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ORIGINAL PAPER

Molecular survey and genetic characterization of tick-borne pathogens in dogs in metropolitan Recife (north-eastern Brazil)

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Abstract To identify DNA of the main tick-borne pathogens in dogs from Recife (Brazil), polymerase chain reactions were carried out on blood samples of dogs treated at the Veterinary Hospital of the Universidade Federal Rural de Pernambuco from March 2007 to June 2008. The detection of DNA was performed using specific primers. Amplicons were analyzed through electrophoresis and sequencing. A phylogenetic tree was constructed using the UPGMA method, revealing that the sequences were closely related to those of strains from other geographic regions. Among the 205 blood samples analyzed, 48.78% was positive for *Anaplasma platys*; 38.04% was positive for *Ehrlichia canis*; 7.31% was positive for *Babesia canis vogeli*; and 0.49% was positive for *Hepatozoon canis* and *Mycoplasma haemocanis*. Coinfection of two or three pathogens was found in 23.9% (49/205) of the dogs. The subspecies *B. canis vogeli* was identified. Infection by *H. canis* and *M. haemocanis* is reported for the first time in dogs in the state of Pernambuco (Brazil). The data indicate that the main tick-borne pathogens in dogs in this region are *E. canis* and/or *A. platys*, followed by *B. canis vogeli.*

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Introduction

Vector-borne diseases are increasingly recognized as the cause of severe clinical illness in dogs (Solano-Gallego et al. 2006). In Brazil, the main tick-borne pathogens described for dogs are *Babesia canis*, *Ehrlichia canis*, *Anaplasma platys*, *Hepatozoon canis*, and *Mycoplasma haemocanis* (Passos et al. 2005; Santos et al. 2007; Forlano et al. 2007; Trapp et al. 2006). The transmission of these pathogens occurs mainly by the brown dog tick, *Rhipicephalus sanguineus* (Labruna and Pereira 2001; Dantas-Torres et al. 2004).

B. canis and *Babesia gibsoni* are the etiological agents of canine babesiosis. The former is the most important in Brazil and is divided into three subspecies (*Babesia canis canis*, *Babesia canis vogeli*, and *Babesia canis rossi*), which are characterized by vector, geographical distribution, pathogenicity, and antigenic property (Uilenberg et al. 1989). At present, only *B. canis vogeli* (Passos et al. 2005) and *B. gibsoni* (Trapp et al. 2006) have been described infecting dogs in Brazil. In the genus *Hepatozoon*, two species have been described infecting dogs, *H. canis* and *Hepatozoon americanum* (Baneth et al. 2000). However, only *H. canis* has been described in Brazil, based on molecular studies (Rubini et al. 2005; Forlano et al. 2007). *E. canis* and *A. platys* are obligatory intracellular rickettsia with tropism for leukocytes and platelets, respectively. Both have wide geographical distribution (Aguirre et al. 2006; Pinyoowong et al. 2007; Yabsley et al. 2008) and are the main blood pathogens that infect dogs in urban areas in Brazil (Santos et al. 2007). There is little epidemiological information on *M. haemocanis* in Brazil (Trapp et al. 2006).

In the routine of veterinary clinics, the microscopic examination of stained blood smears and serological

methods have been used for the diagnosis of many of these pathogens. However, the cyclic parasitemia of many of these organisms (Harrus et al. 1997) and serologic cross-reactivity between related species (Dreher et al. 2005) hinder the diagnosis of these infections (Ferreira et al. 2007).

The aims of the present study were to assess the frequency of infection by *B. canis vogeli*, *A. platys*, *E. canis*, *M. haemocanis*, and *Hepatozon canis* in sick dogs using a molecular tool and to molecularly characterize the strains of the tick-borne pathogens found.

Materials and methods

Biological samples and DNA extraction

Blood samples from 205 dogs from metropolitan Recife (state of Pernambuco, Brazil) treated at the Veterinary Hospital of Universidade Federal Rural de Pernambuco from March 2007 to June 2008 were collected with EDTA and stored at −20°C. Genomic DNA was extracted using the Easy DNA kit (Invitrogen) following the manufacturer's instructions. The quality and concentration of the extracted samples were evaluated through electrophoresis in agarose gel and spectrophotometry. Positive controls were obtained from dogs having tested positive in microscopic blood smear examination. Distilled water was used as negative controls.

