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Molecular diagnosis of *Ehrlichia canis* and *Babesia vogeli*, and serological diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection in dogs from the municipality of Cândido Sales-BA and microregion

[*Diagnóstico molecular de infecção por* Ehrlichia canis *e* Babesia vogeli *e diagnóstico sorológico de infecção por* Neospora caninum *e* Toxoplasma gondii *em cães provenientes do município de Cândido Sales-BA e microrregião*]

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ABSTRACT

The occurrence of *Toxoplasma gondii*, *Neospora caninum*, *Ehrlichia canis* and *Babesia vogeli* in dogs from the municipality of Cândido Sales, Bahia, was investigated. Total and peripheral blood samples were obtained from 131 dogs. Blood smears were performed to check for morulae. *Toxoplasma gondii* and *N. caninum* infections were determined by an Indirect Immunofluorescence Reaction. Nested-PCR and PCR were used for the diagnoses of *E. canis* and *B. vogeli* infections, respectively. Additionally, the risk factors associated with infection by these agents were analyzed. The frequency of infection was 70.2% for *N. caninum*, 67.9% for *T. gondii* and 37.4% for *E. canis*. None of the dogs tested positive for *B. vogeli*. Morulae of *Ehrlichia* spp. were identified in two animals and the piroplasm in one animal. Age (> 3 years) was considered a risk factor for infections by *E. canis* and *N. caninum* and the rural habitat for infections by *N. caninum*. Co-infections were frequent, mainly with *N. caninum* and *T. gondii* (45.03% of dogs). *Ehrlichia canis* infection was significantly associated with *N. caninum* and *T. gondii* infection. These findings indicated a high occurrence of *T. gondii*, *N. caninum*, and *E. canis* in the studied region.

Keywords: babesiosis, epidemiology, ehrlichiosis, neosporosis, toxoplasmosis

RESUMO

Avaliou-se a ocorrência de infecções por Toxoplasma gondii, Neospora caninum, Ehrlichia canis *e* Babesia vogeli *em cães do município de Cândido Sales – BA. Para isso, foram obtidas amostras de sangue total e periférico de 131 cães. Esfregaços sanguíneos foram confeccionados para verificação de mórulas. Infecções por* T. gondii *e* N. caninum *foram determinadas pela reação de imunofluorescência indireta. O diagnóstico das infecções por* E. canis *e* B. vogeli *foi feito por meio de Nested-PCR e PCR, respectivamente. Adicionalmente, foram analisados os fatores de risco associados à infecção por esses agentes. Verificou-se frequência de infecção de 70,2% para* N. caninum, *67,9% para* T. gondii *e de 37,4% para* E. canis. *Nenhum cão foi positivo para* B. vogeli. *Mórulas de* Ehrlichia *spp. foram identificadas em dois animais, e piroplasma em um. A idade (acima de 3 anos) foi fator de risco para infecções por* E. canis *e* N. caninum, *e hábitat rural para* N. caninum. *Coinfecções foram frequentes, principalmente por* N. caninum *e* T. gondii *(45,03%). Infecção por* E. canis *foi associada significativamente às infecções por* N. caninum *e* T. gondii. *Os achados indicam elevada ocorrência de* T. gondii, N. caninum, *e* E. canis *na região.*

Palavras-chave: babesiose, epidemiologia, erliquiose, neosporose, toxoplasmose

INTRODUCTION

The protozoan parasites *Toxoplasma gondii* and *Neospora caninum* belong to the phylum Apicomplexa and are obligate intracellular parasites of major importance in veterinary medicine. They have a wide geographical distribution and are found in several parts of the world (Tenter *et al*., 2000). Dogs, humans, and other species are considered intermediate hosts of *T. gondii*. In these animals, the infection can

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either show no symptoms or have a major clinical presentation, particularly in immunocompromised individuals. The evaluation of the seroprevalence of this agent in canine species is of great importance because the diagnosis in these animals can reveal environmental and human contamination (Ratzlaff *et al*., 2018). In addition, dogs can act as definitive hosts of *N. caninum* which, in areas with cattle, can lead to severe economic losses because infection by this agent is mainly related to episodes of pregnancy loss in cows (Dubey *et al*., 2007).

