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ORIGINAL PAPER

Tick infestation and occurrence of *Anaplasma phagocytophilum* and piroplasms in cattle in the Republic of Serbia

Ana Vasić[1,2] · Marion Nieder[3] · Nemanja Zdravković[1,4] · Jovan Bojkovski[1] · Dejan Bugarski[5] · Ivan Pavlović[4] · Cornelia Silaghi[2,6,7,8]

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Abstract

Ticks transmit important pathogens affecting cattle such as intracellular bacteria of the genus *Anaplasma* or protozoa of the order *Piroplasmida*. This study aimed at assessing tick species present on pastures and cattle and determining occurrence of the tick-borne pathogens *Anaplasma phagocytophilum* and *Babesia* spp. in cattle. During May and June 2013, ticks and EDTA blood were collected from 185 dairy cows at six locations in Serbia. Ticks were also collected directly from the pastures at four of these locations. The occurrence of *A. phagocytophilum* was investigated by serological (immunofluorescence antibody test (IFAT)) and molecular methods (real-time PCR) and of piroplasms by traditional PCR followed by sequencing. The most prevalent tick species on animals was *Ixodes ricinus*, ($n = 167$), followed by *Haemaphysalis punctata* ($n = 146$) and *Dermacentor marginatus* ($n = 122$). On the pasture, however, the most common species was *H. punctata* ($n = 41$), *I. ricinus* ($n = 37$), and *D. marginatus* ($n = 2$). Altogether, 4 out of 163 (2.45%) serum samples of cows were positive for *A. phagocytophilum*-specific antibodies by IFAT. However, the 135 blood samples tested for *A. phagocytophilum* DNA were all negative. Altogether, 5 out of these 135 samples were positive for piroplasm DNA. PCR products were sequenced and identified as a benign *Theileria* spp. with 100% identity with GenBank entries from Italy (*Theileria sergenti*), China (*Theileria* spp.), and Korea (*Theileria buffeli* isolate HS252). The results provide evidence for the presence of several hard tick species infesting cattle in Serbia which can carry pathogens potentially influencing animal health, as well as evidence of contact with tick-borne pathogens *Theileria* spp. and *A. phagocytophilum*.

Keywords Dairy cows · Tick-borne pathogens · *Ixodes ricinus* · *Dermacentor marginatus* · *Haemaphysalis punctata* · Serbia

✉ Ana Vasić
Ana.Vasic@fli.de

1 Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia

2 Institute of Infectology, Friedrich- Loeffler-Institut, Südufer 10, 17493 Greifswald Insel-Riems, Germany

3 Institute of Animal Hygiene and Veterinary Public Health, University of Leipzig, Leipzig, Germany

4 Scientific Veterinary Institute of Serbia, Belgrade, Serbia

5 Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia

6 National Center of Vector Entomology, University of Zürich, Zürich, Switzerland

7 Comparative Tropical Medicine and Parasitology, Ludwig-Maximilians-Universität München, Munich, Germany

8 Ernst-Moritz-Arndt-Universität, Greifswald, Germany

Introduction

Hard ticks are important vectors of many viral, bacterial, and protozoan pathogens such as Crimean-Congo hemorrhagic fever virus, tick-borne encephalitis virus, *Anaplasma* spp., *Rickettsia* spp., *Borrelia* spp., *Francisella tularensis*, *Babesia*, and *Theileria* species (de la Fuente et al. 2008). The tick biodiversity in Serbia includes hard ticks of the genera *Ixodes*, *Dermacentor*, *Hyalomma*, *Haemaphysalis*, and *Rhipicephalus*. The dominant tick species collected from sheep, goats, and dogs was *Ixodes ricinus* followed by occurrence of, for example, *Dermacentor marginatus*, *Dermacentor reticulatus*, *Rhipicephalus sanguineus*, *Rhipicephalus bursa*, *Haemaphysalis punctata*, *and Haemaphysalis inermis*. However, in some geographical areas of Serbia, *R. sanguineus* was described as the most abundant species on dogs (Milutinović and Radulović 2002; Pavlović et al. 2016; Becskei et al. 2015; Potkonjak et al. 2016).

