

MOLECULAR EVIDENCE OF *THEILERIA ORIENTALIS* INFECTION IN CATTLE FROM BOSNIA AND HERZEGOVINA

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Abstract

There are no data on the distribution of oriental theileriosis in cattle from Bosnia and Herzegovina. For the first time, a possible endemic focus of *Theileria orientalis* infection was confirmed in specific areas of Pale municipality, Sarajevo-Romanija region, Bosnia and Herzegovina. Selective sampling included 30 cattle from 10 smallholder farms in several locations in Pale municipality. The total of 30 whole blood samples were screened for the presence of piroplasmids using commercial PCR. Positive PCR products were sequenced in both directions, the sequences were analyzed and a phylogenetic tree was created. Piroplasm (*Babesia/Theileria*-specific) DNA fragments were detected in 13/30 examined cattle (43%). At the farm level, PCR-positive animals were identified in 6/10 examined farms (60%). Upon sequence analysis, the species *Theileria orientalis* was confirmed. This survey reports a high rate of PCR-positive cases of bovine piroplasmidosis and provides the first description of *Theileria orientalis* in Bosnia and Herzegovina.

Key Words: Bovine piroplasmidosis, Bosnia and Herzegovina, PCR, *Theileria orientalis*

INTRODUCTION

In general, the term piroplasmidosis is used for the tick-borne diseases caused by piroplasmids of the genera *Babesia* and *Theileria*. On the other hand, benign or oriental

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theileriosis of cattle is a protozoan disease caused only by the *Theileria orientalis* complex-group (*Theileria orientalis/buffeli/sergenti*). The life cycles of piroplasms from the *Theileria orientalis* group are indirect and involve ticks of the genera *Amblyomma*, *Haemaphysalis* and *Rhipicephalus* (Watts et al., 2016; Hornok et al., 2014; Toma et al., 2017). *Theileria orientalis* is a widespread piroplasm, and literature data describe clinical outbreaks of oriental theileriosis with high economic losses in New Zealand and Australia (McFadden et al., 2013; Kamau et al., 2011). In Europe, recent surveys indicate the presence of *Theileria orientalis* in Italy (Ceci et al., 1997), Greece (Papadopoulos, 1999), Spain (García-Sanmartín et al., 2006), Portugal (Gomes et al., 2013), Romania (Ionita et al., 2013), Hungary (Hornok et al., 2014), the United Kingdom (Fernández de Marco et al., 2016), Serbia (Vasić et al., 2018), Croatia (Jurković et al., 2020) and Russia (Kovalchuk, 2022). Furthermore, *Theileria orientalis* was recorded in asymptomatic cattle, demonstrating a very low prevalence in a cross-sectional study (5/135, 3.70%) from neighbouring Serbia (Vasić et al., 2018). However, a case study from Croatia documented that *Theileria orientalis* as a co-infection with *Anaplasma marginale* and *Anaplasma bovis* contributed to the lethal outcome in three cows (Jurković et al., 2020). Hornok et al. (2014) reported the clinical significance of *Theileria buffeli* (part of the orientalis group) in a herd of beef cattle kept in northeastern Hungary. It seems that oriental theileriosis is not yet an issue in Europe, probably due to endemic stability and high infection rates in cattle populations without clinical cases, but some sporadic cases of diseased animals can be expected (Hornok et al., 2014; Jurković et al., 2020).

Hereby, we report a high prevalence of bovine piroplasmosis in the small geographical area of the Sarajevo-Romanija region (Bosnia and Herzegovina), with molecular confirmation of *Theileria orientalis* presence in grazing cattle from smallholder farms.

MATERIALS AND METHODS

Selective sampling performed in November and December 2021 included 30 cattle from 10 farms in several villages from the municipality (epidemiological unit) of Pale, Sarajevo-Romanija region, Republika Srpska (Bosnia, and Herzegovina). Samples were taken from the animals during a formal epizootiological examination by officials of the Veterinary Institute “Dr Vaso Butozan”. The owners gave consent for the study, and the blood samples were obtained within the regulations for animal welfare (Official Gazette of the BiH, 25/2009 and 9/2018). The study area was determined according to data taken from the official veterinarian. Namely, cases of pyrexia (3/10, 30% farms), cachexia (1/10, 10% farms), reproductive disorders (5/10, 50% farms), locomotor problems (1/10, 10% farms), diarrhoea (1/10, 10% farms) and haemoglobinuria (1/10, 10% farms) were recorded in the locations of Saice (43°44'16.4"N 18°38'01.5"E), Podgrab (43°45'15.6"N 18°41'48.4"E), Pale (43°48'42.0"N 18°33'00.6"E) and Mokro (43°53'30.2"N 18°37'54.5"E). Also, on one farm in Saice, two cows died in 2021 without specific clinical signs, and the causes of the deaths remained undetermined. In 9 of the 10 selected farms, the owners confirmed that their animals were exposed to

ticks during the grazing season. All 10 visited farms were in the smallholding extensive system, with an average number of three animals per household (from 1 to 8). The age of the examined cattle in this survey was from 1 to 10 years. Within-herd, cattle for examination were chosen randomly. None of the examined animals were purchased or introduced from other regions or/and countries. Out of the total of 30 sampled animals, 27 were female, while the three males were sampled from one farm. Only one seven-year-old dairy cow showed clinical signs of disease during the farm visit, while the other 29 remained asymptomatic. The observed clinical symptoms were nonspecific, including pyrexia, leg swelling, lameness, apathy, anorexia and paresis. The animal had been bought from a smallholder in a neighbouring village in the same municipality. Shortly after sampling, this cow died, and necropsy revealed bilateral purulent nephritis caused by *Streptococcus* spp. as the cause of death.

