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A One Health approach to study the circulation of tick-borne pathogens: A preliminary study

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ABSTRACT

Tick-borne pathogens (TBPs) have complex life cycles involving tick vectors and vertebrate hosts. However, there is limited empirical evidence on the zoonotic circulation of TBPs. In this study, we used a One Health approach to study the possible circulation of TBPs in ticks, animals and humans within a rural household in the foothills of the Fruška Gora mountain, northern Serbia. The presence of TBP DNA was assessed using microfluidic PCR (25 bacterial species, 7 parasite species, 5 bacterial genera, 3 parasite genera) in animal, human and tick samples and the presence of tick-borne encephalitis virus (TBEV) RNA was screened for using RT-qPCR on tick samples. In addition, Lyme borreliosis serology was assessed in patients sera. *Rhipicephalus sanguineus* and *Ixodes ricinus* ticks were identified on dogs and *Haemaphysalis punctata* was identified on house walls. *Rickettsia helvetica* was the most common pathogen detected in pooled *R. sanguineus* and *I. ricinus* tick samples, followed by *Hepatozoon canis*. None of the *H. punctata* tick samples tested positive for the presence of TBPs. *Anaplasma phagocytophilum* and *Rickettsia monacensis* were the most frequent pathogens detected in dogs, followed by *Rickettsia felis*, whereas *Anaplasma bovis* was the only pathogen found in one of the goats tested. None of the human blood samples collected from family members tested positive for the presence of TBPs. Although microfluidic PCR did not detect *Borrelia* sp. in any of the tested tick or blood samples, a family member with a history of Lyme disease was seropositive for *Borrelia burgdorferi* sensu lato (s.l.). We conclude that, despite the presence of TBPs in tick and vertebrate reservoirs, there is no evidence of infection with TBPs across various components of the epidemiological chain in a rural Fruška Gora household.

1. Introduction

Ticks (order Ixodida) are obligate blood-feeding ectoparasites of mammals, birds, and reptiles, which are globally important vectors of disease-causing agents that impact both human and animal health [1]. Ticks are second only to mosquitoes in importance as vectors species, but, among all blood-sucking arthropods, they harbor and transmit the widest variety of pathogens, including bacteria, rickettsiae, protozoa and viruses [2]. The incidence of tick-borne diseases (TBDs) is increasing and becoming a serious problem worldwide due to the public health impact and economic losses related to reduced livestock production, particularly for developing countries such as Serbia [3]. In recent years, the growing number of emerging zoonotic TBD cases has led to increased public awareness about the interrelatedness of human and animal health. The World Health Organization (WHO) developed and promotes the "One Health" concept, an interdisciplinary approach to the

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study of the spread of diseases between animals and humans to better protect public health [4]. One Health studies aim to capture the inherent interdependence of human and animal health and the environment [4]. The One Health approach is particularly relevant for the development of strategies to control tick infestations and TBDs.

The number of known tick-borne pathogens (TBPs) has increased dramatically since the 1980s and is now a serious problem worldwide due to its impact on public health, livestock-related economic losses and morbidity for wildlife [5]. The occurrence of TBDs is on the rise due to climate change, globalization, population movements and growth, and modifications of landscapes and natural habitats, as well as to improved surveillance and diagnostic techniques [6–8]. Continuous human exploitation of environmental resources and an increase in outdoor activities have led to more contacts between humans and arthropod vectors, promoting the emergence and resurgence of TBPs [6–8]. Humans are frequently exposed to ticks and TBP infections, some with zoonotic potential, not only during occupational and outdoor recreational activities, but also within the premises of their own households [9].

Serbia has diverse landscapes, with plains in the north, limestone ranges and basins in the east and mountains and hills in the south and southeast. As in the rest of Europe, the most common and widespread human TBD in Serbia is Lyme borreliosis, along with other less frequently diagnosed diseases such as tick-borne encephalitis, human monocytic ehrlichiosis, tick-borne lymphadenopathy, and Crimean-Congo hemorrhagic fever (CCHF) [10–13]. Apart from the endemicity of CCHF [14], little is known about the impact of TBDs on human health in Serbia. We hypothesize that other, less frequent TBDs are underdiagnosed due to restricted access to specialized laboratories that mainly operate within tertiary health care facilities.

