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Original Article

Clinical and pathological factors associated with *Ehrlichia canis* in companion dogs

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Abstract

Introduction: Canine monocytic ehrlichiosis (CME) is a disease caused by the Gram-negative bacteria *Ehrlichia canis*, a bacterium that affects domestic dogs but can also infect humans. The diagnosis implies a challenge due to its diversity in clinical manifestations.
Methodology: The frequency of *E. canis* infection, risk factors, and clinical-pathological parameters associated with seropositivity were calculated with the PROC FREQ TABLES and PROC LOGISTIC procedures of the SAS statistical software.
Results: The study showed a seroprevalence of 26.62% (156/586). Association between seropositivity and risk factors was found. The age and the presence of ticks including clinical signs such as anorexia, seizures, cough, petechiae, epistaxis, and hematochezia, as well as multiple blood and biochemical alterations were analyzed. The logistic regression analysis showed a high predictive power (c = 0.98) for CME for thrombocytopenia, leukopenia, and anemia.
Conclusions: The high prevalence of *E. canis* in endemic areas makes its diagnosis difficult. Thus, clinical signs must be considered, along with blood and biochemical alterations, as a possible predictor of the disease.

Key words: Ehrlichiosis; dogs; hematology; clinical manifestations.

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Introduction

Canine monocytic ehrlichiosis (CME) is an infectious disease that is distributed worldwide and is caused by the Gram-negative bacterium *Ehrlichia canis* that belongs to the Anaplasmataceae family of the Rickettsial order. This bacterial species has a tropism for monocytes, lymphocytes, and macrophages [1] and more than 800 hosts have been reported, including humans. *E. canis* mainly affects the Canidae family, however, human infection has been described in Venezuela, Panama, and recently in North Mexico [2-4]. The pathogen, is transmitted by *Dermacentor variabilis* and the brown dog tick *Rhipicephalus sanguineus* [5]. The clinical signs and severity of the disease depend upon the bacterial strain, the host immunity, and co-infection with other pathogens such as *Babesia canis*, *Rickettsia rickettsii*, and *Hepatozoon canis* [6,7]. CME has an acute subclinical and chronic phase. The incubation period comprises from 8 to 20 days, followed by the acute phase lasting 15 to 30 days. This stage is characterized by fever, depression, splenomegaly, lymphadenomegaly, and a tendency to hemorrhage, which leads to petechiae, ecchymosis, and epistaxis [8]. The main laboratory finding is thrombocytopenia, although this can occur in all stages. In the subclinical phase, clinical signs are rarely present; however, pancytopenia, hyperglobulinemia, and high blood urea nitrogen concentration can be observed. The chronic phase is characterized by increased clinical signs and severity. Additionally, secondary infections occur with marked thrombocytopenia, leukopenia, and anemia [9-11].

The diagnosis of CME is challenging due to non-specific clinical signs and because it is a multistage disease. Diagnostic methods include white layer smear, serology, culture, and molecular techniques. These assays must be complemented with the clinical history and abnormalities in the blood count and chemistry [8].

Enzyme-linked immunosorbent assay (ELISA) has been developed and found helpful in diagnosis. Thus, some commercial tests have emerged such as: Snap3Dx, and Snap4Dx [12]. Although the use of serological tests in areas where the disease is endemic is limited, false positives have been reported since dogs can raise high antibody titers against *E. canis* without clinical signs [9].

Cases of CME have been evidenced in Africa, Europe, Asia, and America. Mexico had documented the disease in canines from several states. In 2005, a seroprevalence of *E. canis* was estimated at 44% in Yucatan [13]. In Sinaloa, the seroprevalence was reported to be 74% [14], and in Coahuila and Durango *E. canis* was detected by PCR in 2017 [15]. *R. sanguineus* of temperate lineage was identified in Chihuahua, and *Ehrlichia* spp was analyzed and identified in 66% of the tick pools [16].

Due to the increase in suspected cases of CME during different seasons of the year, the diversity of clinical signs, the difficulty in diagnosis, and the lack of scientific reports in the entity, the objective of this study was to estimate the frequency, analyze the risk factors and clinical pathological manifestations associated with the disease in dogs treated at veterinary hospitals in Chihuahua.