DNA amplification

The detection of pathogen DNA was performed with polymerase chain reaction (PCR) using a sets of primers (Table 1). A single PCR was used for the detection of *B. canis vogeli*, *Hepatozoon* sp., and *Mycoplasma* sp., and a nested PCR was used for *E. canis* and *A. platys*. The reactions were performed in final volume of 25 μl, containing 10 mM of Tris–HCl (pH 8.3), 50 μM of KCl, 1.5 mM of $MgCl_2$, 0.2 mM of each desoxynucleoside triphosphate, 1.5 U of Taq DNA polymerase (Invitrogen), 11 pmol of each primer, and approximately 100 ηg of genomic DNA. The following parameters were used in the single PCR: 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, annealing at 56°C for *B. canis* and *B. gibsoni*, 50°C for *Mycoplasma* sp. and *Hepatozoon* sp. for 30 s, extension at 72°C for 40 s. In the nested PCR, the primer sets used in the first reaction were 8F and 1448R for *A. platys* and ECC and ECB for *E. canis*. In the second reaction, the PLATYS-F and EHR16S-R primer sets were used for *A. platys*, and the HE and ECA primer sets were used for *E. canis*. The PCR parameters were 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, annealing at 45°C for *A. platys* and 60º C for *E. canis* for 1 min, extension at 72°C for 40 s. In the second reaction, the annealing temperature was 53°C for *A. platys* and 60°C for *E. canis* for 30 s. A final extension step at 72°C for 3 min, prior to stopping the reaction at 4°C, was employed for all reactions. The amplification products were viewed under an ultraviolet light after electrophoresis on agarose gel stained with SyBr Gold (Invitrogen).

To avoid cross-contamination and sample carryover, pre- and post-PCR sample processings were performed in separate rooms. All fluid transfers were carried out with plugged pipette tips to eliminate aerosols.

Sequence analysis

Amplicons were purified from agarose gel using the QIAEX II Gel Extraction Kit (Qiagen) and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI 3130 Genetic Analyzer (Applied

Table 1 Primers used in PCR for detection of DNA of tick-borne pathogens in dogs from metropolitan region of Recife, Pernambuco state, Brazil

Primer	Pathogen	Gene	Sequence 5′- 3′	Base pairs	Reference
8F 1448R	*A. platys*	16S rRNA	AGTTTGATCATGGCTCAG CCATGGCGTGACGGGCAGTGT	–	Martin et al. (2005)
PLATYS-F EHR16S-R	*A. platys*	16S rRNA	GATTTTTGTCGTAGCTTGCTATG TAGCACTCATCGTTTACAGC	678	Martin et al. (2005)
ECC ECB	*E. canis*	16S rRNA	AGAACGAACGCTGGCGGCAAGCC CGTATTACCGCGGCTGCTGGC	478	Wen et al. (1997)
HE ECA	*E. canis*	16S rRNA	TATAGGTACCGTCATTATCTTCCCTAT CAATTATTTATAGCCTCTGGCTATAGGAA	389	Wen et al. (1997)
HepF HepR	*Hepatozoon sp.*	18S rRNA	ATACATGAGCAAAATCTCAAC CTTATTATTCCATGCTGCAG	666	Inokuma et al. (2002)
fHF5 rHF6	*Mycoplasma* sp.	16S rRNA	AGCAGCAGTAGGGAATCTTCCAC TGCACCACCTGTCACCTCGATAAC	659	Messick et al. (1998)
BAB1 BAB4	*B. canis vogeli*	18S rRNA	GTGAACCTTATCACTTAAAGG CAACTCCTCCACGCAATCG	590	Duarte et al. (2008)

