First Molecular Identification of *Ehrlichia chaffeensis* in Dogs

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Abstract. *The species Ehrlichia chaffeensis is important for public health as it is the etiological agent of human monocytic ehrlichiosis (HME). The objective was to detect Ehrlichia chaffeensis in dogs from León, Nicaragua. Thirty-nine dogs with symptoms consistent with hemoparasitosis and tick infestation were evaluated and underwent a blood smear, SNAP® 4Dx® Plus immunochromatography test, and nested PCR. The presence of Ehrlichia spp. morulae in blood smears was detected in 87.2% (95% CI, 72.6–95.7) of the samples, while 53.9% (95% CI, 36.9–70.8) of E. canis / E. ewingii seropositive canines were detected with SNAP. Ehrlichia spp. was detected by PCR in 87.2% (95% CI, 72.6–95.7) of the samples. In the nested analysis, 24.6% (95% CI, 10.7–40.6) of canines were positive for Ehrlichia chaffeensis. This is the first report of molecular detection of this important zoonotic agent causing canine ehrlichiosis in Nicaragua.*

Introduction

Most neglected zoonotic and vector-borne diseases go undiagnosed in humans and animals, often because they are subclinical. In some cases, however, they are associated with serious pathological manifestations. Canine vector-borne diseases are often widespread in tropical and subtropical regions due to climatic conditions favorable for the survivability of arthropod vectors and the development of vector-borne canine pathogens (Kilpatrick and Randolph, 2012; Selim et al., 2021)and many endemic diseases have increased in incidence. Although introductions and local emergence are frequently considered distinct processes, many emerging endemic pathogens are in fact invading at a local scale coincident with habitat change. We highlight key differences in the dynamics and disease burden that result from increased pathogen transmission following habitat change compared with the introduction of pathogens to new regions. Truly in situ emergence is commonly driven by changes in human factors as much as by enhanced enzootic cycles whereas pathogen invasion results from anthropogenic trade and travel and suitable conditions for a pathogen, including hosts, vectors, and climate. Once established, ecological factors related to vector characteristics shape the evolutionary selective pressure on pathogens that may result in increased use of humans as transmission hosts. We describe challenges inherent in the control of vector-borne zoonotic diseases and some emerging non-traditional strategies that may be more effective in the long term.","container-title":"Lancet","DOI":"10.1016/S0140-6736(12.

An increase in *Ehrlichia* infections has been observed due to factors such as the presence and number of animal reservoirs and vector ticks in the endemic area *(Mogg et al.*, 2020 ; Forero-Becerra et al., 2021)506 healthy residents and 114 dogs from four municipalities (Cauca, Colombia. *Ehrlichia chaffeensis*, *E. ewingii*, and *E. canis* species have been identified as causative agents of emerging zoonotic infections in humans (Thomas et al., 2009; Bouza-Mora et al., 2017)including divergent tandem repeat sequences. Nucleotide sequences of dsb and trp36 amplicons revealed a novel genotype of E. canis in blood bank donors from Costa Rica. Indirect immunofluorescence assay (IFA. *E. chaffeensis* is the etiological agent of human monocytic ehrlichiosis (HME) (Guillemi et al., 2019), a disease described in 1987 in the United States. Although *E. canis* was initially implicated as the responsible bacterium (serological cross-reaction) in HME conditions, it is now known that the causal agent is *E. chaffeensis*, due to its isolation in Fort Chaffee, which birthed the name *E. chaffeensis* (Dolz et al., 2013). The diagnosis of ehrlichiosis by PCR has shown greater effectiveness in blood samples, presenting more specific results, since there are no cross-reactions, and it also detects *Ehrlichia* spp. in any of its phases and offers a definitive diagnosis (Franco-Zetina et al., 2019).

