Preventive Veterinary Medicine 109 (2013) 321–326

Contents lists available at SciVerse ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Factors associated with epidemiology of *Anaplasma platys* in dogs in rural and urban areas of Minas Gerais State, Brazil

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ARTICLE INFO

Article history:
Received 4 October 2011
Received in revised form 16 October 2012
Accepted 21 October 2012

Keywords:
Anaplasma platys
Dog
Ticks
Epidemiology
Rural area

ABSTRACT

This epidemiological survey of *Anaplasma platys* was carried out in rural and urban areas of three distinct regions of the State of Minas Gerais, Brazil. EDTA blood samples were collected during the dry season from dogs living on farms with an attempt to resample the same dogs in the subsequent rainy season. Samples were also taken from dogs in urban areas. DNA was extracted from blood samples for real time PCR. Risk factors, such as age, breed, sex, presence of ticks, and packed cell volume were analyzed. During the rainy season, the prevalence of infection by *A. platys* in dogs in the rural areas was significantly higher (13.9%) than that observed in dogs in the urban areas (5.1%). Dogs in the Nanuque region were 3.74 times ($p = 0.001$) more likely to be real-time PCR positive than dogs in the other two studied regions. Dogs infested with ticks showed higher rates of positivity. The results showed that in rural areas of Minas Gerais *A. platys* infection is influenced by climatic conditions. In areas of higher temperature and higher humidity, transmission occurs during both the dry and rainy seasons, while in areas with lower temperature and humidity transmission occurs mainly during the dry season.

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1. Introduction

Anaplasma platys are obligate intracellular rickettsial organisms that infect peripheral blood platelets resulting in basophilic inclusions, so-called morulae, and causing canine infectious cyclic thrombocytopenia (Harrus et al., 1997; Dumler et al., 2001). *Rhipicephalus sanguineus* has been implicated as the major tick vector of *A. platys* (Woody and Hoskins, 1991; Harrus et al., 1997), since *A. platys* has been frequently detected in this tick species (Inokuma et al., 2000).

Little is known about the occurrence, transmission and other epidemiological aspects of canine anaplasmosis in rural regions of Brazil, where *Amblyomma* ticks are found more frequently than *R. sanguineus* (O'Dwyer et al., 2001). However there is no evidence for transmission of *A. platys* by *Amblyomma cajennense*. Brown et al. (2005) suspected that *Heterodoxus spiniger* could be a vector during winter in Australia, since the prevalence of *A. platys* and *H. spiniger* in the cold season is high, unlike the prevalence of *R. sanguineus*.

Most dogs infected with *A. platys* are asymptomatic and rarely show signs of bleeding, even when presenting severe thrombocytopenia (Harvey et al., 1978; Woody and Hoskins, 1991; Chang et al., 1996). However, virulent cases have been reported in Greece and the USA (Kontos et al., 1991; Wilson, 1992). In the chronic phase, platelet numbers can decrease, resulting in cyclic thrombocytopenia associated with sporadic rickettsemia (Harvey et al., 1978; Chang et al., 1996).

A. platys infection is generally diagnosed by the presence of basophilic inclusions in platelets observed in whole

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0167-5877/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.prevetmed.2012.10.011

blood or buffy-coat smears (Harvey et al., 1978; Woody and Hoskins, 1991; Martin et al., 2005). However, this test has low sensitivity, due to cyclic rickettsemia and the low numbers of infected cells (Harvey et al., 1978; Chang et al., 1996). Thus, molecular techniques such as PCR, RFLP-PCR, nPCR, and real-time PCR have been widely used to diagnose *A. platys* infections (Chang and Pan, 1996; Inokuma et al., 2000; Hancock et al., 2001; Dagnone et al., 2003; Martin et al., 2005; Teglas et al., 2005).

A. platys is endemic in Brazil with prevalence rates varying from 1.1% to 41.9%, depending on the geographic region, target population (hospitalised animals, stray dogs or companion animals) and the diagnostic method used (direct or indirect detection) (Moura et al., 2002; Souza et al., 2004). Most studies conducted in Brazil have analyzed cases in hospitalized animals based on blood smear examination. Rodrigues et al. (2004) observed a prevalence of 9.9% of *A. platys* in blood smears from dogs living in rural areas of Juiz de Fora, Minas Gerais. This is the only report of *A. platys* infections in a rural area of Brazil.

The aims of the present study were to determine the prevalence and incidence of *A. platys* infections in rural and urban areas of three regions of the state of Minas Gerais and to verify the existence of risk factors influencing the epidemiology of the infections.