Studies investigating the diversity of ticks and TBDs in humans and domestic animals inhabiting the same area are scarce in Serbia. Here we used a One Health approach to assess the circulation of TBPs within one rural household in northern Serbia, an area where tick infestations in animals and humans are frequently reported. We integrated medical observation and examination of family members with molecular diagnosis to screen for major TBPs in humans and animals, as well as in the ticks collected in the household located on the foothills of Fruška Gora, the largest mountain in the Vojvodina region in northern Serbia.

2. Materials and methods

2.1. Ethics statement

This study was approved by the ethics committee of the Faculty of Medicine in Novi Sad (Ethical approval no. 01-39/206/1) and conducted according to the Helsinki Declaration and the Patient Rights Law of the Republic of Serbia. Written informed consent for publication of this clinical case report was obtained from the patients. Handling of household animals and their blood samples was carried out in accordance with the EU Directive 2010/63/EU for animal experimentation.

2.2. Study precedents and design

2.2.1. Precedents of tick infestation and TBDs in the household

On 1 August 2019, a family with a previous history of tick infestation reported the presence of ticks on the outer walls of their house. This family of four members (father, mother and two daughters under the age of 18) lives in a one-story house of old construction. The family had five dogs and keeps alpine goats near the house for goat milk production (for family consumption). The house is located in the foothills of the Fruška Gora mountain (45.1857°N, 19.8042°E). Following this report, the family was advised to collect tick specimens and contact the Pasteur Institute in Novi Sad, the nearest large city. On 8 August 2019, a member of the family delivered a plastic vial containing 15 engorged ticks to the Novi Sad Pasteur Institute. All family members were examined for the presence of clinical signs of TBDs, but none developed any signs or symptoms within the 6 months following notification of the tick infestation.

On 18 June 2020, all family members ($n = 4$) came to the Novi Sad Pasteur Institute for Lyme borreliosis serology testing upon their request, because one of the daughters developed itching at sites of previous tick bites (upper arms and back). The itching started after a recent tick bite on the lower left leg: the location of itching corresponded to earlier tick bites, not to the recent tick bite. Because the lesions associated with the itching were similar to urticarial lesions, and there were no signs of any TBD at the site of the most recent tick bite, the patient was advised to apply chloropyramine cream (Synopen, Pliva, Croatia) locally. None of the family members reported any history of TBDs, except the father who developed Lyme arthritis in the shoulders and elbows more than 10 years ago. He did not recall any tick bite or lesion similar to erythema migrans before the manifestation of Lyme arthritis. He was hospitalized and treated with ceftriaxone intravenously (Longaceph, Galenika AD, Belgrade, Serbia). After the treatment, he was discharged from the hospital. Despite negative history of TBDs, all other family members reported numerous tick bites, as well as heavy tick infestation in the house and on animals in earlier months.

2.2.2. Study design using a One Health approach

Based on these antecedents, a study was carried out to assess the circulation of TBPs in this rural household located in the Fruška Gora foothills using a One Health approach (Fig. 1). To do so, blood samples were collected from family members and the family's dogs and alpine goats. Ticks from dogs and the walls of the house were also collected. No ticks were found on the goats or the family members during the sampling period. The presence of TBP DNAs was assessed using microfluidic PCR (25 bacterial species, 7 parasite species, 5 bacterial genera, 3 parasite genera) on blood and tick samples and tick-borne encephalitis virus (TBEV) RNA was screened for using quantitative reverse-transcription PCR (RT-qPCR) on tick samples. In addition, Lyme borreliosis serology was carried out on patient sera.

2.2.3. Sample collection and nucleic acid extraction

Blood samples were collected from family members ($n = 4$), the family's dogs ($n = 5$), and the female ($n = 5$) and male ($n = 2$) alpine goats. For each patient, 3 to 3.5 mL of blood was extracted in BD Vacutainer® SST™ or BD Vacutainer® spray-coated K2EDTA tubes (BD, Oakville, USA), respectively. For dogs and goats, 3 mL of blood was collected in BD Vacutainer® spray-coated K2EDTA tubes). Blood DNA was isolated using the Nucleospin Tissue kit (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions.

