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Co-infection by tick-borne pathogens and *Leishmania* spp. in dogs with clinical signs suggestive of leishmaniasis from an endemic area in northeastern Brazil[1]

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ABSTRACT.- Evaristo A.M.C.F., Santos P.T.T., Sé F.S., Collere F.C.M., Silva B.B.F., Cardoso E.R.N., Kakimori M.T.A., Vieira T.S.W.J., Krawczak F.S., Moraes-Filho J., Vieira R.F.C. & Horta M.C. 2024. **Co-infection by tick-borne pathogens and *Leishmania* spp. in dogs with clinical signs suggestive of leishmaniasis from an endemic area in northeastern Brazil.** *Pesquisa Veterinária Brasileira 44:e07437, 2024.* Laboratório de Doenças Parasitárias, Universidade Federal do Vale do São Francisco, Rodovia BR-407 Km 12 Lote 543, Projeto de Irrigação Nilo Coelho, Petrolina, PE 56300-000, Brazil. E-mail: anna.evaristo@ufca.edu.br

The present study aimed to investigate the occurrence of *Leishmania* spp., hemotropic *Mycoplasma* spp., tick-borne pathogens (TBP), and co-infection in dogs with clinical signs suggestive of visceral leishmaniasis (VL). It also aimed to determine the factors associated with infection and to map the distribution of co-infected dogs in an endemic area in the Northeast region of Brazil. Blood samples from 168 dogs were evaluated for serological analysis to *Leishmania* spp., *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., and molecular assays to *Leishmania* spp., *Anaplasma platys*, *Ehrlichia canis*, *Babesia* spp., and hemotropic *Mycoplasma* spp. In serological and molecular analysis, 29.8% and 5.9% of dogs were co-infected. In the regression analysis, seropositivity for *Ehrlichia* spp., *Babesia* spp., and *Leishmania* spp. was significantly associated with the presence of petechiae, young dogs, and weight loss. Serology revealed that co-exposure with *Babesia* spp. and *Ehrlichia* spp. was associated with fever and thrombocytopenia, and there was an association between seropositivity for *Ehrlichia* spp. and *Babesia* spp. in dogs seropositive for *Leishmania* spp. The presence of hemotropic *Mycoplasma* spp. DNA was associated with anorexia. Thus, dogs with clinical VL have co-infection with other pathogens, reinforcing the importance of this study for a better understanding of these co-infections in dogs from endemic areas.

INDEX TERMS: *Ehrlichia*, *Anaplasma*, hemotropic *Mycoplasma*, *Babesia, Leishmania*, diagnosis, dogs, leishmaniasis.

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RESUMO.- [Co-infecção de patógenos transmitidos por carrapatos e *Leishmania* sp. em cães com sinais sugestivos para leishmaniose em uma área endêmica no nordeste do Brasil.] O presente estudo objetivou investigar a prevalência de *Leishmania* spp., *Mycoplasma* spp. hemotrópico, patógenos transmitidos por carrapatos (PTC), e coinfecção em cães com sinais clínicos sugestivos de leishmaniose visceral (LV), determinar os fatores associados à infecção, e mapear a distribuição de cães coinfectados em uma área endêmica no Nordeste do Brasil. Amostras de sangue de 168 cães foram avaliadas por análises sorológicas para *Leishmania* spp., *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., e ensaio molecular para *Leishmania* spp., *Anaplasma platys*, *Ehrlichia canis*, *Babesia* spp., e *Mycoplasma* hemotrópico. Pelas análises sorológicas e moleculares, 29,8% e 5,9% dos cães apresentaram coinfecção, respectivamente. Na análise de regressão, a soropositividade para *Ehrlichia* spp., *Babesia* spp., e *Leishmania* spp. foram significantemente associadas com a presença de petéquias, cães jovens, e perda de peso. O diagnóstico sorológico revelou que a coexposição à *Babesia* spp. e *Ehrlichia* spp. está associada com febre e trombocitopenia, havendo associação entre a soropositividade para *Ehrlichia* spp. e *Babesia* spp. em cães soropositivos para *Leishmania* spp. A presença de DNA de *Mycoplasma* foi associada à anorexia. Desta forma, cães com sinais de LV possuem coinfecção com outros patógenos, reforçando a importância deste estudo para um melhor entendimento dessas coinfecções em cães de áreas endêmicas.

TERMO DE INDEXAÇÃO: *Ehrlichia*, *Anaplasma*, *Mycoplasma* hemotrópico, *Babesia, Leishmania*, diagnóstico, cães, leishmaniose.

INTRODUCTION

Canine visceral leishmaniasis (CanVL) is a major zoonotic disease caused by *Leishmania infantum* and transmitted through sand fly vectors during blood-feeding (Attipa et al. 2018, Medkour et al. 2020). Brazil accounts for the highest number of human visceral leishmaniasis (VL) cases in Latin America (PAHO 2021). The majority of the Northeast region of Brazil is considered endemic (Machado et al. 2021) because of the widespread prevalence of *Lutzomyia longipalpis*, favorable climate, and poverty in many areas (Reguera et al. 2016).

