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Babesia canis vogeli infection in dogs and ticks in the semiarid region of Pernambuco, Brazil[1]

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ABSTRACT.- Araujo A.C., Silveira J.A.G., Azevedo S.S., Nieri-Bastos F.A., Ribeiro M.F.B., Labruna M.B. & Horta M.C. 2015. ***Babesia canis vogeli* infection in dogs and ticks in the semiarid region of Pernambuco, Brazil**. *Pesquisa Veterinária Brasileira 35(5):456-461*. Laboratório de Doenças Parasitárias, Universidade Federal do Vale do São Francisco, Rodovia BR-407 Km 12, Lote 543, Projeto de Irrigação Nilo Coelho s/n, C1, Petrolina, PE 56300-990, Brazil. E-mail: horta.mc@hotmail.com

This study aimed to report the prevalence of *Babesia canis vogeli* in dogs and ticks in the urban and rural areas of Petrolina, Pernambuco. Serum and peripheral blood samples of 404 dogs were tested by indirect immunofluorescence assay (IFA) and by blood smears, respectively. The presence of tick infestation was evaluated, and some specimens were submitted to DNA amplification by polymerase chain reaction (PCR). The presence of antibodies anti-*B. canis vogeli* was determinate in 57.9% (234/404) of dogs. The direct detection of *Babesia* spp was obtained in 0.5% (2/404) dogs by visualization of intraerythrocytic forms. Infestation by *Rhipicephalus sanguineus* sensu lato was observed in 54.5% (220/404) of dogs in both urban and rural areas. DNA of *Babesia canis* vogeli were obtained by PCR in 6% individual (3/50) and 8.7% of pool of ticks (7/80). The risk factors for the presence of anti-*B. canis vogeli* antibodies, as determined through the application of logistic regression models ($P<0.05$), were the following: medium breed size variables ($P<0.001$); contact with areas of forest ($P=0.021$); and access on the street ($P=0.046$). This study describes, for the first time, the confirmation of infection of *B. canis vogeli* in dogs and ticks in the semiarid region of Pernambuco, Brazil.

INDEX TERMS: Babesiosis, *Rhipicephalus sanguineus*, indirect immunofluorescence assay, PCR, Pernambuco.

RESUMO.- [**Infecção por *Babesia canis vogeli* em cães e carrapatos de uma região semiárida de Pernambuco.**] Este trabalho objetivou avaliar a prevalência de *Babesia canis vogeli* em cães e carrapatos de áreas urbanas e rurais do município de Petrolina, Pernambuco, Nordeste do Brasil. Amostras de soro e sangue periférico de 404 cães foram testadas pela Reação de Imunoflorescência Indireta (RIFI), e por esfregaço sanguíneo. A presença de infestação por carrapatos foi avaliada, e alguns espécimes foram submetidos à amplificação do DNA pela Reação em Cadeia pela Polimerase (PCR). A presença de anticorpos anti-*B. canis vogeli* foi determinada em 57,9% (234/404) dos cães. A soroprevalência em áreas urbanas e rurais foi 48,5% e 67,3%, respectivamente. A detecção direta de *Babesia* spp foi obtida em 0,5% dos cães pela visualização de formas intraeritrocitárias. A infestação pelo carrapato *Rhipicephalus sanguineus* foi observada em 54,5% (220/404) dos cães. DNA de *Babesia canis vogeli* obtido pela PCR foi 6% (3/50) em carrapatos processados individualmente e 8,7% (7/80) em pools. Os fatores de risco para presença de anticorpos anti-*B. canis vogeli* utilizando modelo de regressão logística ($P < 0,05$) foram porte médio ($P <0,001$), contato com áreas

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de floresta (P = 0,021), e acesso dos cães à rua (P = 0,046). Este estudo descreve pela primeira vez a confirmação da infecção de *Babesia canis* infectando cães e carrapatos em uma região semiárida de Pernambuco, Brasil.

