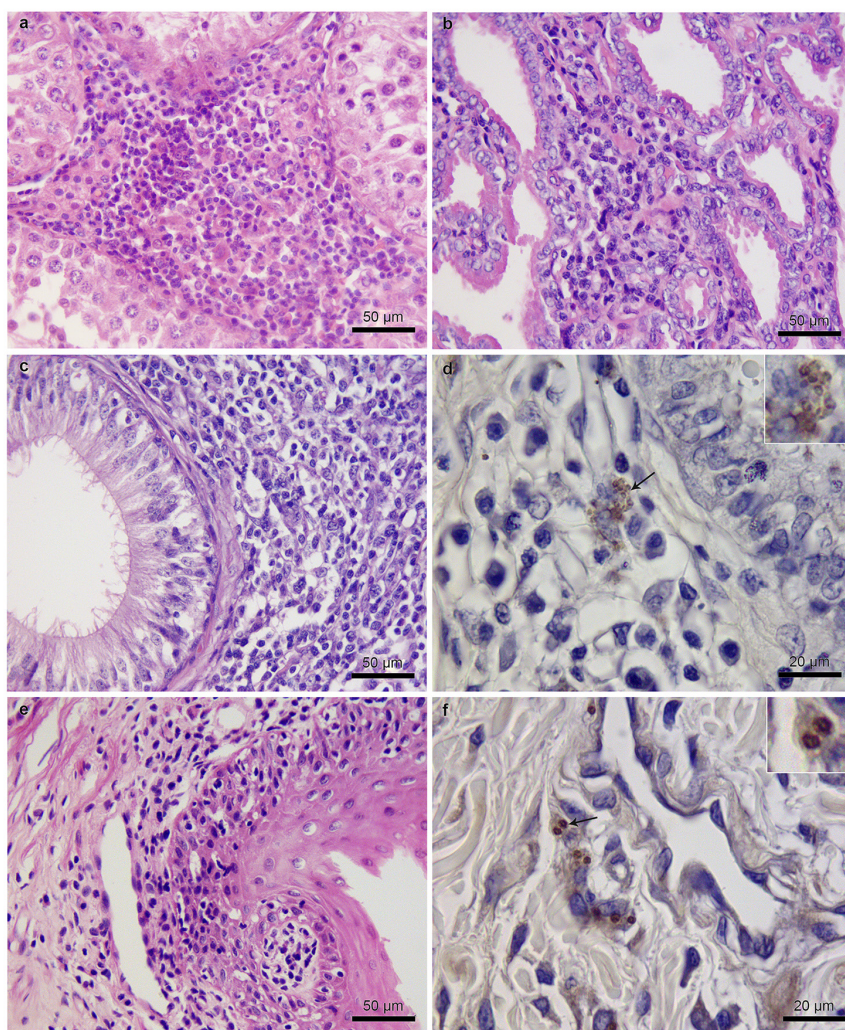

Figure 1. Dogs seropositive for anti-*Brucella canis* antibodies and infected with *Leishmania infantum*. (a) Male #1. Moderate to intense granulomatous interstitial orchitis. (b to d) Male #2. (b) Mild lymphoplasmacytic prostatitis. (c) Moderate to intense granulomatous epididymitis. (d) Brown-stained amastigote forms of *Leishmania* in the cytoplasm of macrophages (arrow and inset) in the epididymis. (e to f) Female #6. (e) Mild vaginitis in the lamina propria and perivascular region consisting of macrophages, lymphocytes and neutrophils. (f) Brown-stained amastigote forms of *Leishmania* in the cytoplasm of macrophages (arrow and inset) in the vulva. Hematoxylin-eosin staining (a, b, c, e). Immunohistochemistry (d, f).

ng in the prostate, from 0.50 to 18.40 gEq/ng in the vulva, from 1.10 to 16.49 gEq/ng in the vagina, and from 0.69 to 14.18 gEq/ng in the uterus.

Considering all dogs with negative anti-*B. canis* antibodies in this study (n = 99, 58 males and 41 females, 67 tested and 32 not tested by qPCR for detection of *L. infantum* and *Brucella* spp. DNA), 89 (90%) had clinical signs compatible with CVL. These clinical signs were lymphadenomegaly (53%), alopecia (46%), onychogryphosis (45%), splenomegaly (39%), skin desquamation (38%), cachexia (13%), thinness (13%), keratoconjunctivitis (12%), and pale mucosae (4%). Ninety-two (93%) of these 99 dogs, 54 males and 38 females, tested positive for *Leishmania* in the genital tract by at least one of the diagnostic methods used.