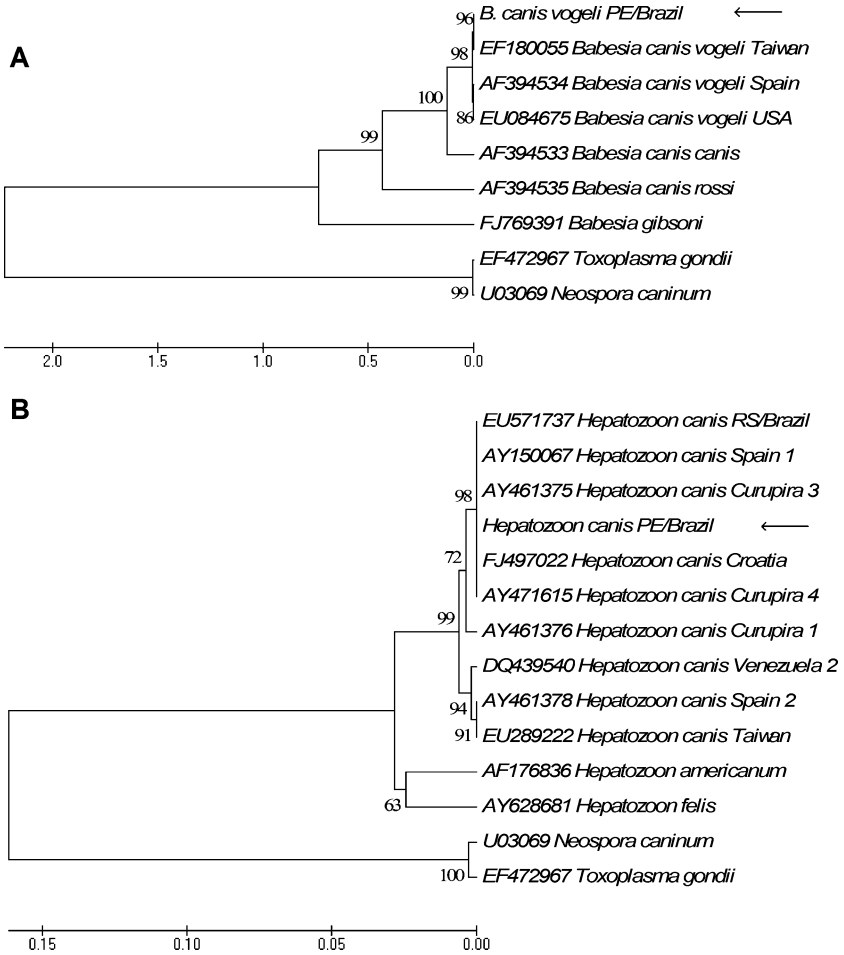

Fig. 1 Phylogenetic tree based on *Babesia* (**a**) and *Hepatozoon* (**b**) 18S rRNA gene sequences. Sequences were compared with the UPGMA method operated by MEGA software (version 4.0). *Scale bar* represents the number of mutations per sequence position. The *numbers* at the nodes indicate the percentage of 500 bootstrap resamplings. New sequences are marked by *arrows*

