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Serological and Molecular Evaluation of Ehrlichiosis, Babesiosis and Leishmaniosis in Concórdia Municipality, Santa Catarina, Brazil

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ABSTRACT

To evaluate occurrences of ehrlichiosis, babesiosis and canine leishmaniasis in the municipality of Concórdia, Santa Catarina, Brazil. Blood was collected from 424 dogs that were attended at the Clinical Practice Center of the Federal Institute of Santa Catarina, Concordia campus, and also in private clinics in the city or in its rural zone. Animals were evaluated first by serological tests, and among them, 191 dogs were proportionally and randomly chosen to undergo the molecular test. The presence of antibodies against *Leishmania infantum* was investigated using the Indirect Immunofluorescent Antibody Test and against *Babesia canis* and *Ehrlichia canis* using the Enzyme-linked Immunosorbent Assay, along with the Polymerase Chain Reaction for the three agents. The chi-square test at the significance level of 5% was used to compare the positive and negative animals in the population sampled. Among the samples evaluated in the serological tests, 43.45%, 43.98% and 14.66% were positive for ehrlichiosis, babesiosis and leishmaniasis, respectively. In the molecular test, only 4.19% of the samples were positive for babesiosis. Anti-*E. canis*, anti-*B. canis* and anti-*L. infantum* antibodies were present in the canine population studied. Due to the serological tests used for the use of IgG immunoglobulin, it is suggested that seropositive animals come into contact with the parasite previously and possibly have developed immunity against them. The polymerase chain reaction indicated that the positive animals had the genetic material of the parasite at that time, indicating that they were possibly at an early stage of the disease.

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Introduction

Canine ehrlichiosis, babesiosis and leishmaniosis are diseases that have high incidence in many countries around the world (Greene, 2012) and can trigger similar clinical-laboratory alterations that may lead to conflicting diagnoses (Miró *et al.*, 2008; Maggi *et al.*, 2014).

Ehrlichiosis is caused by *Ehrlichia canis* and is mainly transmitted by the tick *Rhipicephalus sanguineus* (Dantas-Torres, 2008). Babesiosis is caused by the protozoa *Babesia canis* and *Babesia gibsoni* and is also transmitted by the tick *R. sanguineus* (Ayoob, 2010).

Leishmaniasis is an infectious disease that affects humans and domestic and wild animals. It is caused by obligate intracellular protozoa that are members of the genus *Leishmania* (Baneth and Gallego, 2012). The vectors implicated in the transmission of leishmaniasis are insects called sandflies, among which *Lutzomyia longipalpis* and *Lutzomyia cruzi* are found in Brazil (Lopes *et al.*, 2017). Domestic dogs are the main reservoir of *Leishmania infantum* and play an important role in the transmission of the disease (Castro-Junior *et al.*, 2014). This is not only because of their proximity to humans but also because many dogs are positive and asymptomatic, thus becoming a source of contamination for the vectors (Marzochi *et al.*, 2009).

In this regard, the aim of the present study was to evaluate occurrences of *E. canis*, *B. canis* and *L. infantum* in dogs in the municipality of Concórdia, in the west of the state of Santa Catarina, a region that is considered non-endemic for these diseases.

Materials and methods

Samples

A total of 424 asymptomatic dogs were used (Zuchi *et al.*, 2020), which were investigated for *E. canis*, *B. canis* and *L. infantum* by serological tests, and among them, 191 dogs were proportionally and randomly chosen to be evaluated in the molecular test. The animals were attended at the Clinical Practice Center of the Federal Institute of Santa Catarina, Concordia campus, or at private clinics in the city, or were attended on farms in this municipality's rural zone. From each dog, 5 mL of whole blood was collected and divided into two aliquots. In one, the serum was separated out for the purpose

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of detecting antibodies; the other was used for DNA extraction. These aliquots were stored at -20 ºC until the time of performing the technique. Information regarding these animals' sex, access to the streets, place of residence, age and breed was gathered. This study was approved by the Ethics Committee for Animal Use of the Federal Institute of Santa Catarina, under protocol number 31/2014.

Search for anti-E. canis, anti-B. canis and anti-L. infantum antibodies

To detect anti-*E. canis* and anti-*B. canis* antibodies, commercial Enzyme-linked Immunosorbent Assay (ELISA) kits were used: Imunoteste Ehrlichia and Imunoteste *Babesia*, respectively (both from Immunodot Diagnostics, Brazil). The protocols used followed the manufacturer's recommendations, such that samples with an optical density greater than or equal to the cutoff index were considered positive. Anti-*L. infantum* antibodies were detected via the Indirect Immunofluorescent Antibody Test (IFAT), using the commercial kit Imunoteste *Leishmania* (Immunodot Diagnostics, Brazil) and following the manufacturer's instructions. Green fluorescence on the entire surface of the parasites was considered positive, and the reaction was negative when there was no fluorescence.