Further, diseases transmitted by ticks are of great importance because the same species can transmit several pathogens, favoring the presence of co-infections in animals. Among various hemoparasites, babesiosis and ehrlichiosis are transmitted by ticks. In Brazil, canine babesiosis has great prominence, and despite reports showing sporadic occurrence of other species, like *B. gibsoni* reports in the south of the country, *B. vogeli* is mainly responsible for the disease in dogs (Dantas-Torres *et al*., 2021). Similarly, *E. canis* is of great epidemiological importance, and is transmitted mainly through *Rhipicephalus sanguineus*, which is known as the "brown tick," and in Brazil, uses dogs predominantly as its hosts (Dantas-Torres *et al*., 2004; Vieira *et al.*, 2021). The occurrence of these diseases in dogs in Brazil is highlighted by the tropical climate, which favors the vector's life cycle (Soares *et al.*, 2017).

Moreover, the close proximity between dogs and humans may also favor the transmission of common agents between both species, such as *E. canis* and other hemoparasites (Cunha *et al.*, 2009).

Several coinfections in dogs have been described, which can lead to clinical problems and are common in areas where several etiological agents occur concomitantly. Epidemiological studies related to these pathogens are of great importance because of the growing connection between canine species and humans, which necessitates the establishment of better care and strategic actions for the prevention of zoonoses and other important diseases for animal health (Ratzlaff *et al.*, 2018).

To date, there have been few studies in Bahia on the frequency of infection by *T. gondii*, *N. caninum*, *E. canis*, and *B. vogeli* in dogs, while no studies have been conducted specific to the south-central mesoregion of the state. The objective of this study was to evaluate the occurrence of infection by these agents in the region of Cândido Sales, Bahia, Brazil.

MATERIALS AND METHODS

This study included 131 domesticated domiciled and semi-domiciled dogs from urban and rural areas of the south-central region of Bahia, including the municipality of Cândido Sales and a rural district of Vitória da Conquista (Cercadinho), located near the city of Cândido Sales. At the time of the study, the city of Cândido Sales, Bahia, had no medical veterinary assistance. The methodology was approved by the Ethics Committee on Animal Use of the Universidade Estadual de Santa Cruz – UESC (CEUA/UESC) (protocol 005/21). The inclusion of the animals in the study and the sampling of biological material occurred only after authorization by the tutors and obtaining written informed consent.

After the animals were physically restrained, approximately 5 ml of venous blood was collected by cephalic puncture, which was dispensed into two tubes, one with the anticoagulant ethylenediaminetetraacetic acid (EDTA) and the other with a clot activator for later molecular and serological tests, respectively.

Blood smears were prepared from the whole and peripheral blood samples (ear tips). The slides were stained with fast panoptic dye and analyzed under a binocular optical microscope (1600x Olen K55ba Kasvi) to study and identify hematozoa or other inclusions. Additionally, a questionnaire was completed to investigate the history and management of the animals and verify the risk factors associated with infections.

The blood samples stored in the tubes with clot activator were centrifuged to obtain the serum, which was stored in 2 ml microtubes at a temperature of -20°C until the IFA serological technique was performed to detect the IgG antibodies anti-*N. caninum* and anti-*T. gondii*.

The IFA technique for the detection of anti-*N. caninum* and anti-*T. gondii* antibodies were used as previously described by Gondim *et al*. (2001), and Valadas *et al*. (2010), respectively. To visualize the reactions, anti-Dog IgG-F7884 conjugate (Sigma Aldrich ®) with a 1:128 dilution, labeled with fluorescein isothiocyanate, was used. The slides were examined under an epifluorescence microscope (BX51 ®; Olympus). Reactions in which total peripheral fluorescence was observed in > 50% of tachyzoites were considered positive. Positive and negative controls were obtained from samples from previous studies in the region. Benchmarks were set at 1:50 for *N. caninum* and 1:16 for *T. gondii*.

