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Díaz-Medina et al.
Ehrlichia canis in dogs from Mexico
3(8):251-257,2016

MOLECULAR SURVEY OF *Ehrlichia canis* IN DOGS FROM MEXICO: PREVALENCE OF INFECTION AND POSSIBLE ASSOCIATED FACTORS

Estudio molecular de *Ehrlichia canis* en perros de México: prevalencia de infección y posibles factores asociados

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ABSTRACT. A cross-sectional study was performed to estimate the prevalence of infection of *Ehrlichia canis* in dogs and to explore the factors associated with the presence of the bacteria. The study was carried out in a village in Yucatan, Mexico. Blood samples were obtained from 200 dogs. Samples were analyzed by nested-PCR to detect the presence of *E. canis* DNA and thrombocyte counts were calculated. One-hundred and forty of the dogs (70 %) were found to be infested with ticks. A total of 1 116 ticks were recovered and all were identified as *Rhipicephalus sanguineus*. The prevalence of *E. canis* infection was 69.2 %. None of the variables studied (gender, age, body condition, platelet-related bleeding, thrombocytopenia, and presence of ticks) showed association with *E. canis* infection. In conclusion, there is a high probability that dogs living in Yucatan, Mexico are infected with *E. canis*.
Key words: Dogs, *Ehrlichia canis*, Prevalence, Nested-PCR, Yucatan, Mexico.

RESUMEN. Un estudio de sección cruzada fue realizada para estimar la prevalencia de infección de *Ehrlichia canis* en perros y explorar la presencia de factores asociados con la presencia de la bacteria. El estudio se realizó en una comunidad de Yucatán, México. Muestras de sangre de un total de 200 perros fueron obtenidas. Las muestras fueron analizadas por Nested-PCR para detectar la presencia de ADN de *E. canis* y se calculó el conteo de Plaquetas. 140 perros de 200 estudiados (70 %) estuvieron infestados por garrapatas. Un total de 1 116 garrapatas fueron recuperadas y todas identificadas como *Rhipicephalus sanguineus*. La prevalencia de infección por *E. canis* fue de 69.2 %. Ninguna de las variables estudiadas (género, edad, condición corporal, hemorragias relacionadas con plaquetas, trombocitopenia y presencia de garrapatas) mostraron asociación con la infección por *E. canis*. En conclusión, existe una alta probabilidad que los perros que viven en Yucatán, México, estén infectados con *E. canis*.
Palabras clave: Perros, *Ehrlichia canis*, Prevalencia, PCR anidado, Yucatán, México.

INTRODUCTION

Ehrlichia canis is a tick-borne obligate intracellular Gram-negative bacterium that infects canine monocytes and is the primary etiological agent of Canine Monocytic Ehrlichiosis (CME). The pathogen is transmitted to canines by the brown dog tick, *Rhipicephalus sanguineus*. Experimental inoculations have demonstrated an incubation period of 8-20 d in which the bacteria spread throughout the body via the mononuclear-phagocyte system (Neer and Harrus 2006). Following incubation, *E. canis* infection may progress into three consecutive clinical stages: acute, sub-clinical and chronic. The disease is characterized by severe clinical presentation with hemorrhages, thrombocytopenia and,

Díaz-Medina et al.
Ehrlichia canis in dogs from Mexico
3(8):251-257,2016

in some cases, bone marrow failure (Harrus *et al.* 1997).

The disease is distributed worldwide, but the prevalence is highest in tropical and subtropical regions where the parasite and the vector are present. Mexico is considered an endemic region for canine ehrlichiosis caused by *E. canis* (Rodriguez-Vivas *et al.* 2000). Previous epidemiological surveys from Mexico indicated that the seroprevalence of *E. canis* in apparently healthy dogs was 8.7 % (Jiménez-Coello *et al.* 2009) and 44.1 % (Rodríguez-Vivas *et al.* 2005). Age ≥2 years, platelet-related bleeding and thrombocytopenia were found to be associated with higher prevalence of *E. canis* seropositivity. The actual prevalence of *E. canis* infection may differ from the seroprevalence because the presence of serum anti-*E. canis* antibodies indicates previous exposure to infection and not necessarily active infection (Neer and Harrus 2006). However, dogs with clinical ehrlichiosis may not have identifiable antibodies in the first days after the initial infection, before the development of a detectable antibody titer (Harrus *et al.* 1996, Neer and Harrus 2006).

