Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Epidemiological and molecular study of *Ehrlichia canis* in dogs in Bahia, Brazil

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Genet. Mol. Res. 7 (3): 657-662 (2008)
Received April 7, 2008
Accepted June 24, 2008
Published July 29, 2008

ABSTRACT. The objective of the present article was an epidemiological and molecular study of *Ehrlichia canis* in dogs of Ilhéus and Itabuna in Bahia, as well as an evaluation of associated risk factors. Blood samples were collected from 153 dogs and DNA was extracted and analyzed by the nested-polymerase chain reaction, using one pair of primers to detect *Ehrlichia* bacteria and another pair to detect the presence of *E. canis*. Of the 153 animals, 12 (7.8%) were polymerase chain reaction-positive for *E. canis*, indicating the presence of the parasite in dogs of the Ilhéus-Itabuna microregion. The associated risk factors were exposure to tick-infested habitats and the fact that the dogs lived in the countryside.

Key words: Molecular; Ehrlichiosis; Dogs; Ticks; Rickettsia

INTRODUCTION

Ehrlichia is a *Rickettsia* of the Anaplasmataceae family, Gram-negative intracytoplasmic bacteria, that invade and multiply within leukocytes and platelets in the peripheral blood of various species of domestic and wild mammals. It causes ehrlichiosis (Borjesson, 2000; Mavromatis et al., 2006), a world-wide distributed zoonosis (Perez et al., 2005), concentrated in tropical and subtropical regions due to the geographical distribution of its vector tick Ixodidae *Rhipicephalus sanguineus* (Andereg and Passos, 1999). It can also infect humans (Dantas-Torres et al., 2006). In Brazil, this seems to be the main vector for *Ehrlichia canis* in urban areas, although in rural areas human infection seems to be related to the genus *Amblyomma* (Labruna and Pereira, 2001).

Several species of *Ehrlichia* infect dogs such as *Anaplasma platys*, *E. equi*, *E. ewingii*, *E. risticii*, *E. chaffeensis*, *E. sennetsu*, and *E. canis*. The latter being the main species that infects dogs producing several clinical symptoms (Stiles, 2000) - fever, anorexia, vomiting, loss of weight, enlargement of the liver, spleen and lymph nodes, epistaxis, hemorrhage and thrombocytopenia (Moreira et al., 2003).

Specific diagnosis of ehrlichiosis can be made using a blood smear, indirect immunofluorescence assay, ELISA, Western blot, and polymerase chain reaction (PCR) (Cadman et al., 1994; Iqbal et al., 1994; Hegarty et al., 1997; Carlos et al., 2007).

PCR is a technique used in the early diagnosis of ehrlichiosis and also for identification of the infecting species, helping with the taxonomic schemes (Iqbal et al., 1994). Using PCR, it is possible to isolate DNA and identify *E. canis* from blood samples or tissue from lungs, spleen, lymph nodes, kidneys, brain, and eyes of infected animals (Stiles, 2000).

Molecular diagnosis is based on the analyses of the 16S rRNA gene sequence, which is genetically related to Gram-negative intracellular bacteria (Dagnone et al., 2001), as *Ehrlichia* differs genetically from other *Rickettsias* (Chen et al., 1994).

The 16S rRNA gene of *E. canis* was partially sequenced in August 1996, revealing 1433 bp. The sequencing was finished in 2007 and has been described and deposited in GenBank under accession number CP000107 (Copeland et al., 2007). The complete genome sequence reveals that *E. canis* has a single circular chromosome of 1,315,030 bp to code for 925 proteins, which can be associated with pathogen-host interactions (Mavromatis et al., 2006).

The prevalence of *E. canis*, based on the PCR technique, was reported as follows: 21% in Paraná (Dagnone et al., 2003), 30.9% in São Paulo (Bulla et al., 2004) and 15% in Rio de Janeiro (Macieira et al., 2005).

The objective of the present article was an epidemiological and molecular study of *Ehrlichia canis* in dogs of Ilhéus and Itabuna, in Bahia, Brazil, as well as an evaluation of associated risk factors.

