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GENETICS, EVOLUTION, AND PHYLOGENY - SHORT COMMUNICATION

Epidemiological link between canine monocytic ehrlichiosis caused by *Ehrlichia canis* and the presence of *Rhipicephalus sanguineus* sensu stricto in Argentina

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Abstract

In this work, we analyze data that support an epidemiological link between cases of canine monocytic ehrlichiosis (CME) by *Ehrlichia canis* and the presence of *Rhipicephalus sanguineus* sensu stricto as vector in an endemic area for this tick in Argentina. In a blood sample of a 1-year-old toy poodle with CME compatible clinical signs, which showed CME typical morulae in monocytes in Giemsa-stained blood smear, DNA of *E. canis* was detected by PCR. Further, DNA of *E. canis* was also detected in a female of *R. sanguineus* s.s. collected on the infected dog. *Rhipicephalus sanguineus* s.s. is the only member of the *R. sanguineus* group that prevails in the study area. The results of this study suggest that *R. sanguineus* s.s. may play a more important role in the transmission of *E. canis* than it was assumed so far. The epidemiological link between CME cases and *R. sanguineus* s.s. as vector in temperate areas of Argentina described in this work contrast previous studies which found that *R. sanguineus* sensu lato "tropical lineage" (which is absent in the study area) is competent to transmit *E. canis* but not *R. sanguineus* s.s.

Keywords Canine monocytic ehrlichiosis · *Ehrlichia canis* · *Rhipicephalus sanguineus* · Mendoza · Argentina

Introduction

Bacteria of the genus *Ehrlichia* are obligate intracellular, tick-borne bacteria of the family Anaplasmataceae. The genus *Ehrlichia* consists of six formally described species: *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia minasensis*, *Ehrlichia muris,* and *Ehrlichia ruminantium* (Dumler et al. 2001; Cabezas-Cruz et al. 2016), but different new strains of *Ehrlichia* sp. have been described in the last years also in Argentina (Cicuttin et al. 2020). Therefore, it can be assumed that the number of *Ehrlichia* species is underestimated. One of the most important ehrlichial bacteria in veterinary medicine is *E. canis*, the etiological agent of canine monocytic ehrlichiosis (CME) (Harrus et al. 2012). The course of an infection with *E. canis* can be divided in three phases: acute, subclinical and chronic, and it is characterized by a wide range of clinical signs including fever, depression, lethargy, anorexia, lymphadenopathy, splenomegaly, mucosal pallor, bleeding tendency, ocular abnormalities, ulcerative stomatitis, necrotic glossitis, central

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nervous signs, and death (Harrus et al. 2012). The main vectors of *E. canis* are ticks belonging to the *Rhipicephalus sanguineus* group (Bremer et al. 2005), which are represented in the new world by at least two different taxa, namely *Rhipicephalus sanguineus* sensu stricto (s.s.) and *R. sanguineus* sensu lato (s.l.) "tropical lineage" (Nava et al. 2018). In an experimental study on vector competence, Moraes-Filho et al. (2015) have found that *R. sanguineus* s.l. "tropical lineage" is competent to transmit *E. canis* but not *R. sanguineus* s.s. (named as *R. sanguineus* "temperate lineage"). However, some studies recently performed in Argentina suggest that *R. sanguineus* s.s. could be involved as vector of *E. canis* in cases of CME (Eiras et al. 2013; Cicuttin et al. 2017; Tarragona et al. 2019; Sánchez et al. 2020).

In the province of Mendoza, western Argentina, clinical cases compatible with ehrlichiosis were diagnosed in dogs by serological techniques since the year 2009 (Mera y Sierra et al. 2019), but the presence of *Ehrlichia* spp. was not determined nor confirmed. The tick species that were determined infesting dogs in urban areas of greater Mendoza city are *Rhipicephalus sanguineus* s.s.[1] and *Amblyomma tigrinum* (Fantozzi et al. 2018). In this work, we describe and confirm by molecular tools a clinical case of CME caused by *E. canis* in an endemic area for *R. sanguineus* s.s. in Argentina and analyze data that support the epidemiological link between cases of CME and the presence of this tick species.