TERMOS DE INDEXAÇÃO: Babesiose, *Rhipicephalus sanguineus*, imunofluorescência indireta, PCR, Pernambuco.

INTRODUCTION

Canine babesiosis is an emerging disease of large veterinary importance worldwide (Jefferies et al. 2007), caused by intraerythrocytic protozoans of the genus *Babesia*. The main species that infect dogs are *B. canis* and *B. gibsoni* transmitted by ticks of different genus and species (Dantas-Torres & Figueredo 2006).

The disease in Brazil is predominantly caused by *B. canis vogeli* (Dantas-Torres 2008a), presenting prevalence ranging from 18.8% to 73.3% (Trapp et al. 2006, Maia et al. 2007, Furuta et al. 2009, Spolidorio et al. 2010); associated with high incidence of tick vector *Rhipicephalus sanguineus* (Dantas-Torres 2008b), whose prevalence and intensity of infestation have increased in dogs (Labruna 2004).

The diagnosis can be done by identification of parasites based on the size and morphology of intraerythrocytic forms in peripheral blood smears (Passos et al. 2005, Ungar de Sá et al. 2007). Serological tests are broadly used in the diagnosis of this disease, as the Indirect Immunofluorescence Assay (IFA) (Boozer & Macintire 2003) are useful in the identification of asymptomatic carriers animals and diagnose chronic infections, when the level of parasitemia is generally low or undetectable in blood smear (Furuta et al. 2009). Nevertheless, IFA indicates the exposure to the agent, without providing information regarding the current stage of infection (Dantas-Torres & Figueredo 2006). Therefore, molecular techniques, such as the Polymerase Chain Reaction (PCR), have been applied, showing that detection and identification of the infections with *Babesia* spp was efficient (Birkenheuer et al. 2004, Passos et al. 2005).

The canine babesiosis has a cosmopolitan distribution, however the prevalence of antibodies anti-*B. canis vogeli* in the northeastern region are scarce. In Pernambuco state, few studies have been conducted in the Atlantic Forest areas (Ramos et al. 2010), whereas no information about canine babesiosis in small and medium cities, more specifically in the semiarid region, has been published.

This study aimed to determine the prevalence of *Babesia* spp. in dogs and ticks of urban and rural areas of the municipality of Petrolina, Pernambuco state, by detection of parasites in blood smears, detection of antibodies by IFA, and detection of DNA of *Babesia* spp. in ticks by PCR, analyzing the risk factors associated with infection by the pathogen in this region.

MATERIALS AND METHODS

The study was conducted in urban and rural areas of the municipality of Petrolina, Pernambuco state (S 9°23'55", W 40°30'03"), located in the semiarid region of Northeastern Brazil. Five neighborhoods were chosen randomly within each urban (Areia Branca, Gercino Coelho, José e Maria, São Gonçalo, Vila Eduardo) and rural area (Maria Tereza, N5, N6, N10, Nova Descoberta). According to the Brazilian Institute of Geography and Statistics, the municipality has a population of 294,081 inhabitants; occupying an area of 4,558,537 km^2, which is covered by the polygon of droughts (IBGE 2010). However, there's no data about the canine population in this area.

From August 2011 to January 2012, 404 dogs were evaluated (202 from urban areas and 202 from rural areas), varying gender, breed and age. The number of animals studied was based on an estimated prevalence of 50%, with a confidence level of 95% and a margin of error of 7%. Blood was collected by cephalic venipuncture. About 4 mL of blood was collected from each animal, then the blood was placed into tubes without anticoagulant and maintained at room temperature until the retraction of the clot, and serum was obtained by centrifugation. The samples were stored at -20°C.

All dogs were inspected for the presence of ticks, which, once found, were removed with the aid of anatomical tweezers, placed in tubes containing 70º ethanol solution and identified under a stereomicroscope, according to morphological key provided by Aragão & Fonseca (1961).

