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Frequency of *Anaplasma platys* in dogs from the municipality of Veracruz, Mexico

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Abstract

Infectious canine cyclic thrombocytopenia or canine anaplasmosis is an infectious disease caused by *Anaplasma platys*. In Mexico, cases of human infection have been reported. The present cross-sectional study aimed to determine the frequency of *A. platys* infection in the municipality of Veracruz, Mexico, by nested polymerase chain reaction method. A total of 100 blood samples from dogs living in the municipality were collected and analyzed between March and June 2022. A descriptive analysis of blood samples for hemoparasites frequencies was performed with the free online software VassarStats. The evaluated variables were sex, street access, usage of ticks control methods, and living environment. The free online software WinEpi software was used to calculate odds ratio (OR) and confidence interval (CI: 95.00%). Out of 100 analyzed blood samples, 27 were confirmed positive for *A. platys*. The only risk factor found was the absence of tick prevention methods (OR = 9.81; 95.00% CI: 23.00 - 44.50). In conclusion, the frequency of *A. platys* was 27.00% and no risk factors were identified.

Introduction

Anaplasma platys, an obligate intra-cellular Gram-negative bacterium, is the only rickettsial agent affecting platelets in canids.[1,2] The Anaplasmataceae family includes the genera *Ehrlichia*, *Anaplasma*, *Wolbachia* and *Neorickettsia.*[2] Co-infection by these agents is frequent in dogs, and the presence of *Ehrlichia canis* may exacerbate the severity of disease caused by *A. platys.*[3,4]

Infectious canine cyclic thrombocytopenia is a disease caused *by A. platys*, being transmitted by the bite of the tick *Rhipicephalus sanguineus.*[2,5,6] Dog is the most frequent host, but there are infection reports in cats and other domestic and wild mammals. Anaplasmosis cases described in humans in Venezuela and the United States have suggested some zoonotic potential for this agent.[7]

Anaplasma platys has been reported in different places over time; in Lara, Venezuela, was discovered 16.00% in 43 samples,[8] later, in Italy a 3.70% was communicated in 135 blood samples[9] and finally, in Santiago, Chile a 20.00% was described in 30 samples.[10] Several studies have been carried out by molecular diagnosis; although, cross-reaction with bacteria of the same family has been well-documented.

The objective of this study was to determine the presence and frequency of *A. platys* in dogs from the municipality of Veracruz, Mexico, sampled from March to June 2022 using nested polymerase chain reaction (PCR), as well as the risk factors associated with this infection.

Materials and Methods

The authors declare that the present study does not contain clinical studies or patient data, and informed consent was obtained from all participants included in the study. The study does not require to be reviewed and approved by our Bioethics Committee.

A cross-sectional study with a quantitative approach was used. The study was carried out in the municipality of Veracruz, Mexico, between March and June 2022. This place has an approximate area of 37.24 Km^2. It has a population of 754,166 inhabitants and the climate is warm with average temperatures of 25.00 ˚C and an average annual rainfall of 1,699 mm.

A simple convenience sampling was done on 100 domiciliated dogs (dogs that have an owner) and related information was collected to determine some potential risk factors including age, sex, healthy status, presence of

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ticks, and previous records of tick infestation. Dogs under antimicrobials treatment were excluded from this study. Samples were collected by puncture of the cephalic or jugular veins. Data were collected from each dog including name, age, sex, breed, street access, environment in which the dog lives, and preventive methods against ticks.

The DNA extraction was performed using Chelex-100 chelating resin and to ensure that DNA was obtained, it was quantified by spectrophotometry according to Stangegaard *et al*.[11] Because *A. platys* until 2001 was considered to belong to the genus *Ehrlichia* spp. (*Ehrlichia platys*), it was decided to perform endpoint PCR to identify the genus *Ehrlichia* spp., and samples tested positive were subjected to nested PCR to specifically search for *A. platys*.[12] The primers for PCR were synthesized upon request by the DNA Synthesis and Sequencing Unit of the Biotechnology Institute of the National Autonomous University of Mexico (Mexico City, Mexico). To perform the endpoint PCR technique for the identification of the genus *Ehrlichia* spp., the primers used correspond to the ECB indicator with sequence of 5'-AGAACGAACGCTGGCGGC AA GCC-3 and the ECC indicator with sequence of 5'-CGTATTACCACCGCGGCTGCTGCTGGC-3. Subsequently, to carry out the nested PCR for the identification of *A. platys*, the EPLAT 5 indicator with sequence of TTTGTCGTCGTAG CTTGCTATGAT and the EPLAT 3 indicator with sequence of CTTCTGTGGGTACCGTC were used; these were designed to amplify a fragment of the specific gene for *A. platys*.