Methodology

Location

The study was carried out in Chihuahua city, Mexico, which is located at 28.63° latitude and -106.08° longitude. Chihuahua is the second largest city in the state and its population is 878,062 inhabitants. The city's climate is dry and semi-dry, with temperatures below 0 °C in winter and above 30 °C in summer.

Sample collection and processing

A total of 586 dogs blood samples were collected in two private veterinarian clinics for a period of four years (2014-2017). The inclusion criteria included dogs residing in Chihuahua, presenting clinical signs suggestive of CME or were infested with ticks, and an informed consent signed by the dog´s owner. The present project was approved by the bioethics committee of the University of Ciudad Juárez (CIBE-2016-1-05). Patient data were included in the epidemiological questionnaire: race (pure/Creole), gender (female/male), age (less than 2, 2 to 4, > 4 years), type of coat (short/long), and the presence or absence of ticks. Abnormalities observed at the time of physical examination were also documented, as well as data relating to the clinical history such as apathy, anorexia, weight loss, seizures, fever, lymphadenopathy, dyspnea, cough, petechiae, epistaxis, edema in extremities, ataxia, limp, and erythema. To perform the blood chemistry and complete blood count tests, 3 mL of blood per individual were collected in vacutainer tubes without EDTA. Then the samples were submitted to the 5R+ Vet ® automatic hematology analyzer (KontroLab, Guangxi, China). For the biochemical analysis of the samples, the ES-300 ® Automated Chemistry Analyzer (KontroLab, Guangxi, China) was used. The blood chemistry analytes evaluated were: a) substrates: creatinine (< 2.0 mg/dL), bilirubin (0.01-0.61 mg/dL), urea (15-39 mg/dL); b) proteins: total proteins (5.4- 7.5 g/dL), globulins (2.7-4.4 mg/dL), albumin (2.8-4.0 g/dL); c) enzymes: alanine transaminase (ALT; < 86 mg/dL), aspartate transaminase (AST; < 54 mg/dL), and alkaline phosphatase (ALP; < 200mg/dL). Additionally, the blood cell count (200,000) was carried out, and a cell pack volume below 37% was considered anemia. To determine the type of anemia that the patient presented, the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin (MCHC) were evaluated and classified into eight types: macrocytic-hyperchromic, microcytic-normochromic, normocytic-hyperchromic, macrocytic-normochromic, macrocytic-hypochromic, microcytic-hypochromic, normocytic-normochromic, and macrocytic-normochromic.

The serological diagnosis of *E. canis* was carried out using the commercial dot-ELISA kit (SNAP 4DX ®, IDEXX, Laboratories, Westbrook, ME) following the manufacturer's instructions. Previously reported sensitivity and specificity for this kit are 97.8% and 92.3%, respectively [17].

Statistical analysis

Statistical analyzes were performed in the SAS® version 9.4 (TS1M7) package. The FREQ procedure was used to determine the frequency distribution of positive and negative cases with their respective percentages, and prevalence by year and month. The Chi-square statistic and Fisher's exact test were used to compare proportions by year and month of the year. A p value ≤ 0.05 was considered significant.

To determine the association of seropositivity with clinical signs and risk factors for *E. canis,* contingency tables were constructed and resolved using the Chi-square statistic for independence. The association was present when the statistic was significant ($p \leq 0.05$). The analysis was performed by applying the FREQ procedure with the TABLES statement. The

associations were quantified with odds ratios (OR) and expressed with their 95% confidence intervals (95% CI).