Ticks were found and removed from four of the five dogs ($n = 7$) and collected on the house walls ($n = 15$). All collected ticks were identified with regard to species, sex and life stage, based on morphological features and standard taxonomic keys described in Estrada-Peña et al. [15], and conserved in 70% ethanol at 8 °C until further use. The ticks were pooled resulting in three and four pools for the ticks collected on the walls (five ticks per pool) and the dogs (one pool per dog), respectively.

Pooled ticks were homogenized using a Precellys 24 lyser/homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 3724 ×g for 20 s with 2.8 mm stainless steel beads in 180 µL of Lysis buffer (T1 buffer) and 25 µL of proteinase K from the Nucleospin Tissue kit (Macherey Nagel). Homogenates were incubated for 3 h at 56 °C and DNA was extracted according to the manufacturer's instructions. Purified DNA was eluted in 50 µL elution buffer. *Haemaphysalis punctata* homogenates were also used for RNA extraction using the RNeasy Mini Kit (Qiagen, Germany).

2.3. DNA pre-amplification for microfluidic real-time PCR

To allow for better detection of pathogen DNA, total DNA was pre-amplified using the PreAmp Master Mix (Fluidigm, San Francisco, CA, USA), employed according to the manufacturer's instructions. Primers

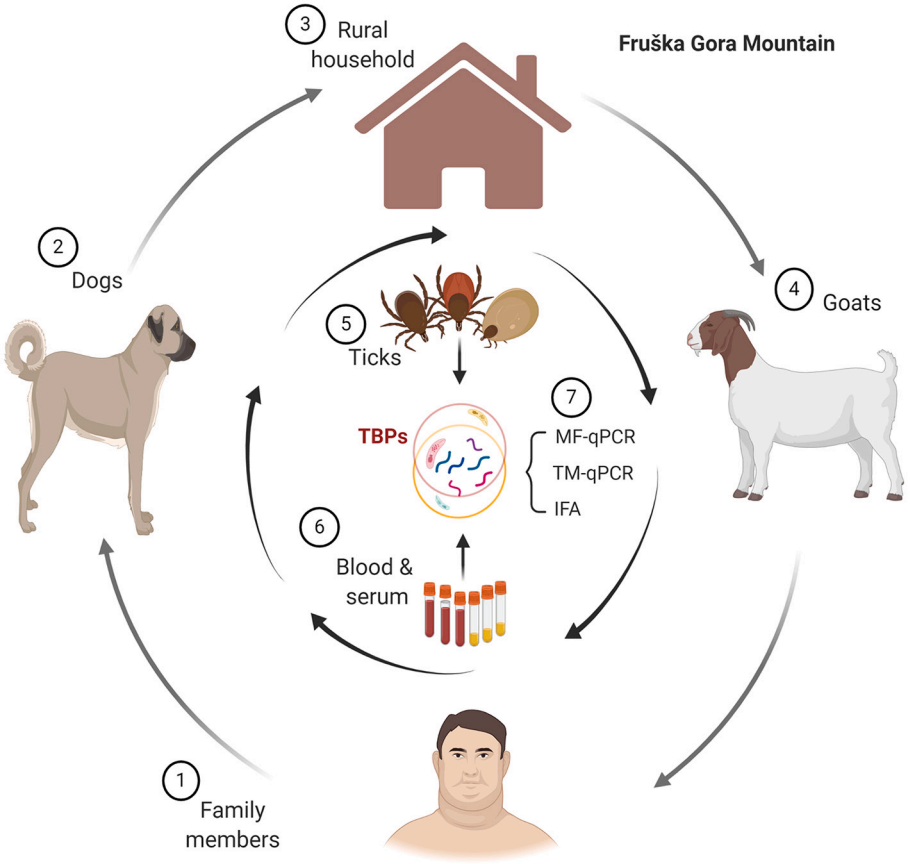

Fig. 1. Schematic diagram of the One Health approach used in this study. An epidemiological study was conducted in a rural household located in the foothills of the Fruška Gora mountain. The analysis included the molecular and serological diagnosis of several TBPs using microfluidic qPCR (MF-qPCR), TaqMan RT-qPCR (TQ-qPCR) and an indirect fluorescent antibody test (IFA). The analyses were performed on blood samples from humans (family members), dogs and alpine goats. In addition, tick samples were directly collected on the dogs and the walls of the house. Created with BioRender.com.