MATERIAL AND METHODS

Study location

This comparative study was conducted on the municipalities of Ilhéus (latitude and longitude 14°47'20" S, 39°02'58" W) and Itabuna (latitude and longitude 14°47'08" S, 39°16'49" W), the Ilhéus-Itabuna microregion, in the south of Bahia State. The two munici-

palities have a population of approximately 200,000 inhabitants each and they are 37 km apart. The climate is warm and humid; the average temperature is 24°C, and the average annual precipitation is 2134 mm.

Sample collection

A total of 153 convenience samples were obtained from the Veterinary Outpatient Clinic at the State University of Santa Cruz (UESC), and from veterinary clinics and home visits in both municipalities.

The animals were evaluated and examined and 3 mL whole blood was collected in tubes with EDTA. The blood samples were identified and kept in isothermal boxes until they could be processed at the Animal Genetics Veterinary Laboratory of UESC.

The analysis of samples was performed using the Epi-Info 6.4 (Dean and Arner, 2007) based on the population of dogs, which was estimated from the human population of the municipalities. To calculate the proportion dog/human we used the 1:10 ratio, which resulted in a total of 40,000 animals. The sample analysis was carried out considering a 95% degree of confidence, the possibility of illness detection considered for 15% of the dogs (Macieira et al., 2005), and a statistical error of 5.65%, resulting in a sample of 153 dogs.

DNA extraction

The genomic DNA of the samples was extracted and isolated from 100 µL of the dog's whole blood using the extraction kit - ChargeSwitch® gDNA 100 µL Blood Kits (Invitrogen®), according to manufacturer instructions, which made it possible to extract small amounts of DNA rapidly and free from contamination. The samples were stored in a freezer at -20°C for later PCR.

Nested-polymerase chain reaction

The forward primer ECC 5'-AGAACGAACGCTGGCGGCAAGC-3' and the reverse one, ECB 5'-CGTATTACCGCGGCTGCTGGCA-3' were used to amplify part of the 16S rRNA gene of *Ehrlichia* spp. Afterwards, the samples were subjected to the forward primer 5'-CAATTATTTATAGCCTCTGGCTATAGGA-3' and the reverse one, HE3 5'-TATAGGTACCGTCATTATCTTCCCTAT-3' (Murphy et al., 1998), specific for *E. canis*.

For the PCR mixture, 6 µL purified DNA was used, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.2 µM of each primer, and 2 U *Taq* DNA polymerase (Invitrogen®) for a total volume of 25 µL reaction mixture. The amplification program used to id entify the genetic sequence of the genus *Ehrlichia* spp consisted of a first step of denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 2 min and extension at 72°C for 2 min. The nested-PCR for the identification of the genetic sequence specific for *E. canis* consisted of denaturation for 3 min at 94°C, followed by 35 denaturation cycles at 94°C for 1 min, annealing at 58°C for 2 min and extension at 72°C for 1.5 min. The PCR products were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide and photographed.

Statistical analysis

The analysis was performed according to the following variables: prevalence between the two municipalities of Ilhéus and Itabuna, gender of the animals (male and female), age group (from 0 to 3 years old, from 4 to 7 years old and over 7 years old), breed (groups of defined and non-defined breed), presence of ticks and animals' location (urban or rural).

The statistical analysis was performed by the chi-square test and the Fisher exact test to determine the relation between the observed variables and the dispersion of these frequencies.

RESULTS AND DISCUSSION

A total of 153 dogs were evaluated, 84 from Ilhéus and 69 from Itabuna, and among them, 12 animals (7.8%) were positive for *E. canis*, 9 animals (10.7%) in Ilhéus and 3 animals (4.3%) in Itabuna. The amplification of the 16S rRNA fragment with specific primers for *E. canis* produced a specific, visible, single band pattern of approximately 400 bp (Figure 1).