Materials and methods

Clinical diagnosis

In December 2019, a 1-year-old male toy poodle living in an urban area of Godoy Cruz, Mendoza, was received for clinical examination in a private veterinary practice due lethargy, anorexia, fever, vomiting, pallor of mucosal membranes, and a profuse tick infestation. It was referred to the laboratory for a complete blood count and serum chemistry. The following hematological parameters were determined in an Abacus Junior Vet® automated hematology analyzer: red blood cell count (RBC), hemoglobin concentration (Hb), packed cell volume (PCV), platelet count (Plt), and total leukocyte count (WBC). A blood smear was fixed with methanol and stained with Giemsa to perform manual relative and differential cell counts to obtain absolute counts for neutrophils, eosinophils, lymphocytes, and monocytes. Reticulocyte percentages were determined on blood smears stained with brilliant cresyl blue. Serum chemistry was performed with an InCCA® auto analyzer. Animal ethics guidelines regarding animal care were strictly adhered. Ethics approval, protocol No. 158, was granted by the Institutional Animal Care and Use Committee (Comité Institucional de Cuidado y Uso de Animales en Investigación y Docencia) of Universidad Juan Agustín Maza, Mendoza.

Molecular diagnosis

DNA was extracted from whole blood sample, taken from the dog, using a standardized chloroform/phenol method. The DNA sample was tested for the presence of a 177 bp fragment of the 16S rRNA gene, specific for the family Anaplasmataceae, by a real-time PCR assay described by Monje et al. (2019). The positive samples in the real-time PCR were then tested by a conventional PCR method detecting the *dsb* gene, which encodes the thio-disulfide oxidoreductase of ehrlichial bacteria, following the methods described in Doyle et al. (2005) and Aguiar et al. (2007). In all PCR reactions, DNA of *Ehrlichia* sp. STRAIN San Luis was used as positive control, while ultra-pure PCR water acted as negative control. Ticks were collected from the dog, stored in ethanol (96%), and morphologically determined following Nava et al. (2018). Morphological determination was confirmed by comparison of sequences of the mitochondrial 16S rRNA gene. DNA extraction from parts of the ticks and PCR amplification of a fragment of the mitochondrial 16S rRNA gene was processed according to the methods described by Mangold et al. (1998). DNA extracted of the ticks was also employed for molecular detection of *Ehrlichia* spp. with the PCR methods described above.

Phylogenetic analyses

Positive PCR samples were purified using a DNA purification kit (Wizard® SV Gel and PCR Clean-Up System, Promega) and sent to INTA Castelar (Genomics Unit, Buenos Aires, Argentina) for sequencing. Obtained partial sequences of the *dsb* gene were edited using BioEdit Sequence Alignment Editor (Hall 1999) with manual edition whenever it was necessary, aligned with the program Clustal W (Thompson et al. 1994), and compared with sequences deposited in GenBank. Phylogenetic analyses were performed with maximum-likelihood (ML) methods by using the program Mega X (Kumar et al. 2018). Best fitting substitution models were determined with the Akaike Information Criterion using the ML model test implemented in MEGA 5 (Substitution model was Tamura and Nei model with invariant sites). Support for the topologies was tested by bootstrapping over 1000 replications and gaps were excluded from the comparisons.

[1] Fantozzi et al. (2018) mentioned the presence of *R. sanguineus* s.l. in Mendoza, but according to the information given by Nava et al. (2012, 2018), it can be inferred that the taxon present in this area corresponds in fact to *R. sanguineus* s.s. (see also results of this work).

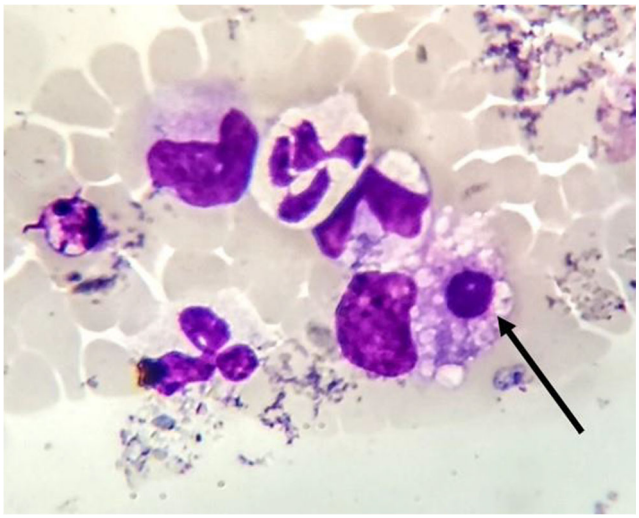

Fig. 1 Blood smear of the canine monocytic ehrlichiosis (CME) case (Giemsa staining). Monocytes with a structure compatible with ehrlichial morulae (see black arrow) and phagocytosis of nuclear material (typical vacuoles)

Results and discussion

A non-regenerative anemia was present with low Hb (2.8 g/dl), RBC (1.77 × 106/μl), PCV (13%), and a reticulocyte count of 0.3%. Thrombocytopenia (157.99 × 103/μl), eosinopenia (0 × 103/μl), and lymphopenia (0.689 × 103/μl) with presence of reactive lymphocytes were observed; band neutrophils were increased (1.68 × 103/μl). Morulae compatible with *Ehrlichia* were observed in monocytes in the stained blood smear (see Fig. 1). Alkaline phosphatase (1423 IU/L) and creatine phosphokinase (365 IU/L) were above reference values and total protein (5.36 g/dl), albumin (1.14 g/dl), and albumin to globulin ratio (0.27) were below reference ranges. The patient was treated with doxycycline with a favorable outcome being healthy 4 months after initial diagnosis.