Dogs are the main domestic reservoirs of CanVL and represent a major source of vector infection (Coura-Vital et al. 2013). The seroprevalence of *L. infantum* among dogs in the Brazilian Northeast region may range from 11 to 55.8% (Lira et al. 2006, Queiroz et al. 2010, Pimentel et al. 2015, Araujo et al. 2016, Mendonça et al. 2017, Silva et al. 2017, Evaristo et al. 2020, 2021). A previous study in the Petrolina municipality, located in a Semi-arid region of northeastern Brazil, found a CanVL seroprevalence of 11.2%, with 60.7% of the evaluated seropositive dogs presenting clinical signs suggestive of the disease at the time of sampling (Araujo et al. 2016). However, some CanVL clinical signs are similar to those of other tick-borne diseases (Chalker 2005, Sainz et al. 2015), which may complicate disease diagnosis, treatment, and prognosis (De Tommasi et al. 2013).

Tick-borne pathogens (TBP), such as *Ehrlichia canis*, *Babesia vogeli, Anaplasma platys,* and hemotropic *Mycoplasma* spp. are globally prevalent among dogs (Izzi et al. 2013, Vieira et al. 2013b, Araujo et al. 2015, 2016, Bouzouraa et al. 2017, Attipa et al. 2018, Toepp et al. 2019, Dantas-Torres et al. 2020, Evaristo et al. 2020). Some of these TBP and hemoplasma species are also of public health concern (Maggi et al. 2013b, Vieira et al. 2013a, Krawczak et al. 2015, Gizzarelli et al. 2019). Additionally, co-infection with more than one pathogen in dogs is a common clinical observation (De Tommasi et al. 2013, Vieira et al. 2013b) owing to the ability of arthropod vectors to host and simultaneously transmit several pathogens (Leitner et al. 2015, Gizzarelli et al. 2019).

Co-infections by TBP in dogs with CanVL can result in more severe clinic pathological abnormalities than in dogs with only CanVL, worsening the clinical status and making veterinary diagnosis difficult (Attipa et al. 2017, 2018). Several studies have described co-infection of *L. infantum* with other TBP in dogs with suggestive clinical signs of leishmaniosis (Cardinot et al. 2016, Baxarias et al. 2018, Attipa et al. 2018, Toepp et al. 2019).

Studies that aim to identify the main co-infections, clinical alterations, hematologic abnormalities, and factors associated with infection by these agents can contribute to a more effective veterinary diagnosis. Consequently, they can improve the understanding of the types of pathogenic co-infections found more frequently in veterinary practice (Attipa et al. 2018), assisting in choosing the best therapeutic approach and prognosis for infected animals (Cardinot et al. 2016).

Although the co-infection with other TBP in dogs infected with *L. infantum* may occur in VL endemic areas, no study has so far focused on detecting these pathogens and their co-infections in dogs in the municipalities of this region. Therefore, this study aimed to detect TBP, hemoplasma, and *L. infantum*, using different serological and molecular techniques, observing spatial distribution and factors associated with infection in dogs from an area endemic to CanVL.

MATERIALS AND METHODS

Ethical approval. This study was approved by the Ethics Committee on the Use of Animals (CEUA) from the "Universidade Federal do Vale do São Francisco" (Univasf) (0009/270619).

Study area. The study was performed in the Petrolina municipality (9°23′55″ S; 40°30′3″ W), located in the Semi-arid region of Pernambuco state, Northeast region of Brazil. The municipality occupies an area of 4,561.72km, with an estimated population of 326,017 in 2010 (IBGE 2010). Petrolina is situated within the Caatinga biome, at an altitude of 420m, and presents stretches of hyper-xerophilic deciduous forest (IBGE 2010). The region has a hot Semi-arid climate (Köppen climate classification BSh), with an average annual temperature of 26.3°C and average rainfall of 443mm/year. The region has a high number of human VL cases (Araujo et al. 2016) and is classified as an area of moderate transmission (Brasil 2019).

Sampling. Non-probabilistic convenience sampling was performed. From March 2019 to August 2021, 168 dogs (78 males and 90 females) were evaluated. The inclusion criterion for this study was that dogs should present one or more clinical manifestations of CanVL (Solano-Gallego et al. 2009). Dogs were physically examined, and the following clinical signs were recorded: Apathy, fever, weight loss, skin lesions, ocular lesions, pale mucous membranes (ocular and oral), petechiae, lymphadenomegaly (evaluation of the main popliteal, prescapular, and submandibular lymph nodes), cachexia, onychogryphosis, muscular atrophy, splenomegaly, hepatosplenomegaly, vomiting, diarrhea, joint pain, polyuria, polydipsia, and lameness. Blood samples were collected by venipuncture of the jugular or

cephalic vein using tubes without anticoagulant and kept at room temperature (25°C) until visible clot formation. The samples were centrifuged at 1,500 × g for 5 min, and serum was separated and stored at -20°C for serological testing. In addition, blood samples were collected in tubes containing ethylenediaminetetraacetic acid (BD Vacutainer Franklin Lakes/NJ, USA) for hematological and polymerase chain reaction (PCR) analysis and stored at -20°C until molecular testing.

A comprehensive epidemiological questionnaire was provided to each dog tutor addressing breed (mongrel or pure breed), sex (male or female), age (<12, ≥12 to <84, or ≥84 months), size (small, medium, or large), living in urban or rural areas, and the presence of ticks at the time of sampling. Additionally, animals were classified according to the clinical staging of CanVL as Stage I (mild disease), Stage II (moderate disease), Stage III (severe disease), or Stage IV (very severe disease), as previously described (Solano-Gallego et al. 2009).

Tick specimens infesting dogs were retrieved and placed in absolute ethanol-labeled tubes for identification according to the morphological taxonomic keys (Šlapeta et al. 2022).