2. Materials and methods

2.1. Location and climatic conditions

The study was carried out in rural and urban areas in three distinct regions (Lavras, Belo Horizonte, and Nanuque) in the state of Minas Gerais, Brazil. Climatic conditions in the regions differ, although all have two well-defined seasons: a dry season from April to September and a rainy season from October to March. The climatic and geographic data for each region are shown in Table 1.

2.2. Sampling procedure

The number of samples to be collected was calculated as a total of 235, based on an estimated prevalence of 42% according to Souza et al. (2004), with a confidence interval of 95% and an error margin of 15% (Centro Panamericano do Zoonosis (Cepanzo), 1973). However, an extra 6% of samples were collected ($n = 250$). During the dry season (April–September), blood samples were collected into EDTA from dogs living on farms in Lavras ($n = 97$), Belo Horizonte ($n = 49$), and Nanuque ($n = 102$). In the subsequent rainy season (October–March), an attempt was made to re-sample these dogs; however, some had died, been transferred to other farms, or were missing. The number of dogs re-sampled in each region was: Lavras, 69; Belo Horizonte, 29; and Nanuque, 67.

The farms where blood samples were collected from dogs were selected based on even distribution inside the region; some host farms were chosen and samples were also collected randomly in the neighboring farms. Most dogs sampled in rural regions were working dogs.

During the rainy season, blood samples were collected from 98 dogs in urban areas of Lavras ($n = 37$), Belo Horizonte ($n = 20$), and Nanuque ($n = 41$). The urban area was divided into four quadrants and in each quadrant the main streets were selected. All homes in the selected streets were visited. Samples were collected from dogs present in these homes under the same conditions as those from rural areas.

During the sampling procedure, all animals were clinically examined and the presence of ticks was recorded. Only clinically healthy animals whose owners consented were enrolled in the study. Animal breed, sex, and age, as given by the owner or estimated by dental examination, were recorded. Ticks were collected and placed into tubes containing 70% alcohol and identified according to Aragão and Fonseca (1961). Larvae and nymphs of the *Amblyomma* genus were not identified to species level. EDTA blood samples were processed for packed cell volume (PCV). The remaining samples were frozen at −20 °C for subsequent DNA extraction.

2.3. Real time PCR

DNA was extracted from 300 μl of blood using a commercial kit (Wizard Genomic DNA Purification, Promega, Madison, USA). Real time PCR was used to amplify 16S rDNA of *A. platys* according to Teglas et al. (2005). The amplifications were carried out with the primer sets Eplat-19f-5′-CGGATTTTTGTCGTAGCTTGCTAT-3′, Eplat-117r-5′-CCATTTCTAGTGGCTATCCCATACTACT-3′, and the probe Eplat-55p-5′TGGCAGACGGGTGAGTAATGCATAGGA-3′. A total volume of 25 μl master mix contained 2 mM Tris–HCl, 10 mM KCl, 0.1 mM DTT, 0.01 mM EDTA, 0.05%

Table 1
Climatic and geographic data of the regions of Lavras, Nanuque, and Belo Horizonte, Minas Gerais, Brazil.

Parameter	Region					
	Lavras		Nanuque		Belo Horizonte	
	Dry season	Rainy season	Dry season	Rainy season	Dry season	Rainy season
Temperature (°C) (mean)	19.6	23.3	25.7	28.5	21.0	23.8
Minimum temperature (°C) (mean)	13.5	19.1	20.2	22.9	16.3	19.6
Maximum temperature (°C) (mean)	25.8	27.5	31.2	34.1	25.7	28.0
Rainfall (mm) (total)	214.1	1266.3	243.0	852.7	263.1	1354.3
Altitude m	1060		159		850	
Latitude S	21°48′		17°70′		19°55′	
Longitude W	44°64′		40°76′		43°56′	

Source: http://www.agritempo.gov.br/agroclima/sumario?uf=MG.

