Full Article

Molecular detection of *Ehrlichia canis* and *Anaplasma platys* in dogs in Southern Brazil

Detecção molecular de *Ehrlichia canis* e *Anaplasma platys* em cães do sul do Brasil

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Abstract

The aims of this study were to determine the occurrence of *Anaplasma platys* and *Ehrlichia canis* infection in dogs in Porto Alegre, Southern Brazil; and to investigate their association with hematological abnormalities. Serum samples from 196 dogs were first tested using dot-ELISA for antibodies against *Anaplasma* spp. and *Ehrlichia canis*. Peripheral blood samples from 199 dogs were subjected to 16S rRNA nested PCR (nPCR) for *A. platys* and *E. canis*, followed by DNA sequencing to ensure pathogen identity. A total of 19/196 samples (9.69%) were positive for *Anaplasma* spp. using ELISA and 28/199 (14.07%) samples were positive for *A. platys* by nested PCR. All the dog samples were negative for *E. canis*, both in anti-*E. canis* antibody tests and in nested PCR. There were no significant differences in hematological parameters between *A. platys*-PCR positive and negative dogs and *Anaplasma* spp. serologically positive dogs, except for basophil counts, which were higher in nPCR-positive dogs. This is the first report showing *A. platys* presence in dogs in Southern Brazil. In conclusion, hematological parameters may not be sufficient to diagnose *A. platys* infection in dogs in Southern Brazil, probably due either to low pathogenicity or to chronic infection. On the other hand, *E. canis* may either have very low occurrence or be absent in dogs in Porto Alegre.

Keywords: Anaplasmataceae, dogs, Southern Brazil, 16S rRNA gene.

Resumo

O objetivo deste estudo foi determinar a ocorrência de *Anaplasma platys* e *Ehrlichia canis* em cães de Porto Alegre, sul do Brasil, sua detecção molecular e associação com anormalidades hematológicas. Amostras séricas de 196 cães foram inicialmente triadas por dot-ELISA para a presença de anticorpos contra *Anaplasma* spp. e *Ehrlichia canis*. Amostras de sangue periférico de 199 cães foram submetidas à nested PCR (16S rRNA) para *A. platys* e *E. canis*, seguido de sequenciamento do DNA para confirmar a identidade do agente. Do total, 19/196 (9,69%) amostras foram positivas para *Anaplasma* spp. por dot-ELISA e 28/199 (14,07%) por nPCR. Todas as amostras dos cães foram negativas para *E. canis* no teste sorológico anti-*E. canis* e também na nPCR. Não houve diferença significativa nos parâmetros hematológicos, exceto a contagem de basófilos, que apresentou valores mais altos em cães positivos na nPCR para *A. platys*. Este é o primeiro relato da presença de *A. platys* no Rio Grande do Sul, e a primeira detecção molecular do agente no sul do Brasil. Em conclusão, parâmetros hematológicos não são suficientes para diagnosticar a infecção por *A. platys* em cães, provavelmente devido sua baixa patogenicidade ou infecção crônica. Por outro lado, *E. canis* parece ter ocorrência baixa ou mesmo nula em cães de Porto Alegre.

Palavras-chave: Anaplasmataceae, cães, sul do Brasil, gene 16S rRNA.

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Introduction

Anaplasma platys and *Ehrlichia canis* are tick-borne bacteria that cause mild to severe disease in dogs (BEALL et al., 2008; GAUNT et al., 2010). Both organisms are transmitted by ticks, mainly by *Rhipicephalus sanguineus*. *Anaplasma platys* - formerly known as *Ehrlichia platys* (DUMLER et al., 2001) - and *E. canis*, from the Anaplasmataceae family, cause canine infectious cyclic thrombocytopenia (CICT) and canine monocytic ehrlichiosis (CME), respectively (HARVEY, 2006; DANTAS-TORRES, 2008).

