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Seroprevalence and Traceback of Animals Suspected of Carrying *Ehrlichia canis*, in Dogs Attended in Veterinary Clinics in Mexicali, Baja California, Mexico

Paulina Haro-Álvarez, Gilberto López-Valencia, Luis Tinoco-Gracia,
Tomas Rentería-Evangelista and Gerardo Medina-Basulto
Instituto de Investigaciones en Ciencias Veterinarias,
Universidad Autónoma de Baja California, Mexicali, México

Abstract: A cross-seccional study was carried out in order to estimate the seroprevalence of antibodies against *Ehrlichia canis* as well as track patients suspected of carrying the disease. A total of 384 blood samples obtained from canine patients of 38 veterinary clinics in the urban area of Mexicali, Baja California, Mexico were randomly collected during 23 months and analyzed with ELISA Helica biosystems® commercial kit. A Traceback was carried out by doing a follow-up of 20% of the dogs that were suspected of the disease. It encompassed a revision of their medical record as well as serum analysis. Seroprevalence found reached 21.6% (83/384). From the 20% of suspect cases (15/75) the 80% (12/15) developed signs of the disease between samplings. Of these, 67% (8/12) were treated for *E. canis*, which resulted in 75% (6/8) being negative in their second sample and 25% remaining as suspected of the disease. All dogs that showed signs of the disease and did not receive treatment (3/12) became positive to *E. canis* in their second sample. Taking into account seroprevalence and the percentage of animals suspected of carrying the disease, at least 40% of the total canine population in the city out been in contact with the bacteria. Taking account of tracking parameters for suspected individuals the prevalence obtained in this study could underestimate the real seroprevalence of Canine Monocytic Ehrlichiosis (CME) in this region. As the disease is a zoonosis it is necessary to estimate the risk factors for its presence. Future studies that include molecular biology are required in order to determine the presence of the etiological agent as well as the detection of other *Ehrlichia* species that show cross reaction with *E. canis*.

Key words: Dogs, *E. canis*, seroprevalence, traceback, veterinary clinics, suspected

INTRODUCTION

Canine Monocytic Ehrlichiosis (CME) has been widely recognized as an important disease of canines (Preziosi and Cohn, 2002; Skotarczak, 2003); it is a zoonosis (Perez *et al*., 1996) and is caused by a gram-positive proteobacteria from the *Ehrlichia* genus (Green, 1998) that is transmitted by the bite of *Rhipicephalus sanguineus* tick, which has worldwide distribution. CME has a high prevalence in hot climates or in environments that favor the preproduction of the tick (Didien and Roux, 1997) as is the case in the Mexican northwest. Serological evidence of the bacteria in this region have been reached (Nunez, 2002; Rodriguez *et al*., 2004) Nevertheless, the real prevalence of CME still unknown in the region. This study attempted to: Estimate the seroprevalence of antibodies against *Ehrlichia canis* (*E. canis*) in the Mexicali urban area and carry out a follow-up of those patients that were suspected of having the disease.

MATERIALS AND METHODS

Time and location: This study was carried out from February of 2005 to December 2006 in the city of Mexicali, Baja California, Mexico, which borders with the state of California, USA to the north and is located at 32°40'0"N, 115°28'0"W. The city has an estimated population of 653,046 (INEGI, 2005) with approximately 151,000 canine pets (Flores-Ibarra and Estarella-Valenzuela, 2004). Climate is dry with some showers in winter and extreme temperature variations during the year. In summer the average temperature reached 39°C (28-50°C), while in winter temperatures are around 10°C (-2-18°C). The average annual rainfall is 75 mm (Garcia and Daualos, 2000).

Study population and inclusion criteria: A total of 384 patients older than one month from different breeds, as well as from both genders, were attended in 38 veterinary

Corresponding Author: Ana Paulina Haro Álvarez, Instituto de Investigaciones en Ciencias Veterinarias, Universidad Autónoma de Baja California, México

clinics established in the Mexicali urban area, were randomly included in this study. The number of dogs sampled from each clinic is proportional to the quantity of patients received each season. Sampling was carried out during 22 months.

Sample size: In this study, the sample size was estimated at 384 dogs. In order to determine the sample size the following parameters were considered: A 50% prevalence (as the real prevalence is unknown), a 95% confidence interval and a 5% error. The following formula was used to determine the sample size: $n = [(N)(Z^2)]*[(p)(q)]/[(N)(d^2)+((z^2)(p)(q))]$; where N is the population (151,000), Z is the confidence interval (0.95), p is the prevalence (0.50), q equals 1-p (0.50) and d^2 is the precision level (0.0025) (Daniel, 2002).

Sample collection: Samples were collected by venipuncture of the cephalic or jugular vein that had been previously cleaned with isopropyl alcohol. A total of 3 mL of blood were collected from each patient and placed in a 5 mL plastic tube without anticoagulant and kept at 4°C for a maximum of 7 days until their processing. Samples were centrifuged at 3500 rpm×10 min, serum was separated from the cell pack in 1.5 mL individual vials and kept at -20°C until serological analysis.

