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Phylogenetic position of *Theileria cervi* detected in *Blastocerus dichotomus* (Artiodactyla: Cervidae) with clinical symptoms from argentina

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ABSTRACT

The results of this study document the molecular detection of *Theileria cervi* in a symptomatic adult marsh deer *Blastocerus dichotomus* (Artiodactyla: Cervidae) from Argentina and characterize the phylogenetic position of the Argentinian strain. The animal was founded with signs of obnubilation, anaemia, and ataxia on Isla Talavera in the Paraná Delta, Argentina. Biochemical, haematological and post mortem histopathological studies resulted in the detection of symptoms associated with *Theileria* infections. Piroplasmid DNA was detected in a blood sample and the complete 18S rDNA gene sequence could be archived. Phylogenetic analyses of the obtained sequence verify the genetic relationship of the Argentinian strain with strains of *T. cervi* found in other deer species in North America. This result, together with reports of *T. cervi* detected in various deer species that inhabit countries from Canada in the North to Argentina in the Western Hemisphere, indicates that this Piroplasmorida possess a low host specificity. Although the majority of *T. cervi* infections results asymptomatic or in mild course of the disease, it must be considered that *T. cervi* is circulating in Argentinian *B. dichotomus* populations and can cause serve course of the disease. Therefore, further studies are needed to investigate its prevalence, distribution and veterinary impact.

Introduction

The marsh deer *Blastocerus dichotomus* (Artiodactyla: Cervidae) is the largest species of the family Cervidae in South America. This deer species was originally distributed from the Andes in the West to the Brazilian Atlantic rainforest in the East and from the Amazon rainforest in the North to the Argentinian Pampa in the South (Weber and Gonzalez, 2003; Duarte et al., 2016). Nowadays, the number of marsh deer is largely reduced to isolated populations in river basins (e.g. Paraná river (Argentina, Brazil, Paraguay) and Guaporé River (Brazil, Bolivia)) and National parks. The International Union for Conservation of Nature and Natural Resources (IUCN) classifies *B. dichotomus* as a vulnerable species (Duarte et al., 2016). Tick-borne protozoa from the genera *Theileria* are obligate intracellular haemoparasites that can infect a broad range of domestic animals and wildlife, within ruminants acting as the main hosts (Mehlhorn and Schein, 1984; Bishop et al., 2004). According to their capability to produce indefinite proliferation of the infected host leukocytes, *Theileria* species can be divided into transforming and non-transforming types (Sivakumar et al., 2014). *Theileria cervi* (Bettencourt et al., 1907) was firstly detected and classified in the USA by Kreier et al. (1962) and Schaeffler (1962) in the white-tailed deer *Odocoileus virginianus* (Artiodactyla: Cervidae). Moreover, *T. cervi* infections are reported in different species of cervids (Artiodactyla: Cervidae), including elk (*Cervus canadensis*), mule deer (*Odocoileus hemionus*), axis deer (*Axis axis*), sika deer (*Cervus nippon*), pampas deer (*Ozotoceros bezoarticus*), and marsh deer (*B. dichotomus*) (Silveira et al., 2011, 2013; Wood et al., 2013). Most reports of the detection of *T. cervi* are derived from North-America (Canada and USA). Further, some studies show the detection of *T. cervi* in Brazil (Silveira et al., 2011) and Mexico (Pavón-Rocha et al., 2020). In Argentina, Orozco et al. (2020) describe the presence of *T. cervi* in *B. dichotomus*. The main vector of *T. cervi* in North-America is *Amblyomma americanum* (Goddard and Varela-Stokes, 2009), but the detection of the protozoa is also reported in *Dermacentor nitens* (Olafson et al., 2020). *B. dichotomus* is known to be parasitized by various tick species of the genera *Amblyomma*, *Dermacentor* and *Rhipicephalus* (Nava et al., 2017; Guglielmone et al., 2021). Although Mans et al. (2015) considered *T. cervi* as a less pathogenic *Theileria* species and

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case reports are rare, clinical symptoms including anaemia, weakness, fever, liver failure, and death could be observed in deer populations that are confronted to a combination of stress factors and high levels of infection (Yabsley et al., 2005; Wood et al., 2013; Haus et al. 2018). The aim of the present study was to report a clinical case of a *Theileria* infection, the molecular detection and the characterization of the phylogenetic position of *T. cervi* in *B. dichotomus* from Argentina.