A SmartSpec® Plus (Bio-Rad Laboratories, San Francisco, USA) spectrophotometer was operated to measure the DNA concentration and calculate the radiation absorbance at wavelengths of 260 and 280 nm.[13] For the analysis of PCR product results from primer standardization, 1.50% agarose gels were prepared. 0.60 g of agarose (Thermo Fisher Scientific, Waltham, USA) was weighed and 40.00 mL of 1.00% tris-acetate-ethylene-diamine tetraacetic acid buffer (Thermo Fisher Scientific) was measured in a test tube; these reagents were added in a flask and heated until the gel was watery in appearance. Then, 5.00 µL of ethidium bromide was added and deposited in an electrophoresis chamber with the comb with the number of wells used, waited for the gel to gel, the comb was removed, and the chamber was placed in the electrophoresis equipment.

For reading the endpoint and nested PCR products, 1.50% agarose gels were prepared in larger chambers with the following amounts of reagents: 1.50 g of agarose, 100mL of 1.00% tris-acetate-ethylenediamine tetraacetic acid buffer, and 10.00 µL of ethidium bromide, and the procedure was followed as described above.

To poop the gels, 5.00 µL of the 1.00 kb DNA ladder base pair marker was used and placed in the first well, the following wells were loaded with 2.00 µL of Blue/Orange Loading DYE 6.00 X buffer and 10.00 µL of the PCR products including positive and negative controls and mixed with the same pipette tip. Once everything was loaded in the corresponding wells, the POWERPC Basic (Bio-Rad Laboratories) equipment was calibrated; the small gels with 100 volts for 40 min and large gels with 80.00 volts for 60 min. Afterwards, they were visualized in the ultraviolet transilluminator VWR Scientific Products and photos were taken in the Bio-Rad Laboratories photoreveler using the Image Lab Software (Bio-Rad Laboratories).

For the frequencies obtained, the 95.00% confidence interval (CI; 95.00%) was calculated with the free online program VassarStats (http://vassarstats.net/) and odds ratios (OR) to determine whether any of the variables behaved as a risk factor, where the CI was also considered in a logarithmic approximation with the free online program WinEpi (http://www.winepi.net/), OR values greater than 1.00 and whose CI limits were greater than 1.00 were considered risk factors.

Results

The most relevant result was that 27 out of 100 samples were positive for *A. platys*, representing 27.00%. The most important risk factor found by this study was the absence of tick control methods.[6] The presence of *A. platys* was confirmed by nested PCR tests in 100 dogs, where 27.00% (CI: 18.80 - 37.00) were positive (Table 1).

Table 1. Frequency of dogs from the municipality of Veracruz, Mexico, being positive for *Anaplasma platys* according to some variables.

Variables	Categories (n)	Number of positive dogs (%)	Odds ratio	Confidence interval (95.00%)
Sex	Male (50)	15 (30.00)	1.35 (0.32)*	18.20 - 45.00
	Female (50)	12 (24.00)	1.00†	13.50 - 38.40
Housed	Stray dogs (35)	6 (17.10)	1.00†	7.10 - 34.20
	House dogs (65)	21 (32.90)	2.30 (0.07)*	21.50 - 45.10
Preventive method	Yes (21)	1 (4.80)	1.00†	0.20 - 26.00
	No (79)	26 (32.90)	9.81 (0.006)*	23.00 - 44.50
Environment	Clean floor (60)	15 (25.00)	1.00†	15.10 - 38.10
	Dirty floor (40)	12 (30.00)	1.28 (0.37)*	17.00 - 47.00
Age (years)	0 - 2 (28)	9 (32.10)	3.15 (0.10)*	16.60 - 52.50
	3 - 5 (29)	7 (24.10)	2.12 (0.25)*	11.00 - 44.00
	6 - 8 (23)	3 (13.00)	1.00†	3.40 - 34.70
	9 - 15 (20)	8 (40.00)	4.44 (0.04)*	20.00 - 63.60

* indicates the associated probability to odds ratio, and † 1.00 indicates odds ratio value for reference in the group inside each variable.

Table 2. Frequency of dogs from the municipality of Veracruz, Mexico, being positive for *Ehrlichia* spp. according to some variables.