Anaplasma spp. are etiologic agents of veterinary diseases affecting domestic ruminants, equines, dogs, and cats worldwide. A zoonotic role has been recognized for *A. phagocytophilum*, the causing agent of granulocytic anaplasmosis in humans, dogs, horses, and cats and of tick-borne fever in cattle and sheep (Silaghi et al. 2017). The main vector species of *A. phagocytophilum* in Europe is the tick *I. ricinus* (Stuen et al. 2013). In cattle, *A. phagocytophilum* infection causes thrombocytopenia, erythropenia, and leukopenia and a decrease in hematocrit and hemoglobin concentration (Pusterla et al. 1997). Furthermore, high fever, a sudden decreased milk production, lower limb edema with stiff walking, eye and nasal discharge, and depression were recorded in recent clinical cases in North-West Germany (Nieder et al. 2012).

In Europe, some piroplasms can cause serious disease in cattle (e.g., *Babesia bovis*, *Babesia divergens*, and *Babesia bigemina*) whereas others are considered benign *(Babesia major*, *Theileria orientalis*/*buffelli*) (Hornok et al. 2014). Typical clinical signs of piroplasmosis in cattle include hemolytic anemia and fever, with occasional hemoglobinuria and even death of animals (Decaro et al. 2013). Especially susceptible are newly introduced naïve animals into regions endemic for piroplasms. The cause of benign or non-transforming theileriosis in cattle is *Theileria orientalis* (*syn*. *T. sergenti*, *T. buffelli*). The infection with *T. orientalis* leads to erythrocyte destruction in affected animals (Watts et al. 2016). All proven vectors of *T. orientalis* belong to the genus *Haemaphysalis* (Uilenberg 1981).

Piroplasms as well as *A. phagocytophilum* have recently been found in the Republic of Serbia as emerging or re-emerging pathogens affecting the health of animals (Potkonjak et al. 2016; Davitkov et al. 2015; Pavlović et al. 2002). For example, *A. phagocytophilum* was detected for the first time in Serbia directly from *I. ricinus* (Milutinović et al. 2008). The first report on the presence of piroplasms in cattle in the territory of Serbia dates back to the first half of the twentieth century when *Theileria hirci* was reported in goat, sheep, and cattle (Dschukowsky and Urodschevich 1924). In neighboring countries, the presence of *Th. orientalis* has also been reported (Papadopoulos et al. 1995; Andersson et al. 2017; Hornok et al. 2014).

Serological surveys on *Anaplasma* spp. and piroplasms in Serbia have mainly been conducted in dogs. The authors reported antibodies against *Ehrlichia canis* (causing agent of canine monocytic ehrlichiosis) and *A. phagocytophilum* by indirect immunofluorescence antibody tests (IFAT) (Potkonjak et al. 2013; Bogicevic et al. 2017; Potkonjak et al. 2015). Furthermore, DNA of *Babesia canis* and *Babesia gibsoni* was confirmed by PCR-RFLP in the blood of 60 symptomatic dogs (Davitkov et al. 2015).

Due to extensive cattle breeding, dairy cows in Serbia are exposed to tick-borne pathogens especially during grazing periods in spring and summer when large populations of ticks are present on the pastures. Thus far, there is no data available on the infestation of cattle with different tick species and on the occurrence of *A. phagocytophilum* and piroplasms in dairy cows in Serbia. Therefore, the aims of this study were (i) to investigate the tick species occurring on dairy cows and their pastures and (ii) to determine the occurrence of *A. phagocytophilum* and piroplasms in dairy cows from different locations in Serbia.