At present, diagnosis using molecular procedures, such as nested-polymerase chain reaction (nested-PCR), is an alternative that has the advantages of higher sensitivity and specificity than serological procedures (Unver *et al.* 2001, Mavromastis *et al.* 2006, Perez *et al.* 2006, Aguirre *et al.* 2008). A large dog population (1.5 dogs per household) has been reported in the study community, representing a risk to other animals, including humans, if the disease is present (Ortega-Pacheco *et al.* 2007). It is important to know the prevalence of *E. canis* in rural communities of Yucatan, where the environment is favorable for the bacteria and the vector, in order to design and implement control measures for dogs. The objectives of this study were to estimate the prevalence of *E. canis* in dogs and to determine possible factors associated with its presence in a rural community in Yucatan, Mexico.

MATERIALS AND METHODS

Study area

The study was carried out in the rural village of Molas, Yucatan, Mexico located south of the capital city, Merida (10° 49' 01" N, 89° 37' 49" W). The climate is hot and sub-humid with summer rains. The average temperature is 26 °C, and relative humidity is 83 % with a minimum of 61 % (INEGI 2007).

Study design, study population and variables

A cross-sectional study was carried in 200 dogs between November 2009 and January 2010. Dogs were randomly selected from a population of 568 distributed in 379 houses. None of the studied animals had been included in vaccination or parasite control programs. Blood samples were collected by jugular venipuncture using 3 ml vacuum tubes containing the anticoagulant ethylenediaminetetraacetic acid, decanted within 30 min following sampling and stored at -20 °C until DNA was extracted.

All animals were clinically examined and variables such as gender, body condition, hemorrhages and presence of ticks were recorded. The age was given by the owner or calculated by dental examination, and the body condition score was assessed using a slightly modified version of the method described by Laflamme (1997).

From each sampled dog infected by ticks, at least 5 adult ticks were collected and transported to the Laboratory of Parasitology of the Autonomous University of Yucatan School of Veterinary Medicine (LP-UADY) for tick classification, following the keys of Rodriguez-Vivas and Cob-Galera (2005).

Platelet counting

Thrombocyte counts were calculated using the method described by Cheesbrough and McArtur (1979). Fewer than 200,000 platelets of blood was considered to be thrombocytopenia (Harrus *et al.* 1997). This procedure was carried out at the LP-UADY.

Díaz-Medina et al.
Ehrlichia canis in dogs from Mexico
3(8):251-257,2016

DNA extraction

DNA was extracted from 200 μl of buffy coat with a commercial kit (QIAamp® DNA Mini and Blood from QIAGEN®), according to the manufacturer's instructions. This extraction was performed in a different room from the nested-PCR procedure.