(targeting all pathogens) were pooled combining an equal volume of each primer for a final concentration of 200 nM for each primer. The reaction was performed in a final volume of 5 µL containing 1 µL PreAmp Master Mix, 1.25 µL pooled primers mix, 1.5 µL distilled water and 1.25 µL DNA. The thermocycling program consisted of one cycle at 95 °C for 2 min, 14 cycles at 95 °C for 15 s and 4 min at 60 °C. At the end of the cycling program the reactions were diluted 1:10 in Milli-Q ultrapure water. Pre-amplified DNAs were stored at −20 °C until needed.

2.4. Microfluidic real-time PCR

To detect major TBPs (25 bacterial species, 7 parasite species, 5 bacterial genera, 3 parasite genera), the BioMark™ real-time PCR system (Fluidigm, San Francisco, CA, USA) was used for high-throughput microfluidic real-time PCR amplification using 48.48 Dynamic Array™ IFC chips (Fluidigm, San Francisco, CA, USA). These chips dispense 48 PCR mixes and 48 samples into individual wells, after which on-chip microfluidics assemble real-time PCR reactions in individual chambers before thermal cycling, resulting in 2304 individual reactions. Briefly, amplifications were performed using 6-carboxyfluorescein (FAM)- and black hole quencher (BHQ1)-labeled TaqMan probes with PerfeCTa® qPCR ToughMix®, Low ROX™ (QuantaBio, Beverly, MA, USA) following the manufacturer's instructions. PCR cycling included 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of two-step amplification of 15 s at 95 °C, and 1 min at 60 °C. One negative water control was included per chip. To determine if factors present in the sample inhibit the PCR, *Escherichia coli* strain EDL933 DNA was added to each sample as an internal inhibition control, and primers and probe specific for the *E. coli eae* gene were used. For more details regarding the development of this new high-throughput tool based on real-time microfluidic PCRs (test of sensitivity, specificity, and controls used), please see ref. [16].

2.5. Validation of microfluidic real-time PCR results using standard PCR and DNA sequencing

To confirm the microfluidic real-time PCR results, all samples positive for infectious agents underwent conventional and nested PCR assays using primers different from those of the BioMark™ system (Table 1). Amplicons were sequenced by Eurofins MWG Operon (Ebersberg, Germany) and assembled using BioEdit software (Ibis Biosciences, Carlsbad). The final nucleotide sequences were analyzed to identify the sequenced microorganisms using the GenBank database through the National Center for Biotechnology Information (NCBI; Bethesda, MD) Basic Local Alignment Sequence Tool (BLAST) search engine (www.ncbi.nlm.nih.gov/blast). Nucleotide sequence data reported in the present study are available in GenBank, EMBL and DDBJ databases under accession numbers MZ146328, MZ151073, and MZ146329.

2.6. Molecular detection of TBEV RNA using RT-qPCR on ticks

The possible circulation of TBEV in the household was evaluated using the ticks collected in the house. The presence of TBEV RNA was assessed using a probe-specific RT-qPCR targeting a 67 bp fragment of the 3′ noncoding region of the TBEV genome with the primers, F-TBE 1 (5' GGGCGGTTCTTGTTCTCC 3′) and R-TBE 1 (5' ACACATCACCTCCTTGTCAGACT 3′), and a TaqMan probe (5' TGAGCCACCATCACCCAGACACA 3′) labeled with FAM [17]. RNA from TBEV isolate Ljubljana 1 [18] and water were used as positive and negative controls, respectively. The qPCR reactions were performed using a StepOne™ Real-Time PCR System (Applied Biosystems, California, USA).

