Detection Of Selected Pathogens In Ixodid Ticks Collected From Animals And Vegetation In Five Regions Of Ukraine

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Abstract

Background Over the last few decades tick-borne diseases have emerged as an increasingly significant public health problem. The distribution and prevalence of zoonotic pathogens infecting ixodid ticks from Western Europe have been extensively examined. However, data on ticks and tick-borne pathogens in Eastern Europe, particularly Ukraine are scarce. The objective of the current study was, therefore, to investigate the prevalence rates of *Anaplasma phagocytophilum*, the Anaplasmataceae family, *Rickettsia* spp., *Babesia* spp., *Bartonella* spp., and *Borrelia burgdorferi* sensu lato in engorged and questing ixodid ticks collected from five administrative oblasts (regions) of Ukraine, namely Chernivtsi, Khmelnytskyi, Kyiv, Ternopil, and Vinnytsia.

Methods The ticks were collected from both wild and domestic animals and from vegetation. Of 524 ixodid ticks collected, 3, 99, and 422 ticks were identified as *Ixodes hexagonus*, *Ixodes ricinus*, and *Dermacentor reticulatus*, respectively. DNA samples individually extracted from 168 questing and 354 engorged adult ticks were subjected to pathogen-specific PCR analyses.

Results The mean prevalence rates in all *I. ricinus* and *D. reticulatus* ticks were, respectively: 10.3% (10/97) and 2.8% (12/422) for *A. phagocytophilum*; 69.1% (67/97) and 52.1% (220/422) for the Anaplasmataceae family; 24.7% (24/97) and 27.7% (117/422) for *Rickettsia* spp.; 3.1% (3/97) and 1.4% (6/422) for *Babesia* spp.; and 9.3% (9/97) and 4.7% (20/422) for *Bartonella* spp. Overall, between the five cities, there was no significant difference in the prevalence rates of any of the pathogens for the respective ticks ($p > 0.05$). The prevalence of *B. burgdorferi* s.l. in the engorged and questing *I. ricinus* ticks varied from 26.7% and 0% to 44.4% and 14.3%, respectively, with no statistical significance identified between the five cities ($p > 0.05$).

Conclusions In addition to reporting the updated data for Kyiv and Ternopil, this study is the first to provide the prevalence rates of the tick-borne pathogens for Chernivtsi, Khmelnytskyi, and Vinnytsia. This investigation is also the first to detect *Ca. N. mikurensis* in ixodid ticks from Ukraine. These new data will be useful for medical and veterinary practitioners as well as public health officials when diagnosing infections and when implementing measures to combat tick-borne diseases in Ukraine.

Background

During recent years tick-borne diseases have emerged as a significant public health problem [1–5]. *Ixodes ricinus* and *Dermacentor reticulatus* are the most abundant and widely distributed ixodid tick species in Europe, that transmit numerous pathogens responsible for medically important diseases (e.g., anaplasmosis, babesiosis, bartonellosis, borreliosis, and rickettsiosis) [6–8].

The Anaplasmataceae family includes intracellular bacteria of the *Anaplasma*, *Ehrlichia*, ‘*Candidatus Neoehrlichia*’, and *Rickettsia* genera. These tick-borne bacteria cause diseases that have become increasingly recognized as potentially fatal [9–11]. *Anaplasma phagocytophilum*, a causative agent of granulocytotropic anaplasmosis, infects humans and a variety of other animals including dogs and cats

[11, 12]. Human granulocytic anaplasmosis has been reported in Asia, Australia, Europe, and the United States [11]. In Europe, Ixodes ticks are the vector for *A. phagocytophilum*. The prevalence rates of *A. phagocytophilum* in Ixodes ricinus ticks collected from Western Europe vary from as low as < 1–20% [13].

'Candidatus Neorhlichia mikurensis' (Ca. *N. mikurensis*) represents a newly emerging pathogen transmitted by Ixodes ticks in temperate regions of the Northern hemisphere [14–18]. This pathogen has the capacity to cause severe disease in humans and dogs [19–22]. Similar to *A. phagocytophilum*, the prevalence rates of Ca. *N. mikurensis* vary greatly between different European regions (1%-20%) [11]. Unfortunately, human infections are likely to remain underdiagnosed and perhaps untreated, primarily because diagnostic assays are not widely available [17].