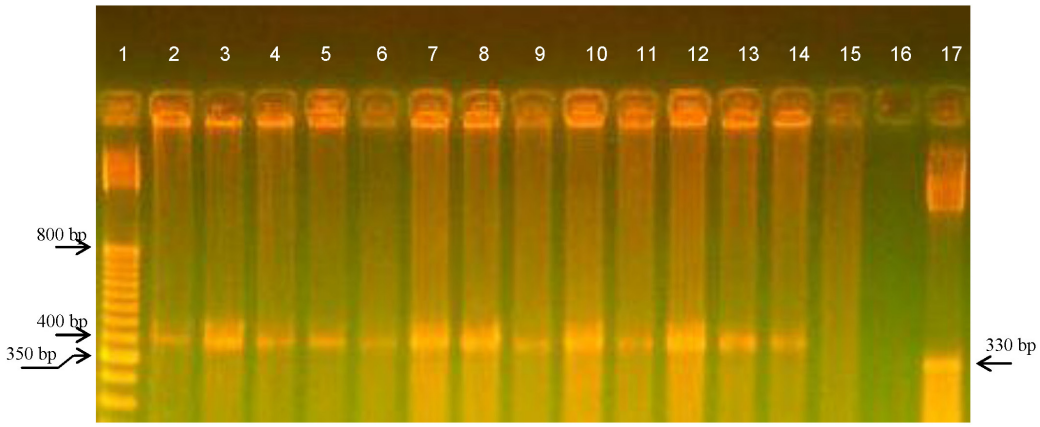

Figure 1. Resolution on agarose gel of the products from nested-polymerase chain reaction with specific primers for *Ehrlichia canis*. Lane 1 = molecular weight, 50-bp DNA ladder; lane 2 = positive control; lanes 3 to 14 = positive animals; lane 15 = negative animal; lane 16 = negative control; lane 17 = molecular weight, 10-bp DNA ladder.

The results of this study showed the presence of *E. canis* in dogs in the microregion of Ilhéus-Itabuna, reporting lower prevalence than in other parts of the country with similar climatic conditions such as Rio de Janeiro (Macieira et al., 2005). This may be due to the lower regional incidence of the disease, which did not seem to be the case, and/or the presence of different species of *Ehrlichia*, such as *E. chaffeensis* and *E. ewingii*, which were not included in the study because of the specificity of the primers used.

There was no significant difference in prevalence (P = 0.24) between the two municipalities, although the number of positive animals in Ilhéus was higher than in Itabuna. This may be explained by the fact that there is a rural neighborhood in Ilhéus, where some samples

were obtained, and a slight difference (Table 1) was observed between rural and urban dogs, confirming the results reported by Dagnone et al. (2002) who found rural dogs with more positivity for parasites than urban dogs. In this rural area, the population is predominantly poor, and their animals are usually infested with ticks. One must also consider that this site is close to the Atlantic Forest, possibly a reservoir for the wild type of the disease, which could contribute to a higher incidence of the parasite.

Table 1. Relation between polymerase chain reaction (PCR)-positive dogs and their location in the municipalities of Ilhéus and Itabuna, Bahia, Brazil.

Location	Dogs		χ^2	P	Incidence rate (OR)	95% Confidence interval
	PCR-positive	PCR-negative				
Rural area	5	17	5.65	0.017[a]	5.21	1.26-21.29
Urban area	7	124				

[a]Chi-square test.

The presence of ticks in dogs was considered to be a risk factor (Table 2), confirming the importance of ticks as a vector for the infection (Groves et al., 1975; Dagnone et al., 2003).

Table 2. Association between polymerase chain reaction (PCR)-positive dogs and the presence of ticks, gender and breed in the municipalities of Ilhéus and Itabuna, Bahia, Brazil.

Variables	Dogs		χ^2	P	Incidence rate (OR)	95% Confidence interval
	PCR-positive	PCR-negative				
No ticks	3	80	3.30	0.069[a]	3.90	0.92-23.34
With ticks	9	61		0.034[b]		
Male	7	83	0.07	0.787[a]	1.02	0.24-3.95
Female	5	58		0.599[b]		
Definite breed	6	99	1.26	0.260[a]	2.36	0.59-9.32
No definite breed	6	42		0.131[b]		

[a]Chi-square test; [b]Fisher exact test.

Analyzing variables, such as sex, breed and age group, no significant differences were reported ($P > 0.26$, $P > 0.78$ and $P > 0.41$, respectively). These results corroborate those of Borba et al. (2002) in Pernambuco, Dagnone et al. (2002) in Paraná, Inokuma et al. (1999) in Japan, and Cocco et al. (2003) in Italy.

Using the PCR technique, it was possible to detect *E. canis* in dogs in Ilhéus and Itabuna with a prevalence of 7.8%. However, further research must be carried out using other primers in order to detect other species of the genus *Ehrlichia* in the region.

ACKNOWLEDGMENTS

Research supported by FAPESB and UESC.

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