The blood samples packed in tubes with an anticoagulant agent were subjected to DNA extraction using the phenol-chloroform-isoamyl alcohol method (Ghaheri *et al*., 2016). The final DNA concentration in each sample was determined by spectrophotometry using a Nanodrop spectrophotometer (260 nm optical density). *E. canis* infection was diagnosed using nested-PCR technique, as previously described by Murphy *et al*. (1998). The following primers were used for the first reaction: ECC (5'-AGAACGAACGCTGGCGGCAAGC-3') and ECB (5'-CGTATTACCGCGGCTGCTGGCA-3'), which amplify a fragment of the 16S gene of the genus *Ehrlichia.* For the second reaction, the primers ECAN (5'-CAATTATTTATAGCCTCTGGCTATAGGA-3') and HE3 (5-'TATAGGTACCGTCATTATCTTCCCTAT-3') were used to obtain a final product of 396 bp of *E. canis* DNA. The sensibility and specificity of this technique was established by Murphy *et al*. (1998). Samples positive for *E. canis* were used as the positive control, while ultrapure water was used as the negative control.

Infection by *B. vogeli* was diagnosed using conventional PCR, in which the primers CAN 626R (5'-GAA CTC GAA AAA GCC AAA CGA-3') and CAN 172F (5'-GTT TAT TAG TTT GAA ACC CGC-3'), specific to *B. vogeli*, were used to amplify a fragment of the 18s ribosomal RNA gene (450 bp) (Inokuma *et al*., 2004). Positive samples for *B. vogeli* were used as the positive control, whereas ultrapure water was used as the negative control.

The amplified products in the nested-PCR reactions for *E. canis* and in the PCR reactions for *B. vogeli* were subjected to 2% agarose gel electrophoresis containing SYBR® Safe DNA Gel Stain (Invitrogen®). The TAE buffer was used for running, and electrophoresis was performed at 75V/200mA for 40 min. A 1 Kb Plus DNA Ladder marker (Invitrogen®) was used to determine the size of the amplified products. The bands were verified using an ultraviolet transilluminator, followed by evaluation using a Locus Biotechnology L-Pix Transilluminator.

Statistical analysis of the risk factors associated with infections was performed with bivariate and multivariate analyses using Epi Info 3.5.2. For bivariate analysis, the chi-square test was used to associate each independent variable with the dependent variables (positive or negative animals). Variables with a p-value ≤ 0.3 were selected for multivariate analysis using unconditional logistic regression. Statistical significance was set at $p < 0.05$.

RESULTS

In the evaluation of blood smears, structures compatible with morulae of *Ehrlichia* spp. were identified in the neutrophils of the whole blood of two animals. Piroplasma was identified in red blood cells in an ear tip blood smear of a dog. Through serological diagnosis, a frequency of 70.2% (92/131) and 67.9% (89/131) was obtained for *N. caninum* and *T. gondii*, respectively. Nested-PCR detected a positivity rate of 37.4% (49/131) in dogs tested for *E. canis*. None of the evaluated animals tested positive for *B. vogeli.* Of the two dogs that presented morulae of *Ehrlichia* spp. in the evaluation of blood smears, only one tested positive for *E. canis* in the molecular diagnosis.

Considering the results obtained by combined serological and molecular techniques, 94.65% (124/131) of the dogs were infected with at least one of the studied agents. Infections by a single etiologic agent occurred in 31.29% (l41/131) of dogs, 19.84% (26/131) by *T. gondii*, 9.92% (13/131) by *N. caninum* and 1.52% (2/131) by *E. canis*. Regarding co-infections in dogs, 45.03% (59/131) presented *N. caninum* and *T. gondii*, 32.82% (43/131) presented *N. caninum* and *E. canis*, and 20.61% (27/131) presented *T. gondii*

and *E. canis* co-infections. Co-infections with three of the agents (*E. canis*, *T. gondii*, and *N. caninum*) were identified in 17.55% (23/131) of the animals.

The presence of *E. canis* infection in this study was significant associated with *T. gondii* infection (p = 0.025; *odds ratio* [OR] (0.3959; 95% CI 0.18–0.84), as well as for *N. caninum* (p = 0.0014; OR 4.8265; 95% CI 1.84–12.62). Although more frequent, co-infections with *N. caninum and T. gondii* showed no statistically significant association (p = 0.218; OR 0.5364; 95% CI 0.22–1.26).

Considering the two dogs that tested positive for the genus *Ehrlichia* spp. in the parasitological diagnosis, both were seropositive for *N. caninum*, and one was seropositive for *T. gondii*. The dog that presented with Piroplasma in red blood cells tested positive for *E. canis* and *N. caninum*.