Ehrlichial DNA was detected in the blood sample of the dog. The obtained partial sequence of the *dsb* gene (358 bp; GenBank accession number: MW201682) matched 100% with different sequences of *E. canis* detected in dogs from Argentina (MN617030), Brazil (GU586135), and Colombia (MK783023–MK783026). Three ticks, one larva, and two females, all identified as *R. sanguineus* s.s. (GenBank accession number of the 446 bp partial sequence of the 16S rDNA: MW202406), were collected on the dog and the two female specimens were deposited in the tick collection of the Instituto Nacional de Tecnología Agropecuaria (INTA; Rafaela, Santa Fe, Argentina) with the collection number INTA 2480. One fed female of *R. sanguineus* s.s. tested positive to *E. canis*. The *dsb* sequence (343 bp; GenBank accession number: MW201683) was identical to *dsb* partial sequence generated from canine blood and available in GenBank (selected sequences from South America with 100% identity: Argentina (MN617030), Brazil (GU586135, KP167596, MG772657), and Colombia (MK783023–MK783026)). These results can be visualized in a phylogenetic context (see Fig. 2). Both sequences clustered with of other sequences of *E. canis*, and the so formed cluster is clearly separated (bootstrap value 100) from other species of the genus *Ehrlichia*, with the nearest relative *E. minasensis*.

The clinical, hematological, and serum chemistry results are compatible with acute CME (Harrus et al. 2012), with the exception of the increase in creatine phosphokinase, which could be due to other undiagnosed concurrent tick-transmitted diseases (Gal et al. 2007), although other factors should not be ruled out. The presence of morulae in the monocytes which prompted the molecular investigation of *Ehrlichia* is an important aid in the diagnosis, yet its presence is quite fortuitous (Harrus et al. 2012). The partial sequence of *dsb* gene amplified from the DNA extracted from the blood sample of the dog was identical to other sequences of *E. canis* from different regions of the world. It must be mentioned that the *dsb* gene has low intraspecific polymorphism (Aguiar et al. 2013). However, it is useful for identification at species level.

CME is a tick-borne disease affecting dogs in different countries of the world, including Argentina. All the clinical cases of CME reported in Argentina were detected in localities

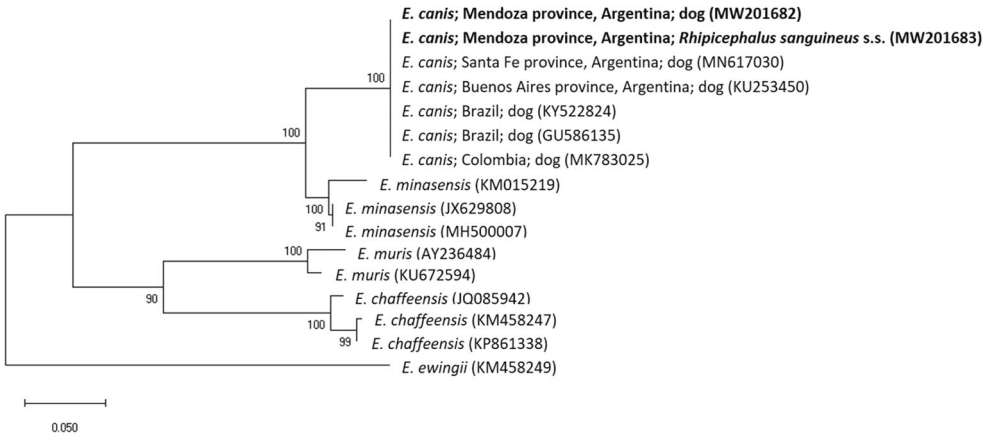

Fig. 2 Maximum-likelihood tree constructed from *dsb* partial gene sequences of different species of the genus *Ehrlichia*. Partial sequences generated in this study are written in bold letters. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are given in brackets