DNA extraction and Polymerase Chain Reaction (PCR)

DNA extraction was performed using the PureLink® Genomic DNA Mini-Kit (Invitrogen, EUA), with 200 µL of whole blood. The material resulting from the extraction was eluted in 50 µl of ultrapure water that was free from DNAse and RNAse and was stored at -20 ºC until the PCR was performed. The positive and negative controls for the reactions were subjected to the same extraction protocol as used for the samples tested. The oligonucleotides used for the amplification reactions are described in Table 1. The PCR reactions for detecting *E. canis* (Harrus *et al*., 2011), *B. canis* (Almeida, 2011) and *L. infantum* (Passos *et al*., 1996) followed the protocols previously cited.

Briefly, a total of 1 µL of the extracted DNA sample was amplified in 25 µL of the final volume, containing buffer solution (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 200 µM dNTP, 20 pmol of each oligonucleotide and 1 U of Taq polymerase. Lastly, $MgCl_2$ was added up to a final concentration of 1.0, 1.5 or 2.0 mM, for the amplification reactions of *E. canis*, *B. canis* or *L. infantum*, respectively.

The PCR amplification cycles were performed in a T100TM thermal cycler (BIORAD), as follows: for *E. canis* there was an initial cycle of 95ºC for 3 min, followed by 33 cycles of 95ºC for 30 sec, 53ºC for 30 sec and 72ºC for 45 sec, and a final extension step of 72 ºC for 5 min; for *B. canis* there was an initial cycle of 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 sec, 58ºC for 30 sec and 72 ºC for 30 sec, and a final extension step of 72ºC for 7 min; and for *L. infantum* there was an initial cycle of 94ºC for 3 min, followed by 34 cycles of 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 30 sec, and a final extension step of 72ºC for 10 min. The PCR products were subjected to electrophoresis on 1% agarose gel, stained with ethidium bromide and viewed with the aid of a transilluminator (LTB-STi UV transilluminator with L-PIX ST-Loccus Photo Documentation Device).

Statistical analysis

The chi-square test at a 5% significance level was used to compare the variables (sex, access to the streets, place of residence, age and breed) between the groups of positive and negative animals within the sample population, using the Minitab® 19 (Minitab, LLC, USA) statistical software.

Results

Out of the 191 samples evaluated, 43.45% (83/191) were considered positive for ehrlichiosis through ELISA, with statis-

Table 1. Oligonucleotides used for the amplification reactions of *E. canis*, *B. canis* and *L. infantum*.

Oligonucleotides	Sequences 5' – 3'	*Amplicon* (pb)	References
EHR 16SD	GGTACCYACAGAAGAAGTCC	345	Harrus *et al*. (2011)
EHR 16SR	TAGCACTCATCGTTTACAGC		
BAB143-167	CCGTGCTAATTGTAGGGCTAATACA	551	Almeida (2011)
BAB694-667	GCTTGAAACACTCTARTTTTCTCAAAG		
LEISH1	GGCCCACTATATTACACCAACCCC	120	Passos *et al*. (1996)
LEISH2	GGGGTAGGGGCGTTCTGCGAA		

Table 2. Investigation of the presence of anti-*E. canis* antibodies through ELISA, and stratification of the results against the different variables tested.

Variables		Dogs	
		Positive (n/%)	Negative (n/%)
Sex	Female[a]	47/83 (56.63)	64/108 (59.35)
	Male[a]	36/83 (43.45)	44/108 (40.74)
Location	Urban area[a]	44/83 (53.01)	82/108 (75.92)
	Rural area[b]	39/83 (46.98)	26/108 (24.07)
Street access	With street access[a]	51/83 (61.44)	50/108 (46.29)
	Without street access[b]	32/83 (38.55)	58/108 (53.70)
Breed	Mongrel[a]	55/83 (66.26)	53/108 (49.07)
	Poodle[b]	3/83 (3.61)	12/108 (11.11)
	Pinscher[b]	2/83 (2.41)	11/108 (10.18)
	Other breeds[a]	23/83 (27.71)	32/108 (29.62)
Age	Less than 1 year[a]	4/83 (4.81)	9/108 (8.33)
	Between 1 and 5 years[a]	40/83 (48.19)	45/108 (41.66)
	Between 5 and 10 years[a]	20/83 (24.09)	24/108 (22.22)
	Over 10 years[a]	14/83 (16.86)	22/108 (20.37)
	Not informed[a]	5/83 (6.02)	8/108 (7.40)