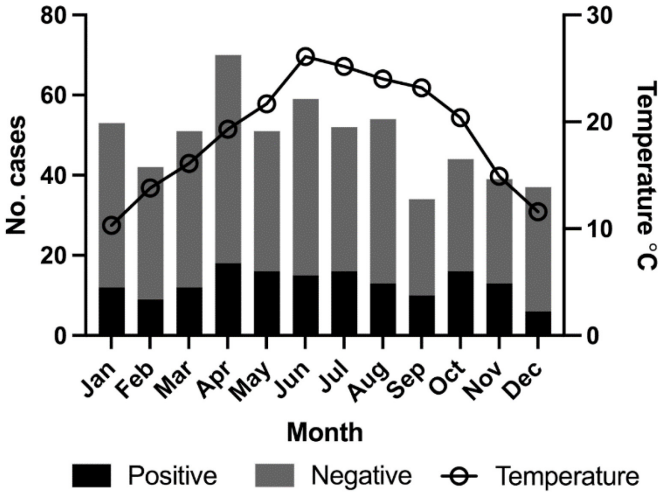
The association between the blood count and the biochemical analytes with the positivity of the test was carried out using univariate and multivariate logistic regression. The LOGISTIC procedure was used in the analysis. The association was quantified with the odds ratio (OR) and expressed with its 95% CI.

Results

The global seroprevalence of *E. canis* in the study was 26.62% (156/586) from January 2014 to January 2017. The annual prevalence was similar in each year: 31.32%, 22.04%, 27.05% (56/207), and 18.9%, respectively. The distribution of *E. canis* cases by month and average temperatures are shown in Figure 1. The risk factors associated with the disease were age and tick infestation. Dogs younger than two years old and those with a history of tick infestation were 2.5 and 113 times, respectively, more likely to be seropositive for the presence of *E. canis* (Table 1). The clinical signs most frequently observed among seropositive patients were apathy 23.7%, anorexia 21.15%, decay 17.31%, and weight loss 16.3% (Table 2), although, the clinical signs associated with seropositivity were anorexia, seizures, cough, petechiae, and epistaxis, with an odds ratio (OR) value of 10, 15.6, 2.7, 18.3, and 3.8, respectively (Table 3).

The results related to hemogram and biochemical analysis, alterations in seropositive dogs (n = 156) were: thrombocytopenia in 96.79% of cases and anemia in 75.64%. The most frequent type of anemia was normochromic normocytic with 78% (Table 4). Other parameters were hypoalbuminemia with 60.39%, uremia with 43%, elevated AST in 40.65%, elevated ALP 35.95%, hypoglobulinemia 33.3%, elevated ALT 31.17% and hyperbilirubinemia in 28.1% (Table 5).

Figure 1. Distribution of *E. canis* cases by months and average temperatures.



Number of cases studied per month and the average temperature of each month in the years covered by the study

Table 2. Frequency of clinical manifestations of dogs diagnosed with anti-*Ehrlichia canis* antibodies (n = 156).

Clinical signs	n (%)
Lethargy	37 (23.72)
Anorexia	33 (21.15)
Weakness	27 (17.31)
Weight loss	25 (16.03)
Seizures	15 (9.62)
Pyrexia	15 (9.6)
Lymphadenomegaly	9 (5.77)
Dyspnea	8 (5.13)
Cough	8 (5.13)
Petechiae	6 (3.85)
Epistaxis	5 (3.21)
Edema in the hind limbs	4 (2.56)
Ataxia	4 (2.56)
Lameness	3 (1.92)
Erythema	2 (1.28)

Table 1. Prevalence, Chi-square and odds ratio (OR) by variable in dogs diagnosed with *E. canis*.

Variable	Prevalence	χ^2	*p*	OR CI 95%
Gender				
Males	31.2% (83/266)	0.65	0.41	1.28 (0.70-2.34)
Females	22.1% (69/311)			1
Age (yrs)				
< 2	35.04% (48/137)	8.56	0.01	2.54 (1.21-5.34)*
2-4	22.64% (12/53)			0.53 (0.18-1.50)
> 4	24.2% (96/300)			1
Tick infestation				
Yes	78.33% (141/180)	184.8	0.01	112.91 (57.1-223.1)*
No	3.69% (15/406)			1

*Associated risk factor.

Table 3. Prevalence, Chi-square, and odds ratio (OR) by clinical signs in dogs diagnosed with *E. canis*.