of Buenos Aires, Entre Ríos, Mendoza, and Santa Fe Provinces and in Buenos Aires city (Eiras et al. 2013, Cicuttin et al. 2016, 2017; Tarragona et al. 2019; Sánchez et al. 2020; this work), where *R. sanguineus* s.s. is the only representative taxon from the *R. sanguineus* group recorded so far (Nava et al. 2012, 2018; Cicuttin et al. 2017; Tarragona et al. 2019; this work). Additionally, Enriquez et al. (2019) could detect *E. canis*—by serological methods—in dogs from the Chaco province, where *R. sanguineus* s.s. also is endemic, although this record deserves further confirmation. In Argentina, both *R. sanguineus* s.s. and *R. sanguineus* s.l. "tropical lineage" are present (Nava et al. 2012, 2018). The tropical lineage of *R. sanguineus* s.l. is distributed in tropical areas of northern Argentina in Formosa and Salta provinces, while *R. sanguineus* s.s. is found in subtropical and temperate areas of Argentina (Nava et al. 2012, 2018). Interestingly, all reported cases of CME in Argentina occurred in areas where *R. sanguineus* s.l. "tropical lineage" is absent while *R. sanguineus* s.s. is endemic (Eiras et al. 2013; Cicuttin et al. 2017; Tarragona et al. 2019; Sánchez et al. 2020; this work). *Ehrlichia canis* was also found in *R. sanguineus* s.s. from USA (Tucker et al. 2020). Laboratory assays to test the vectorial competence of these two taxa from the *R. sanguineus* group showed that *R. sanguineus* s.s. (named as *R. sanguineus* "temperate lineage") has no competence to transmit *E. canis*, while *R. sanguineus* s.l. "tropical lineage" acts a competent vector (Moraes-Filho et al. 2015). Also in a study carried out by Sanches et al. (2018) on *R. sanguineus* s.s. (named as *R. sanguineus* "temperate lineage") form Portugal, the authors could not find *E. canis*. In Argentina, Cicuttin et al. (2015) analyzed the infection with *E. canis* in the both endemic lineage of *R. sanguineus* ("tropical lineage" = sensu lato; "temperate lineage" = sensu stricto; Nava et al. 2018) and could only find *E. canis* infections in *R. sanguineus* s.l. The reports of CME cases in areas of Argentina associated to the presence of *R. sanguineus* s.s. and the evidence presented in this work contrast the results of Cicuttin et al. (2015), Moraes-Filho et al. (2015), and Sanches et al. (2018). Cicuttin et al. (2017) have proposed three hypotheses to explain how *E. canis* could be transmitted in areas where only *R. sanguineus* s.s. is present. First, a dog acquires an infection in a *R. sanguineus* s.l. "tropical lineage" endemic region and maintained it after its transfer from one city to another. Second, *R. sanguineus* s.s. has a low, but not null, vector competence. Or third, *R. sanguineus* s.l. "tropical lineage" enters to *R. sanguineus* s.s. endemic zones with dog that migrated from the north of Argentina and that were kept during the favorable climatic season, not reaching establish themselves as stable populations, but sufficient to transmit *E. canis* to local susceptible hosts. In this study, *E. canis* was detected in a fed female of *R. sanguineus* s.s. Here, it must be mentioned that the detection of a tick-borne pathogen in an engorged tick is not necessarily an indication of the vector competence of the tick to transmit the respective pathogen. It could have been an ingestion of the pathogen by the tick during blood meal. However, due to the absence of *R. sanguineus* s.l. "tropical lineage" in Mendoza province, it could be assumed that *R. sanguineus* s.s. play a role in the natural transmission of *E. canis*, giving support to the second hypothesis of Cicuttin et al. (2017). Based on the results of this study, together with the findings of Eiras et al. (2013), Cicuttin et al. (2017), Tarragona et al. (2019), Sánchez et al. (2020), and Tucker et al. (2020) it can be assumed that *E. canis* does not only circulate in endemic zones of *R. sanguineus* s.s. but also that it is transmitted by this tick species in some areas of Argentina. To confirm this hypothesis, experimental studies regarding the vectorial competence of *R. sanguineus* s.s. to transmit *E. canis* must be performed by using strains isolated in *R. sanguineus* s.s. endemic areas. These studies can help to understand the role of *R. sanguineus* s.s.in the transmission of *E. canis*.

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Compliance with ethical standards

Animal ethics guidelines regarding animal care were strictly adhered. Ethics approval, protocol No. 158, was granted by the Institutional Animal Care and Use Committee (Comité Institucional de Cuidado y Uso de Animales en Investigación y Docencia) of Universidad Juan Agustín Maza, Mendoza.

Conflict of interest The authors declare that they have no competing interests.

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