Anaplasma platys develops inside canine platelets, with no clearly established pathogenic role in dogs (AGUIRRE et al., 2006; HARVEY, 2006). Reports on experimental and natural infections have mostly indicated that *A. platys* causes few or no clinical signs in dogs in the United States (HARVEY, 2006; GAUNT et al., 2010). However, *A. platys* strains are reportedly more virulent in France, Spain, Turkey and Tunisia (BEAUFILS et al., 2002; AGUIRRE et al., 2006; ULUTAS et al., 2007, M´GHIRBI et al., 2009), with clinical signs that are probably influenced by stress, immune status, breed predisposition (AGUIRRE et al., 2006) or strain variability. In contrast, *E. canis* infects monocytes and has been associated with serious and sometimes fatal disease in dogs (NEER; HARRUS, 2006). It has also been suggested to be a zoonotic agent in Venezuela (PEREZ et al., 1996, 2006).

No serological surveys on *A. platys* have been performed to date in Brazil, whereas the seroprevalence of *E. canis* in dogs in Brazil may range from 0.7 to 92.3%, depending on the population, geographical area and diagnostic test used (OLIVEIRA et al., 2000; LABARTHE et al., 2003; VIEIRA et al., 2011). Moreover, molecular detection of *A. platys* and *E. canis* in dogs has been conducted in Brazil and has shown widely variable prevalence, from 7.8 to 88% and from 8.1 to 55%, respectively (DAGNONE et al., 2003, 2009; COSTA Jr., 2007b; RAMOS et al., 2009; SANTOS et al. 2009a).

Although *R. sanguineus* is a commonly found tick species in Southern Brazil (RIBEIRO et al., 1997), it has not yet been fully established whether *A. platys* and *E. canis* cause infection in dogs in the state of Rio Grande do Sul. Accordingly, the aims of the present study were to determine the serological and molecular prevalence of *A. platys* and *E. canis* and to correlate infection with hematological abnormalities in two populations of naturally infected dogs in Porto Alegre, the capital of Rio Grande do Sul, Southern Brazil.

Materials and Methods

Study population and samples

This study was carried out in the city of Porto Alegre (30° 01' 59" S and 51° 13' 48" W), the capital of the state of Rio Grande do Sul, Southern Brazil, with an estimated population of 1,400,000 inhabitants. Blood samples were collected between May 2007 and February 2009, from 53 stray dogs at the city's Zoonosis Control Center and 146 semi-owned dogs (dogs that were living outdoors, with full access to the streets, i.e. free roaming) on a low-income island (Arquipelago district) within the city limits, thus totaling 199 dogs. Only one sample was taken from each dog.

The Arquipelago district is the biggest in Porto Alegre and is formed by 15 islands. Its population is approximately 5,000 inhabitants and most of them rely on garbage recycling. The Zoonosis Control Center did not have precise information about where each dog was initially caught, but it functions throughout the city. The data recorded on each animal included information on breed, age and location. In the Arquipelago district, the dogs were selected by convenience, at times when clients volunteered their dogs during attendance campaigns, without specific inclusion criteria. Only the animals that were receiving antibiotic therapy were excluded from the study. This work was approved by the Ethics Committee for Animal Experimentation and Animal Welfare of Universidade Federal do Rio Grande do Sul (UFRGS) (number 13.313).

Blood collection and hematological analysis

Blood samples were collected and aliquoted into EDTA tubes for hematological analyses and PCR, and into serum tubes for serological tests. Samples from three dogs had insufficient volume for serum tests. Blood cell counts were determined as previously described (RIZZI et al., 2010). Blood smears were examined microscopically for the presence of morulae, and the peripheral blood smear WBC differential was ascertained by counting 100 leukocytes. This was done by a person who was blinded to the serological and nPCR results.