[a,b] Different letters indicate a statistically significant difference

tically significant differences for the variables of location, access to the streets and breed (P ≤ 0.05). Regarding babesiosis, 43.98% (84/191) of the samples were positive through ELISA, with statistically significant differences for the variables of access to the streets and location (P ≤ 0.05). Regarding leishmaniasis, 14.66% (28/191) of the samples were positive through IFAT, with a statistically significant difference only for the variable of access to the streets (P ≤ 0.05).

Among the samples analyzed, 4.19% (8/191) presented seropositivity for all the three diseases. However, PCR showed that only 4.19% (8/191) of the samples were positive for babesiosis, while all the samples were negative for ehrlichiosis and leishmaniasis. The results from the serological tests for ehrlichiosis, babesiosis and leishmaniasis, along with the segregation of the population studied in relation to different variables, are shown in Tables 2, 3 and 4, respectively.

Discussion

Studies carried out in Brazil have shown that the seroprevalence of *E. canis* is similar between this country's regions, for example 31.2% in the north, 42.5% in the central region and 35.6% in the northeast (Saito *et al*., 2008; Silva *et al*., 2010; Souza *et al*., 2010). In the southeastern region, in the city of Jaboticabal-SP, Faria *et al*. (2010) found a seroprevalence of 82.5%. However, in a study carried out in the southern region by Saito *et al*. (2008), only 19 (4.8%) out of 389 dogs were positive for *E. canis*. This is divergent from the present study, in which seroprevalence of 43.45% was observed among the animals, i.e. a prevalence similar to what has been found in other regions of Brazil.

Serological tests are important diagnostic tools during the subclinical and chronic phases of ehrlichiosis (Vieira *et al*., 2011). Thus, it can be proposed that the serologically positive animals in the present study had come into contact with the hemoparasite and possibly were in subclinical stages. Lasta *et al*. (2013) evaluated occurrences of *E. canis* in the municipality of Porto Alegre-RS using the dot-ELISA and nested PCR tests and found absence of positivity in both tests. Malheiros *et al*. (2016) conducted a study in Passo Fundo-RS and found through ELISA that 9% (7/80) of the animals were positive for ehrlichiosis, but that through PCR all the animals were negative. Both of those studies are divergent from the present survey regarding the serological test, since the seropositivity in the present study was 43.45%. However, all of these three studies showed similar results from the molecular test.

The seropositivity for babesiosis found in the present sur-

Table 3. Investigation of the presence of anti-*B. canis* antibodies through ELISA, and stratification of the results against the different variables tested.

Variables		Dogs	
		Positive (n/%)	Negative (n/%)
Sex	Female[a]	52/84 (61.90)	59/107 (55.15)
	Male[a]	32/84 (38,09)	48/107 (44.85)
Location	Urban area[a]	65/84 (77.38)	61/107 (57.01)
	Rural area[b]	19/84 (22.61)	46/107 (42.99)
Street access	With street access[a]	31/84 (36.90)	70/107 (65.42)
	Without street access[b]	53/84 (63.09)	37/107 (34.58)
Breed	Mongrel[a]	46/84 (54.76)	61/107 (57.01)
	Poodle[a]	6/84 (7.14)	9/107 (8.41)
	Pinscher[a]	2/84 (2.38)	11/107 (10.28)
	Other breeds[a]	30/84 (35.71)	26/107(24.30)
Age	Less than 1 year[a]	3/84 (3.57)	10/107 (9.34)
	Between 1 and 5 years[a]	39/84 (46.42)	46/107 (42.99)
	Between 5 and 10 years[a]	24/84 (28.57)	17/107 (15.88)
	Over 10 years[a]	13/84 (15.47)	26/107 (24.30)
	Not informed[a]	5/84 (5.95)	8/107 (7.47)

[a,b] Different letters indicate a statistically significant difference.

Table 4. Investigation of the presence of anti-*L. infantum* antibodies through RIFI and, stratification of the results against the different variables tested.