Rickettsia spp. are also members of the Anaplasmataceae family and are responsible for the following rickettsiosis in Europe: tick-borne lymphadenopathy (TIBOLA), scalp eschar and neck lymphadenopathy (SENLAT), Dermacentor-borne necrosis erythema lymphadenopathy (DEBONEL). These important diseases caused primarily by *Rickettsia slovaca* and *Rickettsia raoultii* are transmitted by Dermacentor ticks [11]. In addition, *Rickettsia helvetica*, a supposedly less pathogenic *Rickettsia* species, is transmitted by *I. ricinus* ticks. Thus, in Europe, *I. ricinus* and *D. reticulatus* serve as a natural reservoir of the Spotted Fever Group (SFG) *Rickettsia* and the prevalence rates of SFG *Rickettsia* spp. in these questing ticks are highly variable: 22.6%-64.0% (*D. reticulatus*) and 2.9%-50.8% (*I. ricinus*) [6, 23–25].

Babesiosis, an important tick-borne disease of humans and animals, is caused by the protozoan parasites of the genus *Babesia* [26]. If left untreated, the disease can be fatal for infected humans and animals [26, 27]. Ixodes ticks are the vectors of more than 100 *Babesia* species; whereas *D. reticulatus* is the most relevant vector for *Babesia canis* [11, 27]. In different regions of Europe, the prevalence rates of *Babesia* spp. in questing *D. reticulatus* ticks vary from 0–14.8% [28, 29].

Another significant tick-borne disease, which is widespread in Europe, is bartonellosis [30]. Over the past 20 years, a total of 13 *Bartonella* species have been identified as potentially pathogenic for humans [11]. Clinical studies indicate transmission of *Bartonella* spp. by ixodid ticks to humans [31, 32]. *Bartonella* species can cause endocarditis in humans and other animals [33–35].

Lyme borreliosis (LB) is one of the most prevalent tick-borne diseases in the Northern Hemisphere [36, 37]. This debilitating disease of humans and other animals (primarily dogs) is caused by the bacterial spirochete of *Borrelia burgdorferi* sensu lato complex (*B. burgdorferi* s.l.) [36–38]. *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto (*B. burgdorferi* s.s.) are the main pathogenic genospecies of *B. burgdorferi* s.l. in Europe [39, 40]. *B. burgdorferi* s.l. is transmitted via bites of infected nymphs and adults of Ixodes ticks [39, 41]. The incidence rates of human LB are highly variable for European countries. Of 17 countries of Western Europe, Sweden and Italy have the highest (464/100,000) and lowest (0.001/100,000) incidences of human LB, respectively [36]. LB leads to tremendous societal costs in many European countries [42]. Similar to the other tick-borne pathogens, the spatial distribution of *B.*

burgdorferi s.l. in questing I. ricinus ticks was shown to considerably vary between different natural ecological regions of Europe [43].

Despite numerous studies on the distribution and prevalence of the above-mentioned pathogens in Western Europe [4, 7, 11, 17, 44–47], the respective information for Eastern Europe is very scarce. Specifically, thus far, only a few studies have showed the prevalence rates of selected tick-borne pathogens in ixodid ticks from Ukraine [48–54]. Moreover, *Ca. N. mikurensis* has not been reported in ixodid ticks from Ukraine. Therefore, the objective of the present study was to examine the prevalence rates of *A. phagocytophilum*, the Anaplasmataceae family, *Babesia* spp., *Bartonella* spp., *B. burgdorferi* s.l., and *Rickettsia* spp. in both engorged and questing *I. ricinus* and *D. reticulatus* ticks collected from urban and rural areas of Ukraine (Chernivtsi, Khmelnytskyi, Kyiv, Ternopil, and Vinnytsia).

Methods

Collection of ticks

In Spring and Fall of 2018, questing ixodid ticks were collected by flagging grass, shrubs, and bushes (up to 1.5 m in height) in various randomly chosen city parks and open areas that are frequented by domestic animals and their owners. In addition, engorged ixodid ticks were directly collected from animal patients including wildlife (using tweezers) that were admitted to different urban and rural veterinary clinics throughout 2019. Most engorged ticks were removed the neck area. Both questing and engorged ticks were collected from five administrative oblasts (referred to here as regions) of Ukraine and their respective cities: Chernivtsi (Novoselytskyi, Sokyrianskyi, and Storozhynetskyi raion (referred to here as districts)), Khmelnytskyi (Kamianets-Podilskyi district), Kyiv (Teremky district of Kyiv; and the Chornobyl Exclusion Zone, specifically the former villages: Stari Shepelichi, Novi Shepelichi, Zavorychi, Buriakovka, and Zapolie), Ternopil (Pidvolochysk district), and Vinnytsia (Vinnytsia district) (Table 1). Identification was based on tick morphology [55, 56]. Upon collection, all the ticks were immersed in 70% ethanol and stored at 4°C until further analyses.