Clinical signs	Prevalence	χ^2	*p*	OR CI 95%
Anorexia				
Yes	71.74% (33/46)	43.32	0.0001	10.09 (5.07-20.09)*
No	22.78% (123/540)			
Seizures				
Yes	78.95% (15/19)	22.51	0.0001	15.67 (5.02-48.86)*
No	24.87% (141/567)			
Lymphadenomegaly				
Yes	37.5% (9/24)	3.34	0.06	2.25 (0.943-5.39)
No	26.16% (147/562)			
Cough				
Yes	42.11% (8/19)	4.44	0.04	2.78 (1.07-7.23)*
No	26.10% (148/567)			
Petechiae				
Yes	85.71% (6/7)	6.84	0.0081	18.34 (2.07-162.13)*
No	25.91% (150/579)			
Epistaxis				
Yes	100% (5/5)	13.9	0.0002	3.84 (3.35-4.41)*
No	25.99% (151/585)			
Hematochezia				
Yes	80% (4/5)	7.35	0.0067	11.28 (1.25-101.79)*
No	26.16% (152/581)			

* Associated clinical signs.

Table 4. Anemia classification by cell morphology and hemoglobin concentration and their frequency in seropositive dogs (Total seropositive dogs with anemia, n = 118).

Type of anemia	MCV/1	MCHC/2	Frequency	%
Macrocytic-hypochromic	High	High	3	2.5
Microcytic- normochromic	Low	Normal	1	0.8
Normochromic-hyperchromic	Normal	High	5	4.2
Macrocytic- hypochromic	High	Low	5	4.2
Microcytic- hypochromic	Low	Low	0	0
Normocytic- normochromic	Normal	Normal	92	78
Macrocytic- normochromic /3	High	Normal	9	7.6

/1 MCV: mean corpuscular volume, stands for mean corpuscular volume. Reference value: 63-72 fL. /2 MCHC: mean corpuscular hemoglobin concentration: Measures the average concentration of hemoglobin in red blood cells. Reference value: 32-36.3 g/dL. /3 Without clinical relevance, this does not represent a real anemia.

Table 5. Frequency and association of hemological and blood chemistry alterations in *E. canis* seropositive dogs, adjusted by logistic regression.

Hematobiochemical Parameter	n	%	χ^2	*p*	OR CI 95%
Leukopenia	14	8.97	17.88	0.001*	5.9 (2.3-15.0)
Thrombocytopenia	151	96.79	450.9	0.001*	489 (183.9-1301.2)
Anemia	118	75.64	195.25	0.001*	17 (11.10-27.3)
High ALT (> 86 mg/dL)	48	31.17	17.42	0.001*	2.4 (1.5-3.7)
High AST (> 54 mg/dL)	63	40.65	33.18	0.001*	3.1 (2.1-4.7)
High FAL (> 200 mg/dL)	55	35.95	18.94	0.001*	2.4 (1.6-3.6)
Hypoproteinemia (< 5.4 mg/dL)	11	7.28	27.47	0.001*	33.7 (4.3-263.4)
Hypoglobulinemia (< 2.7 mg/dL)	51	33.33	25.03	0.001*	2.9 (1.89-4.5)
Hypoalbuminemia (< 2.8 mg/dL)	93	60.39	97.47	0.001*	6.8 (4.5-10.3)
Hyperbilirubinemia (> 0.61 mg/dL)	43	28.10	29.41	0.001*	3.5 (2.1-5.6)
Hypercreatinemia (>2,0 mg/dL)	14	9.27	2.8	0.089	1.8 (0.9-3.6)
Uremia (>15-39 mg/dL)	67	43.2	7.96	0.004*	1.7 (1.17-2.50)

*Associated alterations with seropositivity by χ^2 ($p < 0.05$).

The alterations associated with seropositivity using the Chi-square statistic are shown in Table 5. Regarding the logistic regression analysis, the variables: thrombocytopenia, leukopenia, and anemia were included, obtaining an association of c = 0.98 between the values of predictive probability and observable response.

Discussion

The rickettsial diseases transmitted by *R. sanguineus* have risen significantly due to their wide distribution in the northwestern states of Mexico. They affect both animals and humans. Therefore, several studies have focused on describing the presence of these pathogens in the vector, host (dog) and humans. In Culiacan Sinaloa, the prevalence in patients of veterinary clinics was 74.3% in the spring-summer of 2013 [14]. In Durango and Coahuila at "La Comarca Lagunera" they detected *Ehrlichia* spp DNA by polymerase chain reaction (PCR) in 10% (10/100) of the dogs analyzed [15]. In the Chihuahua state, there are only reports in Juárez city. Escárcega Ávila *et al*. detected *Ehrlichia* spp DNA in 40% of blood samples from street dogs [16]. Another study in the same city and same year estimated a 28% seroprevalence of *Ehrlichia* spp in veterinary professionals [18].