Nested-PCR

Nested-PCR was performed to detect the 16S rRNA gene of *E. canis*, using the primers ECC5'-AGAACGAACGCTGGCGGCAAGCC-3' and ECB5'-CGTATTACCGCGGCTGCTGGC-3' in the first reaction and ECA5'-CAATTATTTATAGCCTC-TGGCTATAGGAAA-3' and HE-3 5'-TATAGGTAC-CGTCATTATCTTCCCTAT-3 in the second reaction, based on previously published work (Murphy *et al.*, 1998). The total volume of each reaction was 50 μl, with 10 μl of extracted DNA in 10 mM Tris-HCl (pH 8.3), 0.2 mM dNTPs, 1.5 mM MgCl$_2$, 50 mM KCl, 0.5 μM of each primer, and 1.25 U of *Taq* DNA polymerase (Qiagen). DNA of *E. canis* (Jake strain) purified from DH/82 cells was used for positive controls, and pure water was used for negative controls. The control used in the first reaction was also used in the second reaction. The nested-PCR was performed in a Multigene Labnet^{TM} thermocycler. The Taq polymerase was activated for 15 min. The initial cycling consisted of 30 cycles of denaturation for 1 min at 94 °C, followed by annealing at 55 °C for 2 min and extension at 72 °C for 2 min. For the second (nested) PCR the mixture of reagents was the same, and 5 μl of the first PCR product was used as the template. The Taq polymerase was activated for 15 min. Thermocycling was in two stages: the first consisted of three cycles with denaturation for 1 min at 94 °C, followed by annealing at 55 °C for 2 min and extension at 72 °C for 1.5 min. The second stage consisted of 37 cycles, with denaturation for 1 min at 92 °C, followed by annealing at 55 °C for 2 min and extension at 72 °C for 2 min. PCR products were analyzed by 1.5 % agarose gel electrophoresis (Apex Bioresearch Products); gels were stained with ethidium bromide. All procedures were carried out at the Infectious and Parasitic Diseases Laboratory of the Autonomous University of Yucatan School of Medicine. An animal sample was considered positive when the amplicon was 389 bp in size.

Data analysis

Data were analyzed using descriptive and analytic statistics. The apparent and true prevalence and confidence interval were estimated according to the formula described by Thrusfield (2007). An initial screening of dogs infected with *E. canis* was performed using 2 x 2 contingency tables of exposure variables. All variables with p < 0.20 were analyzed using a logistic-binomial regression model of fixed-effects performed using SPSS v17.0 (Statistical Package for the Social Sciences 2008) software. For analysis of associated factors, animals infected with *E. canis* were considered dependent variables. Gender (male, female), age (<2 years, 2-5 years, >5 years), body condition (poor, medium, good), platelet-related bleeding (yes, no), thrombocytopenia (yes, no), and presence of ticks (yes, no) were considered independent variables.

RESULTS AND DISCUSSION

One-hundred and forty of the 200 dogs studied (70 %) were found to be infested with ticks. A total of 1 116 ticks (952 females and 164 males) were recovered and all were identified as *R. sanguineus*. This tick is a parasite of dogs that can occasionally parasitize other hosts, including humans. It is the most widespread tick in the world and is a vector of many disease agents, some of them (e.g., *Coxiella burnetii, Rickettsia conorii, R. rickettsii* and *E. canis*) being of zoonotic concern (Dantas-Torres 2010). In Yucatan, Mexico, *R. sanguineus* has been identified as the principal tick species in dogs (Rodríguez-Vivas *et al.* 2010). The apparent prevalence of *E. canis* was 71 % (142/200). Considering the sensitivity and specificity of the test, the true prevalence was 69.2 % with a confidence interval between 63.1 % and 74.6 %. The high prevalence of *E. canis* infection observed in this study using the nested-PCR method is similar to that reported by other researchers in different countries around

Ecosistemas
Y
Recursos
Agropecuarios

Díaz-Medina et al.
Ehrlichia canis in dogs from Mexico
3(8):251-257,2016

the world using the same technique: India 50 % (Lakshmanan *et al*. 2007), United States of America 56 % (Kordick *et al*. 1999), Sudan 87.2 % (Inokuma *et al*. 2006). However, in Brazil, Sales *et al*. (2015) found lower prevalence of *E. canis* in dogs using a nested-PCR. Some studies in Yucatan have reported lower seroprevalences (detection of circulating antibodies) than the prevalence reported in the present study. Rodríguez-Vivas *et al*. (2005) reported in their work, which was carried out in owned dogs from the city of Merida and using a commercial diagnostic kit, a prevalence of 44.1 %. The prevalence in that case may have been underestimated because of the 79 % sensitivity of the test, as reported by Belanger *et al*. (2002), which likely produced some false negative results. Other factors that might have influenced the observed difference are sample size and bias in the selection of study animals for the serological survey. In a study performed by Jiménez-Coello *et al*. (2009) using indirect immunofluorescence, which is considered the reference serological test for CME, a prevalence of 8.7 % was reported. It is important to note that this study was conducted using a closed population from the city pound; this prevalence cannot be extrapolated to any other population. When estimating prevalence, the characteristics of the diagnostic test, the method of selection of the animals, and the characteristics measured must be carefully considered. In the present study, bacterial DNA from blood was measured. Despite the high sensitivity and specificity reported for this method (Aguirre *et al*. 2008), the prevalence is expected to be lower than that using a test that detects antibodies, which usually remain in the host animal longer than bacterial DNA.