The smears were made with blood taken from the ear margin capillary bed, then air-dried and stained (Renylab®), and the entire slides were observed under optical microscope immersion (1,000x).

Immunofluorescence Assay (IFA) using antigens of *B. canis vogeli* obtained from a splenectomized dog that was experimentally infected with isolate from Belo Horizonte-Minas Gerais (Bicalho et al. 2002). The antigen was produced following the methodology described by IICA (1984) with modifications. Briefly, IFA was performed with 5µL of serum samples (using a dilution of 1:40) incubated at 37°C for 30 minutes in slides of blood smears containing parasitized erythrocytes and washed three times for 3 minutes in 1x phosphate buffered saline (PBS, pH 7.2) and finally washed twice with distilled water. Subsequently, each well was added with anti-dog IgG conjugated marked with fluorescein isothiocyanate (FITC - Bethyl Laboratories, Montgomery, TX, USA) diluted 1:150 in Evans blue (1:50 in Tween PBS). Slides were then incubated at 37°C for 30 minutes, washed as previously described, allowed to air dry and, subsequently, examined in microscope with fluorescent light source (Olympus Corporation, Tokyo, Japan) at 20- and 40-x magnification, being considered positive reactions with fluorescence around the parasites in samples of serum titers of 40. The reactions were performed at the Laboratory of Veterinary Protozoology, Department of Parasitology, Institute of Biological Sciences, Federal University of Minas Gerais. The positive control comprised appropriately diluted serum derived from a Brazilian dog that was unambiguously seropositive for *B. canis,* while the negative control consisted of a serum sample from a negative dog (samples were further diluted until 1:640).

A total of 370 ticks were randomly separated and 50 were tested individually and 320 in pools of 4 ticks. The entire tick samples were washed in TE (10mM Tris HCl, 1mM EDTA, pH 8.0) according Horta et al. (2007) and subjected to DNA extraction using the Wizard® Genomic DNA Purification commercial kit (Promega, Madison, USA), according the manufacturer's instructions, to a final volume of 50 µL and 75 µL for individual ticks and pools, respectively. The eluted DNA was kept at -20°C before PCR amplification.

For the Polymerase Chain Reaction (PCR) primers for amplification of DNA fragments of 551 base pairs of the 18S rRNA gene of *Babesia* were used according to Almeida (2011): BAB143-167 (5'-CCG TGC TAA TTG TAG GGC TAA TAC A-3') and BAB694-667 (5'-GCT TGA AAC ACT CTA RTT TCT CAA AG-3'). The PCR mix was prepared for a final volume of 25 µL water-solution containing 1×PCR buffer (Invitrogen, Carlsbad, USA); 1.5mM $MgCl_2$

(Invitrogen, Carlsbad, USA); 0.2 mM dNTPs (GE Healthcare, Buckinghamshire, England); 10 pM each primer; 0.25 U of Taq DNA polymerase (Invitrogen, Carlsbad, USA) and 5 µL of DNA sample. Amplification was performed in a thermocycler (Biocycler®), consisting of an initial denaturation for 5 minutes at 95°C, and 35 repetitive cycles of 30 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C, followed by a 7-minute final extension period at 72°C.

The amplification products obtained were subjected to electrophoresis in 1.5% agarose gel (Invitrogen, Carlsbad, CA), stained with ethidium bromide and visualized under UV transilluminator. Amplicons of the expected size were purified with ExoSap (GE Healthcare Pittsburgh, PA) and sequenced in an automatic sequencer (Applied Biosystems/PerkinElmer, model ABI Prism 3500 Genetic, Foster City, CA), according to the manufacturer's protocol. Partial sequences obtained were submitted to BLAST analysis (Altschul et al. 1990) to determine the closest similarities to corresponding sequences.

For each sampled dog, a questionnaire was applied to the dog owner with the purpose of gaining information about independent variables that could be associated with seroreactivity to *Babesia* spp (dependent variables).