Movilla *et al*., reported an annual seroprevalence of *E. canis* of 51.04% (196/384) in dogs from northwestern Mexico (Baja California, Sonora, Sinaloa, Durango and Chihuahua states) [19]. This data agrees with our study, except for the 74.3% (113/152) reported in Sinaloa. However, it is worth mentioning that, unlike the rest of the studies that included samples collected throughout the year, the present study only analyzed samples collected in spring-summer when the vector is more active and infects more hosts. In addition, in Sinaloa, the temperature range is between 23.7 and 39 °C and average relative humidity is 68%. According to Dantas-Torres higher the relative humidity (35-95%) and temperature (30-35°C), the shorter the cycle of the vector *R. sanguineus*, therefore requiring frequent feeding on the hosts in shorter periods to molt [20]. The results in this study showed the highest prevalence in April (average temperature is 20.4 °C), which could be related to vector activity that decreases in winter and increases in April when the temperature is ideal (20 - 35°C) to continue its cycle [20].

Miró *et al*. observed that dogs under two years of age had an OR of 2.5 times more risk than those that are older, which agrees with the present study [21]. However, most reports differ. Barrantes-González *et al.* estimate an OR of 1.6 in dogs between two and seven years old [22], Piantedosi *et al.* of 2.35 in dogs older than three years [23]; and Movilla *et al.* obtained the same result of OR 2.02 in adult dogs [19]. The authors agree that the exposure to the vector has been greater than in puppies and therefore the probability of exposure to the bacteria is also greater [24]. Nevertheless, in areas where the disease is endemic, dogs could be in constant contact with the pathogen leading to adult dogs developing immunity, which would indicate young dogs being more vulnerable than adults.

Tick infestation was found to be a risk factor associated with *E. canis* seropositivity (OR 112), which had already been reported in several studies [11,19,25]. This indicates that vector density and distribution are closely related to the distribution and prevalence of the diseases that they transmit [26].

CME displays non-specific clinical signs, which could be changed at the cellular level in different organs and tissues making the clinical diagnosis complex. These changes might be due to the pathogenicity, strain variability, the disease stage and the host susceptibility [27] or to co-infection with other pathogens. One of the limitations of this study is that co-infections with other pathogens were not evaluated. It has been reported that *R. sanguineus* is the vector of several rickettsial bacteria. Furthermore, this insect circulates in the northern part of the country and has one of the greatest distributions, which could worsen the clinical presentation of the individuals in this study. The most frequently reported clinical signs include pyrexia, lethargy, apathy, bleeding, and lymphadenomegaly [11].

The most frequent clinical signs found in seropositive dogs were: apathy, anorexia, seizures, and petechiae; consistent with previous reports, except for epistaxis and fever which were observed in 3.2% and 9.6% of the seropositive dogs. Although, 97% of the analyzed dogs presented thrombocytopenia, other types of bleeding resulted in hematochezia and petechiae as associated risk factors (Table 3).

Cough was an associated clinical sign in our study. Although this sign has not been frequently reported in previous studies, Nair *et al*. observed discrete pulmonary microgranulomas scattered in the lung tissue of dogs infected with *E. canis* [28].

Frank *et al.* conducted a retrospective study on 62 dogs with ehrlichiosis in North Carolina and Virginia, USA. They associated hemorrhages and inflammatory processes with spinal cord and central nervous system disorders [29]. When there is a neurological disorder, the individual usually suffers from epileptic episodes,

which alter brain activity, resulting in seizures. In this study, this condition was also strongly associated with positivity (OR 15.67).