Material and methods

Sampling sites and tick collection

Collection of host-seeking ticks on pastures and tick infestation of cattle as well as blood sampling from dairy cows (n = 185) was carried out one time per location during May and June 2013 in six geographically different locations in Northern and Central Serbia (Fig. 1). Blood was sampled according to the Animal Welfare Law (Official Gazette of the Republic of Serbia 41/2009).

Ticks were collected from all six locations directly from animals and by the flagging method using 1m^2 white linen, from pastures at four locations: Zasavica, Mošorin, Gruža, and Susek (Fig. 1). Ticks were collected at the center of the pasture as well as under scattered vegetation present at the locations. The chosen locations differed concerning altitude, soil type, and vegetation (mountain terrain in Gruža, 240 m above sea level; plain pastures in continuation of agricultural ecosystems in Mošorin, 111 m, and in Susek, 96 m; desiccated ground with steppe type of vegetation in Banatski Karlovac, 99 m; hills in Požarevac, 97 m; riverside with few trees on pastures in Zasavica, 78 m). Dairy cows in Serbia freely graze on traditional community pastures from March to November. Pastures are not treated with insecticides, and usually, cattle share the pastures with other animal species depending on the location (e.g., domestic animals such as dogs, cats, sheep, donkeys, and wildlife species such as several species of wild birds, roe deer, wild boars, and rodents).

The ticks present on animals were collected manually at the time of blood sampling early in the morning before the cows were released to the pastures. The whole animal body was checked for ticks by two co-authors of this study.

The collected ticks were stored in 70% ethanol, separated by collection date, location, and individual animal, examined under a stereomicroscope, and separated by species, sex, and stage. Tick identification to species level was done using standard determination keys (Hillyard 1996; Estrada-Peña 2004).

The study population of 185 cattle was kept under extensive production system, and they varied according to breed (51 Simmental, 40 Holstein-Friesian, 7 Holstein-Friesian red, 1 Hereford; and 86 domestic breed in type of Simmental) and age (1 to 8 years old). The blood samples