Material and methods

On 25th July 2020, an adult female specimen of *B. dichotomus* (70 kg) was found with signs of obnubilation, anaemia, and ataxia on Isla Talavera in the Paraná Delta, Argentina (Campana; S 34° 00′00″; W 58°59′20″). After capturing, the animal was transferred to the animal hospital of the Temaikèn foundation (Fundación Temaikèn, Bioparque, Belén de Escobar, Buenos Aires, Argentina). Haematological and biochemical analyses were carried out by private laboratory. The animal died on 28th September 2020 and post mortem histopathological studies were performed by the veterinarians. Whole blood samples were taken and sent to Instituto de Investigación de la Cadena Láctea (IDICAL-Rafaela, Argentina) for the molecular detection of Apicomplexa haemoparasites. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. To detect piroplasmid DNA, a PCR assay described by Soares et al. (2011), amplifying a 600 kb fragment of the 18S rRNA gene, was applied. To archive a complete sequence of the 18S rRNA gene, a battery of three PCR assays was carried out according to Thompson et al. (2018). In all PCR runs, DNA of *Babesia bovis* was used as positive control, while ultrapure water acted as negative control. Positive PCR amplicons 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. The obtained DNA sequences were edited using BioEdit Sequence Alignment Editor (Hall, 1999) with manual edition whenever it was necessary and aligned with the program Clustal W (Thompson et al., 1994). Phylogenetic analyses were carried out with the maximum-likelihood (ML) method. The best-fitting substitution model was determined with the Akaike Information Criterion using the ML model test implemented in MEGA X (Kumar et al., 2018). Branch support was tested by bootstrap analysis using 1000 replicates and gaps were excluded in the pairwise comparisons. A 18S rDNA sequence of *T. equi* (GenBank accession number: MW872317) was chosen as outgroup.

Results and discussion

According to the results of the biochemical and haematological analyses, the female specimen of *B. dichotomus* presented erythropenia, leukopenia and a low haematocrit value (see Table 1). These findings can be related with the observed symptoms of obnubilation, anaemia, and ataxia. Further, the merozoites of *Theileria* spp. were observed in blood smear (Fig. 1). Post mortem histopathological studies determined that the animal died of multiple organ failure, due to advanced deterioration of the general condition, caused by chronic damage of the brain, with generalized atrophy of the brain and cerebellum. The observed symptoms can be related to the findings by Yabsley et al. (2005). Piroplasmid DNA could be detected in the blood sample of the analysed *B. dichotomus*. A 561 bp fragment of the 18S rRNA gene (GenBank accession number: will be created in case of the acceptance of the manuscript) could be sequenced and identified as *T. cervi*. Hereby, sequence similarity up to 98.57 % with *T. cervi* strains detected in white-tail deer and elks (e.g. GenBank accession numbers: AY735122 and AY735128) and in *D. nitens* larvae collected on white-tail deer in the USA (GenBank accession numbers: MW008519 and MW008528) were observed. The complete 18S rRNA gene (1677 bp) could be constructed by combining the partial sequences of three fragments previously amplified by PCR (GenBank accession number: will be created in case of the acceptance of the manuscript). This sequence also shows sequence similarities up to 98.39 % with the same *T. cervi* strains named above. The phylogenetic analysis of the complete 18S rDNA sequences is illustrated in Fig. 2. It can be observed that the sequence generated in this study forms part of a clade together with *T. cervi* strains detected in white-tailed deer and elk in Canada and the USA as well as in *D. nitens* in the USA. This clade separates with a bootstrap-value of 89 from the other *Theileria* species. Within the *T. cervi* clade, two subclades are present (bootstrap-values 76 and 85). The separation of this clade could be explained with the findings of Chae et al. (1999) that describe the presents of two different types of 18S rDNA sequences in *T. cervi* in North-American strains. These two types – F and G (with subtypes G1, G2 and G3) – show a difference of approximately 45 base pairs from each other. According to this classification, the Argentinian strain belongs to the F type of *T. cervi* sequences (GenBank accession number: U97054). Another clade of *Theileria* sp.[1] could be localized in close phylogenetic relation to *T. velifera* (GenBank accession number: JN572703) found in African buffalo (*Syncerus caffer*) from South Africa (Chaisi et al., 2013). All these strains were detected in *Cervus nippon* from China and named as *T. cervi* by the authors (He et al., 2012; Liu et al., 2016). Already in (2002), Gubbels et al. hypothesized that *Theileria* sp. found in *C. nippon* from China and Japan are certainly different from the *T. cervi* strains found in the Western Hemisphere. This hypothesis can be corroborated by the results of the phylogenetic analyses carried out in this study. Unfortunately, only partial sequences of the 18S rRNA gene from *T. cervi* strains detected in Brazil by Silveira et al. (2011, 2013) and Mex-

Table 1
Biochemical and haematological values of *Blastocerus dichotomus* infected with *T. cervi* from Isla Talavera in the Paraná Delta, Argentina.