The high *E. canis* prevalence in the community of Molas could also be due to environmental conditions that allow the completion of the life cycle of the vector *R. sanguineus* (70 % of the studied dogs were infected by *R. sanguineus*) and the agent *E. canis*, such as temperature, humidity, lack of vector control strategies and the wandering of dogs around the town. The possibility of the dogs coming into contact with other reservoirs of the disease (wildlife) is also present (Dantas-Torres 2010). The results in our study agree with those reported by Carvalho *et al*. (2008), who found a higher *E. canis* prevalence in dogs from a rural community when compared to dogs from an urban area.

The frequencies of the studied variables are shown in Table 1. 6.5 % (13/200) of the dogs presented hemorrhages and 21.0 % (42/200) had thrombocytopenia. Thrombocytopenia and hemorrhages are the main hematological abnormalities observed in dogs with CME (Dagnone *et al*. 2003, Rodriguez-Vivas *et al*. 2005). In this study a low frequency of those two variables was observed. No epidemiological associations or statistically significant differences (p < 0.05) in gender, age, body condition, platelet-related bleeding, thrombocytopenia, or presence of ticks occurrence were observed been dogs that were positive and negative for *E. canis* infection. These results differ from those reported by Bulla *et al*. (2004), Rodríguez-Vivas *et al*. (2005) and Santos *et al*. (2009), who found that seropositive and infected dogs had abnormal platelet counts with a statistically significant association between this variable and the presence of the disease. On the other hand, Santos *et al*. (2009) reported that 46.7 % of dogs exhibiting thrombocytopenia were not infected with *E. canis*, indicating the probability of another etiology. A possible explanation for the low frequency of thrombocytopenia in the dogs in the present study is that a high percentage of the infected dogs may have been in the sub-clinical stage of the disease at the time of sampling. The same could apply to the low observation of cutaneous and ocular hemorrhages, which are usually seen during the acute and chronic stages of CME (Santos *et al*. 2009).

In the primary screening, age and body condition had p < 0.20; however, neither variable showed association with *E. canis* infection because their confidence interval included the unit when analyzed by logistic regression. In contrast to the report by Rodriguez-Vivas *et al*. (2005), no significant association between age and seropositivity was found. However, these results are consistent with those re-

Díaz-Medina et al.
Ehrlichia canis in dogs from Mexico
3(8):251-257,2016

Tabla 1. Variables observed in 200 dogs in a rural community in Yucatan, Mexico, included in the detection of *Ehrlichia canis* by PCR.

Variable		Total animals	Positives	Frequency of infection (%)
Gender	Male	145	100	68.9
	Female	55	42	76.36
Age	<2 years	58	37	63.79
	2-5 years	118	85	72.03
	>5 years	24	20	83.33
Body condition	Poor	78	59	75.64
	Medium	112	78	79.64
	Good	10	5	50.00
Platelet-related bleeding	Yes	13	11	84.61
	No	187	131	70.05
Thrombocytopenia	Yes	42	28	66.66
	No	158	114	72.15

ported by Carvalho *et al.* (2008) who also found no significant association. The findings of the present study may indicate that infection of animals with *E. canis* can occur at any age when the animal is in contact with the vector and the bacterium.