Statistical evaluation of risk factors associated with *E. canis* infection in dogs showed that only age (> 3 years old) was significant in the bivariate and multivariate analyses (p = 0.011; OR 0.3176; 95% CI 0.13–0.74 and p= 0.0079; OR 0.3176; 95% CI 0.1363–0.7403, respectively) (Table 1a and 1b, Supplementary Material).

Table 1a. Factors associated with *E. canis* infection in positive dogs

Variable		N	Positive dogs	Frequency %	OR	95%CI	P-value
Gender	Male	67	27	40.30%	1.2886	0.63 -2.62	0.6032210227
	Female	64	22	34.38%			
Age	<3 years old	43	9	20.93%	3.15	1.35-7.38	**0.0113507327**
	>3 years old	88	40	45.45%			
Breed	No definite breed	107	42	39.25%			
	With definite breed	24	7	29.17%	1.5692	0.59 -4,10	0.4905287771
Habitat	Urban area	72	26	36.11%	0.8847	0.43–1.80	0.8756183165
	Rural area	59	23	38.98%			
Outdoor access	Yes	91	34	37.36%	0.9942	0.46 -2.14	1.0000000000
	No	40	15	37.50%			
Presence of ticks	Yes	76	33	43.42%	1.8706	0.89–3.91	0.1362326291
	No	55	16	29.09%			
Ectoparasite control	Yes	29	8	27.59%	0.5668	0.22–1.40	0.3073090445
	No	102	41	40.20%			
Contact with other animals	Yes	109	42	38.53%	1.3433	0.50 – 3.5	0.5527448634
	No	22	7	31.82%			

Table 1b. Association between dogs positive for *E. canis* and the age variable

Variable	Odds ratio	Confidence interval 95%	P valor (<0.05)
Age	3.15	1.35-7.33	**0.0079**

P= 0,0065 Likelihood: 0,0052

Regarding the evaluation of risk factors related to *N. caninum* infection, there was a statistically significant difference in the bivariate analysis related to age above 3 years (p = 0.020; OR 0.3715; 95% CI 0.17–0.81) and the animal's habitat, given that animals from the rural area had a higher risk of infection than animals from the urban area (p = 0.019; OR 0.3601; 95% CI 0.16–0.80). The values were confirmed by multivariate analysis (p = 0.0042; OR 0.2922; 95% CI 0.12–0.67 for age, and p = 0.0046; OR 0.2861; 95% CI 0.12–0.68 for habitat) (Tables 2a and 2b, Supplementary Material).

No risk factors associated with *T. gondii* infection were identified in dogs in the study region (Table 3, Supplementary Material).

Table 2a. Factors associated with *N. caninum* infection in positive dogs

Variable		N	Dogs	Frequency	OR	95%CI	P-valor
Gender	Male	67	46	68.66%	0.8571	0.40–1.81	0.8324554528
	Female	64	46	71.88%			
Age	<3 years old	43	24	55.81%	2.69	1.23–5.88	**0.0204057711**
	>3 years old	88	68	77.27%			
Breed	No definite breed	107	77	71.96%	1.5400	0.60–3.89	0.5033144929
	With definite breed	24	15	62.50%			
Habitat	Urban area	72	44	61.11%	2.78	1.24–6.23	**0.0198472528**
	Rural area	59	48	81.36%			
Outdoor access	Yes	91	68	74.73%	1.9710	0.89–4.34	0.1361952816
	No	40	24	60.00%			
Contact with other animals	Yes	109	77	70.64%	1.1229	0.41–3.01	1.0000000000
	No	22	15	68.18%			
Contact with dogs	Yes	98	71	72.45%	1.5026	0.65–3.46	0.4608060352
	No	33	21	63.64%			
Contact with bovine	Yes	22	18	81.82%	2.0676	0.65–6.57	0.3207241959
	No	108	74	68.52%			
Hunting habit/raw meat intake	Yes	38	31	81.58%	2.3232	0.92–5.85	0.1083827233
	No	93	61	65.59%			

Table 2b. Association between dogs positive for *N. caninum* and the variables age and habitat