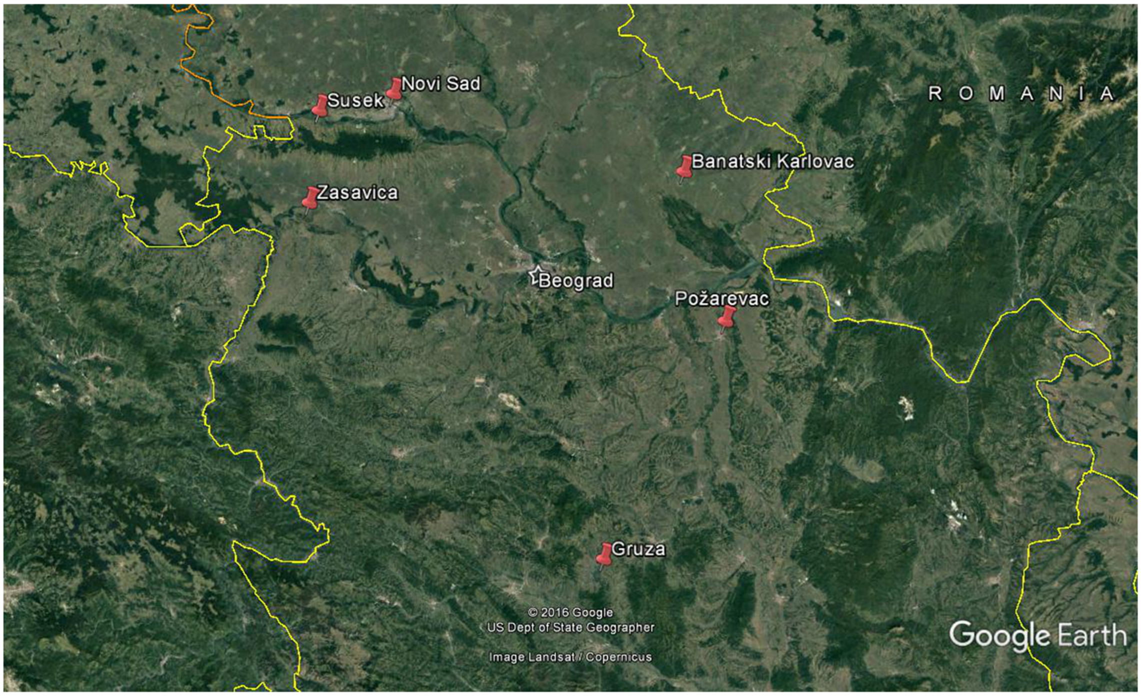

Fig. 1 Sampling locations of ticks and blood from dairy cows in 2013, Republic of Serbia (Map data: Google Earth, US PDept of State Geographer)

from dairy cows were taken by punction of *Vena coccygea*. General health parameters (inner body temperature, respiration) were recorded at the time of sampling.

Microscopic examination of blood smears

Thin blood smears (two for each sample) were carried out immediately after blood sampling, air-dried, stained with Giemsa, and examined with a microscope (Olympus SZX10, Tokyo, Japan) under × 40 and × 100 magnification.

Molecular detection of *A. phagocytophilum* and piroplasms

Total DNA was extracted from EDTA blood samples of 135 dairy cows with the Qiagen DNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA was tested for quality and quantity with a spectrophotometer (NanoDrop® ND-1000, PeqLab, Erlangen, Germany).

Detection of *A. phagocytophilum* DNA was carried out as described in Courtney et al. (2004) and Silaghi et al. (2011). A species-specific real-time PCR targeting the partial *msp2* gene (77 bp) was done using the Applied Biosystems real-time PCR system AB7500 (Thermo Fisher Scientific Inc., Waltham, USA). All samples were tested with positive and negative controls.

Detection of DNA of piroplasms was carried with a conventional PCR targeting the partial *18S rRNA* gene (411–452 bp length) with primers BJ1: 5′-GTC TTG TTA TTG GAA TGA TGG-3′ and BN2: 5′-TAG TTT ATG GTT AGG ACT ACG-3′, followed by agarose gel electrophoresis stained with GelRed™ (Biotium, USA) and visualization under UV light (Schorn et al. 2011; Casati et al. 2006).

The PCR products were purified using the QIAquick Minelute PCR Purification Kit (Qiagen, Hombretikon, Switzerland) and sequenced with forward and reverse primers at Microsynth AG (Balgach, Switzerland). The chromatograms were analyzed using Finch TV 1.4 (finchtv.software.informer.com), the sequences aligned with ClustalW2 (EMBL-EBI), and identity search of obtained sequences with GenBank entries was done using Blastn (https://blast.ncbi.nlm.nih.gov).

A. phagocytophilum IFAT

The indirect immunofluorescence antibody test using MegaScreen® Fluoanaplasma ph. slides (MegaCor, Hörbranz, Austria) and anti-bovine IgG-conjugate in a dilution of 1:80 (Sigma-Aldrich, Taufkirchen, Germany) was done on 163 dairy cow sera. A titer of 1:50 was considered as borderline and a titer of 1:100 as positive.

Results

A total of 435 ticks were collected from the animals and a total of 80 ticks were collected from the pastures. Altogether, three different tick species (*D. marginatus*, *H. punctata*, *and I. ricinus*) were identified both on animals and pastures. The number of collected ticks and tick species from dairy cows and pastures is shown in Table 1. Gender and stage distributions

Table 1 Sampling locations and numbers and species of collected ticks in Serbia, 2013

			Ticks on cows				Ticks on pastures			
Location	No. of dairy cows sampled	No. of dairy cows with ticks	Total no. of ticks	*Dermacentor marginatus*	*Haemaphysalis punctata*	*Ixodes ricinus*	Total no. of ticks	*D. marginatus*	*H. punctata*	*I. ricinus*
Banatski Karlovac	62	55	243	106	137	0	0	NT	NT	NT
Sušek	20	6	8	1	1	6	2	0	1	1
Gruža	15	14	24	0	1	23	33	0	0	33
Mošorin	50	6	8	2	6	0	40	0	40	0
Zasavica	14	14	18	8	2	8	5	2	0	3
Požarevac	24	6	134	6	0	128	0	NT	NT	NT
Total	185	101	435	123	147	165	80	2	41	37