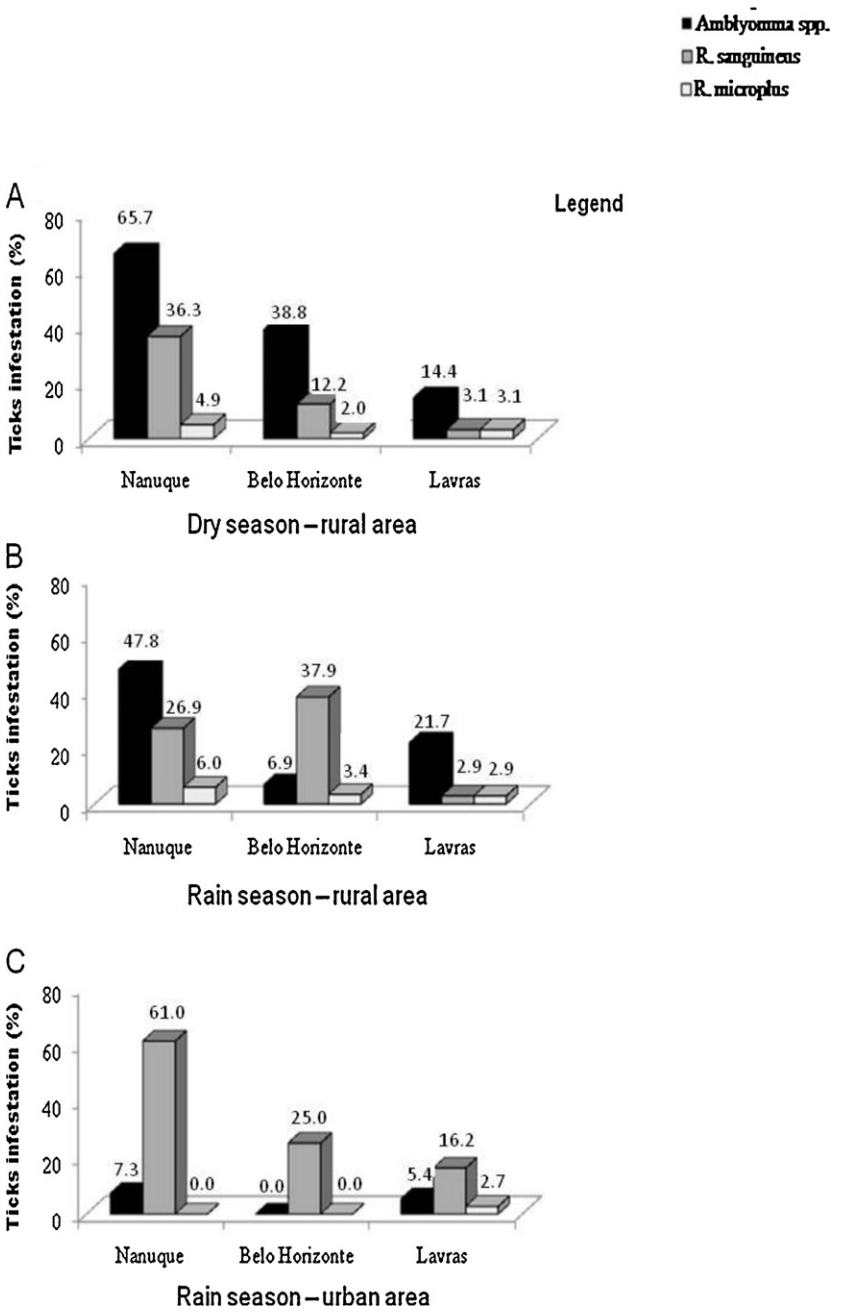


Fig. 1. Tick infestation in dogs from rural region in dry season (A) and raining season (B) and urban regions in raining season (C) of Nanuque, Belo Horizonte, and Lavras, Minas Gerais, Brazil.

Nonidet P-40, 0.05% Tween 20, 5% glycerol, 5 mM $MgCl_2$ (Qiagen, Hilden, Alemanha), 1.25 U of HotStar Taq Polymerase (Qiagen), 200 μM of each dNTP (Fermentas, St. Leon-Rot, Germany), 4.0 μM of each primer (Thermo Electron, Dreieich, Germany), 0.08 μM of probe (Thermo Electron), and 250 ng of DNA. DNA extracted from blood of dogs naturally infected with *A. platys* was used as positive control and DNA extracted from blood of uninfected dogs was the negative control. Positive and negative controls were included in each PCR batch.

The amplification was performed in a thermal cycler (iCycler, Biorad, Munich, Germany) with an initial Taq polymerase activation step of 10 min at 95 °C followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min and 30 s.

2.4. Statistical analysis

The results were analyzed using $2 \times K$ contingency tables of exposure variables. The outcome variable was

Table 2
Prevalence of *Anaplasma platys* by real time PCR in dogs from rural (dry season and rainy season) and urban areas (rainy season) in Lavras, Nanuque, and Belo Horizonte, Minas Gerais, Brazil.

