Anais da Academia Brasileira de Ciências (2017) 89(1): 301-306
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201720150556
www.scielo.br/aabc

Molecular survey of *Anaplasma platys* and *Ehrlichia canis* in dogs from Campo Grande, Mato Grosso do Sul, Brazil

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Manuscript received on July 29, 2015; accepted for publication on September 15, 2016

ABSTRACT

This study investigated the frequency of infection by *Anaplasma platys* and *Ehrlichia canis* in dogs submitted to animal health centers in Campo Grande, state of Mato Grosso do Sul, Brazil. *E. canis* and *A. platys* showed infection frequencies of 55.75% and 16.96%, respectively. The identity of the two species was confirmed by DNA sequencing.

Key words: epidemiology, nPCR, DNA, Midwestern Brazil.

INTRODUCTION

Anaplasma platys and *Ehrlichia canis* are Gram-negative, obligatory intracellular bacteria and members of the Anaplasmataceae family (Dumler et al. 2001). These organisms are often found in dogs, commonly infecting platelets and leukocytes, forming colonies called morulae. *Ehrlichia* organisms are mainly transmitted through the bite of an infected tick. This explains the higher prevalence of ehrlichiosis in tropical and subtropical regions due to the geographical distribution of vectors (Andereg and Passos 1999). *E. canis*, which is mostly transmitted by *Rhipicephalus sanguineus* (Groves et al. 1975), has been detected throughout Brazil (Oliveira et al. 2009, Ueno et al. 2009). *E. canis* infection in Brazil was reported for the first time in 1973 (Costa et al. 1973). Although the disease is currently described nationwide, prevalence data differ with respect to population, geographic area, presence of the vector and the diagnostic test employed. The presence of known competent tick vectors as well as reservoir hosts usually determines where ehrlichiosis is found (Moraes-Filho et al. 2015).

Rhipicephalus sanguineus is widespread in Brazil (Labruna and Pereira 2001) and can also transmit other dog blood parasites such as *Babesia canis*, *Babesia vogeli*, *Babesia rossi*, *Rickettsia* spp, *Hepatozoon canis*, *H. americanum* among others (Dantas-Torres 2008, Kamani et al. 2013, René-Martellet et al. 2015).

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E. canis-infected dogs and co-infected with *Anaplasma* sp. are frequently found (Dagnone et al. 2009, Ramos et al. 2010). *Anaplasma platys* causes the so-called canine cyclic thrombocytopenia. Dogs are the primary reservoir host for *A. platys*, although this organism has been shown to infect humans (Maggi et al. 2013, Arraga-Alvarado et al. 2014). *A. platys* infections are often found in the same geographic regions as *E. canis* and evidence of exposure to or infection with both organisms is often detected in the same dog (Harrus et al. 1997, Gal et al. 2008, Cardoso et al. 2010, Diniz et al. 2010, Aktas et al. 2015). Although both organisms are found on every continent in the world, they are more prevalent in tropical and subtropical climates (Stich et al. 2008, Yabsley et al. 2008).

Several methods with varying degrees of sensitivity and specificity can be used to detect *Ehrlichia* and *Anaplasma* organisms. They are usually identified using light microscopy to identify elementary bodies, initial bodies or morulae in the host-cell cytoplasm of Romanowsky-stained blood smears (Hildebrandt et al. 1973). However, this technique unfortunately lacks sensitivity and specificity. Diagnostic accuracy has been greatly enhanced by the introduction of culture and molecular techniques.

These bacteria are among the blood cell parasites most frequently diagnosed in dogs in various regions of Brazil (Ramos et al. 2010, Lasta et al. 2013), but there are few reports about the quantitative frequency of infection by these organisms in dogs in Campo Grande, State of Mato Grosso do Sul (MS), detected mainly by molecular tools (Dagnone et al. 2009, Souza et al. 2013). Due to the zoonotic potential of these infectious agents, their accurate identification and genetic characterization in dogs is important, as it allows one to estimate the risk of transmission to humans and to identify the presence of genetic variants best adapted to a specific host.

Thus, the aim of this study was to use polymerase chain reaction (PCR) to estimate the frequency of infection by *A. platys* and *E. canis* in dogs submitted to veterinary clinics and hospitals in Campo Grande, Mato Grosso do Sul, in the period from 2007 to 2009. And also to assess the genetic similarity, based on 16S rRNA gene, between local microorganisms found (Campo Grande, MS) and the bacteria (*A. platys* and *E. canis*) identified in other regions.

MATERIALS AND METHODS

A non-probability sample was obtained by collecting blood from 181 dogs submitted to veterinary clinics in the municipality of Campo Grande, MS, from 2007 to 2009, exhibiting pale mucous membranes, fever, loss of appetite, as well as presence or prior history of tick infestation. The samples were drawn into tubes containing EDTA and stored at -16°C for subsequent DNA extraction using the methodology described by Araújo et al. (2009). The integrity and concentration of the DNA samples extracted in 1% agarose gel stained with SYBR Gold (Invitrogen) were assessed, respectively, by electrophoresis and spectrophotometry (260/280 nm).

To eliminate the possibility of obtaining false negative results due to the presence of PCR inhibitors in DNA samples, all the samples were subjected to PCR with primers for the canine *β-actin* gene, as described by Wang et al. (2007).