Risk factor analysis was performed in two steps: univariate and multivariate analysis. Univariate analysis was performed using the chi-square test or Fisher's exact test, and those variables that presented $p \leq 0.20$ were used for multiple logistic regression. The multivariate analysis was then performed, using the stepwise forward method (Hosmer & Emeshow 2000). The significance level in multivariate analysis was 5%. The tests were performed using the SPSS for Windows software package, version 13.0.

The study was approved by the Ethics in Human and Animal Studies at the Federal University of Valley do São Francisco under protocol number 29041107.

RESULTS

The tick infestation was found in 54.5% (220/404) of the dogs. Rural dogs developed a higher percentage of tick infestation 61.4% (124/202), compared with the dogs in urban areas 47.5% (96/202). A total of 1,511 ticks were collected (70 nymphs, 655 males, and 786 females); in both urban (n=652) and rural (n=859) areas. All ticks (100%) were identified as *Rhipicephalus sanguineus* (Table 1).

Intraerythrocytic forms for *Babesia* spp. were observed in only two samples (0.5%) from rural areas, by microscopic analysis of blood smears.

Anti-*B. canis vogeli* antibodies were detected in 57.9% (234/404) of dogs by IFA. The seroprevalence in urban areas and rural areas were 48.5% (98/202) and 67.3%, (136/202), respectively.

DNA of *Babesia* spp. was determinate by PCR in 6% of individual adult ticks (3/50); and in 8.7% of pools (7/80). All positive individual ticks were from rural areas; and all positive pools were from urban areas. All samples submitted to DNA sequencing showed identical sequences 100% identical with *Babesia canis vogeli* 18S ribosomal RNA gene, partial sequence (GenBank KJ494656.1).

In the univariate analysis, variables area, breed, age, breed size, street access, contact with forest/caatinga, presence of ticks and veterinary care were associated ($P<0.20$) with the prevalence of *B. canis vogeli* and then were selected for the multivariate analysis (Table 2). As shown in Table 3, when these independent variables were subjected to the multivariate analysis, the following were identified as risk factors for canine babesiosis, significantly associated with the rate of antibodies to *Babesia canis vogeli* : medium breed size (OR=2.98; $P<0.001$), contact with areas of forest (Caatinga biome) (OR=2.22; P=0.021), access to street dogs (OR=1.51; P=0.046).

DISCUSSION

In the present study the tick infestation was observed in 54.5% of dogs. Rural dogs showed a higher percentage of infestation (56.3%), when compared with urban rural dogs (43.5%). Although the *Rhipicephalus sanguineus* is often found in urban areas (Labruna & Pereira 2001), this tick infestation in rural dogs has also been verified (Szabó et al. 2001, Shimada et al. 2003, O'Dwyer et al. 2009).

Higher prevalence of *R. sanguineus* in rural dogs was also observed by Labruna et al. (2001) in a study of dogs from northern Paraná, although their frequency was lower compared to previous studies carried out in urban areas. The behavior of the dogs, and the rest area in the same place would make this tick species become established in rural areas. High prevalence of *R. sanguineus* in a rural environment of Petrolina, Pernambuco, could be justified by the lifestyle of these dogs, once the great majority was created semi-confined, and having their resting places along the residences of their owners, by facilitating parasitism of this species.

In the microscopic analysis of blood smears, piriform intraerythrocytic forms for *Babesia* spp were observed in only two samples (0.5%) of dogs from the rural area. This low parasitism, found in blood smears, is in accordance with the findings of Guimarães et al. (2004) and Miranda et al. (2008) in dogs in the city of Goytacazes, state of Rio de Janeiro, considered enzootic for canine babesiosis; and of O'Dwyer et al. (2009) in dogs from rural areas of the state of São Paulo. The diagnosis by blood smear has a good specificity, but low sensitivity; committing to early stages of

Table 1. Percentage by area of tick infestation, presence of intraerythrocytic forms *Babesia* spp in blood smears seropositivity and detection of DNA of *Babesia* spp in individual and pools of ticks infesting dogs from the urban and rural areas of semiarid region of Pernambuco, Brazil