One of the clinical signs associated with *E. canis* that facilitate the diagnosis of the disease is thrombocytopenia, which occurs in more than 90% of cases and is considered a good indicator of CME diagnosis [9]. In this particular study, 97% of seropositives presented this anomaly, similar to other studies [11,30,31]. It showed a correlation with seropositivity and an odds ratio of 489. This could be due to thrombocytopenia occurring in different stages of the disease. In the acute phase, it is attributed to the immediate consumption of platelets due to the inflammatory process in blood vessels, to the increase in splenic sequestration of platelets and to the immunological destruction or injury that results in a decrease in the immune-mediated platelet half-life [32]. Also, other studies have characterized the platelet migration inhibitory factor, and its concentration in dogs infected with *E. canis* has been inversely proportional to platelet count. In the chronic phase, in addition to thrombocytopenia, platelet dysfunction has been observed, in which the low platelet count contributes to the hemorrhages observed in CME [8]. Thrall *et al.* also mention that, in the chronic stage, the agent causes bone marrow aplasia with a subsequent decrease in the platelet line [33].

Another haematological parameter associated with the seropositivity of *E. canis* found in our study was anemia, with a frequency of 75.6%. These results agree with non-regenerative normochromic normocytic anemia. The results obtained are consistent with other reports of similar studies [28,34,35]. This result differs from Bai *et al*. who reported a higher frequency of hypochromic normocytic anemia in dogs with a positive diagnosis of *E. canis* [11]. In our study, epistaxis and petechiae were strongly associated with seropositivity (OR 3.84 and 18.34, respectively; Table 3). This could be due to the hemorrhages that patients present during the infection, in addition to the immunological mechanisms involved, in which the production of antibodies and their binding to the membrane of erythrocytes trigger their destruction [36].

A high percentage of patients with liver enzyme abnormalities (ALT, AST, and ALP) was observed within the positive group, reflecting the hepatobiliary damage already associated with the disease. Other studies report liver histopathological lesions in dogs infected with *E. canis* and in dogs infected with *A. platys* [11,28].

Another associated parameter was hypoproteinemia (OR = 33.7). This is another parameter that we found to be associated (OR = 33.7). This can be explained by the low levels of albumin and globulin, also associated with positivity. As mentioned earlier, these proteins are produced in the liver, thus the hypoproteinemia could be related to the reported hepatic failure in *E. canis* infection

Albumin can be slightly decreased in tissue injuries or inflammations since it is considered a negative acute phase reactant. However, the decrease in total proteins does not usually occur since there is compensation for the increase in positive acute phase proteins such as some globulins. Although low albumin levels could also suggest kidney failure [37], few studies report kidney failure caused by *E. canis*. Qurollo *et al.* reported urinary sediment, proteinuria, and other factors associated with kidney failure in dogs positive for *E. ewingii*. They concluded that this could be due not only to the pathogen but also other host factors such as age (immune-senescence) [38]. Our study observed that dogs that presented uremia were 1.7 times more likely to be positive for *E. canis*. Unfortunately, no urinalysis was performed to test for possible renal failure caused by *E. canis* infection.

The clinical diagnosis using serological tests can be complex. The study area is considered an endemic zone and had high seroprevalence of this disease. These factors could lead to false positives since dogs from these areas could present high IgG titers without clinical signs [18,39].

A logistic regression analysis contemplates predicting the disease. Variables highly associated with positivity in our study were included, and these have been associated with the disease in previous studies [9,11,40]. Thrombocytopenia, leukopenia and anemia displayed high association (c = 0.98) predictive of the logistic regression model by using the observable responses of these three abnormal haematological tests to positive results for *E. canis.*

Conclusions

Rickettsial diseases have recently increased in Northern Mexico and thus the need for research to help understand its behavior, both clinically and epidemiologically. This study is the first report of seroprevalence of canine ehrlichiosis in dogs in Chihuahua, Mexico. Likewise, the clinical signs and the pathological parameters associated with the disease are described. This could be helpful to veterinary clinicians in providing an accurate diagnosis for ehrlichiosis, especially due to the complexity for its diagnosis in

endemic areas. Therefore, it is important to design new techniques with greater sensitivity and specificity that allow to distinguish between sick and healthy individuals in endemic areas, where most individuals are exposed to the pathogen, and will also help reduce the use of antibiotics indiscriminately to avoid antimicrobial resistance.

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