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Two novel *Ehrlichia* (Rickettsiales: Anaplasmataceae) strains detected in ticks (Ixodida, Ixodidae) and opossums (Didelphimorphia: Didelphidae) in Argentina.

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ABSTRACT

The aim of this study is to determine if there is circulation of microorganisms of the genus *Ehrlichia* in opossums *Didelphis albiventris* and their ticks from the Humid Chaco in Argentina. Blood samples of 15 specimens of the opossum *D. albiventris* were analysed. Immature stages of the ticks *Amblyomma ovale* (Larvae=26; Nymphs=10), *Amblyomma sculptum* (Larvae=86; Nymphs=6) and *Ornithodoros* sp. cf. *O. mimon* (Larvae=90) were also analyzed. DNA was extracted individually from blood samples and ticks. Molecular detection of *Ehrlichia* agents was performed targeting two different loci: 16S rRNA and *dsb* gen. The phylogenetic analyses showed that the *Ehrlichia* sp. detected in *D. albiventris* in this study is identical to *Ehrlichia* sp. strain Natal previously detected in two marsupials from Brazil. Furthermore, a new *Ehrlichia* strain was amplified from an *A. ovale* nymph (named as *Ehrlichia* sp. strain El Bagual) which is phylogenetically closely related to a strain of *Ehrlichia* sp. detected in *Bradypus tridactylus* in Brazil. The findings of the current study represent the first report of these two strains of *Ehrlichia* for Argentina, showing that the diversity of *Ehrlichia* spp. is greater than previously assumed. Further studies should determine the epidemiological relevance of these findings.

KEYWORDS

Ehrlichia; *Amblyomma ovale*; *Didelphis albiventris*; Argentina

1- Introduction

Bacteria of the genus *Ehrlichia* are obligate intra-leukocyte microorganisms transmitted by hard ticks (Acari: Ixodida: Ixodidae) of importance in public and veterinary health. Six species of *Ehrlichia* are formally recognized: i) *Ehrlichia canis*, causative agent of canine monocytotropic ehrlichiosis (CME) transmitted by ticks from the *Rhipicephalus sanguineus* complex; ii) *Ehrlichia chaffeensis*, causative agent of human monocytic ehrlichiosis (HME) whose main vector is *Amblyomma americanum*; iii) *Ehrlichia ewingii*, a causative agent of canine and human granulocytic ehrlichiosis also transmitted by *A. americanum*; iv) *Ehrlichia muris*, affecting rodents and humans, associated to *Haemaphysalis flava and Ixodes ovatus*; v) *Ehrlichia minasensis*, involved as causative agent of Brazilian bovine ehrlichiosis and of unknown vector; and vi) *Ehrlichia ruminantium*, the causative agent of heartwater ruminant disease whose main vectors are *Amblyomma variegatum* and *Amblyomma hebraeum* (Dummler et al. 2001, Aguiar et al., 2019). Furthermore, in South America, several *Ehrlichia* sp. strains have been reported which still remain not formally named (Widmer et al. 2011, Almeida et al. 2013; Bezerra et al., 2017; Cicuttin et al., 2017, 2020, 2022; Soares et al., 2017; Lopes et al., 2018; Osorio et al., 2018; Monje at al., 2019; Muñoz-Leal et al. 2019; Eberhardt et al., 2020; Fargnoli et al., 2020; Felix et al., 2021a, b; Muraro et al., 2021;). Particularly in Argentina, five species of hard ticks have been reported infected with different strains of *Ehrlichia*: *Amblyomma tigrinum* and *Amblyomma parvum* with *Ehrlichia* sp. strain San Luis (closely related to *E. chaffeensis*) (Cicuttin et al., 2017; Monje et al., 2019), *A. tigrinum* with *Ehrlichia* sp. strain Iberá, *A. parvum* with *Ehrlichia* sp. strain Cordoba (Cicuttin et al., 2017; Eberhardt et al., 2020) and *Ehrlichia* sp. cf. *E. chaffeensis* (Tomassone et al., 2008), *Amblyomma triste* with *Ehrlichia* sp. strain Delta (closely

related to *E. chaffeensis*) (Cicuttin et al., 2020), and *Amblyomma neumanni* infected with *Ehrlichia* sp. strain La Dormida (Fargnoli et al., 2020).

