

MOLECULAR DIAGNOSIS OF LEPTOSPIROSIS IN BLOOD OF DOGS NATURALLY INFECTED*

DIAGNÓSTICO MOLECULAR DE LEPTOSPIROSE NO SANGUE DE CÃES NATURALMENTE INFECTADOS

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ABSTRACT. Meira C.D., Wenceslau A.A., Carvalho F.S., Albuquerque, G.R. & Dias R.C. **Molecular diagnosis of Leptospirosis in blood of dogs naturally infected.** [Diagnóstico molecular de leptospirose no sangue de cães naturalmente infectados]. *Revista Brasileira de Medicina Veterinária*, 33(1):7-11, 2011. Programa de Pós-Graduação em Ciência Animal, Universidade Estadual de Santa Cruz, Rodovia Ilhéus-Itabuna, km 16, Ilhéus, BA 45662-900, Brasil. E-mail: wenceslau@uesc.br

Leptospirosis is an acute and systemic infectious disease, which affects men and animals and is caused by pathogenic spirochetes of the genus *Leptospira* spp. The early diagnosis is important for the adoption of the appropriate therapy and prevention against the installation of critical symptomatologic forms. The Polymerase Chain Reaction (PCR) is being used for the diagnosis of infectious diseases showing efficiency for the diagnosis of the disease before the development of antibodies. The aim of this work was to evaluate the PCR technique with the primers G1 and G2 that amplify a fragment of 285 base pairs, specific of the region of gene secY of *Leptospira* spp, on 200 blood samples from domestic and stray dogs and determination of the risk factors associated from the Municipality of Ilhéus in the State of Bahia. Nine animals (4.5%) presented positive results for leptospirosis. There was no statistically significant difference between the prevalence of leptospirosis in domiciled or stray dogs ($p= 1.0$), sex ($p= 0.455$), age ($p=0.644$), access to the street ($p= 0.113$) and presence and/or contact with rodents ($p= 0.362$).

KEY WORDS. DNA, *Leptospira*, Zoonosis, dogs.

RESUMO. A Leptospirose é uma doença aguda e infecciosa sistêmica, que acomete homens e animais e é causada por espiroquetas patogênicas do gênero *Leptospira* spp. O diagnóstico precoce é importante para a adoção da terapêutica adequada e prevenção contra a instalação de formas sintomatológicas críticas. A Reação em Cadeia da Polimerase (PCR) está sendo usada para diagnóstico de doenças infecciosas, mostrando eficiência para o diagnóstico da doença antes do desenvolvimento de anticorpos. O objetivo deste trabalho foi avaliar a técnica de PCR com os iniciadores G1 e G2, que amplificam um fragmento de 285 pares de base, específicas da região do gene secY de *Leptospira* spp., em 200 amostras de sangue de cães domiciliados e vadios e à determina-

ção do fatores de risco associados do município de Ilhéus, Estado da Bahia. Desses animais, nove (4,5%) apresentaram resultados positivos para leptospirose. Não houve diferença estatisticamente significativa entre a prevalência de leptospirose em cães domiciliados ou errantes ($p = 1,0$), sexo ($p = 0,455$), idade ($p = 0,644$), o acesso à rua ($p = 0,113$) e a presença e/ou contato com roedores ($p = 0,362$).

PALAVRAS-CHAVE. DNA, *Leptospira*, zoonose.

INTRODUCTION

Leptospirosis is a zoonosis of worldwide distribution caused by pathogenic spirochetes of the genus *Leptospira*

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ra (Levett 2001). In Brazil it is considered an endemic disease and constitutes a serious public health problem. The maintenance of the agent in urban and rural area is favored by the tropical climate associated to the inadequate socio-economic and sanitary situations of the population (Ko et al. 1999, Costa et al. 2001, Pappachan et al. 2004).

Various species - domestic, sinantropic and wild - may become carriers and contribute to the spreading of the disease in the environment (Langoni et al. 1999, Corrêa et al. 2004, Delbem et al. 2004). The elimination of *Leptospira* by urine of carriers may occur for a period of time that may vary from a few weeks to various months, for the domestic animals, and for all their lives in the case of rodents (Webster et al. 1995, Tesseroli et al. 2005).

The disease has a seasonal character that coincides with periods of intense pluviometric precipitation and consequent floods. This increases the risk of infection in humans and animals due to high exposition of the mucosa and skin with water contaminated mainly with rodent's urine (Pappachan et al. 2004).

For dogs the clinical signs of leptospirosis may depend on factors such as age, immunological state and virulence of the serovar. The animals may present fever, jaundice, vomit, depression, anorexia, prostration, diffuse hemorrhages mainly in the lungs and digestive system, renal insufficiency and dehydration. However, cases of sub-clinical or chronic infections may happen, case in which the disease remains asymptomatic (Adin et al. 2000, Santim et al. 2006).