Table 1
Primer sets and PCR conditions used for the validation of microfluidic real-time PCR results.

Pathogen	Primer sequences (5′ – 3′)[a]	Target gene	Amplicon size	PCR conditions[b]	References
Hepatozoon canis	Outer primers ATACATGAGCAAAATCTCAAC CTTATTATTCCATGCTGCAG	18S rRNA	660 bp	35 cycles: 10 s 98 °C; 30 s 50 °C; 30 s 72 °C	[16]
	Inner primers GGTATGGTATTGGCTTACC CGAGCTTTTTAACTGCAACA		309 bp	35 cycles: 10 s 98 °C; 30 s 51 °C; 30 s 72 °C	[17]
Anaplasma spp. *Ehrlichia* spp.	Outer primers GAACGAACGCTGGCGGCAAGC AGTAYCGRACCAGATAGCCGC	16S rRNA	693 bp	35 cycles: 10 s 98 °C; 30 s 60 °C; 30 s 72 °C	[18]
	Inner primers TGCATAGGAATCTACCTAGTAG AGTAYCGRACCAGATAGCCGC		629 bp	35 cycles: 10 s 98 °C; 30 s 55 °C; 30 s 72 °C	
Rickettsia spp.	Outer primers GTCAGCGTTACTTCTTCGATGC CCGTACTCCATCTTAGCATCAG	*Omp*B	475 bp	35 cycles: 10 s 98 °C; 30 s 57 °C; 30 s 72 °C	[19]
	Inner primers CCAATGGCAGGACTTAGCTACT AGGCTGGCTGATACACGGAGTAA		267 bp	35 cycles: 10 s 98 °C; 30 s 58 °C; 30 s 72 °C	

[a] Y: T/C; R: A/G.

[b] All PCR reactions: 30 sec 98°C initial activation; 10 min 72°C final extension.

2.7. Screening of human serum samples for the presence of anti-Borrelia antibodies

A fraction of the human blood samples was placed in vials and the blood allowed to clot. After 2000 ×*g* centrifugation for 10 min, serum samples were collected and inactivated at 56 °*C. sera* were used for the detection of anti-*Borrelia* IgM and IgG. Antigens from *Borrelia afzelli*, *Borrelia garinii*, and *Borrelia burgdorferi* strains CH and USA were used in a commercial immunofluorescence assay carried out as per the manufacturer's instructions (Euroimmun, Lübeck, Germany; Cat. No. FI 2138-1010-2 G and FI 2138-1010-2 M). Visible fluorescence reactions using sera diluted to ≥1:100 for IgG and to ≥1:10 for IgM were considered as positive. Fluorescence was analyzed on a microscope (Leica DM 3000, Wetzlar, Germany) with a mercury bulb light source using an N2.1 filter (Leica, Wetzlar, Germany) with an excitation wavelength of 515–560 nm.

3. Results

3.1. Identification of ticks from dogs and house walls

A total of 22 ticks were collected from the outer house walls ($n = 15$) and four dogs ($n = 7$). Following morphological characterization of the ticks, *H. punctata* (15 females, 68%) collected on the house walls was the most abundant tick species in this rural household , followed by *Rhipicephalus sanguineus* (4 males, 18%) and *Ixodes ricinus* (3 nymphs, 14%), which were found on the dogs. *A H. punctata* specimen collected from the house walls is presented in Supplementary Fig. 1. In this study, the dogs were identified as dog I-V. Mixed infestations with *I. ricinus* (nymphs) and *R. sanguineus* (adult males) were observed in dogs I, II, and III, and only *R. sanguineus* (adult male) was found on dog V. No tick infestation was observed on dog IV. Detailed information about tick species, developmental stage, tick size and location is available in Supplementary Table S1. No tick infestation was observed on the goats or the family members.

3.2. Evaluation of the presence of TBEV RNA in H. punctata ticks

Because the *H. punctata* ticks sampled from the house were engorged and this tick species was not found on dogs, we suspected that they fed on the goats nearby. Accordingly, once the goats were fenced off away from the house, ticks no longer appeared on the house. Considering that goats are known for their ability to act as a reservoir for TBEV circulation in nature, we tested for the presence of TBEV in the *H. punctata* ticks. However, none of the *H. punctata* samples tested positive for the presence of TBEV by PCR, whereas a positive signal was detected in the positive control sample (TBEV Ljubljana 1 isolate).