E. chaffeensis has been extensively studied as a cause of acute febrile illness and an emerging tick-

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borne zoonosis present throughout the Americas (Chikeka et al., 2016)the etiologic agent of human monocytic ehrlichiosis (HME. In the United States, the main vector of *E. chaffeensis* is the tick of the *Amblyomma americanum* species; however, other species that can transmit this bacteria have also been described, such as *Amblyomma parvum*, which has already been found in domestic animals in Nicaragua (Düttmann et al., 2016)3299 were parasitized, which represent 68 % of the bovines and 67 % of the equines in study: 59 cows and 25 horses were parasitized by more than one species. In addition, 280 specimens of the entomological museum in León were examined. The ticks found on cattle were Rhipicephalus microplus (75.2 % of the ticks collected. In addition, serological evidence of *E. chaffeensis* infection in humans has been reported (Chikeka et al., 2016) the etiologic agent of human monocytic ehrlichiosis (HME, that could be associated with the role of dogs as a zoonotic reservoir for human infection. Despite these various reports, there are no studies that report the presence of anti-bodies against *E. chaffeensis* or its DNA in canines, thus suggesting the need for the study. The objective was to detect *Ehrlichia chaffeensis* in dogs from León, Nicaragua.

Materials and methods

Blood sampling and *Ehrlichia* spp. screening

An observational study was carried out on 39 tick-infested dogs that attended the UNAN-León veterinary clinic, with a mean age of 48 months (Range 24–84), 49% of Creoles and 51% of other breeds. Following the previously described protocol (Overall, 2013), blood sampling was performed by experienced veterinarians according to established routine practice for laboratory diagnosis. Venous blood (3 mL) was collected aseptically from the cephalic vein and mixed with ethylenediaminetetraacetic acid (EDTA). A blood smear was performed for an examination of intracytoplasmic inclusion bodies compatible with *Ehrlichia*. The SNAP® 4Dx® Plus immunochromatographic assay (IDEXX Laboratories, Inc., Westbrook, Maine) was also applied for antibody detection.

Molecular diagnosis

For molecular detection, DNA extraction was performed using the QIAamp DNA Mini Kit QIAGEN (Hilden, Germany), according to the manufacturer's instructions. For the first PCR reaction, the primers described in Table 1 were used in a reaction volume of 20 µL, containing 8.5 µL of nuclease-free water, 10 µL of Master Mix 2X (Promega, EE. UU), 0.5 µL (5000 nmol) of Forward (F) primer for *Ehrlichia* spp., 0.5 µL (5000 nmol) of Reverse (R) primer for *Ehrlichia* spp. and 0.5 µL of extracted DNA.

Positive samples of the first reaction underwent the nested analysis. A volume of 25 µL was prepared, containing 10.5 µL of nuclease free water, 12.5 µL of Master Mix 2X (Promega, USA), 0.5 µL (5000 nmol) of primer Forward (F) for *E chaffeensis*, 0.5 µL (5000 nmol) of Reverse (R) primer for *E. chaffeensis*, and 1 µL of DNA from the primary PCR product was used. Amplification was performed at 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 55°C for 30 seconds, a final extension at 72°C for 10 minutes and 4°C until the moment of disassembling the reactions. The amplified products were separated using electrophoresis on a 1.2% agarose gel that was stained with ethidium bromide. Negative and positive controls were included in each DNA extraction and PCR reaction.

Ethical consideration

Dogs included in this study were managed according to the *Law for the protection and welfare of domestic animals and domesticated wild animals (747)* (Normas Jurídicas de Nicaragua, 2011), and this study was previously approved by the Research Commission of the School of Agricultural and Veterinary Sciences (ECAV), National Autonomous University of Nicaragua, Leon (UNAN-Leon). Informed consent was obtained from each dog owner.