Serological test: dot-ELISA-based assay

Samples were tested for antibody reactivity against *E. canis* and *Anaplasma* spp. using a commercial rapid in-clinic ELISA assay (SNAP 4Dx® test kit, IDEXX Laboratories, Inc., Westbrook, Maine, USA), according to the manufacturer's instructions.

DNA extraction, nested PCR and DNA sequencing

DNA was isolated from 200 μL of EDTA blood using the QIAamp DNA Blood Mini kit (Qiagen, Valencia, California, USA), following the manufacturer's instructions. Negative control purifications using ultra-pure water were performed to monitor cross-contamination for each batch of 10 samples. To verify the existence of amplifiable DNA in the samples, a PCR assay for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as previously described (SANTOS et al., 2009b).

Samples were initially screened using the universal primer fD1 and the genus-specific primer EHR16SR (INOKUMA et al., 2001). These primers amplify 760 bp of the partial sequence of the 16S rRNA gene of *Ehrlichia* and *Anaplasma* species. Briefly, 5 μL of DNA was used as a template for the primary amplification, in a total reaction mixture of 25 μL containing 1.5 mM of $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.25 U of *Taq* polymerase (Go *Taq* Fexi Promega, Madison, WI, USA) and 0.1 mM of each primer. After initial denaturation at 94 °C for 1 min, the amplification consisted of 35 cycles of 1 min each at temperatures of 94 °C, 55 °C and 72 °C for denaturation, annealing and extension, respectively.

The second amplification (nested PCR) was carried out using the primers CANIS and GA1UR for *E. canis* (504 bp), and PLATYS F and PLATYS R for *A. platys* (408 bp) with a few modifications (INOKUMA et al., 2001), which included: 1) PCR products were diluted 1:5 with nuclease-free water; and 2) 1 μL of the solution was used as the template DNA for the second reaction. The conditions for the PCR amplification were the same as for the first round of PCR, except for the annealing temperature for *E. canis* (57.6 °C) and the number of cycles (39). The positive controls were 10-fold dilutions of a plasmid containing starting concentrations of 5.47×10^9 and 6.18×10^{10} copies/μL of the 16S rRNA gene of *E. canis* and *A. platys*, respectively, until reaching less than 1 copy/μL. In each PCR run, the negative control consisted of all reagents (excluding sample) and ultra-pure water. The negative control from the first PCR run was also used as the negative control in the second run. Positive controls, including the control with the lowest copy number (determined as the detection limit for each assay) were included in every run.

The amplified PCR products were subjected to gel electrophoresis in 1.5% agarose gels for one hour at 100 V, followed by ethidium bromide staining (1 μg/mL), and were viewed under a 312 nm UV light transilluminator. The gels were subsequently photographed using Epi Chem II Darkroom® (UVP, Inc., Upland, California, USA). In order to minimize potential risks of contamination, DNA extractions, PCR preparation, PCR amplification, and agarose gel electrophoresis were performed in separate rooms.

All the nPCR products were purified using the QIAprep Spin Miniprep kit (QIAGEN), and the amplicons were directly sequenced with both forward and reverse primers (Purdue Genomics Core Facility, Purdue University, West Lafayette, Indiana, USA). Primers were deleted from DNA sequences obtained from the *Anaplasma platys* 16S rRNA gene and were compared with those of the GenBank® database using the BLAST® nucleotide (ALTSCHUL et al., 1990) in order to search for identicalness.

Positive controls

Positive controls were obtained from dogs naturally infected by *E. canis* and *A. platys* in the city of Londrina, state of Paraná, Southern Brazil. To construct reliable positive controls, nPCR for *E. canis* and *A. platys* detection was performed as described above. Amplicons (504 bp fragment of the 16S rRNA gene for *E. canis* and 408 bp fragment for *A. platys*) were purified from gel (Zymoclean DNA Gel Recovery, Zymo Research, Orange, California, USA), and were cloned into the pGEM-T Easy Vector (Promega, Madison, Wisconsin, USA) followed by transformation in JM 109 Competent Cells (Promega, Madison, Wisconsin, USA). Plasmids with inserts were isolated, grown and purified using a commercial kit (Miniprep, QIAGEN, Valencia, California, USA).