Didelphis albiventris (Didelphimorphia: Didelphidae) is distributed from northeast and central Brazil to eastern Bolivia, Paraguay, Uruguay and central-northern Argentina (Gardner, 2008). *Didelphis albiventris* lives in rural, suburban, and even urban areas, which determines a substantial contact with man and domestic animals (Cáceres and Monteiro-Filho, 2006). For this reason, this species has the potential to act as a link between wild and domestic epidemiological cycles, which makes it of special importance for the ecology of tick-borne zoonotic diseases (Horta et al., 2009). In Argentina, this species is the host of different tick species: adults and immature stages of *Ixodes loricatus*, *Ixodes luciae*, *A. parvum*, *Amblyomma ovale*, and larvae of soft ticks (Acari: Ixodida: Argasidae) belonging to the genus *Ornithodoros* (Tarragona et al., 2018).

Although *D. albiventris* specimens naturally infected with *Ehrlichia* spp. were reported in Brazil (Lopes et al., 2018), the role of these hosts in the cycle of these microorganisms is unknown. The aim of this study is to determine if there is circulation of microorganisms of the genus *Ehrlichia* in opossums *D. albiventris* and their ticks from the Humid Chaco in Argentina.

2- Material and methods

2.1. Study area

Data were obtained from samples of *D. albiventris* specimens trapped at two sites in Formosa province, Argentina. One site was “Estancia El Bagual” (EB) (26°17'39.48''S;

58°50'55.32''W), a cattle farm. The second sample site was located at the "Reserva Privada El Bagual" (RPB) (26°17'19.32''S; 58°49'45.839''W), a protected area without entry of cattle for more than 30 years. Both sites belong to "Chaco Húmedo con Bosque y Cañadas" from the "Provincia Fitogeográfica Chaqueña" according to the phytogeographic definitions given Oyarzabal et al. (2018).

2.2. Sample collection

Didelphis albiventris specimens were captured by using Tomahawk live-traps on five occasions (2018: April, June, August, December and 2019: December). The traps were baited with chicken, apple and honey. The captured individuals were anesthetized at the sample site with ketamine 15 mg/kg and diazepam 0.5 mg/kg intramuscularly (Tarragona et al., 2014). Blood samples were obtained by puncture of the caudal vein in tubes containing sodium citrate anticoagulant. The age of the hosts was determined by using the formula of the upper jaw, which considers the eruption and tooth wear (Schweigmann et al., 1999). Each individual was sexed, measured, weighed and examined for ticks. After examination, the animal was released. Engorged ticks were collected alive, transferred to the laboratory and kept at 25 ºC and 83-86% RH for moult and morphological identification. Morphological identification of nymphs was made following Martins et al. (2014), Nava et al. (2017) and larvae according to Famadas et al. (1997), Barbieri et al. (2008), and relied by comparisons of partial sequences of the 16S rRNA gene according to Mangold et al. (1998) in cases where it was necessary.

2.3. Molecular detection of *Ehrlichia* sp.

DNA was extracted individually from blood samples of *D. albiventris* and ticks collected from hosts using the DNeasy Tissue Kit (Qiagen, Inc., Chatsworth, CA, USA). All DNA samples were processed individually, in the first instance, by real-time PCR assay amplifying a 177 bp fragment of the 16S rRNA gene from bacteria of the Anaplasmataceae family (Monje et al., 2019). Positive samples were further tested by conventional PCR to amplify a 345 bp fragment of the 16S rRNA gene (Parola et al., 2000) and a 409 bp fragment of the specific *dsb* gene of bacteria of the genus *Ehrlichia* (Doyle et al., 2005). In all PCR runs, DNA of *E. canis* was used as positive control, while ultrapure water acted as negative control. Positive All amplicons were purified and sequenced. The sequences 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 X. Substitution models were HYK and GTR (G + I) for 16S rRNA and *dsb,* respectively. Support for the topologies was tested by bootstrapping over 1,000 replications and gaps were excluded from the comparisons.

The study protocol was approved by the Animal Ethical Commission of the CICUAE-CERSAN of Instituto Nacional de Tecnología Agropecuaria (protocol no. P19-0012).

3- Results

A total of 15 specimens of *D. albiventris* (10 females, 5 males; 8 juveniles, 7 adults) were captured, and blood samples were collected from all specimens. Of the 15 individuals, 10 were parasitized with ticks.