Histopathology in these 99 dogs revealed granulomatous inflammation in the vulva (63.4%), epididymis (60.3%), testes (51.7%), vagina (36.5%), uterus (14.6%), and prostate (3.4%); lymphoplasmacytic inflammation in the prostate (27.5%), vagina (14.6%), vulva (14.6%), testes (3.4%), epididymis (3.4%), and uterus (2.4%); seminiferous tubule degeneration (48.2%) and atrophy (37.9%) in the testes; fibrosis and glandular atrophy in the prostate (12.0%), and fibrosis (6.8%) and necrosis (1.7%) in the testes.

DISCUSSION

The detection of anti-*B. canis* antibodies was low, in agreement with most studies conducted on dogs in Brazil, in which the frequencies of seropositivity ranged from 0.8 to 14% (Rodrigues et al. 2016). In contrast, few studies found frequencies of seropositivity higher than 20% in Brazil (Rodrigues et al. 2016). This variation in *Brucella* seropositivity might be related to the different geographic regions analyzed and the different diagnostic tests used in these

studies (AGID, rapid plate agglutination test with and without 2-mercaptoethanol, and tube agglutination test).

A comparative study of serological techniques for the diagnosis of canine brucellosis was conducted on 127 dogs using the results of blood culture, culture of semen/vaginal swab, and PCR in blood and semen/vaginal swab as reference standards (Keid et al. 2009). That study demonstrated that, although AGID using the same antigen from the same manufacturer as employed in the present study has limited sensitivity (52.9%), its specificity is high (100%). Considering the high specificity of AGID, the negative qPCR result for *Brucella* spp. in the genital tract of dogs that tested seropositive for this bacterium may be due to the absence of bacteremia, although infection persisted in these dogs (Gordon et al. 1985, Wanke 2004, Keid et al. 2009). Additionally, active infection with this bacterium may have occurred in tissues other than those examined, such as spleen, lymph nodes and intervertebral discs (Hollett 2006). It is also possible that *B. canis*-seropositive dogs did not have active infection, but that anti-*B. canis* antibodies persisted in blood since the AGID using cell wall antigens can detect these antibodies for up to 4 months after the animal is abacteremic (Wanke 2004).

The AGID using cell wall antigens of *B. ovis* or *B. canis* and serological tests using *B. ovis* antigens are considered to have low specificity (Hollett 2006, Cosford 2018), while the AGID used in the present study shows high specificity (Keid et al. 2009). This discrepancy between the results may be related to the use of different protocols and *B. ovis* strains for antigen production and to the application of different reference standards in the evaluation of specificity of the serological tests (Keid et al. 2009).

Cosford (2018) recommends PCR combined with serology for the detection of *B. canis* because

of the possibility of false-negative results by culture, which is used as a confirmatory test. Considering the high sensitivity of qPCR (Keid et al. 2009, Hensel et al. 2018) and its combination with AGID in this study, the possibility of false-negative *Brucella* spp. results is low in the 67 *B. canis*-seronegative dogs in which qPCR was also performed. However, we cannot rule out the possibility of false-negative results in the 32 *B. canis*-seronegative dogs that were tested only by AGID due to the low sensitivity of this test (Keid et al. 2009). In these 32 dogs, the use of PCR and culture of blood and/or genital organs would have helped confirm the absence of *B. canis* infection.

Granulomatous inflammation observed in the genital tract is compatible with *L. infantum* and *B. canis* infections (Hollett 2006, Boechat et al. 2020). This fact highlights the importance of including canine brucellosis in the differential histological diagnosis of CVL in the genital tract. However, the negative qPCR result for *Brucella* and detection of *L. infantum* in the genital tract of six of the seven dogs seropositive for *B. canis* suggest that granulomatous inflammation was associated with *L. infantum* infection. Furthermore, seropositivity for *Brucella* spp. does not appear to have influenced the frequency and type of clinical signs or *L. infantum* load since these results were similar in dogs with and without anti-*B. canis* antibodies. However, the participation of *Brucella* should not be completely ruled out since the *L. infantum* load in the prostate of one dog seropositive for *B. canis* (13.27 gEq/ng) was higher than that found in the prostate of dogs infected only with *L. infantum* (1.10 to 8.76 gEq/ng).

In conclusion, the low frequency of anti-*B. canis* antibodies and the lack of detection of DNA of this bacterium in the canine population studied suggest that *L. infantum* infection does not increase the susceptibility to infection with

B. canis. However, seropositivity for *B. canis* demonstrates the exposure of dogs to this bacterium in a visceral leishmaniasis-endemic area and is an alert to include canine brucellosis in the differential diagnosis of CVL.

Acknowledgments

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Author contributions

VCB and RCM conceived and designed the study; VCB, RCM, AAVMJ, AFV, LFCM, LCF, JM, MGD, ROR, RTF and RVB performed the experiments; VCB and RCM analyzed the data; VCB, RCM, SAP and FBF wrote the manuscript; RCM, FBF and SAP secured funding and resources; RCM supervised the study; all authors read and approved the final manuscript.