Data analysis

In statistical analysis, descriptive statistics are reported first. Laboratory results were treated as categorical variables and reported as frequency and 95% confidence intervals (95% CI). Continuous variables (e.g., hematological laboratory values) were reported as mean. To determine hematological parameters associated with *Ehrlichia* positive test results, the Student *t* test for independent samples was employed, and statistical significance was determined if $P < 0.05$. To determine concordance between the

Table 1. Primers used in the nested PCR for the detection of *Ehrlichia* spp. and *Ehrlichia chaffeensis*

Pathogen	Expected product	Primers	Reference
Ehrlichia spp.	490 pb	Primary: ECC (5′AGAACGAACGCTGGCGGCAAGCC) Primary: ECB (5′-CGTATTACCGCGGCTGCTGGCA)	(Gleim et al., 2016) (Dawson et al., 1994) (Anderson et al., 1992)
Ehrlichia chaffeensis	380 pb	Secondary: HE1 (5′CAATTGCTTATAACCTTTTGGTTATAAAT) Secondary: HE3 (5′TATAGGTACCGTCATTATCTTCCCTAT) (51)	(Gleim et al., 2016) (Dawson et al., 1994) (Anderson et al., 1992)

blood smear, SNAP and PCR tests, the Cohen Kappa concordance test was used.

Results

Of the 39 canines in this study, intracytoplasmic morulae were observed in the blood smears of 87.2% (95% CI, 72.6–95.7) (Fig. 1). These were observed in neutrophils (43.6%, 17/34), platelets (35.9%, 14/34), and monocytes (7.7%, 3/34).

SNAP 4Dx plus technique. Additionally, 6 dogs co-infected with *Ehrlichia* spp. / *Anaplasma* spp., 2 dogs co-infected with *Ehrlichia* spp., *Anaplasma* spp. and *Diroflaria immitis* were also observed.

Ehrlichia spp. were detected by PCR in 87.2% (34/39) of dogs, of which 10 (29.4%) were positive for *E. chaffeensis* DNA. Of the 10 dogs positive for *E. chaffeensis*, 9 presented monocytosis as a common hematological alteration.

No hematological parameters were associated withn *Ehrlichia* positive and negative animals by blood smears, PCR tests for *Ehrlichia* spp., or PCR tests for *E chaffensis*, (Table 2).

The test concordance between blood smear and PCR for *Ehrlichia* spp. demonstrated that 29 samples were positive in both tests, 5 dogs were negative by smear but positive by PCR, and 5 dogs were positive in smear but negative in PCR. No sample was negative in both tests (Kappa = –0.147, P = 0.358).

From the concordance of SNAP serology vs PCR for *Ehrlichia* spp. diagnosis, 18 samples were positive in both tests, 17 canines were negative by SNAP but positive in PCR, 3 dogs were positive in SNAP but negative in PCR, and 2 samples were negative in both tests (Kappa = –0.033, P = 0.768) (Table 3).

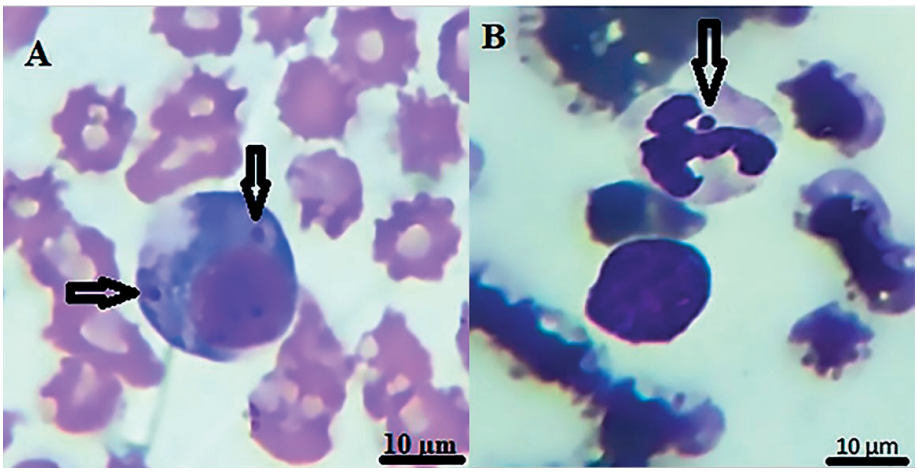

Fig. 1. Morulae of Ehrlichia in blood smear of canine (100 X)
Arrows indicate morulae of monocytic *Ehrlichia* (A) and granulocytic *Ehrlichia* (B)