3.3. Tick-borne pathogens detected in tick and blood samples

The molecular diagnosis was performed on collected blood and tick samples using high-throughput microfluidic real-time PCR. For detection of pathogen DNA, the ticks were pooled, resulting in three pooled samples of *H. punctata*, and three of *R. sanguineus* and *I. ricinus* and one with *R. sanguineus* (Supplementary Table S1). Overall, five (31%, 5/16) blood and three (43%, 3/7) pooled tick samples were positive for at least one of the pathogens included in our detection system. A total of six different pathogens were identified in blood and tick samples. Positive signals were detected for the *Rickettsia helvetica* 23S—5S internal transcribed spacer (23S—5S ITS) in the pooled samples of *R. sanguineus* and *I. ricinus* ticks removed from dogs II and III and in the sample of *R. sanguineus* removed from dog V. The detection of *R. helvetica* 23S—5S ITS in the tick samples from dogs II, III and V concurred with positive signals for the *Rickettsia* spp. *gltA* gene in the microfluidic system. The other pathogen detected was *Hepatozoon canis*, present only in the *R. sanguineus* sample from dog V. The presence of *H. canis* was confirmed by positive detection of *H. canis* 18S rRNA, Apicomplexa 18S rRNA in the microfluidic system and by sequencing a fragment of *H. canis* 18S rRNA (accession number MZ146329). The tick infesting dog V tested positive for a mixed infection with *R. helvetica* and *H. canis*. None of the pooled *H. punctata* samples collected from the house walls tested positive for TBPs included in our assay.

Regarding the blood samples, *Anaplasma phagocytophilum* (confirmed by detection of *A. phagocytophilum msp2*, and *Anaplasma* spp. 16S rRNA in the microfluidic system) and *Rickettsia monacensis* (confirmed by detection of *Rickettsia* spp. *gltA* in the microfluidic system and sequencing of *R. monacensis Omp*B fragment (accession number MZ151073)) were detected in two of the dogs, whereas *Rickettsia felis* (confirmed by detection of *Rickettsia spp.* gltA and R. felis orfB in the microfluidic system) was detected in only one of the dogs. *Anaplasma bovis* was the only pathogen found in the goats tested. The presence of *A. bovis* was confirmed by detection of *Anaplasma* spp. 16S rRNA in the microfluidic system and by sequencing an *A. bovis* 16S rRNA fragment (accession number MZ146328). Dog II was diagnosed with the

concomitant infection of *A. phagocytophilum* and *R. felis*, and dogs III and V were PCR-positive for *R. monacensis* and *A. phagocytophilum* infection, respectively. The occurrence of single and mixed infections of pathogens found in PCR-positive samples is summarized in Table 2. None of the blood samples collected from family members tested positive for the presence of TBPs.

3.4. Presence of anti-Borrelia antibodies in human samples

Using an immunofluorescence test, we found IgG seroreactivity against *B. burgdorferi* sensu stricto (s.s.) (i.e. strains USA and CH) antigens in the serum sample of one of the family members (i.e. father) at a titer of 1:400. The same sample was seronegative for IgM (cut-off titer value, 1:10) against *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii* antigens. Other family member samples were seronegative for IgM (cut-off titer value, 1:10) and IgG (cut-off titer value, 1:100) against *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii* antigens.