Hematological analysis. For blood cell count, the samples were analyzed using an automatic cell counter (Automatic Hematology Analyzer, BC-5000Vet Mindray®) (Schalm 2010). Blood smears were stained using a Romanovsky-type stain (Renylab®, Barbacena/MG, Brazil). They were directly examined in each smear for observing TBP, hemoplasmas and differential counts of WBC using light microscopy at 1,000 × magnification.

Serological testing. Anti-*Leishmania* immunoglobulin IgG antibodies were detected using a rapid immunochromatographic test (ICT) (DPP® Dual Path Platform rapid test, Bio-Manguinhos, RJ, Brazil) (sensitivity = 100%; specificity = 87.5-91.7%), officially used by the "Ministério da Saúde" (Brazilian Ministry of Health) to diagnose CanVL, according to the manufacturer's instructions.

Anti-*Ehrlichia* spp. IgG antibodies in dog serum samples were evaluated using an indirect immunofluorescent antibody assay test (IFAT) with *Ehrlichia canis* crude antigens (São Paulo strain); samples were considered positive at a dilution ≥1:80 (Krawczak et al. 2015). It was performed with FITC-labeled anti-dog IgG (Sigma-Aldrich®) previously titrated to the best working dilution (1:400), as described by Aguiar et al. (2007). A nonreactive and a reactive serum sample (endpoint titer of 640) were included as negative and positive controls on each slide, respectively. The control serum samples were derived from studies by Krawczak et al. (2012) and Paula et al. (2022).

Anti-*Babesia* spp. antibodies were detected through IFAT using antigens obtained from a splenectomized dog inoculated with *Babesia vogeli*, as previously described (Trapp et al. 2006) with modifications (Vieira et al. 2013b). Serum samples with fluorescent protozoa at a dilution ≥1:80 were considered positive. Ten microliters of fluorescein isothiocyanate-conjugated rabbit anti-dog IgG (Sigma-Aldrich, St. Louis/MO) were applied to the slide at 1:1000 dilution in 0.01% Evans blue. Serum samples with fluorescent protozoa at dilution ≥1:80 were considered positive. Dog samples known to be infected with *B. vogeli* (Vieira et al. 2013b) and nuclease-free water were used as positive and negative controls, respectively.

All dogs were also tested for the presence of anti-*Ehrlichia* spp., anti-*Anaplasma* spp., and anti-*Borrelia burgdorferi* antibodies using a commercial rapid enzyme-linked immunosorbent assay (ELISA) test kit (SNAP® 4Dx Plus® Test, IDEXX Laboratories, Maine, USA), according to the manufacturer's instructions.

Molecular analysis. Blood samples were subjected to DNA extraction using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega, Madison/WI, USA), according to the manufacturer's instructions.

Samples were initially screened using a previously described conventional polymerase chain reaction (cPCR) assay targeting a fragment (145 bp) of the kDNA gene of *Leishmania* sp. (Le Fichoux et al. 1999, Lachaud et al. 2002). Dog DNA samples were tested using a previously described PCR assay targeting a fragment (~551 bp) of the 18S rRNA gene of *Babesia* spp. (Spolidorio et al. 2011, Araujo et al. 2015). All samples were additionally screened using a universal hemoplasma SYBR Green real-time polymerase chain reaction (qPCR), as previously described (Willi et al. 2009). The standard curve was calibrated using serial dilutions of gBlock™ (Integrated DNA Technologies, Coralville/IA, USA). All parameters were analyzed according to the standards established by Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin et al. 2009). Samples with cycle threshold (CT) values <32 were considered positive (Vieira et al. 2015). For *E. canis*, species-specific TaqMan qPCR (Doyle et al. 2005) was performed targeting a fragment of the *E. canis dsb* gene, as previously described (Labruna et al. 2004). For *Anaplasma platys*, the primers used were 18S rRNA genes (Khatat et al. 2017). The reactions were performed in 96-well plates and subjected to thermal variations, corresponding to an initial cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min (Labruna et al. 2004). The genes were amplified, and data were acquired and analyzed using a multicolor detection system for real-time PCR (7500 Real-Time PCR Systems; Applied BioSystems, Foster City/CA, USA). For each PCR reaction, a negative sample (nuclease-free water) and a known positive sample (positive control) were used for each TBP (*A. platys*, *Babesia* spp., *E. canis*, *Leishmania infantum*, and hemotropic *Mycoplasma* spp.).

Statistical analysis. A univariate analysis was initially performed, wherein each independent variable underwent an association analysis about the dependent variable (positivity in serological and molecular tests) to analyze the factors associated with TBP infection and determine the presence of co-infection. Variables with a *P*-value ≤0.2 in the Chi-square test or Fisher's exact test were selected for multivariate analysis using the Poisson regression model. Collinearity between independent variables was verified using a correlation analysis. For variables with strong collinearity (correlation coefficient >0.9), one of the two variables was excluded from multivariate analyses, according to biological plausibility (Dohoo et al. 2003). The Pearson Chi-square test was used to assess the model fit, and the significance of the model was verified using the Omnibus test. The significance level adopted in the multivariate analyses was 5%, and the software used was SPSS® for Windows version 20.0.

Spatial analysis. The geographical location of each dog evaluated in this study was determined using the QGIS® v. 2.18 software.