Nested PCR reactions for *A. platys* and *E. canis* were conducted following the methodologies described by Martin et al. (2005) and Wen et al. (1997), respectively. Samples were considered positive when specific band sizes were observed (678 bp for *A. platys* and 389 bp for *E. canis*) after electrophoresis.

For the genetic analysis, five animals positive for each pathogen were randomly selected. Amplified fragments were purified using a QIAEX II kit (Qiagen) and sequenced in both directions

in an ABI-3130 automated sequencer (Applied Biosystems). Three sequences were made of each sample, and a consensus sequence was built using the SEQUENCHER v 4.1.4 program (Gene Codes). The consensus sequences (514 bp for *A. platys* and 388 bp for *E. canis*) were then subjected to a homology search using the BLASTn program (Altschul et al. 1990). The consensus sequence of each bacterium was deposited in the NCBI GenBank database.

RESULTS AND DISCUSSION

Among the 181 samples collected, 108 (59.66%) were positive for *E. canis*, 28 (15.46%) for *A. platys*, and 18 animals (9.94%) showed co-infection by the two agents. All the samples showed amplification for the canine constitutive gene *β-actin* (237 bp), confirming the absence of PCR inhibitors in the samples.

The frequencies of infection by *E. canis* and *A. platys* in dogs, assessed by molecular tools, varied according to the region and population under study. Canine monocytic ehrlichiosis, the disease caused by infection with *E. canis*, is endemic in Brazil and highly prevalent among dogs throughout the country (Vieira et al. 2011). Nevertheless, in a study in the southern region of Brazil (Porto Alegre, RS), Lasta et al. (2013) found a frequency of 14.07% of dogs infected by *A. platys* and no animal infected by *E. canis* out of 199 homeless or partly domiciled animals.

In another study, prevalence of *E. canis* infection in dogs from different areas and from selected hospital populations in southeastern Brazil ranged from 15% (Macieira et al. 2005) to 44.7% (Costa Jr et al. 2007). In general, higher frequencies of infection are expected in regions where the tick vector is more abundant, such as Campo Grande, MS (Almeida et al. 2013), in relation to regions with lower infestation rate by ticks, such as Porto Alegre, Rio Grande do Sul, Brazil (Ribeiro et al. 1997). On the other hand, in some regions with high rates of tick infestation, such as Mato Grosso (Almeida et al. 2012), small frequencies of *E. canis* infection have been found (15.6% of dogs from urban area, and 14.4% of dogs from rural area) (Santos et al. 2013). In addition, in a previous serological survey conducted by Melo et al. (2011), 74.3% of dogs from urban areas and 67.5% from rural areas showed antibodies against *E. canis*. The presence of *R. sanguineus* ticks on the dogs studied was not considered a risk factor for *E. canis* infection (Melo et al. 2011).

Thus, it is evident that factors other than tick infestation rate have been associated to the frequency of infection by *E. canis* and possibly other Rickettsiaceae. For example, Nava et al. (2012) have observed genetic divergence between different strains of *R. sanguineus* from South America. In a recent study, Moraes-Filho et al. (2015) has observed that some of these strains showed different levels of vector competence for *E. canis*, allowing us to conclude that not only the presence but also the vector competence of the tick strain influence the prevalence of the agent in a region.

In a recent study conducted in Campo Grande, MS, using 60 dogs serologically positive for *Leishmania infantum*, the frequency of infection with *E. canis* was 45%, and only 1.6% with *Anaplasma* sp., using PCR (Souza et al. 2013), which again illustrates the higher frequency of infection in this municipality.

However, when sampling involves animals submitted to veterinary clinics and hospitals, which represent points of convergence of sick individuals, frequencies of infection of up to 48.78% have been recorded for *A. platys* and of 38.04% for *E. canis*, according to Ramos et al. (2010). These authors analyzed 205 samples from dogs treated at a veterinary hospital in the city of Recife, Pernambuco. Dagnone et al. (2009) reported similar findings (42.30% for *A. platys* and 38.46% for *E. canis*) among 26 animals treated at a veterinary

hospital in the city of Campo Grande, MS. In the present study the percentage of infected animals was also high (59.66% for *E. canis*), since the samples were obtained from sick dogs at veterinary clinics.

The DNA sequences obtained in this study were deposited at GenBank under access numbers JX118827 (*E. canis*) and JX118826 (*A. platys*). A search for homology using the BLASTn tool indicated that all the sequences (*E. canis* and *A. platys*) showed 99 to 100% identity with their respective DNA sequences deposited in GenBank, thus confirming the specificity of the nested PCR used here.

The high frequency of dogs infected with these bacteria indicates a high tick infestation, especially by *R. sanguineus*, which is their main vector in urban areas (Almeida et al. 2013). In addition, the proximity between humans and dogs, and the possibility of human tick infestation, according to several reports in Brazil (Dantas-Torres et al. 2006, Louly et al. 2006), and in other regions of the world (Carpenter et al. 1990, Felz et al. 1996, Manfredi et al. 1999, Uspensky and Ioffe-Uspensky 2002) represent possible risk factors for human infection with *E. canis* and *A. platys*. Several reports of human infection with *A. platys* have been published (Maggi et al. 2013, Breitschwerdt et al. 2014), including in South America (Arraga-Alvarado et al. 2014).

Data obtained in this report corroborate previous studies from several parts of Brazil that reported high frequencies of *E. canis* infection in dogs, and also confirmed that the Ehrlichia species that is circulating among dogs from Campo Grande, Mato Grosso do Sul is *Ehrlichia canis*.

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