Area	% tick infestation (positive/total)	% blood smears (positive/total)	% IFA (positive/total)	% *Babesia* spp. DNA in ticks: individual (positive/total); pool (positive/total)
Urban	47.5 (96/202)	0 (0/202)	48.5 (98/202)	0 (0/50); 8.7 (7/80)
Rural	61.4 (124/202)	0.5 (1/202)	67.3 (136/202)	6.0 (3/50); 0 (0/80)
Total	54.5 (220/404)	0.2 (1/404)	57.9 (234/404)	3.0 (3/100); 4.4 (7/160)

Table 2. Univariate analysis for risk factors associated with *Babesia canis vogeli* infection in the semiarid region of Pernambuco, Brazil

Independent variables	Category	Total number of dogs	Positives (%)	*P*
Area	Rural	202	136 (67,3)	< 0,001*
	Urban	202	98 (48,5)	
Breed	Purebred	77	31 (40,3)	0,001*
	Mixed-breed	327	203 (62,1)	
Age (years)	< 1	84	37 (44,0)	0,006*
	1 - 3	194	121 (62,4)	
	3 - 8	110	70 (63,6)	
	> 8	16	6 (37,5)	
Gender	Female	142	80 (56,3)	0,712
	Male	262	154 (58,8)	
Breed size	Small	125	54 (43,2)	< 0,001*
	Medium	212	149 (70,3)	
	Large	67	31 (46,3)	
Street access	No	179	92 (51,4)	0,023*
	Yes	225	142 (63,1)	
Contact with other animals	No	122	69 (56,6)	0,798
	Yes	282	165 (58,5)	
Contact with forest / caatinga	No	350	193 (55,1)	0,006*
	Yes	54	41 (75,9)	
Historical tick	No	78	49 (62,8)	0,396
	Yes	326	185 (56,7)	
Presence of ticks	No	184	99 (53,8)	0,152*
	Yes	220	135 (61,4)	
Veterinary care	No	307	188 (61,2)	0,022*
	Yes	97	46 (47,4)	
Use of acaricide	No use	181	112 (61,9)	0,204
	Only animal	173	91 (52,6)	
	Animal + environment	47	30 (63,8)	
	Only environment	3	1 (33,3)	

* Variables selected for the multivariate analysis (P<0.20).

Table 3. Risk factors associated with *Babesia canis vogeli* infection in dogs of the semiarid region of Pernambuco, Brazil

Risk factor	Odds ratio (95% CI)	*P*
Medium breed size	2.98 (1.87 – 4.74)	< 0.001
Contact with forest /caatinga	2.22 (1.13 – 4.37)	0.021
Street access	1.56 (1.01 – 2.41)	0.046

disease, where it has a high level of parasitemia, with false negative results under low parasitemia (Krause et al. 1996, Guimarães et al. 2004, Passos et al. 2005).

Detection of anti-*B. canis vogeli* antibodies occurred in 57.9% of the analyzed dogs; 48.5% in dogs of the urban area and 67.3% of rural area. Guimarães et al. (2009) found seropositivity to IFA in 73.3% dogs from the municipality of Lavras, state of Minas Gerais. However, a lower prevalence (18.8%) was determinate in a canine population in a semiarid area of Porteirinha, Minas Gerais, which has climatic conditions similar to those of the present study (Maia et al. 2007).

Babesia canis is considered endemic in urban areas with high tick infestation of dogs, (Passos et al. 2005), but less frequent in rural areas. The present study, which analyzed dogs who live in rural areas, indicated a seropositivity higher than that observed by Costa-Júnior et al. (2009) for three rural municipalities of Minas Gerais, obtaining a frequency of 28.7%. Vieira et al. (2013) had a higher prevalence (60.3%) of anti-*B. canis vogeli* antibodies in dogs from urban areas of the State of Paraná, which were 3.1 times more likely to acquire anti-*B. canis vogeli* antibodies than dogs from rural areas. However, in this study, the dogs from rural areas had a higher prevalence of antibodies in relation to dogs in urban areas, which may be justified by the high prevalence of the tick vector, *R. sanguineus* in dogs from rural areas.