RESULTS

There were 59 (35.1%) mongrels and 109 (64.9%) pure-breed dogs [Poodle (25), Pinscher (13), American Pit Bull Terrier (14), Shih Tzu (11), Husky (7), Dachshund (6), Labrador (6), German Shepherd (6), Spitz (3), Yorkshire (3), American Bully (2), Boxer (2), French Bulldog (2), Chow-chow (2), Coker Spaniel (2), Pekingese (2), Beagle (1), Blue Heeler (1), and Maltese (1)]. The size of the dogs varied from 45.3% small (76/168), 44% medium (74/168), to 10.7% large (18/168), with the majority of the dogs aged between ≥12 to <84 months (93/168, 55.4%), followed by <12 months

(39/168, 23.2%), and ≥84 months (36/168, 21.4%). The majority of the dogs, 98.8% (166/168), were from urban areas, while only 1.2% (2/168) were from rural areas. Forty-seven out of 168 (27.9%) dogs were parasitized by the tick *Rhipicephalus linnaei* (former *R. sanguineus sensu lato* (s.l.), tropical lineage). The most frequent clinical signs were weight loss (28.6%, 48/168), lymphadenomegaly (26.2%, 44/168), papular dermatitis (22%, 37/168), and fever (22%, 37/186), followed by apathy (18.4%, 31/168), pale mucous membranes (10.7%, 18/168), cachexia (4.8%, 8/168), onychogryphosis (4.8%, 8/168), vomiting (4.8%, 8/168), diarrhea (4.8%, 8/168), muscular atrophy (3%, 5/168), splenomegaly (3%, 5/168), hepatosplenomegaly (1.2%, 2/168), joint pain (1.2%, 2/168), polyuria (0.6%, 1/168), polydipsia (0.6%, 1/168), and lameness (0.6%, 1/168).

A total of 77/168 (45.8%) dogs were anemic, 109/168 (64.9%) showed thrombocytopenia, and 28/168 (16.7%) showed leukocytosis. During blood smear evaluation, 11/168 (6.5%) dogs showed morula structures corresponding to *Ehrlichia* spp. in monocytes, while 10/186 (5.9%) showed the presence of *Babesia* spp. in the red blood cells and 1/168 (0.6%) showed *Anaplasma*-like structures in the platelets. *Leishmanina* sp. and hemotropic *Mycoplasma* were not found in the tested samples.

A total of 48/168 (28.6%) dogs were seropositive for *Leishmania* spp. Anti-*Ehrlichia* spp. antibodies were detected in 111/168 (66.1%) and 25/168 (14.9%) dogs using IFAT and commercial rapid ELISA tests, respectively. Anti-*Babesia* antibodies were found in 36/168 (21.4%) dogs, whereas anti-*Anaplasma* spp. antibodies were found in 2/168 (1.2%) of the dogs.

In relation to the seropositive dogs for *Leishmania* spp., classification according to clinical stage revealed that 43.7% (21/48) of the dogs classified as Stage I, 52.1% (25/48) as Stage II, 4.2% (2/48) as Stage III; none of the dogs classified in Stage IV. Serology results do not show that dogs are infected but indicate the presence of antibodies against the agents tested.

The PCR revealed that 22/168 (13.1%) and 11/168 (6.5%) dogs tested positive for *Leishmania* sp. and *Babesia* sp., respectively. The qPCR analysis showed that 35/168 (20.8%), 11/168 (6.5%), and 2/168 (1.2%) samples tested positive for *Ehrlichia canis*, hemotropic *Mycoplasma* spp., and *Anaplasma platys*, respectively. A total of 10/168 (5.9%) dogs were co-infected with at least two pathogens, of which 6/10 (60%) were positive for *Leishmania* and *E. canis*, 2/10 (20%) for *E. canis* and hemotropic *Mycoplasma* spp., 1/10 (10%) for *Leishmania* spp. and *Babesia* spp., and 1/10 (10%) for *Leishmania* sp., *Babesia* sp., and hemotropic *Mycoplasma* spp.

Serology

Multivariate regression analysis showed that seropositivity for *Ehrlichia* spp. and *Leishmania* spp. was significantly associated with the presence of petechiae (32/115, 27.8%, P=0.026) and weight loss (15/48, 31.2%, P=0.008), respectively. Regarding animal characteristics, only the age range (12 to 84 months) was associated with dogs seropositive for *Babesia* spp. (75%, 27/36, P=0.002) (P<0.05) (Table 1).

The hematological abnormalities of monocytosis (10.4%, 12/115, P=0.020) and thrombocytopenia (12.2%, 17/115, P=0.039) were significantly associated with seropositivity for *Ehrlichia* spp. (Table 1). The presence of hematological abnormalities and seropositivity for *Leishmania* spp. (P=0.186), *Babesia* spp. (P=0.246) and *Anaplasma* spp. (P=0.473) were not associated.

PCR-positivity

The presence of anorexia (45.4%, 5/11, P=0.009) was significantly associated with positivity for hemotropic *Mycoplasma* spp. (Table 1). No significant variable was observed in the multivariate analysis (P<0.05) among dogs PCR-positive for *E. canis*, *Babesia* spp., and *Leishmania* spp. in the presence of hematological abnormalities and clinical signs.

Exposure to selected TBP

Approximately twenty-nine percent (29.8%, 50/168) of the dogs were co-exposed to different TBP (Fig.1). An association (P<0.05) (30%, 15/50) was observed (P=0.001, CI=0.017-3.02) between seroreactivity to *Ehrlichia* spp. and *Babesia* spp. (Table 2). The main clinical signs and hematological abnormalities observed in dogs co-infected with *Babesia* spp. and *Ehrlichia* spp. were apathy (93.3%, 14/15), anorexia (86.6%, 13/15), fever (66.6%, 10/15), normocytic normochromic anemia (73.3%, 11/15), and thrombocytopenia (66.6%, 10/15). However, only fever (P=0.03, CI=1.03-2.23) and thrombocytopenia (P=0.02, CI=1.09-4.07) were significantly associated with these co-infections.