Parameter[1]		Parameter[1]	
Haematogram		Biochemical blood profile	
Haematocrit (%)	27	Urea (mg/dl)	40
Erythrocytes/mm^3	4,900,000	Creatinine (mg/dl)	2.29
Leukocytes/mm^3	3,200	AST/ASA/GOT (IU)	104
Haemoglobin (g/dl)	9.5	ALT/ALA/GPT (IU)	11
MCV (μm^3)	55.1	Alkaline phosphatase (IU)	32
MCH (%)	193.87	Amylase (IU)	183
MCHC (g/dl)	351.85	Albumin (g%)	2.3
		Cholesterol (mg/dl)	60
Relative leukocyte formula	Calcium (mg/dl)	7.3	
Neutrophils (encased, %)	0	Magnesium (mg/dl)	3.86
Neutrophils (segmented, %)	48	Lactate dehydrogenase (LDH, IU)	289
Eosinophils (%)	2	Bilirubin (total, mg/dl)	0.8
Basophils (%)	0	Bilirubin (direct, mg/dl)	0.2
Lymphocytes (%)	46	Bilirubin (indirect, mg/dl)	0.6
Monocytes (%)	4	Phosphate (mg/dl)	5.9
Platelets/mm^3	807,000	Proteins (total, g%)	6.7
Blood smear	Maltese cross		
		Serum ionogram	
		Sodium (mEq/l)	133
		Potassium (mEq/l)	4.3
		Chlorine (mEq/l)	98
		Leptospirosis (serological test)	no reaction

[1] IU = international units; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; AST/ASA/GOT = aspartate aminotransferase; ALT/ALA/GPT = alanine aminotransferase.

[1] Although the authors deposited these sequences in GenBank as *T. cervi*, this sequence does not belong to *T. cervi*, as evidenced in Fig. 2.

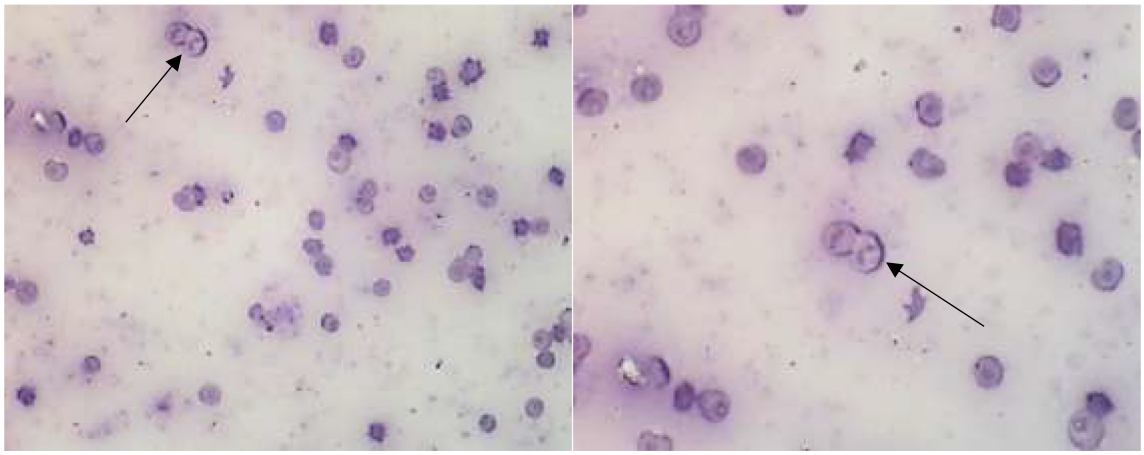

Fig. 1. Merozoites of *Theileria* (black arrows) in blood smear of *Blastocerus dichotomus* from Isla Talavera in the Paraná Delta, Argentina (Giemsa staining).