The early diagnosis is essential for the adoption of the appropriate therapy and prevention against the installation of critical symptomatologic forms. Different laboratory techniques based on the detection of the agent or genetic material may be used (Faine et al. 1999, Riediger 2007). The technique recommended by the World Health Organization (WHO) is the microscopic serum-agglutination (SAM) with live antigens, the most used in the world. Its only disadvantage is its lack of sensitivity for detecting the antibodies in the first two weeks of the disease (Faine et al. 1999, Levett 2001).

The Polymerase Chain Reaction (PCR) is being widely used for the diagnosis of infectious diseases caused by microorganisms, showing efficiency for the diagnosis of the disease before the development of antibodies or when the titers are still low and the clinical course was not characterized (Kee et al. 1994, Wangroongsarb et al. 2005). Teixeira et al. (2008) have shown the importance of molecular biology. They reported a clinical case in which a dog with symptoms suggesting leptospirosis presented negative serology but positive PCR. Its limitation lies in its inability of identify the infecting serovar. However, it may

differentiate pathogenic species of saprophytes (Branger et al. 2005).

The aim of this work was to evaluate the PCR technique for the detection of *Leptospira* spp. in blood samples of dogs and determination of the risk factors associated from the municipality of Ilhéus, Bahia, Brazil.

MATERIAL AND METHODS

Area and study animals

Dogs from the Municipality of Ilhéus, in the South Coast of the State of Bahia (14° 47' south latitude, 39° 02' west longitude) were analyzed. The climate is tropical and the average annual rainfall is 1600–1800mm. The vegetation is formed mainly by the Atlantic Forest. The population is estimated in 219,710 inhabitants (IBGE 2008). The sample was calculated based on the total population of dogs of the municipality from the human population. For the calculation of the proportion dog/man the ration of 1:10 was used, which resulted in approximately 22,000 animals. The calculation of the samples was made by means of the program Epi Info 3.5, considering a trust level of 95%, possibility of detection of the disease of 50% and statistical errors of 7%, resulting in the sample N of 196 that was increased to 200 animals. Half the samples were obtained from domiciled dogs attended at the Veterinary Hospital in the Universidade Estadual de Santa Cruz (HV-UESC) and the other half came from stray dogs and was obtained at the Center for Zoonosis Control (CCZ), Ilhéus, BA. The blood collection was made regardless of the medical state, sex or age of the animal, by jugular or cephalic venopunction, transferred into tubes with EDTA, and kept at -20°C, until total DNA extraction.

DNA extraction and PCR

A quantity of 500 µL of blood was transferred into a microtube with 500 µL of miliQ water. The samples were homogenized, and then centrifuged at 10000 rpm for 5 m. The supernatant was discarded. 500 µL was added from the extraction buffer (Tris-HCL 10mM; EDTA 0,5M; Proteinase K 100 µg/µL; 1% Triton X-100). Each sample was taken to the vortex and heated in double boiler at 50°C for 30 m. After this period, 300 µL Sodium Dodecyl Sulphate (SDS) 0.5 % was added, and another time to the vortex and double boiler at 50°C for 30 m. Phenol/Chloroform/Alcohol Isoamilic was added, homogenized and centrifuged at 14000 rpm for 6 m. The supernatant was transferred into another microtube. The washing with chloroform was repeated. The samples were centrifuged again at 14000 rpm for 8 m. The supernatant was transferred into another microtube. The

DNA was precipitated with ammonium acetate 5M and ethanol 100%. The samples were centrifuged at 14000 rpm for 10 m, the supernatant was discarded and the precipitate was washed with ethanol 95%. It was again centrifuged at 14000 rpm for 15 m. The supernatant was discarded; the pellet was dried up in room temperature and eluted in TE.

For the PCR, specific primers were used for the amplification of a fragment of the gene *secY* from the genome of the pathogenic species *Leptospira* spp., except for *L. kirschneri* (Gravekamp et al. 1993). The primers used on the amplification reaction had the following nucleotide sequences: G1 [direct] - 5' CTG AAT CGC TGT ATA AAA GT 3'; G2 [reverse] - 5' GGAAA CAAATG GTC GGAAG 3'. The amplification of the DNA was carried out in a final volume of 35 μ L. The reaction mixture consisted of 200 μ M dNTP, 5.0 mM MgCl₂, 2 U of Taq DNA polymerase (Ludwig®), 1.6 x amplification buffer (supplied by the manufacturer) and 0.20 mM of each initiator (G1 and G2). The volume of the reaction mixture was corrected with ultrapure water for PCR to 16 μ L, following to that 19 μ L of extracted DNA were added. Thermocycler MJ96G-Biocyler was used. The first cycle consisted of denaturation at 95°C for 5 m, annealing of the primers at 68°C for 1 m and extension at 72°C for 2 m. The 34 following cycles consisted of denaturation at 94°C for 1.5 m, annealing of primers at 55°C for 1 m and extension at 72°C for 2 m. There was a final phase of 5 m at 72°C. The PCR product rates and molecular weight marker 50 pb (Invitrogen®) were submitted to agarose gel electrophoresis at 2%, colored with ethidium bromide and exposed to ultraviolet light in a transilluminator (L-PIX Loccus Biotecnologia). The gel was photo documented and the image was scanned. The definition of positive and negative results was based on the visual identification of the bands colored with ethidium bromide in the agarose gel.