Table 2. Comparison of blood parameters with respect to the detection of hemoparasites in the blood smear, *Ehrlichia* spp. and *Ehrlichia chaffeensis*

Blood parameter	Hemo-parasites	Blood smear		PCR-*Ehrlichia* spp.		PCR-*E. chaffeensis*	
		Means	P^*	Means	P^*	Means	P^*
Hematocrit (%)	Negative	39.30	0.427	39.20	0.440	36.26	0.483
	Positive	34.94		34.96		33.31	
White blood cells count (10^3 cells/mL)	Negative	9.44	0.272	8.15	0.148	12.93	0,417
	Positive	14.24		14.43		15.66	
Red blood cell count (10^6 cells/mL)	Negative	3.65	0.184	4.70	0.537	5.17	0.374
	Positive	5.92		5.77		6.98	
Lymphocytes (%)	Negative	7.14	0.729	7.20	0.747	7.17	0.327
	Positive	8.17		8.16		10.56	
Neutrophils (%)	Negative	58.46	0.749	65.86	0.324	62.20	0.077
	Positive	60.55		59.46		54.70	
Eosinophils (%)	Negative	5.18	0.917	6.30	0.659	5.28	0.796
	Positive	5.43		5.26		5.74	
Monocytes (%)	Negative	21.20	0.798	18.66	0.479	20.60	0.126
	Positive	22.86		23.23		28.59	

Table 3. Concordance analysis between the smear/SNAP/PCR techniques for the diagnosis of Erhlichia spp. in dogs

		PCR-*Erhlichia* spp.		Total	Kappa
		Negative	Positive		
Smear	Negative	0	5	5	–0.147
	Positive	5	29	34	
	Total	5	34	39	
SNAP	Negative	2	16	18	–0.033
	Positive	3	18	21	
	Total	5	34	39	

Discussion

In this observational study of canines in Nicaragua, a high prevalence (87.2%) of ehrlichiosis was documented. Furthermore, *E. chaffeensis* was specifically identified by PCR in a high percentage of dogs, the species with the greatest zoonotic involvement. Prior to this study, there had been no reports of molecular detection of *E. chaffeensis* in ticks or domestic animals from Central America. Despite this lack of evidence in vectors and animal hosts, *E. chaffeensis* was previously highlighted as an unrecognized cause of acute febrile illness in humans in Nicaragua (Chikeka et al., 2016)the etiologic agent of human monocytic ehrlichiosis (HME. Additionally, 5 humans with a history of tick bites and general arthralgia were diagnosed with *E. chaffeensis* infection by PCR in neighboring Costa Rica (Rojas et al., 2015). This new evidence confirms *E. chaffeensis* in canine populations in Central America and highlights the need for heightened awareness of ehrlichiosis epidemiology in the veterinary and human health domains.

This study also indicates a high seroprevalence of *E. canis* / *E. ewingii* according to the SNAP® 4Dx® Plus. This is higher than the 38.20% found in Costa Rica (95% CI: 32.8–43.4%), which also reported the highest prevalence, reaching up to 62%, was in the province of Guanacaste, the area closest to Nicaragua (Montenegro et al., 2017). This finding is similar to a prior report of 62.90% canine seroprevalence in Nicaragua, with authors attributing the high frequency to the fact that the dogs were sampled in the western parts of Nicaragua, the same origin of the canines analyzed in this study. This area is characterized by less rainfall and higher human population density than elsewhere in the country (Springer et al., 2018).