4. Discussion

Tick species identified on dogs in this study had previously been identified on domestic dogs in the Vojvodina region, in which *I. ricinus* and *R. sanguineus* were the most prevalent tick species [19]. *I. ricinus* and *R. sanguineus* are among the most common ectoparasites found infesting dogs and goats in Serbia, as well as in other countries in the Balkans such as North Macedonia, Montenegro, Romania and, Bosnia and Hercegovina [20–23]. Infestations with *R. sanguineus* in houses is frequently reported in the literature [24–27]. In contrast to urban environments, a rural household frequently includes several animal species and potential tick hosts, thereby increasing the risk of human exposure to diverse tick species that use domestic animals as hosts. *H. punctata* has previously been reported to have peaks of abundance in spring and autumn in Serbia and Bosnia, with adult female ticks being more prevalent than males (64.22% female ticks vs 35.78% male ticks prevalence) in one field study [28]. Although it is known that *H. punctata* infests goats [29], it is not common for them to be found on house walls. Anecdotally, the members of the family of the present study, had used a non-labeled substance to treat the tick infestation on the goats. It is possible that the substance was a repellent instead of a tick-killing formulation. Consequently, engorged ticks may have escaped the goat paddock to hide on the outer house walls for oviposition. Of note, the outer house walls on which the ticks were found faced the goat paddock. After the goats were fenced off in different area, no additional ticks were found on the walls of the house.

Table 2
Tick-borne pathogens detected using microfluidic real-time PCR.

Tick-borne pathogens	Total
Pooled tick samples ($n = 7$)	
Total infected pooled tick samples ($n = 3$)	
Rickettsia helvetica	3
Hepatozoon canis[a]	1
Single infections ($n = 2$)	
R. helvetica	2
Mixed infections with two pathogens ($n = 1$)	
R. helvetica + *H. canis*[a]	1
Animal blood samples ($n = 16$)	
Number of infected blood samples ($n = 5$)	
Anaplasma phagocytophilum	2
Anaplasma bovis[a]	1
Rickettsia monacensis[a]	2
Rickettsia felis	1
Single infections ($n = 4$)	
A. phagocytophilum	1
A. bovis[a]	1
R. monacensis[a]	2
Mixed infections with two pathogens ($n = 1$)	
A. phagocytophilum + *R. felis*	1

[a] Species identified according to sequencing results.

Although *H. punctata* has much lower importance for TBEV circulation in Central and Western Europe compared with *I. ricinus* [30], it is considered as a competent TBEV vector [31,32]. Knowing that TBEV foci are present in Fruška Gora [19], and that infected goats can act as reservoirs [33], engorged *H. punctata* may participate in the further dissemination of the virus. Here, TBEV RNA was not detected in engorged *H. punctata* ticks, suggesting that TBEV does not currently circulate in this rural household. However, prior to RNA extraction, *H. punctata* ticks were stored in ethanol, which may affect RNA quality and therefore hinder the detection of TBEV RNA. Further epidemiological screening for the presence of *H. punctata* in rural households in Serbia are required to address the risk posed by this tick species to human health.

In this study, *A. phagocytophilum* infection was confirmed in the family''s dogs. Domestic dogs are considered as potential reservoirs for this pathogen in Serbia [34]. *A. phagocytophilum* is the causative agent of granulocytic anaplasmosis in humans and animals. The occurrence of this pathogen has been reported worldwide, mainly in areas of the northern hemisphere where is naturally transmitted by *Ixodes* ticks [35]. In Serbia, the presence of *A. phagocytophilum* was reported for the first time in a population of *I. ricinus* ticks, and the pathogen was subsequently identified in *Haemaphysalis concinna* and *Dermacentor reticulatus* ticks [36,37]. Furthermore, *Rickettsia* of the spotted fever group (SFG), *R. monacensis* and *R. helvetica* have previously been reported in ticks in Serbia [37]. *R. helvetica* and *R. monacensis* have also been described in *I. ricinus* ticks in Serbia [38], whereas *R. felis* was recently reported in a human blood sample [39].

Dogs cohabit with humans, and thereby confer a high risk of tick encounters and TBP infections in humans. Accordingly, dogs serve as sentinels or reservoirs and play an important role in the epidemiology of some zoonotic TBPs, including several *Rickettsia* spp. [40]. The three members of the SFG rickettsiae identified in this study have been associated with infections in dogs and ticks in many countries throughout Europe [41,42]. Interestingly, only *R. felis* and *R. monacensis* were detected in dogs, and *R. helvetica* was detected in pooled tick samples containing both *R. sanguineus* and *I. ricinus* ticks (pools from dogs II and III) or only *R. sanguineus* ticks (tick from dog V). These findings suggest that the ticks did not acquire *R. helvetica* from the tested dogs.