Seropositivity for *Leishmania* spp. was observed in 70% (35/50) of the co-infected dogs. The results for serology of

Table 1. Multivariate analysis (Poisson regression model) with the factors statistically significant associated with *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp., *Leishmania* spp. and hemotropic *Mycoplasma* spp. by serological and molecular analysis in dogs with clinical signs suggestive of leishmaniasis from northeastern Brazil

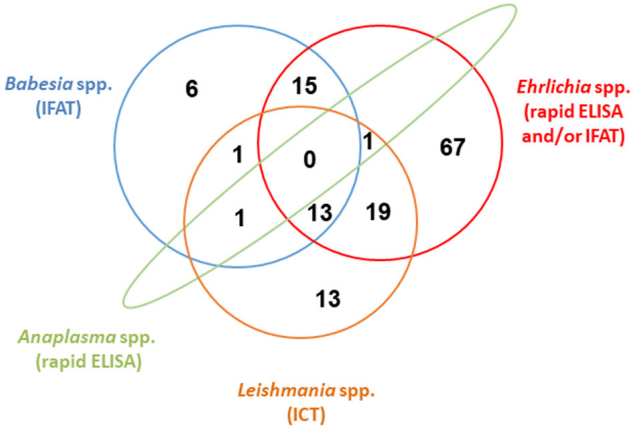
Agents	Variable category	Coefficient estimates	Standard error	Wald Chi-square	Confidence interval (95% CI)	*P*-value*
Serology						
Ehrlichia spp.	Petechiae	2.986	1.341	4.958	1.43; 274.1	0.026
	Monocytosis	1.701	0.729	5.438	1.30; 22.90	0.020
	Thrombocytopenia	0.236	0.115	4.239	1.01; 1.59	0.039
Babesia spp.	Age (≥12 to <84)	2.315	0.760	9.265	2.88; 44.92	0.002
Leishmania spp.	Weight loss	1.492	0.560	7.104	1.48; 13.31	0.008
Molecular						
Mycoplasma spp. hemotropic	Anorexia	2.873	1.093	6.909	2.07; 150.6	0.009

* *P*-values statistically significant (P<0.05).

Babesia spp., *Ehrlichia* spp., *Anaplasma* spp., and co-infections observed in dogs seropositive for *Leishmania* spp. are shown in Table 2. An association was observed (P<0.05) between seroreactivity for *Leishmania* spp. and positivity for *Ehrlichia* spp. and *Babesia* spp. (37.1%, 13/35, P=0.001, CI=0.017-3.02) (Table 2).

The most frequent clinical signs among dogs seropositive for CanVL were lymphadenomegaly (80.7%), onychogryphosis (65.2%), skin lesions (52.9%), and weight loss (49.7%). Weight loss was significantly associated with seropositivity for *Leishmania* spp. (P=0.008, CI=1.48-13.31) (Table 1). The most common blood count abnormalities among dogs positive for *Leishmania* were thrombocytopenia (71%, *P=0.358*) and normocytic normochromic anemia (61.3%, P=0.993). However, their presence was not statistically significant (P<0.05).

Ehrlichia spp. (seropositivity in IFAT and/or rapid ELISA) were frequently observed in dogs seropositive for *Leishmania* spp. (P=0.011, CI=2.40-5.49) (Table 2), with most of the animals classified as having clinical Stage II (32%, 8/25, P=0.005, CI=2.6-214.5) (Fig.2). The most prevalent clinical signs observed in dogs at clinical Stage II were lymphadenopathy (60%, 15/25, P=0.150), onychogryphosis (36%, 9/25), and arthritis (28%, 7/25, P=0.104). However, their occurrence was not statistically significant (P<0.05).



1

Fig.1. Results of co-seropositivity among the pathogens *Leishmania* spp. (ICT), *Anaplasma* spp. (rapid ELISA), *Ehrlichia* spp. (rapid ELISA and/or IFAT) and *Babesia* spp. (IFAT), present in dogs with clinical signs suggestive of leishmaniasis from northeastern Brazil.

When evaluating co-infection based on the presence of pathogen DNA in dogs, it was observed that 5.9% (10/168) of the animals were co-infected (Fig.3), with apathy (66.6%, 4/6) and anorexia (33.3%, 2/6) being the most prevalent clinical signs observed in dogs co-infected with *E. canis* and *Leishmania* spp. However, none of the clinical signs were significantly associated with co-infection (P<0.05). Molecular analysis revealed that, among the positive dogs for *Leishmania* spp., 36.3% (8/22) were co-infected.

The co-infected dogs in this study were widely distributed in the urban areas of the municipality (Fig.4).

The total of dogs positive by cytology (looking at stained blood smears by microscopy) for *Ehrlichia*, *Anaplasma* and *Babesia* and by PCR and/or serology can be observed in Table 3.

DISCUSSION

This study demonstrated the occurrence of *Leishmania* spp., *Ehrlichia* spp., *Babesia* spp., *Anaplasma* spp., and hemotropic *Mycoplasma* spp. in dogs with clinical signs suggestive of VL showing active infection of some hemotropic pathogens by molecular assays and prior exposure by serological techniques in dogs from an endemic area for CanVL in northeastern Brazil. The presence of anti-*Leishmania* spp. antibodies were observed in 28.5% of the dogs, similar to a previous study in the same area (Araujo et al. 2016). Moreover, the occurrence of *Leishmania* spp. was lower by PCR (13.1%), indicating that the animals may come into contact with the parasite, elevating the risk of new infections (Carvalho et al. 2018).