The DNA concentration of positive controls was quantified by means of scanning UV spectrophotometry (NanoDrop® ND-1000 UV/Vis Spectrophotometer, Thermo Fisher Scientific Inc., Wilmington, Delaware, USA) to determine the number of copies/μL. The detection limit for the nPCR was determined by using serial 10-fold dilutions of the positive plasmid controls, spiked in Herring Sperm DNA (KPL, Gaithersburg, Maryland, USA) as the DNA template.

Statistical analysis

Statistical univariate analysis on associations between seropositivity and PCR results and hematological parameters was conducted using the chi-square test. Fisher's exact test was used to evaluate associations between seropositivity and nPCR positivity status when the expected frequency was less than five, using the Stata 11.1 software (Stata Corp, College Station, Texas, USA). Statistical significance was defined as $p < 0.05$.

Results

The nPCR assays were able to amplify the control template diluted to as few as 10^1 and 10^3 gene copies per microliters for *E. canis* and *A. platys*, respectively, and all the samples were positive for GAPDH, thereby confirming the presence of amplifiable DNA in the samples.

All the samples were negative for the presence of antibodies for *E. canis* and *E. canis* 16S rRNA DNA. Among the dog serum samples, 19/196 (9.69%) showed antibodies that were reactive to *Anaplasma* spp. using the SNAP 4Dx® test. *A. platys* DNA (16S rRNA gene) was amplified by nPCR in 28/199 dogs (14.07%). There was no positive association between the PCR and ELISA results ($p > 0.05$).

Among the 28 *A. platys* nPCR-positive dogs, 7 (n = 53) were from the dogs from the city's Zoonosis Control Center (urban area) and 21 (n = 146) from the Arquipelago district (suburban area). No statistical difference between PCR-positivity and location of the dogs was observed (*p*-value = 0.448). Nineteen (67.8%) of the nPCR positive dogs were anemic (RBC < 5.5 x 10^6/μL) and 16 (61.5%) were thrombocytopenic (platelets < 200 × 10^3/μL) (Table 1). Two positive samples had fibrin-clot formation and the data from these samples were not included in the analyses. There was no association between a positive serological test or PCR and other CBC abnormalities except for the basophile count, which was higher the reference value for the leukocyte differential in PCR-positive dogs (p = 0.015).

The *A. platys* 16S rRNA gene partial sequences obtained in this study were 100% identical to each other. A partial 16S rRNA gene sequence of 408 bp, representative of all samples, was deposited in GenBank® database as the Porto Alegre isolate, under the accession number JF418996. Sequence comparisons on the 408 bp fragment from Porto Alegre revealed that the amplicon sequences were identical (100%) to *A. platys* samples from other Brazilian areas such as Campo Grande/MS/Center-West Brazil (JX118826) and Ribeirão Preto/SP/Southeast Brazil (EF052622), and from other countries: Philippines (Q8947792), Cape Verde (GQ395385), Croatia (JQ396431), Italy (EU439943), Malaysia (JF683610), Portugal (EU004823), Spain (AY530806), Japan (AF288136), Venezuela (HE856819) and Thailand (EF13945). The lowest identity (99.75%) was with an *A. platys* sample identified in dogs in Venezuela (AF399917). A lower sequence identity of 98% was found in relation to a German sample of *A. phagocytophilum* (HM480383).

Table 1. Anemia and thrombocytopenia associated with positive PCR for *Anaplasma platys* or serology for *Anaplasma* spp. from dogs.