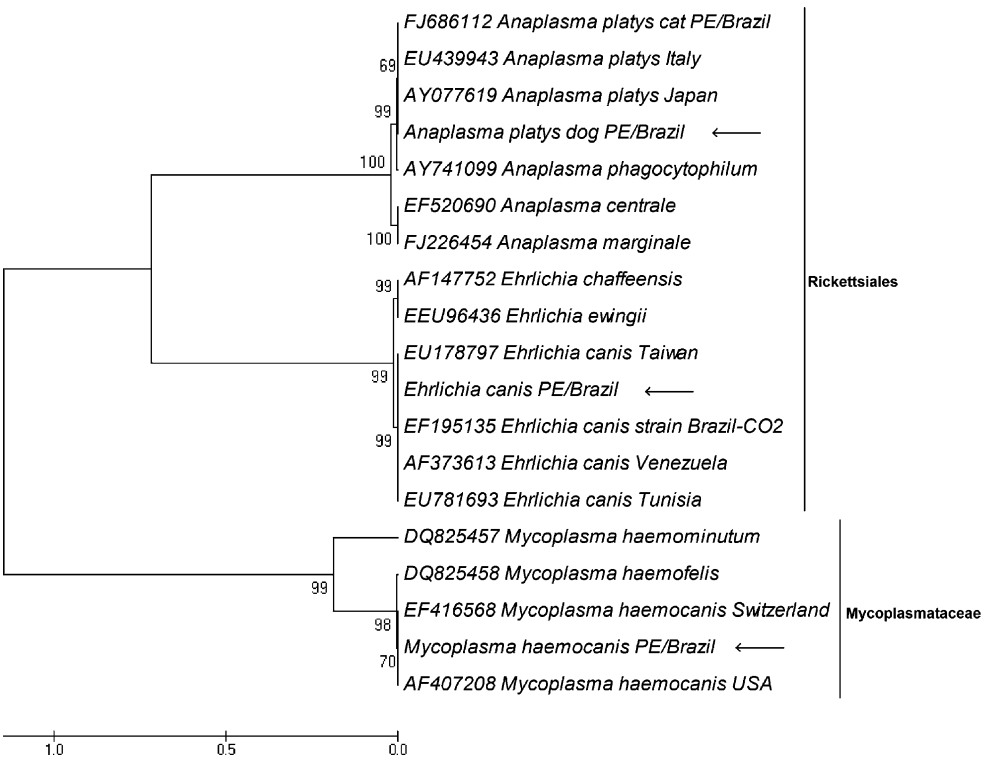

Fig. 2 Phylogenetic tree based on *Anaplasma*, *Ehrlichia*, and *Mycoplasma* 16S rRNA gene sequences. Sequences were compared with the UPGMA method operated by MEGA software (version 4.0). *Scale bar* represents the number of mutations per sequence position. The *numbers* at the nodes indicate the percentage of 500 bootstrap resamplings. New sequences are marked by *arrows*

Table 2 Percentage of infections and coinfections between pathogens studied in dogs from metropolitan region of Recife, Pernambuco state, Brazil

Infection	Percentage (%)	Co-infection	Percentage (%)
A. platys	48.78 (100/205)	*E. canis*/*A. platys*	16.09 (33/205)
E. canis	38.04 (79/205)	*E. canis*/*B. canis*	2.92 (6/205)
B. canis vogeli	7.31 (15/205)	*A. platys*/*B. canis*	1.95 (4/205)
H. canis	0.48 (1/205)	*A. platys*/*M. haemocanis*	0.48% (1/205)
M. haemocanis	0.48 (1/205)	*A. platys*/*B. canis*/*E. canis*	1.95% (4/205)
		A. platys/*E. canis*/*H. canis*	0.48% (1/205)

Biosystems). Five randomly selected *E. canis*, *A. platys*, and *B. canis vogeli* positive PCR products were sequenced. For *Hepatozoon* and *Mycoplasma*, only one animal was positive, and at least four distinct amplicons were sequenced.

Sequence chromatograms were evaluated and edited using the Sequencher v 4.1.4 program (Gene Codes), and consensus sequences were submitted to a BLASTn (Altschul et al. 1990) search (http://www.ncbi.nlm.nih.gov) to determine the sequence identity in order to find orthologous sequences available in the GenBank database. A phylogenetic tree was constructed using the UPGMA method (Sneath and Sokal 1973). The sequences used in this study for the construction of the phylogenetic tree were available in the GenBank database (Figs. 1 and 2). Bootstrap resampling (500 replicates) was performed for the statistical support of the reliabilities of the nodes on the trees (Felsenstein 1985) using the MEGA program, version 4.0 (Tamura et al. 2007).

Results

Table 2 displays the frequency of dogs positive for tick-borne pathogens in metropolitan Recife. In the DNA sequence analysis, the sequences were identical between the amplicons sequenced for each pathogen in this study. Specific identity of 99% to 100% was found between consensus sequence of local isolates and sequences that exhibited the highest level of homology in the BLASTn search (GenBank: *E. canis* EU178797, *A. platys* EU439943, *B. canis vogeli* EF180055, *M. haemocanis* EF416568, and *H. canis* FJ743476). Partial consensus sequences of the 16S rRNA gene (*E. canis*, *A. platys*, and *M. haemocanis*) and 18S rRNA gene (*B. canis vogeli* and *H. canis*) obtained in this study were deposited in the GenBank database under accession numbers FJ943579, FJ943580, FJ911910, FJ588003, and FJ943578, respectively (Table 3).

A subspecies-specific PCR using a primer set developed by Duarte et al. (2008) was successfully used in the present study for the diagnosis of *B. canis vogeli*, as confirmed by the sequence analysis. In the phylogenetic analysis of the partial 18S rRNA sequence from *H. canis* (Fig. 1), the PE/Brazil isolate was grouped in a cluster with the isolates Curupira 1 (no. AY461376), Spain 1 (no. AY150067), and others, while isolates Venezuela 2 (no. DQ439540) and Spain 2 (no. AY461378) were grouped in an independent clade (Fig. 1b). The phylogenetic tree for *B. canis vogeli* (Fig. 1a), *A. platys*, *E. canis*, and *M. haemocanis* (Fig. 2) demonstrates that variability between Brazilian strain of these pathogens and those from other geographic regions is low. Coinfections of two or three pathogens occurred in 23.9% (49/205) of the animals (Table 2). The most frequently coinfections were by *E. canis* and *A. platys*.