The application of SNAP® 4Dx® Plus identified canines with serological evidence of multiple concomitant exposures (6/39 (15%) for *Ehrlichia*/*Anaplasma* and (2/39 (5.12%) for *Ehrlichia*/*Anaplasma*/*Diroflaria*), unlike in the study of Montenegro et al. (2018) where double exposure to pathogens was only 8.9%. These results support the high seroprevalence of hemoparasites in dogs from Western Nicaragua, as well as the reproducibility when SNAP® 4Dx® Plus is applied.

A high percentage of dogs had morulae compatible with *Ehrlichia* spp., compared with what has been reported by others in Nigeria (1.5%) (Daramola et al., 2022)Nigeria by microscopy and nested PCR. Blood samples were collected from 205 dogs, thin smears were made, field-stained, and DNA was extracted from the blood samples. A partial region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR and Thailand (36.7%) (Rucksaken et al., 2019). This could be attributed to the fact that we sampled dogs with symptoms compatible with ehrlichiosis and tick infestations, biasing our selection toward clinically ill canines. No significant differences were observed in hematological parameters between positive and negative dogs for *Ehrlichia* spp. by PCR. This lack of association could be due to the small number of the analyzed samples, while other studies demonstrated a higher percentage of monocytes in canines positive for *Ehrlichia* spp. and *E. chaffeensis* (Lara et al., 2020; Thongsahuan et al., 2020)tick-borne rickettsial pathogens of dogs that may cause life-threatening diseases. In this study, we assessed the usefulness of PCR and a widely used commercial antibody-based point-of-care (POC.

PCR is one of the most sensitive and specific techniques for the diagnosis of *Ehrlichia* spp., and has even been considered the gold standard (Franco-Zetina et al., 2019). By comparison to PCR, the blood smear technique had a low concordance, reflecting a low accuracy for the detection of *Ehrlichia*. Due to its low cost, microscopy in Nicaragua and most countries is the only method available for routine use for diagnosing hemoparasites in dogs (Harrus & Waner, 2011)caused by the rickettsia Ehrlichia canis, an important canine disease with a worldwide distribution. Diagnosis of the disease can be challenging due to its different phases and multiple clinical manifestations. CME should be suspected when a compatible history (living in or traveling to an endemic region, previous tick exposure; nevertheless, this technique lacks specificity due to the need for experienced examiners to distinguish between *Ehrlichia* spp. infections and other cytoplasmic inclusions (Kaur et al., 2020). Although the blood smear is a simple, rapid, and inexpensive technique to routinely detect the bacteria, visualization of morulae in peripheral

blood cells is also the least sensitive and nonspecific technique, because of the low circulating amounts of bacteria, morulae are not detected in the blood smear, and sometimes it is possible to find inclusions not related to *Ehrlichia* spp. That can cause diagnostic confusion such as the identification of false positives (Dolz et al., 2013). Its low sensitivity could be a factor why some studies have reported low prevalence of canine *Ehrlichia* (Happi et al., 2018; Daramola et al., 2022) Nigeria by microscopy and nested PCR. Blood samples were collected from 205 dogs, thin smears were made, field-stained, and DNA was extracted from the blood samples. A partial region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR. No concordance was observed between PCR *vs* SNAP, a rapid test that has become essential to improve diagnosis. However, similar to the natural history of infection with many pathogens, antibodies are generally absent during the first two weeks and may persist after removal of the agent from the body (Wong et al., 2011)there was 85.2% concordance. The 24 discordant results between serology and PCR occurred in tests involving Ehrlichia canis (14. Cross-reactions between members of the Anaplasmataceae family that result in false positive results are also possible (Dolz et al., 2013).

This study documents an important veterinary and zoonotic pathogen in dogs of Central America and provides new evidence that dogs may be a source of *E. caffessis* infection in Nicaragua. Veterinary and human public health professionals should pay special attention to its zoonotic potential, and clinicians should consider ehrlichiosis in acute febrile conditions in humans if other causes have been ruled out.

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