Follow-up of suspected animals: In order to carry out the follow up, blood samples were taken from 20% of the animals that had resulted as suspected in the initial ELISA test. Samples were processed according to the aforementioned methods. This second sampling was done within a period no greater than 21 months after the first sampling.

Data collection: Tracking was carried out by medical follow-up, including revision of medical records, of 20% of the animals that had resulted as suspected in the initial ELISA tests. Half of these patients were receiving treatment whereas the other half did not. Animals that were considered treated were those that received therapy against *E. canis* using one of the following drugs: Doxycycline, tetracycline, oxytetracycline, chloramphenicol, minocycline, amicarbalide or Imidocarb dipropionate (Green, 2006).

Serological analysis: The Enzyme Linked Immunosorbent Assay to detect IgG antibodies against *E. canis* using the Helica® commercial diagnosis kit (Helica Biosystems Inc.®, Fullerton, CA, USA) was the diagnostic test used following manufacturers recommendations. ELISA plates with 96 wells were used, with the first well as the negative control by placing 100 μL of serum from a patient not reactive to *E. canis* (included in the diagnostic package) and the 2nd well as the positive control by placing 100 μL of the positive control solution (from a dog positive to *E. canis*, included in the diagnostic kit). At different stages the following solutions were used: Buffering solution (phosphate buffered saline solution, pH 7.4 and 0.05% Tween 20, reconstituted in 1 liter of distilled water), anti-canine heavy chain IgG conjugate from rabbit labeled with horseradish peroxidase, buffered substrate tampon solution (contains urea peroxide and 3,3'5,5' tetramethylbenzidine) and a Antigen-Antibody (Ag-Ab) reaction stop solution (diluted phosphoric acid). Each serum sample was thawed at room temperature and diluted with the buffering solution to 1:100. A 100 μL of the diluted serum were placed in each well and incubated for 15 minutes at room temperature (20-25°C). The plates were washed 4 times with the saline buffer solution, 66 μL of the conjugate were added and incubated and washed as described before. Later 66 μL of substrate solution were added and incubated for 10 min at room temperature and then 66 μL of Ag-Ab stop solution were added to each well to stop the reaction. In order to compare the reaction with the positive and negative controls the plates were placed in an ELISA reading spectrophotometer with a 450 nm filter (Bio-rad® Laboratories, Hercules, CA, USA) to read the absorbency. Following manufacturer's instructions, all samples that showed an optic density equal or greater than 0.5 were considered positive, all those with values between 0.301 and 0.499 were considered as suspected to have the disease and those with 0.300 or less were considered as negative.

Data analysis: The prevalence and confidence intervals were estimated with the formulas described by Daniel (2002). A chi-square test (X^2) was used in order to assess if the presence of the disease was associated to the season. Furthermore, the Odds Ratio (OR) was calculated for with a 95% confidence interval. All statistical analysis were carried out in MedCalc® (2006).

RESULTS AND DISCUSSION

The seroprevalence found reached 21.6% (83/384) to *E. canis*, while 19.5% (75/384) were suspected of carrying the disease and 58.9% (226/384) were negative to antibodies against *E. canis*. The results from the ELISA test can be found in Table 1. Seroprevalence in this study is similar to reports from other countries such as Spain, Brazil, Italy, Israel and Egypt (Sainz *et al*., 1996; Labarthe *et al*., 2003; Torina and Caracappa, 2006; Baneth *et al*., 1996; Botros *et al*., 1995) although some of these

Table 1: Results for CME using the ELISA test in dogs attended in 38 veterinary clinics in the city of Mexicali

Result	n	(%)	95% CI
Positive	83	21.6	17.7-26.0
Suspected	75	19.5	15.8-23.7
Negative	226	58.9	53.8-63.6
Total	384	100	

Table 2: Results from the follow-up of patients suspected of carrying Canine Monocytic Erlichiosis according to ELISA test (n = 15)

Months between samples	ELISA[a]	Signs at 1st sampling	Signs at 2nd sampling	Tx[b]	Months between TX and ELISA
9	Positive	No	Yes	No	-
13	Positive	No	Yes	No	-
13	Positive	No	Yes	No	-
21	Suspect	No	Yes	Yes	1
4	Suspect	No	No	No	9
23	Suspect	Yes	No	Yes	10
21	Negative	Yes	Yes	Yes	36
21	Negative	Yes	Yes	Yes	21
12	Negative	No	Yes	Yes	12
11	Negative	Yes	No	Yes	12
12	Negative	No	No	No	-
5	Negative	No	No	No	-
5	Negative	Yes	No	No	-
4	Negative	No	Yes	Yes	3
23	Negative	Yes	No	Yes	3