Variable	Odds ratio	Confidence interval 95%	P valor (<0.05)
Age	3.42	1.47-7.95	**0.0042**
Habitat	3.50	1.47-8.30	**0.0046**

P=0.007 Likelihood: 0.0005

Table 3. Factors associated with *T. gondii* infection in positive dogs

Variable		N	Positive dogs	Frequency%	OR	95%CI	P-valor
Gender	Male	67	47	70.15%			
	Female	64	42	65.63%	1.2310	0.59– 2.56	0.7133505849
Age	<3 years old	43	31	72.09%	1.3362	0.60-2.97	0.6080975703
	>3 years old	88	58	65.91%			
Breed	No definite breed	107	75	70.09%			
	With definite breed	24	14	58.33%	1.6741	0.67– 4.16	0.3822955696
Habitat	Urban area	72	49	68.06%	1.0120	0.48– 2.11	1.0000000000
	Rural area	59	40	67.80%			
Outdoor access	Yes	91	62	68.13%	1.0294	0.46– 2,27	1.0000000000
	No	40	27	67.50%			
Contact with other animals	Yes	109	76	69.72%	1.5944	0.62– 4.09	0.4687974907
	No	22	13	59.09%			
Contact with cats	Yes	38	24	63.16%	0.7385	0.33– 1.63	0.5869817177
	No	93	65	69.89%			
Hunting habit/raw meat intake	Yes	38	24	63.16%	0.,7385	0.33– 1.63	0.5869817177
	No	93	65	69.89%			

DISCUSSION

The low frequency of *Ehrlichia* spp. in parasitological diagnosis may be due to the low parasitemia presented by the animals, possibly due to the stage of infection, in addition to the low sensitivity of the technique. On the other hand, in some animals, morulae suggestive of *Ehrlichia* spp. can be observed in non-mononuclear leukocytes, and the animal may still test negative for *E. canis* using molecular techniques, as observed in one of the animals in this study. In this case, the findings can be interpreted as another species of *Ehrlichia* spp. or can also be characterized as other types of intracytoplasmic inclusions, such as azurophilic granules, platelets, and material from phagocytosis (Mylonakis *et al*., 2003).

The frequency of *E. canis* infection found in this study is similar to that described by Ramos *et al*. (2010) in Recife, Pernambuco, who reported positivity in 38.04% (78/205) of dogs evaluated using the PCR technique, and by Santos *et al.* (2009) in Ribeirão Preto, São Paulo, who found a prevalence of 38.9% (86/221) using the nested-PCR technique. In the state of Bahia, the present study demonstrated the highest frequency of *E. canis* infection through molecular diagnosis, compared to the findings described by Cordeiro *et al*. (2020), who reported a prevalence of 16.54% in Itabuna; Guedes *et al*. (2015), who observed a prevalence of 25.6% in Ituberá; and

Carvalho *et al*. (2008), who reported a prevalence of 10.7% and 4.3% in Ilhéus and Itabuna, respectively.

Previous studies conducted in Brazil reported a low prevalence of infection by *B. vogeli* in dogs (below 10%), using the molecular diagnosis technique (conventional PCR) (Ramos *et al*., 2010; Bahiense *et al*., 2020). Despite the absence of positivity for *B. vogeli* by PCR in this study, the observation of Piroplasma in the blood smear of an animal that tested negative for *B. vogeli* in the molecular diagnosis indicates the occurrence of other species or subspecies of *Babesia* spp. in the studied region.

The frequency of *N. caninum* infection found in the animals in this study was higher than that reported in other studies carried out in the last 20 years in Brazil, including the study conducted by Benetti *et al*. (2009), which reported an infection prevalence of 67.56% (25/37) in dogs in the rural area of the southwest region of Mato Grosso using the serological technique. This result is highly important for the Cândido Sales region, especially for rural properties, as it warns about the possibility of infection in cattle, which can cause episodes of pregnancy loss in this species, resulting in economic losses.

The frequency of *T. gondii* infection found in the present study was similar to the results obtained by Valadas *et al*. (2010) in Pará, Amazonas, and by Barbosa *et al*. (2003) in Salvador, Bahia, who reported a prevalence of 69.8% (90/120) and 63.55% (143/225), respectively. The high frequency of anti-*Toxoplasma gondii* antibodies found in dogs in the region of Cândido Sales warns of the possible risk to humans, since dogs and humans can share the same infection sources.