	Rural areas		Urban areas +/*n* (%)
	Dry season +/*n* (%)	Rainy season +/*n* (%)	
Nanuque	20/102 (19.6)	18/67 (26.9)	4/41 (9.7)
Lavras	7/97 (7.2)	0/69 (0.0)	0/37 (0.0)
Belo Horizonte	2/49 (4.1)	5/29 (17.2)	1/20 (5.0)
Total	29/248 (11.7)	23/165 (13.9)	5/98 (5.1)

positivity to *A. platys* and the independent variables were: age (<2 years, 2–5 years or >5 years old), sex, tick presence or absence, species of tick (*R. sanguineus*, *Amblyomma* spp., or *Rhipicephalus microplus*), breed (mixed breed or pure-bred), and PCV (below or above 30%) according to Moreira et al. (2003), Watanabe et al. (2004) and Rodriguez-Vivas et al. (2005). The Chi-square test was used to evaluate any associations between prevalence and incidence among regions and infestation with ticks; a probability of less than 0.05 was considered to be significant.

Data from all dogs from rural regions in the dry season were used for epidemiological analysis. Odds Ratios (OR) and *p*-values were calculated separately for each variable by univariate analysis using BioEstat software (Ayres et al., 2003) to check for significant associations between categorical risk factors and disease status. Risk factors with *p*-value < 0.20 in univariate analysis were selected for a multivariable model (logistic regression) using the program Minitab 14 for Windows. The manual backward elimination process was done to identify which variables remain significant for the final model.

3. Results

3.1. Tick infestation

In rural areas the percentage of dogs infested with any species of tick was 48.0% in the dry season and 29.8% in rainy season ($p > 0.05$). In dry season the infestation rate of dogs from Nanuque (76.5%) was statistically higher ($p < 0.05$) than dogs from Lavras (19.6%) and Belo Horizonte (44.9%). In all regions there was a predominance of *Amblyomma* spp., mainly *A. cajennense*, followed by *R. sanguineus* (Fig. 1). In rural areas of the region of Nanuque, there was no difference regarding species distribution or infestation between dry and rainy seasons. However, in the Belo Horizonte region there was a significant reduction in *Amblyomma* spp. and an increase in *R. sanguineus* infestation in the rainy season compared to the dry season (Fig. 1).

In urban areas of all three regions there was a predominance of *R. sanguineus* that was statistically higher in dogs from Nanuque (61.0%) ($p < 0.05$) than in dogs from Belo Horizonte (25.0%) and Lavras (16.2%) (Fig. 1).

3.2. Prevalence of A. platys

The prevalence of infection with *A. platys* in dogs in rural areas of Minas Gerais in dry (11.7%) and rainy seasons (13.9%) was not significantly different ($p > 0.05$). However in the rainy season the infection in dogs from rural areas was significantly higher ($p < 0.05$) than the prevalence observed in animals from urban areas (5.1%) (Table 2). Dogs from rural areas exhibited regional variations in *A. platys* prevalence ($p < 0.05$), with those from rural Nanuque being 3.71 times ($p = 0.00$; IC 95%, 1.61–8.53) more likely to be infected with *A. platys* than animals from rural areas of the other two regions. Dogs infested with either tick species (OR = 3.74; $p = 0.00$; IC 95% 1.53–9.11) and those infested only with *R. sanguineus* (OR = 4.38; $p = 0.00$; IC 95% 1.92–10.00) were more likely to be infected with *A. platys* than uninfested dogs (Table 3). However no quantitative data relating to tick control were recorded.

There were no significant differences ($p < 0.05$) in the prevalence of *A. platys* among urban areas of Lavras (0.0%), Nanuque (9.7%), and Belo Horizonte (5.0%). No variable studied was shown to be a risk factor for infection of *A. platys* in urban areas of Minas Gerais.

3.3. Incidence of A. platys

Incidence of *A. platys* in rural areas of Minas Gerais was 12.3% (18/146). In Lavras region no dog among the 62 negatives found in the dry season became positive in the rainy season. Incidence of *A. platys* in Nanuque and Belo Horizonte was 22.8% (13/57) and 18.5% (5/27). These values were significantly higher ($p < 0.01$) than those observed in Lavras.

4. Discussion

The prevalence of canine anaplasmosis varies according to climatic conditions (Keefe et al., 1982) and epidemiological factors such as the distribution of vectors, dog survival, average age and habitat, and management practices (Inokuma et al., 2003, 2006; Ramos et al., 2009, 2010; Santos et al., 2009).