Ticks collected on *D. albiventris* were determined as *A. ovale* (Larvae=26; Nymphs=10), *Amblyomma sculptum* (Larvae=86; Nymphs=6) and *Ornithodoros* sp. cf. *O. mimon* (Larvae=90) (Table 1). The partial 16S rDNA sequences of representative immature specimens of *A. ovale* and *A. sculptum* were deposited in GenBank by the following accession numbers: OP281207 and OP281206, respectively. The first showed 98.98% similarity with *A. ovale* from Argentina (NC050255) and 99.23% with that sequence of A. *ovale* from Brazil (KU894381). The second showed 97.57% identity with *A. sculptum* from Brazil (MG523424) and 98.3% with *A. sculptum* from Argentina (KT820361). The larvae of *Onithodoros* sp. cf. *mimon* have been morphologically compared with material deposited in ¨Tick Collection of Instituto Nacional de Tecnología Agropecuaria¨.

Ehrlichial DNA was detected in one blood sample of *D. albiventris* and in one nymph of *A. ovale.* Partial sequences of the *dsb* gene were obtained for both positive samples, while a 16S rRNA gene sequence was only generated for the positive blood sample of *D. albiventris* (Table 1). The ML phylogenetic tree for the 16S rRNA fragment shows that the *Ehrlichia* sp. detected in *D. albiventris* during this study (GenBank accession number: OP242179) is related to *Ehrlichia* sp. also detected in *D. albiventris* in Brazil[1] (91% bootstrap support, see Figure 1). According to the ML phylogenetic tree constructed with the *dsb* fragment (Figure 2), *Ehrlichia* sp. found in *D. albiventris* in Argentina (GenBank accession number: OP270482) is identical to *Ehrlichia* sp. strain Natal

[1] Although the authors deposited this sequence in GenBank as *E. ruminantium*, this sequence does not belong to *E. ruminantium* s.s., as evidenced in Figure 2.

previously detected in *D. albiventris* and *Gracilinanus agilis* from Natal (state of Rio Grande do Norte) and Caatinga (state of Pernambuco) of Brazil, respectively.

The phylogenetic analysis performed with *dsb* sequences demonstrates that the strain of *Ehrlichia* detected in the *A. ovale* nymph in this study named as *Ehrlichia* sp. strain El Bagual (GenBank accession number: OP270483) constitutes a clade (99% bootstrap support) with a strain of *Ehrlichia* sp. detected in *Bradypus tridactylus* from the state of Pará, Brazil (Figure 2).

4- Discussion

The findings of the current study represent the first report of *Ehrlichia* sp. strain Natal in *D. albiventris* from Argentina, and a new strain of *Ehrlichia* was found in an *A. ovale* nymph collected on *D. albiventris*. This is the first report of *A. ovale* associated with *Ehrlichia* sp. in the Neotropics. These results show a possible association that denotes specificity between *Ehrlichia* sp. strain Natal and Didelphidae family, because the same *Ehrlichia* was found in the same host species but with a wide geographical distance (more than 3,000 km), for the case of *D. albiventris* and of another marsupial as well as *G. agilis* (Lopes et al., 2018; Oliveira et al., 2020). Although André et al. (2022) recently reported populations of *D. albiventris* naturally infected with *Ehrlichia* sp. strian Natal in Mato Grosso do Sul, Brazil, by observation of *Ehrlichia* morulae in blood smears and DNA amplification. Since the amplified product was a different 16S rRNA fragment, it could not be compared with the sequence of *Ehrlichia* sp. Natal Argentina obtained in our study. Taking into account that in South America *D. albiventris* was reported as a potential reservoir of different pathogens that cause zoonosis like leishmaniasis,

leptospirosis, Chagas disease, toxoplasmosis, neosporosis, salmonellosis, brucellosis (De La Vega and Carrillo, 1979; Yai, 2003; Casagrande et al., 2011; Nantes et al., 2021) as well as a potential amplifying host of *Rickettsia rickettsii* (the causative agent of Brazilian Spotted Fever) (Horta et al., 2009), future studies are necessary to characterize the enzootic cycle of *Ehrlichia* sp. strain Natal and determine its potential pathogenicity for humans and animals.

Sequences analyses of *dsb* have showed that the bacteria detected in *A. ovale* ticks form a phylogenetic clade with *Ehrlichia* sp. strain detected in *B. tridactylus* from Brazil (Soares et al., 2017). It is probable that these two *Ehrlichia* sp. strains represent the same species, but further characterization is needed. In this case, the opposite seems to occur to the hypothesis proposed for the association *Ehrlichia* sp. stain Natal - Didelphidae. Two similar *Ehrlichia* are found in a tick associated to an opossum and in a large mammal (*B. tridactylus*) not phylogenetically related to opposums and from ecologically different sites. Furthermore, no infestation of *B. tridactylus* with *A. ovale* has been described so far (Guglielmone et al., 2021).