No statistically significant association was found between gender and the presence of *E. canis*, which is in agreement with previous reports by Carvalho *et al.* (2008), Leiva *et al.* (2005), and Rotondano *et al.* (2015). Moreover, no statistically significant association was found between body condition and the presence of *E. canis*, indicating that

this variable probably does not influence infection. Finally, it is important to point out that one of the limitations to consider when studying associations in cross-sectional epidemiological studies is that they cannot measure causality. It is not possible to determine which event occurred first, the associated factor or the disease. In conclusion, there is a high probability that dogs living in Yucatan, are infected with *E. canis*. It is therefore important to implement vector control measures in the community to reduce the risk of infection.

LITERATURA CITADA

Aguirre E, Tesouro M, Amusategui I, Rodríguez-Franco F, Sainz A (2008) Comparison between different polymerase chain reaction methods for the diagnosis of *Ehrlichia canis* infection. Annals of the New York Academic Sciences 1149: 118-120.

Belanger M, Sorenson HL, France MK, Bowie MV, Barbet AF, Breitschwerdt EB, et al. (2002) Comparison of serological detection methods for diagnosis of Ehrlichia canis infection in dogs. Journal of Clinical Microbiology 40: 3506-3508.

Bulla C, Kiomi TR, Pessoa AJ, Trinca LA, Souza LR, Wiedmeyer CE (2004) The relationship between the degree of thrombocytopenia and infection with *Ehrlichia canis* in an endemic area. Veterinary Research 35: 141-146.

Carvalho FS, Wenceslau AA, Carlos RS, Albuquerque GR (2008) Epidemiological and molecular study of *Ehrlichia canis* in dogs in Bahia, Brazil. Genetic Molecular Research 7: 657-662.

Cheesbrough M, McArtur J (1979) Manual de laboratorio para hospitales rurales en zonas tropicales. México DF: Continental 293p.

Dagnone AS, De Morais HS, Vidotto MC, Jojima VO (2003) Ehrlichiosis in anemic, thrombocytopenic, or tick-infested dogs from a hospital population in South Brazil. Veterinary Parasitology 117: 85-290.

Dantas-Torres F (2010) Biology and ecology of the brown dog tick, *Rhipicephalus sanguineus. Parasites &* Vectors 3: 1-26.

Díaz-Medina et al.
Ehrlichia canis in dogs from Mexico
3(8):251-257,2016

Harrus S, Wane, T, Avidar Y, Bogin E, Peh H, Bark H (1996) Serum protein alterations in canine ehrlichiosis. Veterinary Parasitology 66: 241-249.

Harrus S, Bark H, Waner T (1997) Canine monocytic ehrlichiosis: an update. Compendium Continuing Education Practicing Veterinarian 19: 431-444. INEGI (2002) Anuario estadístico del Estado de Yucatán, México. Instituto Nacional de Estadística, Geografía e Informática, México. 450p.

Inokuma H, Oyamada M, Davoust B, El Boni M, Dereure J, Bucheton B, *et al.* (2006) Epidemiological survey of *Ehrlichia canis* and related species infection in dogs in Eastern Sudan. Annals of the New York Academic Sciences 1071: 461-463.

Jiménez-Coello M, Pérez-Osorio C, Vado-Solis I, Rodríguez-Buenfil JC, Ortega-Pacheco A (2009) Serological survey of *Ehrlichia canis* in stray dogs from Yucatan, Mexico, using two different diagnostic tests. Vector-Borne Zoonotic Disease 9: 209-211.

INEGI. Instituto Nacional de Estadística y Geografía. (2007). Censo Agrícola, Ganadero y Forestal. http://www.inegi.org.mx. Fecha de consulta 23 de febrero de 2014.

Kordick SK, Breitschwerdt EB, Hegarty BC, Southwick KL, Colitz CM, Hancock SI, *et al.* (1999) Coinfection with multiple tick-born pathogens in a walker hound kennel in North Carolina. Journal Clinical Microbiology 37: 2631-2638.

Laflamme DP (1997) Development and validation of a body condition score system for dogs. Canine Practice 22: 10-15.

Lakshmanan B, John L, Gomathinayagam S, Dhinakarraj G (2007) Molecular detection of *Ehrlichia canis* from blood of naturally infected dogs in India. Veterinary Archives 77: 307-312.