Whole blood (3 ml of EDTA blood) was sampled from the cattle by aseptic coccygeal vein puncture. No ticks were recorded during blood sampling. Samples were sent to the Laboratory for Virology and Molecular Diagnostics (PI Dr. Vaso Butozan Veterinary Institute, Banja Luka, Bosnia and Herzegovina). Nucleic acid extraction from whole blood samples was performed using Indi Spin Pathogen Kit (Qiagen GmbH for Indical Bioscience) according to the manufacturer's instructions. Piroplasm (*Babesia/Theileria*) DNA was detected by PCR using previously described primers (BJ 5'-GTC TTG TAA TTG GAA TGA TGG-3' and BN2: 5'-TAG TTT ATG GTT AGG ACT ACG-3'). Visualized PCR products were in the form of a 452bp band corresponding to the specified fragment of the small subunit ribosomal RNA gene (Casati et al., 2006; Hornok et al., 2014). A DNA extract of sheep blood, previously confirmed positive for *Babesia ovis* by sequencing (GenBank acc. no. MZ853105), served as the positive PCR control. Representative positive PCR products (strong bands of DNA on agarose gel) were sent for sequencing to the Center for Human Molecular Genetics, Faculty of Biology, the University of Belgrade, Serbia. The obtained sequences were analyzed in both directions, and consensus sequences were created to be used for further analysis (Stevanovic et al., 2022). Phylogenetic analysis was performed in MEGA 7 using the Neighbor-Joining method (Tamura-Nei model) and a bootstrap value of 1000 replications (Kumar et al., 2016).

RESULTS AND DISCUSSION

Babesia/Theileria-specific DNA fragments were recorded in 13 of 30 examined cattle (43%). At the farm level, PCR-positive animals were detected on 6 of the 10 examined farms (60%). Furthermore, piroplasm DNA was found in cattle originating from three farms (3/3, 100%) with a history of pyrexia cases. Also, piroplasm DNA was determined in one cow from a farm with previously registered cases of haemoglobinuria, as well as on all farms where the owners stated that cattle were exposed to ticks on pasture. The within-herd piroplasm prevalence ranged from 0 on farms in Mokro and Pale to 62.5% on the farm in Saice. Most PCR-positive farms were located in Saice and

Podgrab (6/6, 100%). Moreover, the clinically suspect dairy cow (which later died), originating from the farm in Saice with the highest within-herd prevalence of bovine piroplasmosis, was PCR-positive.

The obtained sequences of the small subunit ribosomal RNA gene fragments were analyzed using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), which confirmed the detected piroplasm species as *Theileria orientalis*. The nucleotide homology of the analyzed sequences with previously reported sequences from Croatia, South Korea, Australia, Turkey, Pakistan, India Myanmar, Bangladesh, Russia and Iraq ranged from 99 to 100%, so our sequences were grouped together with these on the phylogenetic tree (Figure 1). These partial *Theileria orientalis* sequences from Bosnian cattle were submitted to GenBank (ON148460, ON148461, ON148462 and ON148463).

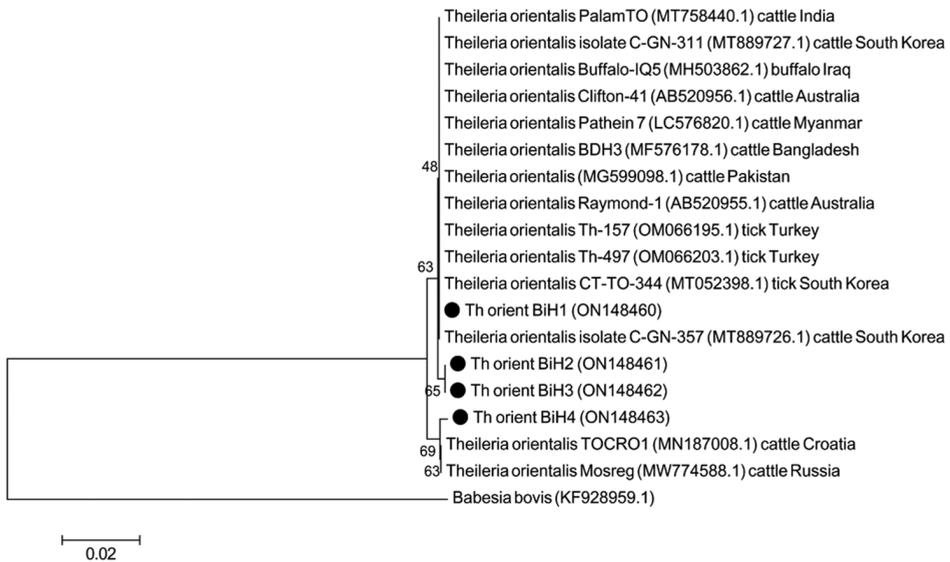


Figure 1. Phylogenetic tree based on the small subunit ribosomal RNA gene fragment of *Theileria orientalis* from Bosnia and Herzegovina (marked by black circles) and analogous sequences retrieved from the GenBank database. *Babesia bovis* was used as the outgroup.