These results not only show that the diversity of *Ehrlichia* spp. is greater than previously assumed for South America, as has already been pointed out in previous studies, but also demonstrates the need to explore the evolutionary aspects related to the diversity of *Ehrlichia* spp. as well as the nature of the *Ehrlichia*-host relationship. Some authors suggest that some ehrlichial species exhibit substantial diversity (e.g. *E. chaffeensis* and *E. ruminantium*) while others are highly conserved (e.g. *E. canis*), which might be associated with the diversity of hosts and vectors of the organisms (Yu et al., 2007).

Future studies should be focused on these aspects in order to elucidate the pattern of *Ehrlichia* evolution and ecology.

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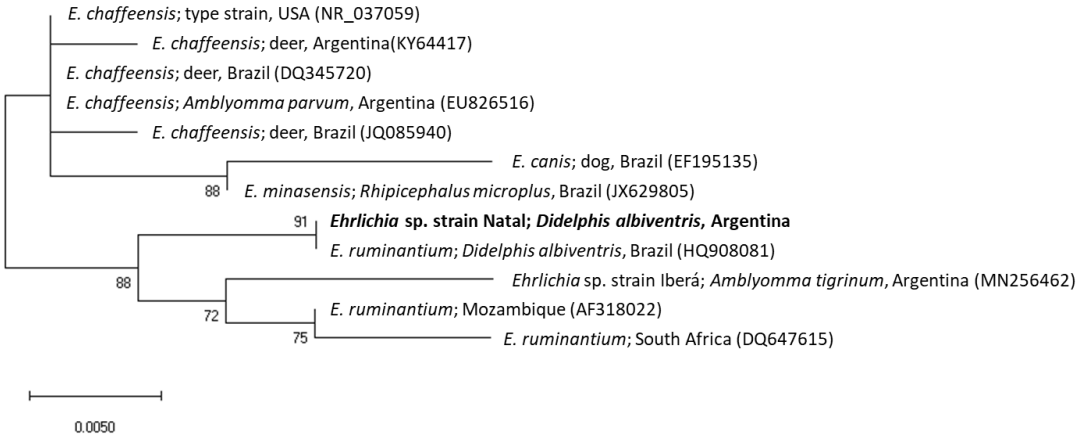

Figure 1. Maximum-likelihood tree constructed from 16S rRNA partial gene sequences of different species of the genus *Ehrlichia* (Substitution models were HYK). Partial sequence generated in this study is written in bold letters. Numbers represents bootstrap support generated from 1.000 replications. GenBank accession numbers are given in brackets.