Variables		Dogs	
		Positive (n/%)	Negative (n/%)
Sex	Female[a]	17/28 (60.71)	94/163 (57.66)
	Male[a]	11/28 (39.28)	69/163 (42.33)
Location	Urban area[a]	21/28 (75.00)	105/163 (64.41)
	Rural area[a]	7/28 (25.00)	58/163 (35.58)
Street access	With street access[a]	10/28 (35.71)	91/163 (55.82)
	Without street access[b]	18/28 (64.28)	72/163 (44.17)
Breed	Mongrel[a]	20/28 (71.42)	88/163 (53.98)
	Poodle[a]	2/28 (7.14)	13/163 (7.97)
	Pinscher[a]	2/28 (7.14)	11/163 (6.74)
	Other breeds[a]	4/28 (14.28)	51/163 (31.28)
Age	Less than 1 year[a]	-	12/163 (7.36)
	Between 1 and 5 years[a]	13/28 (46.42)	72/163 (44.17)
	Between 5 and 10 years[a]	6/28 (21.42)	35/163 (21.47)
	Over 10 years[a]	8/28 (28.57)	32/163 (19.63)
	Not informed[a]	1/28 (3.57)	12/163 (7.36)

[a,b] Different letters indicate a statistically significant difference.

vey corroborates the results from a study carried out in Paraná by Trapp *et al.* (2006), who found positivity of 35.7% through using the IFAT technique. In the molecular test, 4.19% (8/191) of the animals were positive for *B. canis*, a result similar to those found by Silva *et al.* (2012) in Maranhão (3.33%) and Ramos *et al.* (2010) in Recife (7.31%).

The eight animals that were found to be positive through PCR did not have evident clinical signs, or any presence of the vector. In addition, five of these animals were also positive according to the serological test. It can be suggested that serologically positive animals had previous contact with the parasite and subsequently developed immunity and cure. The animals that are positive according to PCR could be showing parasitemia at the time of diagnosis, as concluded by Hernández (2010), who found that 51.6% of their animals were positive according to serological tests and only 5.5% according to the molecular test.

The seropositivity found for leishmaniasis was 14.66% (28/191). This result is similar to what was found by Maziero *et al.* (2014), who conducted a study on 252 animals in the west of the state. Among their animals, 43 (about 17%) presented seropositivity for *L. infantum*, through IFAT.

The serological examination determines that the animal came into contact with the pathogen (Miró *et al.*, 2008) and the PCR performed on peripheral blood is more effective for detecting the early stages of infection (Paltrinieri *et al.*, 2010). Thus, it can be suggested that animals that presented seroconversion came into contact with the parasite at a given time and were supposedly have developed immunity. Because they were not positive in the molecular diagnosis of blood samples, they were possibly not in the early stages of the infection. This fact may also be due to cross-reactivity with another agent present in the region, in addition to the possibility of false negative in peripheral blood and greater parasitic load on the spleen and bone marrow. However, these assessments have not been carried out.

In a study by Nunes *et al.* (2016) in the city of Alfenas-MG, a region that is considered non-endemic for leishmaniasis, 2.3% of the samples were seroreactive through IFAT and the whole blood samples were negative according to PCR. The latter result was equivalent to what was observed in the present investigation. However, PCR of spleen and liver samples gave rise to results that were positive for leishmaniasis (Nunes *et al.*, 2016).

Cross-reactions between antibodies are questionable aspects of the diagnosis of parasitic blood diseases. Troncarelli *et al.* (2009) identified occurrences of cross-reactions between *Leishmania* spp. and *Trypanosoma cruzi* in 16.5% of the animals tested, and proved this through molecular tests. Ferreira *et al.* (2007) reported that cross-reactivity with *E. canis* was present among animals that were seropositive for leishmaniasis. Mettler *et al.* (2005) identified cross-reactivity with *B. canis* in cases of leishmaniasis. However, a study carried out in endemic areas found through serological tests that there was no cross-reaction between *E. canis*, *B. canis* and *L. infantum*, but that co-infection was occurring (Krawczak *et al.*, 2015). In the present study, one of the possibilities for the results of seropositive animals is to cross-react with other agents present in the region, however other tests were not carried out so that this could in fact be stated. In order to determine whether cross-reactivity or co-infection was occurring, it would be necessary to perform PCR on spleen or bone marrow samples. However, this is unfeasible in an epidemiological study because of the invasive nature of this sample collection.

Conclusion

Through the present study, it can be suggested that animals that were positive according to serological tests had previously had contact with hemoparasites and developed immunity or were cured. Another possibility is that they could have cross-reactivity with other agents. Animals that were positive according to the molecular test could be in the early stages of the disease. Still, due to the finding of seropositive animals for leishmaniasis, it would be opportune to investigate the presence of the vector in the city, given the importance of epidemiological surveillance in cases of zoonoses in non-endemic regions.

Conflict of interest

Authors declared no conflict of interests exist.

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