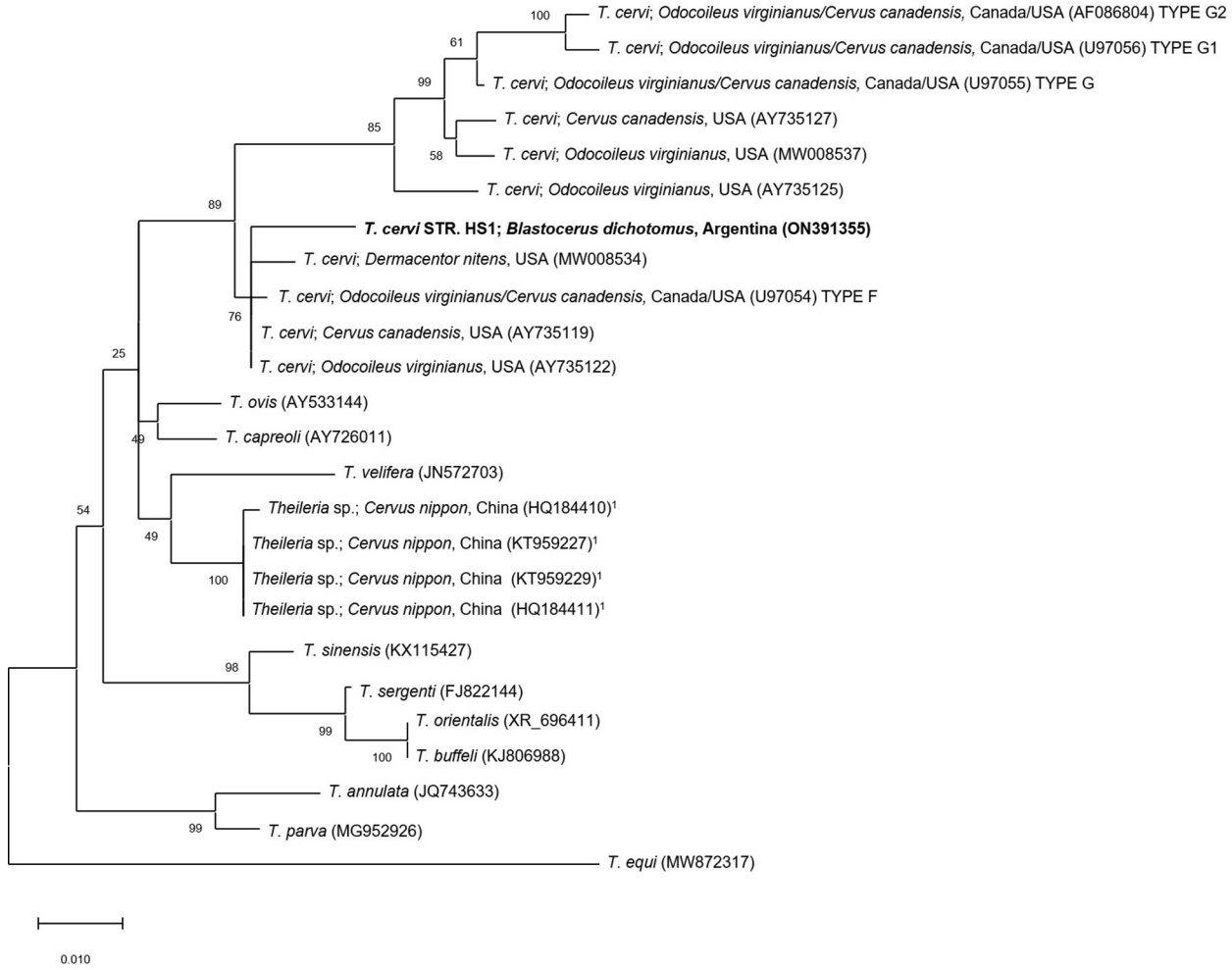

Fig. 2. Maximum-likelihood tree constructed from 18S rDNA sequences for different *Theileria* species (Substitution model: Generalised time-reversible model with Gamma distribution and invariant sites). Complete sequence generated in this study is written in bold letters. Numbers represents bootstrap support generated from 1000 replications. GenBank accession numbers are given in brackets. [1] Sequences deposited as *T. cervi* by the authors.

ico (Pavón-Rocha et al., 2020) are deposited in the GenBank database. Thus, it was not possible to include these strains in the phylogenetic analysis. However, sequence comparison of the partial sequence of the Argentinian strain, resulted in the detection of sequence homologies from 96.3 % with *T. cervi* detected in *Odocoileus virginianus* (Mexico; GenBank accession number MT373536) to 98.3 % with *T. cervi* detected in *Ozotoceros bezoarticus* (Brazil; GenBank accession number JX274285). Recently, Orozco et al. (2020) presented the first results