The high rate of infections by these agents in dogs in the studied region may also be related to the lack of veterinary assistance and consequent lack of knowledge on the part of owners about the prophylaxis of these infections. Aspects related to sanitary management and animal life habits, as well as contact with other species, such as cattle and cats, feeding habits, ectoparasite control, lack of environmental hygiene, basic sanitation, and water treatment, can also favor the transmission and persistence of agents in the environment.

The occurrence of co-infection with *T. gondii* and *E. canis* observed in the dogs in this study was similar to that found by Deiró *et al.* (2018). These researchers used IFA and ELISA serological techniques to diagnose infection by *T. gondii* and *E. canis*, respectively, in 353 dogs from Bahia and found co-infection by these agents in 20.1% (71/353) of the evaluated animals. The relationship between co-infection by *E. canis* and *T. gondii* or *N. caninum* may be due to the opportunistic characteristics of the parasites (Ratzlaff *et al*., 2018).

The co-infection rate of *N. caninum* and *T. gondii* observed in this study was much higher when compared to the rates obtained by Mineo *et al*. (2001), who described only 3.1% (5/163) of reactive samples for both parasites in dogs with neuromuscular signs in Uberlândia, Minas Gerais, and Acosta *et al*. (2016), who described only one animal with co-infection by *N. caninum* and *T. gondii* in Espírito Santo. *N. caninum* infection, however, did not present a statistically significant association with the simultaneous occurrence of *T. gondii*, as was also previously described by Bresciani *et al*. (2007) in a serological study conducted in the city of Araçatuba, São Paulo. Although there was no significant correlation, the high occurrence of this co-infection may contribute to the clinical manifestation of the disease (Girardi *et al*., 2014). The high rate of concomitant infection with *N. caninum* and *T. gondii* observed in this study suggests that both etiological agents should be considered in differential clinical diagnoses, especially in dogs with neuromuscular, respiratory, and/or gastrointestinal disorders (Mineo *et al*., 2001).

In this study, age (> 3 years) was considered a risk factor for infection by *E. canis* and *N. caninum*, respectively, corroborating with the studies carried out by Deiró *et al.* (2018) and Souza *et al*. (2002) who described that adult dogs may have a higher prevalence of infection due to longer exposure to pathogens.

In addition, rural habitat was considered a risk factor for *N. caninum* infection, and this result corroborates those obtained by Cunha Filho *et al*. (2008) in a survey carried out in Pelotas, Rio Grande do Sul. The greater risk of infection in dogs from rural environments can be explained by the animals' easier access to sources of

infection, with a greater possibility of ingestion of carcasses, aborted bovine fetuses, and placental remains (Souza *et al*., 2002; Cunha Filho *et al*., 2008).

In this study, no variable was identified as a risk factor for *T. gondii* infection. However, according to Fábrega *et al.* (2020) the risk of infection increases according to the animals' lifetime, due to greater exposure to pathogens.

Furthermore, it should be considered that risk factors related to infections by different pathogens in dogs can be influenced by inherent characteristics of each region. Socioeconomic and environmental issues, for example, may favor different risks for infections (Fábrega *et al*., 2020).

CONCLUSIONS

The results of this study confirm the presence of high rates of infection and co-infections with *N. caninum*, *T. gondii*, and *E. canis* in the region of Cândido Sales, Bahia, with no occurrence of *B. vogeli.* Age (> 3 years old) was considered a risk factor for infection by *E. canis* and *N. caninum.* In addition, the (rural) habitat was considered a risk factor for *N. caninum* infection. These findings reaffirm that specific diagnostic techniques should be used in veterinary practice to reduce the high rate of infections and/or co-infections caused by immunosuppressive or opportunistic agents in animals. The risk factors described should be observed and prophylactic measures should be taken to avoid the persistence and transmission of these agents in the study region. Further studies with a larger number of animals are needed to elucidate the risk factors, especially for *T. gondii* infections, and to confirm the absence or rare occurrence of *B. vogeli* in dogs in the Cândido Sales-BA region.

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