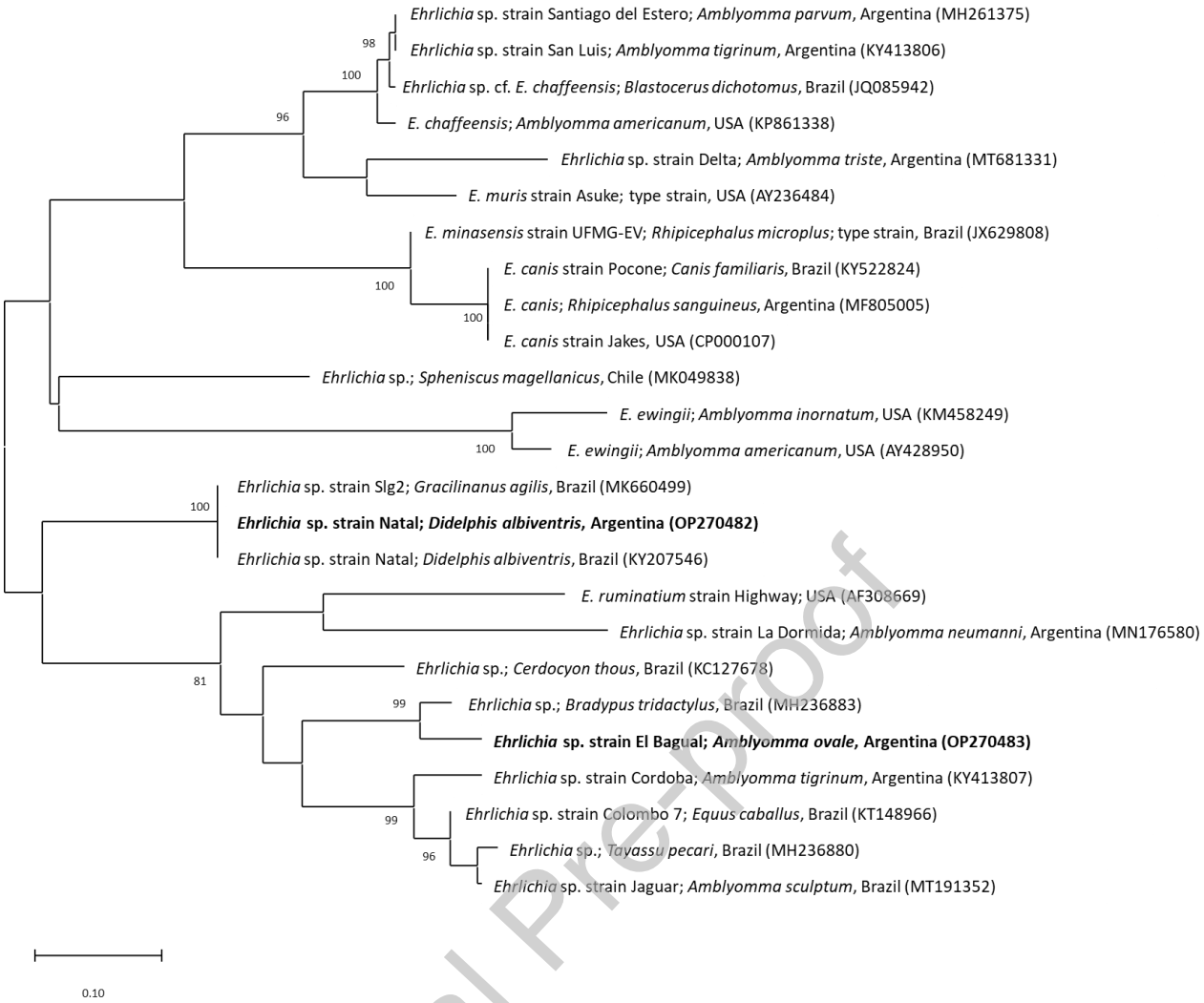

Figure 2: Maximum-likelihood tree constructed from dsb partial gene sequences of different species of the genus Ehrlichia (Substitution models were GTR (G + I)). Partial sequences generated in this study are written in bold letters. Numbers represents bootstrap support generated from 1000 replications. GenBank accession numbers are given in brackets.

Table 1. Tick specimens collected on *Didelphis albiventris* in "Estancia El Bagual" (EB) a farm with bovine and buffalo cattle and the "Reserva Privada El Bagual" (RPB) without cattle from "Chaco Húmedo con Bosque y Cañadas" of the "Provincia Fitogeográfica Chaqueña" according to Oyarzabal et al. (2018), Argentina. Age and sex (J: juvenile; A: adult; f: females; m: males) of each examined host is also indicated and results of the DNA pathogen detection in hosts blood and ticks are given.

					Amblyomma ovale		*Amblyomma sculptum*		*Ornithodoros* sp. cf. *O. mimon*		Blood sample PCR*		Ticks sample PCR*	
Date	ID	Site	Age	Sex	Larvae	Nymphs	Larvae	Nymphs	Larvae	Nymphs	16S rRNA	*dsb*	16S rRNA	*dsb*
2018-04-07	01/1º	RPB	J	F	7	3	4				0	0	0	1N†
2018-04-07	02/1º	RPB	A	F	12	3	2		31		0	0	0	0
2018-04-07	03/1º	RPB	A	F		1			39		0	0	0	0
2018-04-08	04/1º	RPB	A	F					4		0	0	0	0
2018-04-08	05/1º	RPB	J	M		1	2		2		1	1	0	0
2018-06-29	02	RPB	A	F		1	78		8		0	0	0	0
2018-08-31	03	RPB	A	F		1		3	6		0	0	0	0
2019-12-05	05	RPB	A	F				3			0	0	0	0
2019-12-05	06	RPB	J	M	3						0	0	0	0
2019-12-05	07	RPB	J	M	4						0	0	0	0
2019-12-05	08	RPB	J	M							0	0	0	0
2019-12-05	09	RPB	J	F							0	0	0	0
2019-12-06	10	EB	A	F							0	0	0	0
2019-12-07	11	RPB	J	F							0	0	0	0
2019-12-07	12	RPB	J	M							0	0	0	0

*Polymerase chain reaction. 16S rRNA and *dsb*= gene of bacteria of the Anaplasmataceae family and *Ehrlichia* gen, respectively. 0: negative PCR result; 1: positive PCR result. Specimens of *Ornithodoros* sp. cf. *O. mimon* were excluded of the *Ehrlichia* detection.

†*A. ovale* nymph; N= Nymph.