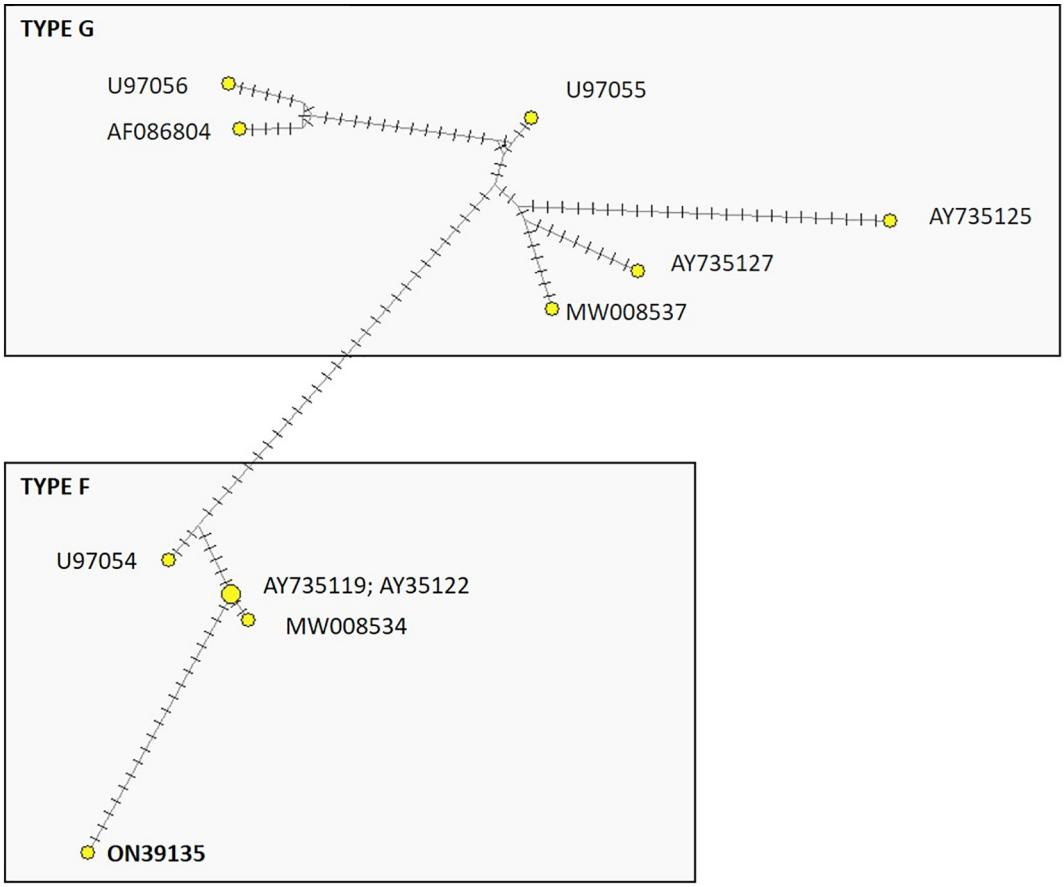

Fig. 3. DNASP analyses of the different 18S rDNA sequence types of *Theileria cervi*. The GenBank accession numbers are equal to them used for the construction of the Maximum-likelihood tree (see Fig. 2). Complete sequence generated in this study is written in bold letters.

on *B. dichotomus* morbidity and mortality surveillance in Argentina. Within this study, the authors report the molecular detection of *T. cervi* in 21 of 40 tested marsh deer (52.5 %), but no DNA sequences are available so that these reports from Argentina also could not be included in our analysis. High infection rates as in the Argentinean study, were also reported in the USA in white-tailed deer (57 %, Robinson et al., 1967; 97.6 % in wild and 40.4 % in farmed animals; Cauvin et al., 2019). The fact that these high infection rates, together with stress factors as malnutrition, secondary infection, or high population densities, can result in severe clinical diseases should be considered important in the surveillance of *T. cervi* in deer populations (Cauvin et al., 2019). The reports of *T. cervi*, detected in different deer species that inhabit countries from Canada in the North to Argentina in the South of Western Hemisphere, indicate that this Piroplasmorida possess a low host specificity (Fig. 3).

Conclusion

The results of this study document the first detection of *T. cervi* in *B. dichotomus* in Argentina based on the complete 18S rDNA sequence associated with the symptomatic case and observation of the etiological agent in erythrocytes by blood smear. Phylogenetic analyses showed that the Argentinian strain is closely related to *T. cervi* strains from North-America. This result, together with the findings by Orozco et al. (2020), suggests that *T. cervi* is endemic in Argentina. Further studies focusing on the prevalence, the geographical distribution, the potential vectors, and the genetic variety must be carried out to archive a more complete overview about *T. cervi* infections in Argentina.

Ethical statement

The authors assert that all procedures contributing to this work comply with the ethical standards of International Guiding Principles for Biomedical Research involving Animals.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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