Clinical manisfestation	nPCR positive	nPCR negative	p	Serology positive	Serology negative	p
Anemia	n = 28	n = 171		n = 19	n = 177	
Yes	67.8% (19)	33.9% (58)	0.85	36.8% (7)	33.8% (60)	0.79
No	32.1% (09)	66.0% (113)		63.1% (12)	66.1% (117)	
Thrombocytopenia	n = 26*	n = 169*		n = 17*	n = 175*	
Yes	61.5% (16)	40.8% (69)	0.45	58.2% (10)	44.5% (78)	0.28
No	38.4% (10)	59.1% (100)		41.1% (7)	55.4% (97)	

*Two positive samples had fibrin-clot formation.

Discussion

All the samples tested negative for *E. canis* by PCR and the SNAP 4Dx® test, thus suggesting that there was lower occurrence of *E. canis* in Porto Alegre than in other Brazilian regions. A previous study in Southern Brazil using serological tests showed low occurrence of *E. canis* in asymptomatic dogs, of 4.8% (SAITO et al., 2008). However, higher prevalence was found in other Brazilian regions, ranging from 24.8% to 44.7%, using similar populations of dogs (AGUIAR et al., 2007; COSTA Jr et al., 2007a, SILVA et al., 2010; SOUZA et al., 2010). Although the tick vector *R. sanguineus* is abundant throughout all urban areas of Brazil (SAITO et al., 2008), including in the state of Rio Grande do Sul (RIBEIRO et al., 1997), it is important to consider that different populations of this tick species are found in Brazil (MORAES-FILHO et al., 2011). Thus, the negative results for *E. canis* found in the present study may have been due to differences in the vector competence of the *R. sanguineus* (SZABÓ et al., 2005). Although peripheral blood samples were used in this study, it may be postulated that false-negative results from nPCR for *E. canis* detection occurred because the agent could be sequestered in the spleen and bone marrow during the subclinical or chronic phase of the disease (MYLONAKIS et al., 2003). However, we did not find any seropositive (exposed) dog, which leads us to conclude that occurrence of *E. canis* in dogs in this work is very low or absent.

Six dogs showed positive results in both tests (Table 2). Thirteen dogs (13/19, 52.6%) that were serologically positive for *Anaplasma* sp. were negative according to nPCR, which suggests that there was a likelihood of previous exposure to the agent. Also, the cyclic parasitemia in dogs infected with *A. platys* may explain this discrepancy between serological and molecular results, as previously observed (FERREIRA et al., 2007, 2008b). Although *A. phagocytophilum* antigens are used in the commercial SNAP 4Dx® test kit, *A. platys* antibodies cross-react with the *A. phagocytophilum* spot in this test (CHANDRASHEKAR, et al., 2010). The kit uses a synthetic peptide based on p44 *A. phagocytophilum* immunodominant protein and detects IgM and IgG antibodies. Because *A. phagocytophilum* and *A. platys* are closely related and share epitopes, the kit can be used for *A. platys* diagnosis (FERREIRA et al., 2008a.) On the other hand, many dogs (19/25, 76%) that were positive by *Anaplasma* sp. nPCR were negative by serological tests for this agent (Table 2), which suggest that negative serological findings do not imply absence of organism infection (FERREIRA et al., 2008a). Positive *Anaplasma* sp. nPCR

Table 2. *Anaplasma* spp. serology and *Anaplasma platys* nPCR results in dogs.

	Anaplasma spp. Dot-ELISA		Total
	Negative	**Positive**	
***Anaplasma platys* nPCR**			
Negative	158	13	171
Positive	19	6	25*
Total	177	19	196

*Samples from three dogs had insufficient volume for serum tests.

results with negative serological results might also indicate an early stage of infection. *Anaplasma* sp. nPCR was able to detect 10^3 gene copies per reaction, but it is also possible that dogs have lower numbers of copies of *A. platys* DNA, thus resulting in false negative results.