Variables	Categories (n)	Number of positive dogs (%)	Odds ratio	Confidence interval (95.00%)
Sex	Male (50)	23 (46.00)	1.65 (0.15)*	32.00 - 60.50
	Female (50)	17 (34.00)	1.00†	21.50 - 49.00
Domiciliated	Street access (35)	9 (25.70)	1.00†	13.10 - 43.50
	Home restricted (65)	31 (47.70)	2.63 (0.025)*	35.30 - 60.50
Preventive method	Confirmed usage (21)	5 (23.00)	1.00†	9.10 - 47.50
	Absence of usage (79)	35 (44.00)	2.55 (0.07)*	33.20 - 56.00
Environment	Cement floor (60)	25 (41.00)	1.19 (0.41)*	29.30 - 55.00
	Dirt floor (40)	15 (37.50)	1.00†	23.10 - 54.20
Age (years)	0 - 2 (28)	12 (42.90)	2.13 (0.17)*	25.00 - 62.50
	3 - 5 (29)	12 (41.40)	2.0 (0.19)*	24.10 - 61.00
	6 - 8 (23)	6 (26.10)	1.00†	11.10 - 48.70
	9 - 15 (20)	10 (50.00)	2.83 (0.09)*	27.90 - 72.10

* indicates the associated probability to odds ratio, and † 1.00 indicates odds ratio value for reference in the group inside each variable.

On the other hand, 40 of 100 samples were found positive for *Ehrlichia* spp., representing 40.00% of the individuals sampled with active infection in blood circulation. In the risk analysis, it can be observed that dogs without access to the street was a risk factor associated with *Ehrlichia* spp. infection because they are 2.60% times more likely to contract the infection than those individuals with street access. Out of the other factors analyzed, no significance was found for sex, since the parasite can infect both females and males; also, the infection can occur at any stage of life, ruling out any risk of infection according to age. Likewise, there was no difference in dog's housing conditions, since animals can contract the infection when housed either on dirty or cement floor (Table 2).

Discussion

Infectious canine cyclic thrombocytopenia is a disease of great importance because the presence of the vector, the tick *R. sanguineus*, is very common in this municipality, and is favored by climatic conditions facilitating its reproduction. Nevertheless, the risk of zoonosis has been reported as mentioned above; the close relationship of dogs as companion animals with humans could be considered as a possibility for contracting an infection by *A. platys* due to the tick bite.[6]

Regarding diagnosis, infectious canine cyclic thrombocytopenia is often misdiagnosed and confused with canine ehrlichiosis. The current diagnosis is based on blood smear staining to observe intra-platelet inclusions; however, serology such as rapid tests identifying antibodies and the nested PCR technique, seem to be the most efficient techniques. PCR was selected and used to recognize the distribution of *A. platys* and *Ehrlichia* spp. in the municipality. Due to the pathogenesis of infectious canine cyclic thrombocytopenia, the diagnosis is extremely complex between the clinical manifestations and the existing co-infection with other infectious agents such as *Ehrlichia* spp.[14]

According to previous studies, the prevalence of this pathogen varies in dogs in several places. In Minas Gerais, Brazil, a 11.70% was described in 248 samples during dry season; on the other hand, a 13.90% was recorded in 165 dogs,[15] in Malaysia a 13.30% was announced in 30 animals,[16] a 21.40% was found in Panama in 201 dogs,[17] a 15.38% was documented in French Guiana in 65 dogs,[18] a 7.20% was disclosed in Buenos Aires, Argentina, in 223 dogs,[19] later, a 3.00% was reported in Coahuila and Durango, Mexico in 100 samples[20], also, a 6.00% was revealed in Thrace, Türkiye in 400 animals,[21] then, a 11.40% was exposed in Taiwan in 175 samples,[22] and finally, a 6.40% was informed in Qalyubia, Gharbia, and Kafr El Sheikh, Egypt in 500 dogs.[4]

In conclusion, overwhelming evidence demonstrates that this agent has worldwide impact and distribution. Therefore, this study is unprecedented since there is no information on any other investigation previously conducted in the municipality of Veracruz, Mexico, and this turned out to be the main difficulty in carrying out this research. This is the first molecular identification study confirming the presence of *A. platys* circulating in dogs (27.00%) in the municipality of Veracruz, Mexico. No risk factors were identified so they merit further study. In this case, the most important factor was the absence of the use of tick control methods, but sample size was small.

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Conflict of interest

The authors declare that this study was conducted without any commercial or financial relationships that could potentially create a conflict of interest.

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