NT not collected

of the ticks were 329 females (136 *I. ricinus*, 116 *H. punctata*, 77 *D. marginatus*) to 106 males (29 *I. ricinus*, 31 *H. punctata*, 46 *D. marginatus*) on animals and 40 females (18 *I. ricinus*, 21 *H. punctata*, 1 *D. marginatus*), 34 males (14 *I. ricinus*, 19 *H. punctata*, 1 *D. marginatus*), and 6 nymphs (5 *I. ricinus*, 1 *H. punctata)* on pastures. Table 1 shows the tick species distribution differing from area to area. The most abundant tick species on animals in Banatski Karlovac was *H. punctata* (137 out of 243), followed by *D. marginatus* (106 out of 243), while no *I. ricinus* ticks were observed on animals. In Požarevac, no *H. punctata* ticks were registered on animals at all, but the abundance of *I. ricinus* (128 out of 134) ticks was high.

There were no animals with clinical signs of disease. None of the Giemsa-stained blood smears were found positive for *A. phagocytophylum* morulae nor for *Theileria* spp. or any other blood parasites. No DNA of *A. phagocytophilum* was detected by real-time PCR in any of the samples, but a total of 4 out of 163 (2.45%) sera were positive for the presence of *A. phagocytophilum*-specific antibodies by IFAT. Another six sera had borderline results. The titers ranged from 1:100 to 1:200 (Table 2).

Five out of 135 samples from Banatski Karlovac showed positive DNA results for piroplasms from two different stables. From these five positive cattle, three animals from two different stables were infested by ticks. Two animals had two ticks (two females; and one female and one male tick) per animal and one had only one female tick, all determined as *H. punctata*. The obtained partial sequences were identified as *Theileria* sp., with 100% identity with GenBank entries from Italy (*T. sergenti*, accession No. KX375823), China (*Theileria* spp. JW-2014 clone 6, accession No. KJ020546), and Korea (*T. buffeli* isolate HS252, accession No. KX965722). Sequences from this study were deposited in GenBank under accession nos. KY310672, KY310673, KY310674, KY310675, and KY310676.

Discussion

High numbers of ticks on pastures and animals are a well-known problem in vector activity periods. Infestation of sheep, cattle, and dogs was recorded in previous studies with seven different hard tick species present (Milutinovic and Radulovic 2002; Mihaljica et al. 2012). In our study, species compositions were different according to geographical area. In Banatski Karlovac, high numbers of *H. punctata* were registered, while in Požarevac, *I. ricinus* was the most frequent tick species. Even though these two areas are geographically close to each other, they are separated by the Danube River

Table 2 Results of IFAT for *Anaplasma phagocytophilum*-specific antibodies in dairy cows in Serbia, 2013

Location	No. of tested sera	No. of positive sera	% of positive sera	1:100	1:200
Banatski Karlovac	62	0	0	0	0
Sušek	20	0	0	0	0
Gruža	15	2	13.3	1	1
Mošorin	28	0	0	0	0
Zasavica	14	0	0	0	0
Požarevac	24	2	8.3	1	1
Total	163	4	2.5	2	2

and thus form two completely different habitats with different vegetations. In two other examined localities (Susek, Gruža), *I. ricinus* ticks were highly present on both animals and pastures with no geographical connection between these two localities. Findings of *I. ricinus* in meadows and forests were previously shown in Savić et al. (2010). Interestingly, in the natural reservation area of Zasavica, the presence of *D. marginatus*, *H. punctata*, and *I. ricinus* was determined on cattle, but *H. punctata* was not found on the pastures. The presence of several tick species can be explained with the high biodiversity in the area and presence of different hosts. At the locality of Mošorin, *H. punctata* was the most abundant on animals followed by *D. marginatus*. On the pastures, *H. punctata* was the only species. From the results, we can consider that the tick species distribution differs from area to area and that the three major species present are *I. ricinus*, *D. marginatus*, and *H. punctata*. This is in correlation with available literature data from Serbia (Pavlovic et al. 2002; Becskei et al. 2015; Milutinovic et al. 2008).