The difference in the positivity of the results obtained when we compare the serological (indirect diagnosis) and molecular (direct diagnosis) analyses can be explained by the

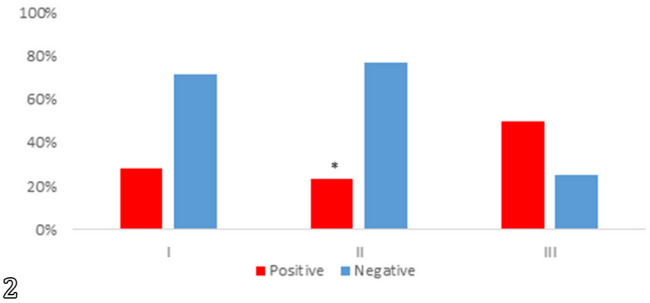


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Fig.2. Percentage of seropositivity for *Ehrlichia* spp. by IFAT and/or rapid ELISA according to clinical stage classification of leishmaniasis in dogs from northeastern Brazil. Asterisk = P<0.05.

Table 2. Number of seropositive dogs for *Leishmania* spp. (ICT) co-seropositive for *Babesia* spp. (IFAT), *Anaplasma* spp. (rapid ELISA) and *Ehrlichia* spp. (rapid ELISA and/or IFAT) from northeastern Brazil

Agents	Positive dogs (n = 48)	*P*-value	Confidence interval (95% CI)
Ehrlichia spp.	19	P = 0.011*	2.40-5.49
Babesia spp.	1	P = 0.063	0.06-1.07
Anaplasma spp.	0	P = 0.278	0.51-9.85
Ehrlichia spp. and *Babesia* spp.	13	P = 0.001*	0.017-3.02
Ehrlichia spp. and *Anaplasma* spp.	1	P = 0.072	0.89-14.17
Anaplasma spp. and *Babesia* spp.	1	P = 0.206	0.41-1.22

ICT = immunochromatographic test, IFAT = immunofluorescent antibody assay test, ELISA = enzyme-linked immunosorbent assay; * *P*-values statistically significant (P<0.05).

fact that the animal may have eliminated the agent through the immune system or treatment. However, the antibodies indicating exposure and circulation of the pathogen in the area may remain detectable for months to years. This can be clearly demonstrated in the situation of *Ehrlichia canis*, where an animal can present antibodies up to two years after the agent has been eliminated from the body (Harrus & Waner 2011).

The detection of clinical signs is critical for the early diagnosis of suspected CanVL cases (Carvalho et al. 2018). In our study, weight loss was significantly associated with seropositivity for CanVL. Poor nutritional status of the animal can decrease its immunity, making it more susceptible to pathogenic infections (Ciaramella et al. 1997, Koutinas et al. 1999, Baneth et al. 2008).

Ehrlichia was the most prevalent species, per serologic analysis, indicating its widespread regional distribution. Infection by *Ehrlichia* spp. has been reported in several TBP studies conducted in the tropical regions of Brazil (Vieira et al. 2011, Souza et al. 2013, Andrade et al. 2014, Dantas-Torres et al. 2018). However, the dogs were sick with clinical signs suggestive of infection with *Leishmania* spp. and attended veterinary clinics, which may have increased the occurrence of infection (Dantas-Torres et al. 2018).

Multivariate analysis revealed that dogs seropositive for *Ehrlichia* spp. showed petechiae as the main clinical sign. Dogs may present vascular disorders, which promote the presence of bleeding, mainly petechiae and ecchymosis on the skin (Lima et al. 2021). Regarding the observed hematological abnormalities, monocytosis was associated with seropositivity. This is an anticipated finding in dogs infected with monocytic ehrlichiosis since monocytes are commonly parasitized by *Ehrlichia* morulae (Lima et al. 2021).

In the results of serology for *Ehrlichia* spp., it was observed that IFAT showed a higher prevalence than ELISA. This was expected since IFAT is considered the gold standard test for the serological diagnosis of *E. canis* (Harrus & Waner 2011). Besides, in the IFAT, the antigen used is the crude DH82-infected cells of a Brazilian strain of *E. canis*. The snap SNAP®4Dx® employs synthetic peptides derived from the major immunodominant *E. canis* proteins P30 and P30-1 as antigens for *E. canis* antibody detection (O'Connor et al. 2006). Thus, it is expected that dogs from Brazil show higher immune responses to local antigens when compared to those used in North American kits such as Snap.

Seropositivity for *Ehrlichia* spp. was significantly associated with positivity for *Babesia* spp. (30%). Co-infection with *Ehrlichia* spp. is a common occurrence in dogs with babesiosis (Rojas et al. 2014) since these diseases are transmitted by the tick *Rhipicephalus linnaei* (Araujo et al. 2015, Nogueira et al. 2021), which may be infected with multiple pathogens (Shaw et al. 2001, Rautenbach et al. 2018). In addition, Krawczak et al. (2015) suggested that this type of co-infection is not a cross-reaction since it involves phylogenetically distant and different pathogens, including protozoa (*Babesia* spp.) and bacteria (*Ehrlichia* spp.) (Oliveira et al. 2008).

Multivariate analysis indicated that dogs ≥12 to <84-month-old were more predisposed to infection with *Babesia* spp. by serology. This may be due to the immune system of dogs associated with the possible acute phase of the infection with higher production of antibodies (Alvar et al. 2004, Izzi et al. 2013).