[a]Positive =0.500, suspect = 0.301-0.499, negative = 0.300, [b]Tx = treatment with doxycycline and/or Imidocarb dipropionate

Table 3: Odds Ratio (OR) between the season and positive samples to CME

Season of the year	Samples analyzed	CME positive cases (%)	OR	p-value
Autumn-winter	153	31 (20.2)	Ref	0.87
Spring-summer	231	52 (22.5)	NS	
Total	384	83 (21.6)		

reports the test used was Immunofluorescence Antibody (IFA) (Harrus *et al*., 2002). The similarity could be due to the fact that their climate is similar to that in our region. Nevertheless, in Mexico several studies have established prevalence between 33 and 70% (Nunez, 2002; Rodriguez *et al*., 2004; Campos *et al*., 2006). The differences between those studies and the present research could be due to:

- The geographical region studied.
- Climate.
- Presence and abundance of the vector in each study region.
- Sampling duration.
- Sampling regime.
- Sample size.
- Season when the studies were carried out.
- Due to include not-healthy animals.

These variables are important as it has been established that the frequency of CME is related to the season in which the vector proliferates (Didier and Roux, 1997), nevertheless, in this study no relationship between the season and prevalence could be observed, reflecting the random nature of this study (Table 3). Other studies carried out in Mexico have used Snap 3Dx® ELISA (IDEXX, USA) which uses 2 recombinant proteins specific to *E. canis* (rp30 and rp30-1) as the antigen, compared to the Helica Biosystems Inc.® which uses the whole antigen. The use of different sources of antigen can affect reactivity and in consequence affect sensitivity and specificity of the tests (Baneth *et al*., 1996.). Furthermore, seroconversion only indicates the presence of antibodies, which can be explained as either;

- Evidence of a past infection.
- Previous contact with the bacteria but no development of an infection.
- Presence of an active infection at the time of sampling.
- Cross-reaction with other species such as *E. ewingii, E. chaffeensis* and *E. equi,* as well as other microorganisms such as *Neorickettsia helminthoeca* and *Rickettsia ricketssi* (Balanger *et al*., 2002).

Therefore, seroconversion should be considered as the first step towards diagnosis of a disease caused by rickettsias (Parola *et al*., 2005). Taking into consideration the percentage of individuals suspected of carrying the disease (19.5%: 75/384) is similar to those that were positive and while the presence of antibodies does not necessarily indicate that the animal has the disease, (Harrus and Bark, 1997) 20% of these were followed up (15/75). The results obtained from the follow-up are shown in Table 2. The observations in the medical follow-up of this study are different from expectations because the 80% (12/15) of the animals suspected of carrying the disease showed signs of the disease between the time of the first and second sampling. This would indicate that indeed they could be infected by the bacteria at the time of the first sampling either in an acute or subclinical stage, of these who developed signs 67% (8/12) received treatment against *E. canis* with either doxycycline and/or Imidocarb dipropionate. Of those patients that were treated, 75% (6/8) were seronegative in the follow-up test indicating a successful treatment that eliminated the bacteria and the remaining 15% (2/8) remained as suspected to have the disease which could indicate either evidence of contact with the disease, subclinical carriers of the agent or a cross reaction with another microorganism. All the dogs that had signs of the disease and did not receive treatment (3/12) resulted as seropositive to *E. canis* in the second serological test. Antibodies persisted in two of the three patients treated for the disease which could be due to the fact that in the majority of dogs antibody titers progressive decrease and

patients sometimes become seronegative up to 6 or 9 months after treatment. Some patients can remain as asymptomatic carriers after treatment and retain high titers against *E. canis* for years. Therefore, it is not possible to always determine if the bacteria or antibody persist clinically in animals that have been treated. It can be assumed that the etiological agent has been eliminated when hyperglobulinemia, as well as other clinical signs and laboratory findings are progressively better after treatment (Harrus *et al*., 1997). A definitive diagnosis of the presence or absence of *E. canis* can only be done through molecular biology techniques (Jiraporn *et al*., 2001; Roger *et al*., 2002).

CONCLUSION

Prevalence of Canine Monocytic Ehrlichiosis was 21.6% in dogs treated in veterinary clinics of Mexicali. There is no significant difference in prevalence among the seasons where the sample was taken. Due to the numerous situations when a dog can have antibodies against *E. canis*, serology should be consider as the first step towards diagnosing CME. Taking into account that 19.5% of the samples were suspected of having the disease it is suggested that approximately 40% of the canine population of the city has come into contact with the bacteria or has a cross reaction with another agent. In view of our follow-up results, the prevalence obtained in this study underestimates the real CME prevalence in this region. As the disease is a zoonosis it is necessary to estimate the risk factors for its presence. Future studies, that include molecular biology are required in order to determine the presence of the etiological agent as well as the detection of other *Ehrlichia* species that show cross reaction with *E. canis*.

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