Statistical analysis

For the determination of the risk factors associated to leptospirosis a epidemiologic questionnaire was conducted with the owners of the animals. The data refer to sex, age, breed and risk factors such as contact with rodents and access to the street. The questions were answered when the blood samples were collected. In the case of the stray animals sheltered by CCZ only sex, presence of clinical symptomatology and estimate age were recorded. The results obtained after the study were submitted to the statistical analysis by Qui-square (χ^2) and Fisher's Exact Test of the program EpiInfo 3.5.

RESULTS

From the 200 dogs evaluated in this study, nine (4.5%) were positive for leptospirosis – five domiciled and four strays dogs from CCZ-Ilhéus, Bahia. The PCR of the blood samples of the positive animals generated a specific band, visible and compatible with the 285 base pairs (bp) of the DNA fragment of *Leptospira* amplified by the primers G1 and G2, indicating the presence of the bacteria in the blood circulation (Figure 1).

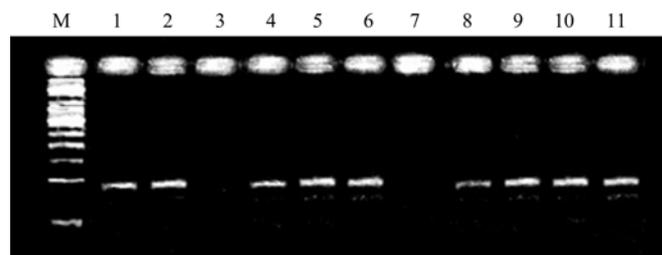


Figure 1. Agarose gel electrophoresis 2.0% of the PCR products. M: Molecular markers 50bp; 1: positive control; 2-4-5-6: positive domiciled animals; 8-9-10-11: positive stray animals; 3-7: negative control.

There was no statistically significant difference between the prevalence of leptospirosis in domiciled or stray dogs ($p= 1.0$), sex ($p= 0.455$), age ($p=0.644$), access to the street ($p= 0.113$) and presence and/or contact with rodents ($p= 0.362$).

DISCUSSION

Various diagnosis techniques based on the detection of the genetic material of *Leptospira* spp. are being used. Among them is the PCR, a quick, sensitive and specific method that enables the diagnosis from a small DNA sample. However, it may present sensitivity and specificity variations depending on some factors such as the type, conservation and storage time of the biological sample and initiators and reagents used (Bal et al. 1994, Kritski et al. 1997, Veloso et al. 2000).

There are few studies in Brazil related to the diagnosis of leptospirosis in dogs by means of the PCR. Most of the epidemiologic studies is based on serology, that indicate a variation of 2.6% to 85% of positive results (Jouglard & Brod 2000, Viegas et al. 2001, Blazius et al. 2005, Batista et al. 2005, Magalhães et al. 2006).

The leptospirosis seroepidemiologic screening in dogs, in various Brazilian states, show a major variability as regards frequency. This fact may be explained by the multiplicity of factors that influence the occurrence of the disease, such as the climate, being higher in tropical regions, pluviometric rates and sanitary conditions, such as inadequate waste collection, mud build-up and numerous population of rodents (Alves et al. 2000, Blazius

et al. 2005). Both stray and domestic dogs, for their close contact with human, are an important source of infection participating of the epidemiology of the disease (Farrington & Sulzer 1982).

Vicari et al. (2007), using the PCR technique in Italy with 64 dogs selected at random obtained 7.8% positive animals, a result close to this study, once that the molecular diagnosis allows that only the animals really infected present positive results, differently from the serology that may result in false-positive animals, mainly due to factors such as cross reactions among serovars and vaccination titers.

The beginning of the acute phase of the disease is also considered as an important factor on the interpretation of laboratorial results, and the serologic tests must be carried out with the epidemiologic data, as well as the information obtained during anamnesis and physical examination (Bolin 1996).

The absence of significant statistical difference between the prevalence of leptospirosis in domestic dogs (5%) and stray dogs (4%) deny the results obtained by Viegas et al. (2001) and Blazius et al. (2005), where the serologic tests showed that stray dogs have higher incidence of infections caused by *Leptospira* spp. than domestic dogs. According to the interview with the owners, most of the domestic animals had free access to the street, being regularly exposed to infection risks. Dogs that have more access to the street suffer higher infection risk by direct or indirect contact with other animals or wetlands (Querino et al. 2003, Batista et al. 2005).

The results obtained in the present study indicated that the presence and/or contact with rodents and access to the street did not present significant difference, maybe due to the small number of samples. However, all positive animals had access to these variables. It is probable that the habit of hunting rodents and access to the street are linked, favoring the spreading of leptospirosis among dogs (Querino et al. 2003). There were no positive animals among the dogs without access to the street.

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