Rapid ELISA and qPCR detected *Anaplasma platys* in only 1.2% of the dogs. Only one dog presented with simultaneous serological evidence of exposure to *Anaplasma* spp. and *Ehrlichia* spp., according to the findings of Diniz et al. (2010), Izzi et al. (2013), Ybañez et al. (2018), and Low et al. (2018). This seems to be the first study evaluating *Anaplasma* spp. among dogs in the municipality; hence, further studies are needed to better understand its epidemiology and co-infections in the region.

To our knowledge, this study is the first to report infection with hemotropic *Mycoplasma* spp. among dogs in a Semi-arid region of Brazil. The DNA of this species has earlier been detected among dogs from other regions of the country, with

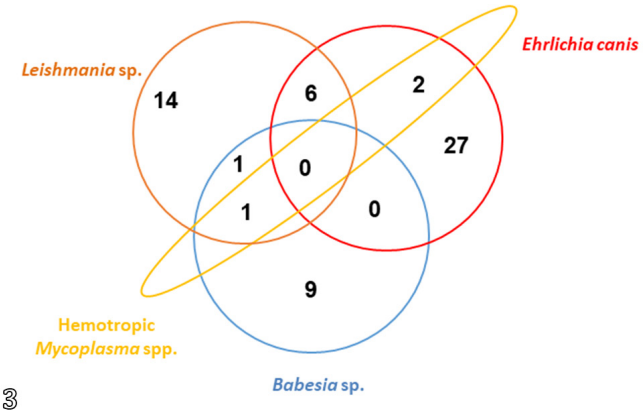

Fig.3. Results of co-infection by molecular analysis among the pathogens (*Anaplasma platys*, *Babesia* spp., *Ehrlichia canis*, hemotropic *Mycoplasma* spp. and *Leishmania* spp.) present in dogs with clinical signs suggestive of leishmaniasis from northeastern Brazil.

Table 3. Total of dogs positive by cytology (looking at stained blood smears by microscopy) for *Ehrlichia*, *Anaplasma* and *Babesia* and by PCR and/or serology

Blood smears % (+/total)	cPCR % (+/total)	qPCR % (+/total)	rapid ELISA % (+/total)	RIFI % (+/total)
Ehrlichia spp. 6.5% (11/168)	-	90.9% (10/11)	45.4% (5/11)	45.4% (5/11)
Babesia spp. 5.9% (10/168)	90% (9/10)	-	-	45.4% (5/11)
Anaplasma spp. 0.6% (1/168)	-	100% (1/1)	100% (1/1)	-

cPCR = conventional polymerase chain reaction, qPCR = quantitative real-time polymerase chain reaction, ELISA = enzyme-linked immunosorbent assay, RIFI = indirect immunofluorescence reaction.

their prevalence ranging from 1.8-44.7% (Valle et al. 2014, Soares et al. 2016, Sousa et al. 2017, Lashnits et al. 2019, Barbosa et al. 2021, Di Cataldo et al. 2021). Although only the tick *R. linnaei* was identified in this study, other studies have indicated that the transmission of hemotropic *Mycoplasma* spp. is still unclear, and some arthropod vectors, such as fleas, may be involved (Sykes 2010, Willi et al. 2010, Soto et al. 2017). There may also be iatrogenic transmission through blood transfusion (Sykes et al. 2004, Messick & Harvey 2015) due to aggressive interactions between animals that can lead to blood contact (Willi et al. 2010) or vertical transmission (Lashnits et al. 2019).

In the present study, anorexia was associated with hemoplasma infection (Willi et al. 2010). In addition, dogs with hemotropic mycoplasmosis were also co-infected with *E. canis*, *Leishmania* spp., and *Babesia* spp. (Andersson et al. 2017, Bouzouraa et al. 2017, Hofmann et al. 2019). Concomitant infections in dogs positive for hemotropic *Mycoplasma* spp. reinforce the importance of the studied canine population because of the zoonotic potential of hemoplasmas (Maggi et al. 2013a, Vieira et al. 2015) and *Leishmania* (Araujo et al. 2016), indicating the need for preventive measures to control these diseases and their vectors in the region (Andersson et al. 2017).

The majority of *Leishmania* spp.-positive dogs were co-infected with TBP. The occurrence of co-infections with TBP in dogs in Brazil is known (Vieira et al. 2013b, Gizzi et al. 2014, Krawczak et al. 2015, Rotondano et al. 2017), and these co-infections can increase disease severity in animals