The racial pattern, age and mixed breed animals (SRD) showed higher prevalence of presence of *B. canis vogeli* antibodies, as also reported by Maia et al. (2007). These are more likely to acquire the infection by *Babesia* spp compared to animals of breed, since these are usually raised in confinement, more restricted environments, with little contact with other animals and external environments, thus reducing exposure to vector. Dogs over one year of age have a higher probability of seroconversion, with a higher chance of acquiring tick infestation and consequently a greater chance of acquiring the disease, becoming chronically infected (Ribeiro et al. 1990, Solano-Gallego et al. 2008).

The multivariate analysis showed that dogs of medium breed size were 2.9 times more likely to acquire anti-*Babesia canis vogeli* antibodies than dogs of the small and large breed size. Access on the street and contact with the dogs with areas of forest (Caatinga) were identified as risk factors for canine babesiosis. These results indicate that the tick vector of *B. canis vogeli* in the study region prefers to live in rural areas, especially in areas with vegetation (forest, caatinga). Additionally, univariate analyses indicated a significant association ($P < 0.20$) of *B. canis vogeli* seroreactivity with dogs living in rural areas and with dogs mixed breed animals (Table 2).

Gender, breed, and age were not associated with the risk of seropositivity amongst the canine population, a finding that is in agreement with of the epidemiological studies on canine babesiosis (Ribeiro et al. 1990, Maia et al. 2007, Costa-Junior et al. 2009).

Detection of *Babesia* infection in ticks has been widely used in epidemiological studies in various countries (Inokuma et al. 2003, Matjila et al. 2005, M'Ghirbi & Bouattour 2008, Majláthová et al. 2011). However, in Brazil, few studies have been performed and little is known about the aspects of infection with *Babesia* spp. in ticks, being unprecedented in the municipality of Petrolina, PE. Silva et al. (2012) obtained a prevalence of 2.6% *R. sanguineus* infected with *B. canis vogeli* in a study conducted in ticks from dogs from rural and urban areas of western region of Maranhão, lower than the one obtained in this study. M'Ghirbi & Bouattour (2008) obtained a prevalence of 0.6% in Tunisia. Higher prevalence was found in 35.6% ticks in Slovakia (Majláthová et al. 2011).

DNA of *B. canis vogeli* was observed in 6% of individual ticks and 8.7% of pools of ticks. Some seronegative dogs (57.1%) were infesting by positive ticks. These ticks were, probably, collected from dogs before the inoculation of the agent by ticks, which requires an average three days of blood feeding for transmission, or because the diagnosis by IFA be compromised the initial stage of occurring disease

because the parasite appears in the blood before there is a detectable antibody level.

Although the municipality of Petrolina, Pernambuco presents favorable climatic conditions for the development of the *R. sanguineus*, the dynamics of tick populations depends on climatic conditions, thus affecting the transmission of *Babesia* and hence its maintenance in nature (Friedhoff 1988). Considering the hematophagous habitat, ticks have blood of their hosts and positive PCR results in the possibility of tick infection or infected dog. However, it provides information on the prevalence of these agents in the canine population of the region (Inokuma et al. 2003).

CONCLUSIONS

The presence of canine babesiosis was confirmed in both urban and rural areas of the municipality of Petrolina, Pernambuco, by detection of antibodies anti-*Babesia canis* in canine sera; and detection of *Babesia canis vogeli* in ticks infesting dogs.

The risk factors for the presence of anti-*B. canis vogeli* antibodies were medium breed size variables; contact with areas of forest; and access to the street.

This study describes, for the first time, the confirmation of infection of *Babesia canis vogeli* in dogs and ticks in the semiarid region of Pernambuco.

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