No DNA of *A. phagocytophilum* was detected by real-time PCR in any of the blood samples from cattle. However, 4 out of 163 (2.45%) sera were positive for the presence of *A. phagocytophilum*-specific antibodies by IFAT. This implies that the agent is present in at least two of the tested locations (Požarevac and Gruža) since there was no evidence that the animals were transported to or from another site before in their lives. It is not surprising that *A. phagocytophilum* DNA was not detected, as PCR positivity only occurs for few days after infection whereas titers can last for several months (Pusterla et al. 1997; Woldehiwet 2010). The sampling time in the spring can also explain low seroprevalence since data from other study show increasing seroprevalence during summer and autumn compared to that during spring (Lempereur et al. 2012).

On both locations, *I. ricinus* was the most abundant tick species, which implies that this tick species may be responsible for *A. phagocytophilum* transmission also in the examined locations. The prevalence of *A. phagocytophilum* in questing *I. ricinus* ticks was 17.6% in the northwestern region of Serbia (wide region of Zasavica location in our study) and 15.4% in central Serbia (wide region of Gruža) by Milutinovic et al. (2008) implying that the pathogen may also be present in *I. ricinus* ticks in the region of our study. The presence of *A. phagocytophilum* DNA by molecular methods in cattle with no clinical cases attributable to *Anaplasma* spp. was shown in northeastern Hungary (Hornok et al. 2014).

Sequencing of the positive samples of the piroplasm PCR showed the presence of *T. orientalis* (*syn. T. sergenti*, *T. buffelli*) in the area of Banatski Karlovac without obvious clinical disease in cattle. In the same area on the animals, we determined the presence of *H. puntata* ticks which belongs to the genus proven to be vector of *T. orientalis* (Uilenberg 1981). These results are in accordance with neighboring countries. In the northern Macedonian region in Greece, *Theileria* spp. was observed in blood smears of 33 out of 50 cattle, without clinical symptoms in the animals. Serologically, species-specific titers against *T. orientalis* were detected in 41.4% of animals, while only 2% had a positive reaction for *T. annulata*. *Haemaphysalis punctata* was present in 17 out of 28 localities of animals affected with *T. orientalis* (Papadopoulos et al. 1995). In Romania, a recent study showed the presence of *T. buffeli* in *Rh. bursa* ticks collected from cattle (Andersson et al. 2017). In Hungary, in a herd of 88 beef cattle, bovine piroplasmosis was diagnosed in nine animals through the examination of blood smears or by molecular methods. *B. major* was identified in five animals, two of which died. In addition, four cattle harbored *T. buffeli* and one of these animals was anemic. Despite their presence, a contributory role of *Anaplasma marginale* and *A. phagocytophilum* could not be established in the disease cases in that study (Hornok et al. 2014).

Conclusion

In conclusion, we showed the presence of three different tick species (*D. marginatus*, *I. ricinus*, and *H. punctata*) on cattle and their pastures in Serbia. Antibodies against *A. phagocytophilum* were found in cattle in areas of Gruža and Požarevac, but no direct detection of the pathogen could be established. Specific sequences of *T. orientalis* in the absence of clinical signs of infection in cattle were shown to be present in cattle in Banatski Karlovac. Due to lack of clinical signs of diseases at the time of sampling, the impact of *T. orientalis* on the health of cattle at the examined locations is yet to be determined.

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures on animals were done according to the Animal Welfare Law (Official Gazette of the Republic of Serbia 41/2009).

Conflict of interest The authors declare that they have no conflict of interest.

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