The possibility that dogs in our study were infected or co-infected with *A. phagocytophilum* cannot be ruled out, since dogs may be asymptomatic carriers for both agents. In a survey of zoonotic vector-borne diseases conducted previously in Botucatu, state of São Paulo, 198 sick dogs with clinical signs of tick-borne disease showed no evidence of *A. phagocytophilum* exposure or infection (DINIZ et al., 2007). In fact, *A. phagocytophilum* was recently detected in dogs by real-time PCR, in a survey conducted in the state of Rio de Janeiro (SANTOS et al., 2011), and in wild birds in the states of São Paulo and Goiás (MACHADO et al., 2012). Moreover, new genotypes of Anaplasmataceae agents have been reported in wild animals in Brazil (ANDRÉ et al., 2010, 2012; SACCHI et al., 2012).

Although dogs in Porto Alegre were infected by *A. platys* in the same way as described in other Brazilian regions (FERREIRA et al., 2007, 2008b; COSTA Jr, 2007b; DAGNONE et al., 2009; RAMOS et al., 2009), only two isolates have been molecularly characterized to date in Brazil (CARDOZO et al., 2007). Sequence comparisons on the partial 16S rRNA gene in the samples from dogs in Porto Alegre revealed that the sequences were identical (100%) to those from other locations in Brazil (Campo Grande/MS/ Center-West Brazil and Ribeirão Preto/SP/Southeast Brazil), as well as to those from other strains around the world (INOKUMA et al., 2000, 2001; AGUIRRE et al., 2006; PINYOOWONG et al., 2008; GÖTSCH et al., 2009; DYACHENKO et al., 2012).

Anemia and thrombocytopenia are common findings associated with *A. platys* infection, even in asymptomatic dogs (HARVEY, 2006; FERREIRA et al., 2008b). However, occurrences of anemia

or thrombocytopenia did not show any association with positive PCR or serological findings in the present study (Table 1). It can be explained by bad nutritional status, since most of these dogs are fed food scraps, garbage or low quality diet. These co-finding factors might have interfered with the statistical analyses.

The WBC varied among the dogs in the present study, which is consistent with previous reports of *A. platys* infection showing WBC within the reference range (AGUIRRE et al., 2006) or greater than this range (BEAUFILS et al., 2002; ULUTAS et al., 2007). The basophil count above the reference values for the leukocyte differential was the only significant difference (p = 0.015) associated with nPCR positive dogs. Although no previous study has reported basophilia in association with *A. platys* infection, basophils are known to chemotactically respond to bacterial products (RIZZI et al., 2010).

This was the first molecular study to survey the presence of the vector-borne pathogens *E. canis* and *A. platys* in domestic dogs in Porto Alegre, Southern Brazil. The present results indicate that CICT caused by *A. platys* may be endemic in this area. Although *A. platys* is considered to be less pathogenic than other species of the Anaplasmataceae family, such as *E. canis*, the impact of *A. platys* infection on animal health should not be underestimated, since infection may increase the risk of other diseases (CARDOZO et al., 2009; GAUNT et al., 2010). Our findings should be further investigated in order to fully establish the impact of canine cyclic thrombocytopenia in dogs and their potential reservoir role and co-infections with *A. platys* in Southern Brazil.

Conclusions

In this study, we reported on occurrences and molecular detection of *A. platys* in naturally infected dogs in Southern Brazil for the first time. Our results showed that for an accurate diagnosis, serological and molecular methods should be combined, since there was no correlation between PCR and serological findings, and no hematological abnormalities were associated with *A. platys* infection.

Although dogs are commonly infected with *E. canis* in Brazil, all the dogs from the Zoonosis Control Center and from Arquipelago of Porto Alegre, Southern Brazil, tested in this study were negative for *E. canis* infection. Thus, the prevalence of *E. canis* in these areas is either low or absent. The ELISA test for these organisms has not previously been validated for strains of *Anaplasma* and *Ehrlichia* in Brazil, and despite its widespread use in routine clinical analyses in Brazil, it may be flawed with regard to identifying native species.

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