The most problematic task for the field veterinarian is to assess the clinical significance of an established pathogen in animals with non-specific signs of disease. This was also evident upon analyzing the presence of piroplasms from the *Theileria orientalis* group in the investigated geographical location. *Theileria* piroplasms are generally not eliminated from circulation after they cause infection, and parasitaemia persists in infected animals for prolonged periods, even for life if the carrier state is established (Watts et al., 2016). *Theileria buffeli* was found in anaemic beef cattle kept on pasture (Hornok et al., 2014); however, based on our field observations, it is difficult to associate the appearance of anaemia in cattle kept in extensive conditions with pathogens of low virulence, since animals on our mountain pastures are exposed to parasitism and poor nutrition,

affecting their health status in different ways. Although our study describes a high number of PCR-positive piroplasmosis cases in cattle originating from farms with a history of febrile episodes, the clinical significance of such findings is challenging to interpret due to the lack of specific clinical presentation and laboratory tests.

This report has epidemiological significance because it helps to fill the current knowledge gap concerning the distribution of bovine piroplasmosis in Bosnia and Herzegovina and the Balkans. The high number of positive cattle in the locations of Saice and Podgrab indicates the possible presence of an endemic focus of bovine piroplasmosis. Furthermore, the large percentage of infected cattle without any recorded increase in clinical incidence in these areas could be an indicator of the endemic stability of oriental theileriosis (Hornok et al., 2014), or the relatively low pathogenicity of the *Theileria orientalis* genotypes present.

The presence of clinically significant infections with *Babesia divergens* and haemotropic mycoplasmas transmitted by *Ixodes ricinus* ticks has already been described in other areas of Bosnia and Herzegovina (Stevanović et al., 2020). Since small farm owners often refuse to pay for additional diagnostic procedures, local veterinarians are advised to empirically apply oxytetracycline along with the available acaricidal therapy in suspected cases of oriental theileriosis. Although the effectiveness of oxytetracycline against *Theileria orientalis* is questionable (Yam et al., 2018), there are no alternative options for veterinarians from our region. More effective drugs (buparvaquone and parvaquone) for the therapy of oriental theileriosis are not officially registered in Bosnia and Herzegovina.

CONCLUSION

This report provides the first finding of *Theileria orientalis* in Bosnia and Herzegovina. The high number of PCR-positive animals in an isolated geographical area indicates that these types of piroplasms are likely widespread and remain underreported, but there is no evidence of disease emergence or re-emergence. It is necessary to continue further investigations to assess the epidemiological and clinical significance of bovine piroplasmosis in a broader area of Bosnia and Herzegovina.

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Authors' contributions

Oliver Stevanovic: sampling, epidemiological analysis, PCR, writing the manuscript; Andrea Radalj: phylogenetic analysis and editing the final version of manuscript;

Competing interests

Authors declare that they have no competing interests.

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MOLEKULARNI DOKAZ INFEKCIJE SA *THEILERIA ORIENTALIS* KOD GOVEDA U BOSNI I HERCEGOVINI

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Kratak sadržaj

Nema podataka o rasprostranjenosti orijentalne teilerioze kod goveda iz Bosne i Hercegovine. Prvi put je potvrđeno moguće endemsko žarište infekcije *Theileria orientalis* na pojedinim područjima opštine Pale, Sarajevsko-romanijske regije, Bosne i Hercegovine. Selektivnim uzorkovanjem obuhvaćeno je 30 goveda sa 10 malih gazdinstava na nekoliko lokacija u opštini Pale. Ukupno je pregledano 30 uzoraka pune krvi pr na prisustvo piroplazmi pomoću komercijalnog PCR-a. Pozitivni PCR proizvodi su sekvencionirani u oba smera, sekvence su analizirane i formirano je filogenetsko stablo. Fragment DNK piroplazmi (*Babesia/Theileria* – specifičan) otkriven je kod 13/30 pregledanih goveda (43%). Na nivou farme, PCR pozitivne životinje su identifikovane u 6/10 ispitanih farmi (60%). Analizom sekvence potvrđena je vrsta *Theileria orientalis*. Ovo istraživanje izveštava o visokoj stopi PCR pozitivnih slučajeva goveđe piroplazmoze i predstavlja prvi opis *Theileria orientalis* u Bosni i Hercegovini.

Ključne riječi: piroplazmoza goveda, *Theileria orientalis*, PCR, Bosna i Hercegovina