Discussion

The frequency of dogs positive for *E. canis* and *A. platys* is highly variable in different regions of the world. The percentage of positive animals in the present study—*A. platys* (48.78%) and *E. canis* (38.04%)—is higher than the 15.84% described by Ferreira et al. (2007) for *A. platys* in Rio de Janeiro (Brazil) and the 38.9% and 14.9% described by Santos et al. (2007) for *E. canis* and *A. platys*, respectively, in Ribeirão Preto (Brazil). It was also higher than that described for *A. platys* by Huang et al. (2005; 16%) in Venezuela and De La Fuente et al. (2006; 4%) in

Table 3 Pathogen, specific identity, and GenBank accession number

Pathogen	Identity (%)	Best hit	GenBank[a]
E. canis	99	*E. canis* (EU178797)	FJ943579
A. platys	99	*A. platys* (EU439943)	FJ943580
B. canis vogeli	99	*B. canis vogeli* (EF180055)	FJ588003
M. haemocanis	100	*M. haemocanis* (EF416568)	FJ911910
H. canis	99	*H. canis* (FJ743476)	FJ943578

[a] Sequence of GenBank accession numbers obtained in this study

Sicily, Italy. However, the percentage in the present study is similar to that described by Wen et al. (1997) for *E. canis* in the USA (44%).

According to Labarthe et al. (2003), approximately 20% of dogs treated in veterinary hospitals and clinics in the southern, south-eastern, central-western, and north-eastern regions of Brazil are serologically positive for *E. canis*. The variation in the prevalence of infection may be related to differences in the dog population studied, geographical differences in vector exposure, and potential differences in the diagnostic methods employed (Solano-Gallego et al. 2006). In the present study, the high frequencies found were likely due to the fact that the dog population sampled was clinically suspected of having tick-borne diseases and that the test used (nPCR) has high sensitivity (Wen et al. 1997; Martin et al. 2005). The frequency of positive animals for *B. canis vogeli* (7.31%) was higher than that described for Spain (0.01%) and Sudan (0.025%), as described by Oyamada et al. (2005). *Amblyomma* ticks have been implicated in the transmission of *H. canis* Curupira 1 and Spain 1, and *R. sanguineus* is implicated in the transmission of other isolates (Criado-Fornelio et al. 2007). This may explain the low frequency of positive dogs to *H. canis* in the present study (0.48%), as the brown dog tick, *R. sanguineus*, has been the only species found parasitizing dogs in metropolitan Recife (Dantas-Torres et al. 2004).

In Brazil, canine hepatozoonosis is mainly reported in rural areas, where the main ticks are *Amblyomma* spp. (Labruna and Pereira 2001; Rubini et al. 2008). A recent molecular survey found 53.3% prevalence in dogs in the rural areas in the state of São Paulo (Rubini et al. 2008).

M. haemocanis, formerly classified as a *Haemobartonella* species, has recently been positioned within the genus *Mycoplasma* by 16S rRNA analysis (Messick et al. 2002). This group of bacteria attaches to the surface of red blood cells, where it then grows. Infection by *M. haemocanis* has been documented in dogs in the USA, Europe, Canada, the United Kingdom (Seneviratna et al. 1973; Chalker 2005), and Brazil (Trapp et al. 2006), although few epidemiological data are found. The PCR assay used in the present study was originally developed for the detection of *Mycoplasma haemofelis* in cats (Messick et al. 1998) and was later successfully used to detect *M. haemocanis* in a dog (Brinson and Messick 2001). This PCR assay can be used for the detection of other *Mycoplasma* species such as *Candidatus Mycoplasma haemominutum* and *Candidatus Mycoplasma haematoparvum* (Foley et al. 1998).

There are descriptions of co-infection by tick-borne pathogens of dogs in China (Hua et al. 2000), the Caribbean (Yabsley et al. 2008), Venezuela (Suksawat et al. 2001), and Brazil (Santos et al. 2007). The high prevalence of *A. platys* combined with *E. canis* is evidence that *R. sanguineus* may be the same vector for both. If *R. sanguineus* is a competent vector, *A. platy* is likely to be found in the same geographic areas as *E. canis* (Yabsley et al. 2008). The present study also presents the first description of infection by *H. canis* and *M. haemocanis* in dogs in the state of Pernambuco, Brazil.

Conclusions

The data from the present study indicate that the main tick-borne pathogens of dogs in metropolitan Recife are *E. canis* and/or *A. platys*, followed by *B. canis vogeli.* These findings are important to the understanding of the epidemiology of tick-borne pathogens of domestic dogs in this region, which will assist in the management of these pathogens.

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