Climatic conditions represent an important factor in population dynamics of ticks. At temperatures below 17 °C, ticks exhibit delay in oviposition, interfering with the hatching of eggs and increasing duration of the parasitic phase, which results in a reduced number of generations within the year (Sonenshine, 1993). The highest average prevalence of *A. platys* was found in Nanuque (overall mean of 23.2%, 19.6 and 26.9% in the dry and rainy seasons, respectively), followed by Belo Horizonte (overall mean of 10.6, 4.1 and 17.2% in the dry and rainy seasons, respectively), both presenting highest average annual temperature and highest tick infestation. A lower tick prevalence was found in Lavras (overall mean of 3.6, 7.2

Table 3
Prevalence and logistic regression to detect factors associated with *Anaplasma platys* infections in dogs in dry season from rural regions of Minas Gerais, Brazil, 2004.

Variable	Serology			
	% Prevalence (+/*n*)	OR	*p*-value	95% CI
Age[b]				
<2 years	13.7 (17/124)	Ref.	Ref.	Ref.
2–5 years	6.4 (4/62)	0.50	0.22	0.16–1.53
>5 years old	12.9 (8/62)	1.11	0.82	0.43–2.85
Sex				
Female	11.8 (6/68)	Ref.	Ref.	Ref.
Male	11.7 (21/180)	0.90	0.82	0.36–2.25
Tick[a,b]				
Absent	5.6 (7/126)	Ref.	Ref.	Ref.
Present	18.0 (22/122)	3.74	0.00	1.53–9.11
Specie of tick[b]				
Absent	5.6 (7/126)	Ref.	Ref.	Ref.
R. sanguineus	28.3 (13/46)	4.38	0.00	1.92–10.00
Amblyomma spp.	17.0 (17/100)	1.72	0.20	0.74–3.99
R. microplus	22.2 (2/9)	1.93	0.47	0.32–11.60
Breed				
Pure breeds	11.8 (6/51)	Ref.	Ref.	Ref.
Mongrel	11.7 (23/191)	0.92	0.87	0.33–2.59
PCV (%)				
>30	11.1 (22/199)	Ref.	Ref.	Ref.
<30	14.3 (7/49)	0.67	0.42	0.26–1.76
Overall prevalence	11.7 (29/248)			

+: number of positive animals; n: number of samples per variable; OR: odds ratios; *p*-value: probability; 95% CI: 95% confidence interval; Ref.: variable used as a reference value.

[a] Ticks observed during blood collection.

[b] Variables used in multivariable model.

and 0.0% in dry and rainy seasons, respectively), which recorded the lowest average annual temperature and lowest tick infestation. These data suggest the occurrence of tick diapause in the Lavras area, which possibly interferes with the transmission of pathogens by these ticks in the dry season. This is further corroborated by the dry season absence of *A. platys* in Lavras, while high incidences were observed in Nanuque (22.8%) and Belo Horizonte (18.5%).

The data on the incidence of infection with *A. platys* in the canine population in rural areas confirms the importance of temperature in transmission of the agent. Lavras had the coldest period, with an average minimum dry season temperature of 13.5 °C associated with an interruption in the cycle of *R. sanguineus* and no new infections with *A. platys*. In Nanuque, during the coolest period of the year, at a minimum average temperature of 20.2 °C, the biological cycle of *R. sanguineus* continued and there was an *A. platys* incidence of 22.8%, indicating transmission throughout the year.

Reports on the prevalence of *A. platys* in Brazil, determined by molecular methods, range from 9.5% to 55.0% (Dagnone et al., 2004, 2009; Souza et al., 2004; Ramos et al., 2009, 2010), depending on the population studied. The cited studies were performed on dogs from urban areas and generally used samples obtained from veterinary hospitals, which explains the high rates. In the present study, the *A. platys* prevalence in urban areas was 5.1% in randomly sampled dogs, not identified as diseased, from cities of lower population.

The prevalence of dogs infected with *A. platys* observed in rural areas (13.9%) was statistically higher than in urban areas (5.1%) ($p < 0.05$). This suggests that dogs from rural areas are more frequently exposed to tick vectors. This difference was more evident due to the high infestation with *R. sanguineus* in Nanuque in all seasons (Table 2).

Epidemiological data from this study support the hypothesis that *R. sanguineus* is the major vector of *A. platys*, since we observed a strong association between animals from rural areas infested with *R. sanguineus* and infection with *A. platys* (OR = 4.38; $p = 0.00$; IC 95% 1.92–10.00).

Acknowledgements

The authors thank CNPq (The Brazilian National Council for Scientific and Technological Development) for awarding a PhD scholarship to Livio M. Costa-Júnior, CAPES and DAAD for the financial support given to the project (Project 182/04) and Dr Lesley Bell-Sakyi for revising the manuscript.

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