Leiva M, Naranjo C, Peña T (2005) Ocular signs of canine monocytic ehrlichiosis: a retrospective study in dogs from Barcelona, Spain. Veterinary Ophthalmology 8: 387-393.

Neer TM, Harrus S (2006) Canine monocytotropic ehrlichiosis and neorickettsiosis (*E. canis, E. chaffeensis, E. ruminatium, N. sennetsu* and *N. risticii* infection). In: Greene CE (ed.) Infectious diseases of the dog and cat. St. Louis, USA, Saunders Elsevier. pp: 203-217.

Mavromastis K, Kuyler DC, Lykidis A, Ivanova N, Francino MP, Chain P, *et al.* (2006) The genome of the obligately intracellular bacterium *Ehrlichia canis* reveals themes of complex membrane structure and immune evasion strategies. Journal Bacteriology 188: 4015-4023.

Ortega-Pacheco A, Rodríguez-Buenfil JC, Bolio-González ME, Sauri-Arceo CH, Jiménez-Coello M, Forsberg CL (2007) A survey of dog populations in urban and rural areas of Yucatan, Mexico. Anthrozoos 20: 261-274.

Pérez M, Bodor M, Zhang C, Ziong Q, Rikihisa Y (2006) Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. Annals of the New York Academic Sciences 1078: 110-117.

Rodríguez-Vivas RI, Albornoz REF, Bolio-González ME (2005) *Ehrlichia canis* in dog in Yucatan. Seroprevalence, prevalence of infection and factors associated. Veterinary Parasitology 127: 75-79.

Rodriguez-Vivas RI, Cob-Galera LA, Dominguez-Alpizar JL (2000) Hemoparásitos en bovinos, caninos y equinos diagnosticados en el laboratorio de parasitología de la Facultad de Medicina Veterinaria y Zootecnia de la Universidad Autónoma de Yucatán (1984-1999). Revista Biomédica 11: 277-282.

Díaz-Medina et al.
Ehrlichia canis in dogs from Mexico
3(8):251-257,2016

Rodríguez-Vivas RI, Manrique-Saide P, Ramírez-Cruz GT, Cob-Galera L, Rosado-Aguilar JA, Bolio-González M (2010) Insectos y ácaros de importancia médica y veterinaria. En: Diversidad y desarrollo humano en Yucatán. CICY, CONABIO, SEDUMA. Mérida, México. pp: 303-305.

Rotondano TE, Almeida HK, Krawczak F S, Santana VL, Vidal IF, Labruna MB, *et al.* (2015) Survey of *Ehrlichia canis*, *Babesia* spp. and *Hepatozoon* spp. in dogs from a semiarid region of Brazil. Revista Brasileira de Parasitologia Veterinária 24: 52-58.

Sales MRRP, Ignacchiti MDC, Mendes Junior AF, Suhett WG, Porfírio LC, Marins M, *et al.* (2015) Prevalence of *Ehrlichia canis* using the nested-PCR, correlation with the presence of morulae and thrombocytopenia in dogs treated in Veterinary Hospital of the Federal University of Espirito Santo. Revista Brasileira de Medicina Veterinária 37: 47-51.

Santos F, Coppede J, Pereira JA, Oliveira L, Roberto P, Benedetti R, *et al.* (2009) Molecular evaluation of the incidence or *Erlichia canis, Anaplasma platys* and *Babesia* spp. in dogs from Ribeirão Preto. The Veterinary Journal 179:145-148.

(SPSS) Statistical Package for the Social Sciences V 17.0 (2008) SPSS Inc. Chicago, Illinois.

Thrusfield M (2007) Veterinary Epidemiology. Oxford, UK: Blacwell Publishing. 53p.

Unver H, Perez M, Orellana N, Huang H, Rikihisa Y (2001). Molecular and antigenic comparision of *Ehrlichia canis* isolates from dogs, ticks and human in Venezuela. Journal Clinical Microbiology 39: 2788-2793.