In Serbia, the occurrence of *H. canis* has been previously described in epidemiological surveys conducted on dogs [43], red foxes [44] and *I. ricinus* [19]. This study reports the presence of *H. canis* in *R. sanguineus* for the first time in the country. Considering that *R. sanguineus* is the main vector of *H. canis* [45], the results suggest that *H. canis* is transmitted by *R. sanguineus* in the studied area. Similarly, Gianelli et al. [46] also indicated that *I. ricinus* is not a biological vector of *H. canis*, because *H. canis* sporogony does not occur in *I. ricinus* ticks, but in *R. sanguineus*.

Here, we also provided the first evidence of the presence of *A. bovis* is an obligate intracellular bacterium of the genus *Anaplasma* (family Anaplasmataceae) and infects circulating monocytes [47,48]. This pathogen is commonly reported worldwide and has been associated with subclinical symptoms in small ruminants such as sheep and goats [49,50]. In addition, *Anaplasma ovis*, also asymtomatic in goats [50-52], and *A. bovis* can coinfect small ruminants [50]. However, *A. bovis* was not detected in the goats sampled in this study. However, *A. bovis* infection, similarly to *Anaplasma ovis* infection [47], is asymptomatic in goats [45–47]. Recently, Jurković et al. [53] reported the first molecular confirmation of lethal cases of bovine anaplasmosis caused by *Anaplasma marginale* with concurrent infection of *A. bovis* and *Theileria orientalis* in Croatian cattle. Given that Croatia is a neighboring country of Serbia, and that goats have been described as a reservoir host of *A. bovis*, further studies should evaluate the impact of *A. bovis* in small ruminants and cattle in Serbia.

One of the family members showed IgG reactivity to *B. burgdorferi* s.l. complex antigens. Namely, the father was positive for *B. burgdorferi* s.s.,

a bacterial species known for its tropism for joints [54]. This bacterial species probably caused the previous disease (i.e. Lyme arthritis) reported by the father. Accordingly, the serum sample was reactive only for IgG, which is the dominant antibody isotope in late-stage *Borrelia* infection [55]. The bacterial members of the *B. burgdorferi* s.*l. complex* are the causative agents of Lyme borreliosis, described as a multisystemic TBD with high morbidity rates in humans. In Serbia, *B. burgdorferi* s.l. have been identified in competent vectors of the genus *Ixodes*, mainly *I. ricinus*, as well as in human serum samples [23,39]. The risk factors associated with human infection by *Borrelia* includes the presence and abundance of competent tick vectors in different types of habitats, a high prevalence of *B. burgdorferi* s.l. in ticks, and extended periods of possible exposure to tick bites [56]. The absence of *Borrelia* in the data collected from the molecular analysis of tick and animal samples echoes the low anti-*Borrelia* antibody seroprevalence within this family, despite a history of tick bites in this family. However, more extensive sampling of questing *I. ricinus* is required to obtain a better assessment of *Borrelia* infection prevalence in the region.

5. Conclusions

The study provides evidence of the effectiveness of a One Health approach (i.e., testing all the possible components of a localized epidemiological chain) for the assessment of zoonotic TBDs. To the best of our knowledge, this is the first report of SFG rickettsiae *R. felis* and *R. monacensis* infections in dogs in Serbia, as well as *R. helvetica* and *H. canis* in *R. sanguineus ticks* These results extend our knowledge of the potential vector spectrum and diversity of TBPs affecting human and animal health in Serbia. Further larger-scale epidemiological studies including new geographic regions are necessary to determine the spatial and temporal distributions of tick populations to improve local and regional tick control programs. We conclude that, despite the presence of TBPs in tick and vertebrate reservoirs, there is no chain of TBP infection across the epidemiological chain in the rural Fruška Gora household studied here. The integration of the One Health approach in surveillance programs will improve our understanding of the circulation of zoonotic TBPs in different epidemiological settings.

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Conflict of interest

The authors declare no competing interests.

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