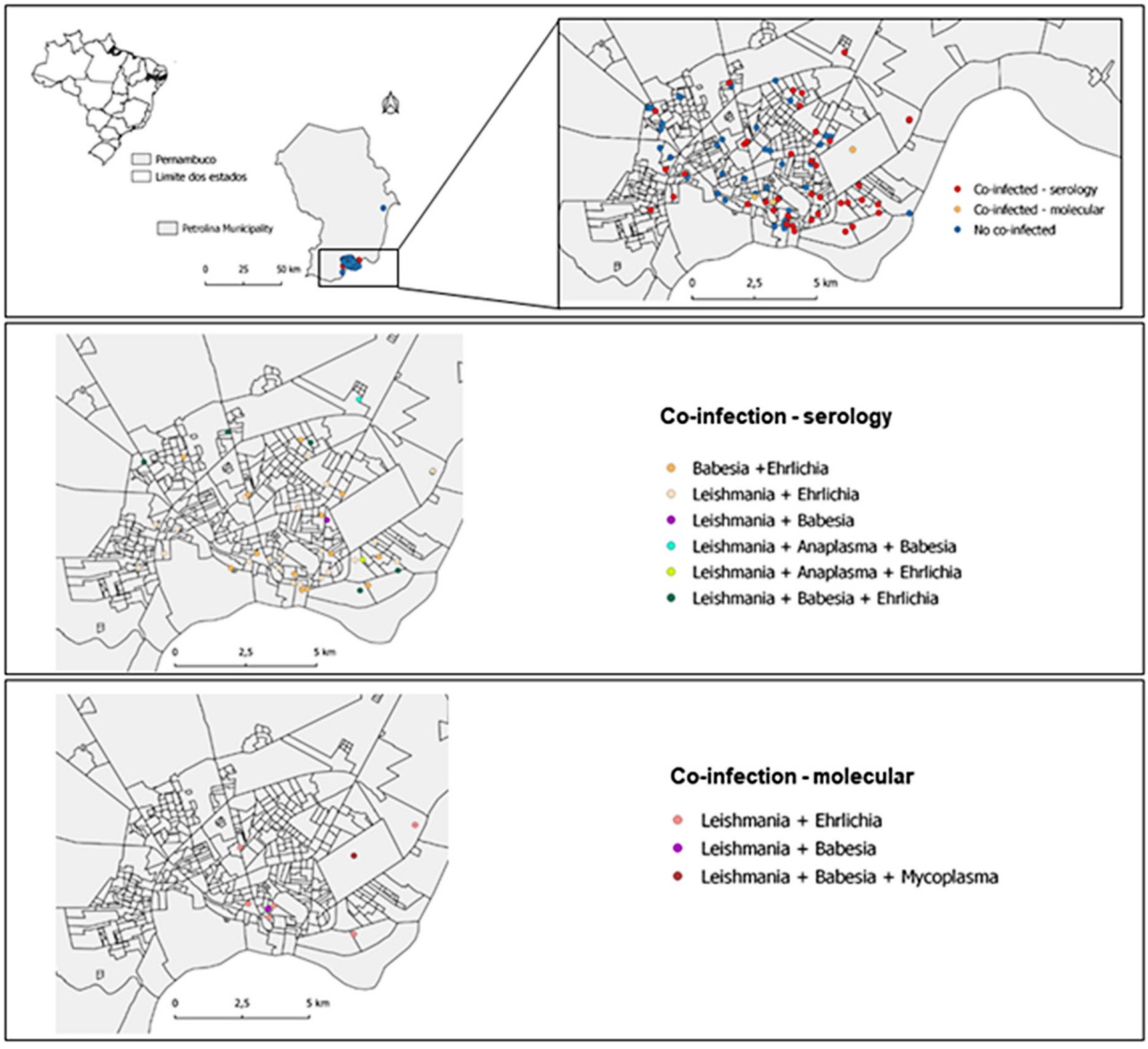

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Fig.4. Distribution of dogs that were co-infected and/or co-exposure with *Leishmania*, tick-borne pathogens and hemoplasma in the serologic and molecular analysis in the municipality of Petrolina, a Semi-arid region of Pernambuco, northeastern Brazil.

co-infected with leishmaniasis, which may impair the clinical progression for these animals (Toepp et al. 2019).

In this study, dogs seropositive for *Leishmania* spp. showed seropositivity mainly with *Ehrlichia* spp. and *Babesia* spp. The coexistence exposure of these pathogens triggers cytokine production, which activates the pathogenesis of these species; this can prevent their clinical improvement and treatment success (De Tommasi et al. 2013). In addition, the clinical signs of fever and thrombocytopenia were significantly associated with these types of co-infection. These alterations are common in infections caused by vector-borne pathogens, particularly those transmitted by ticks (Rojas et al. 2014, Araujo et al. 2015).

The clinical Stage II of CanLV was associated with the presence of antibodies against *Ehrlichia* spp., presenting clinical signs such as lymphadenomegaly, anorexia, and weight loss, common in both diseases (Solano-Gallego et al. 2009), which may have influenced this association.

In general, co-infected dogs were widely distributed in the urban area of the municipality, indicating a high prevalence of vectors in this region, which reinforces the need for measures to control these diseases and their vectors in the area studied (Evaristo et al. 2020).

CONCLUSION

Our results demonstrate that, besides visceral leishmaniasis (VL) being a critical health concern in humans and animals, tick-borne pathogens (TBP) also represents risks for dogs and can infect animals concomitantly, which may potentiate the clinical condition and complicate diagnosis. Thus, the importance of using different diagnostic methods is reinforced to understand and diagnose these diseases and their co-infections better, thereby designing strategies for their prevention and control.

Authors' contributions.- Evaristo A.M.C.F.: Wrote and revised the manuscript and participated in the entire methodological part. Santos P.T.T.: Participated in the entire methodological part. Sé F.S.: Assisted in the collection of the animal's blood and serological diagnosis. Collere F.C.M.: Participated in the molecular diagnosis. Silva B.B.F.: Participated in the serological and molecular diagnosis. Cardoso E.R.N.: Participated in the serological and molecular diagnosis. Kakimori M.T.A.: Participated in the molecular diagnosis. Vieira T.S.W.J.: Wrote and revised the manuscript and participated in the molecular diagnosis. Krawczak F.S.: Wrote and revised the manuscript and participated in the serological and molecular diagnosis. Filho J.M.: Wrote and revised the manuscript and participated in the molecular diagnosis. Vieira R.F.C.: Wrote and revised the manuscript and participated in the molecular diagnosis. Horta M.C.: Provided guidance, participated in the delimitation of the study and reviewed the manuscript.

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