DNA extraction

Ticks were individually transferred into 1.5-ml Eppendorf tubes and crushed with sterile scalpels. DNA samples were then isolated from each of 522 collected ixodid adult ticks by using the DNeasy Blood & Tissue Kit (Qiagen, Inc., Germany) according to the manufacturer's instructions with some modifications. Specifically, crashed ticks were incubated with proteinase K for 1-3 hours at 56°C. DNA was then eluted in 75 µl of the elution buffer and then stored at –20°C until PCR analyses.

Polymerase Chain Reaction (PCR)

A total of 522 adult ticks (25 questing and 72 engorged adults of *I. ricinus*; 141 questing and 281 engorged adults of *D. reticulatus*; and 2 questing and 1 engorged adults of *I. hexagonus*) were individually analyzed by pathogen-specific PCRs detailed below. Specifically, both *I. ricinus* and *D. reticulatus* ticks were examined for the DNA presence of *A. phagocytophilum*, the *Anaplasmataceae* family, *Babesia* spp., *Bartonella* spp., and *Rickettsia* spp. In addition, *I. ricinus* ticks were PCR-analyzed for *B. burgdorferi* s.l. The PCR primers used in the current study are listed in Table 3. Importantly, previously sequenced DNA samples of each pathogen and nuclease-free water were utilized as positive and negative controls, respectively. Resultant PCR products were analyzed by electrophoresis in 1.5% agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Germany) and visualized via UV light with MiniBIS Pro gel doc system (DNR Bio-Imaging Systems, Israel).

In order to detect DNA of *Babesia* spp. and *B. burgdorferi* s.l., each PCR reaction was carried out, by utilizing T-personal Thermocycler (Biometra, Germany), in a 25- μ L-reaction volume that contained 12.5 μ L of 2x Color OptiTaq PCR Master Mix (EURx, Poland), 2 μ L of sample DNA, 0.5 μ L of respective forward and reverse primers with a final concentration of 0.2 μ M of each primer, and 9.5 μ L of nuclease-free water. The master mix consisted of 1.25 units of OptiTaq DNA polymerase, 0.2 mM of each dNTP, 0.5 μ L of respective forward and reverse primers with a final concentration of 0.2 μ M of each primer, and 1x reaction buffer with 1.5 mM MgCl₂.

To test for the DNA presence of *A. phagocytophilum*, *Bartonella* spp., *Anaplasmataceae*, and *Rickettsia* spp., PCR reactions were performed by using MyCycler thermal cycler (BioRad, USA). Each PCR was carried out in a 25- μ L-reaction volume, which contained 5 μ L of 5x FIREPol Master Mix (2 μ M MgCl₂, 200 μ M of each dNTPs, HOT FIREPol® DNA polymerase; Solis BioDyne, Estonia), 1 μ L of each respective primer (a final concentration of each primer was 0.4 μ M), 5 μ L of sample DNA, and 13 μ L of nuclease-free water.

The thermocycler conditions were adapted from previous publications [57-61]. Specifically, for the *Babesia*-specific PCR, the conditions were as follows: initial denaturation at 94°C for 4-5 min, followed by 40 cycles with denaturation at 94°C for 20 s, annealing at 57°C for 30 s, extension at 72°C for 45 s, and the final extension at 72°C for 7 min [57]. For the *B. burgdorferi* s.l.-specific PCR, the programming consisted of initial denaturation at 94°C for 3 min, 40 cycles of: denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and the final extension at 72°C for 3 min [58]. The conditions of *Rickettsia*-specific PCR comprised denaturation at 95°C for 45 s, annealing at 59°C for 45 s, extension at 65°C for 60 s, and the final extension at 72°C for 7 min [59]. For the *A. phagocytophilum*-specific PCR, the programming included initial denaturation at 95°C for 5 min, followed by 35 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 60 s, and the final extension at 72°C for 5 min [60]. Finally, the conditions of the *Bartonella*- and the *Anaplasmataceae* family-specific PCRs consisted of initial denaturation at 95°C for 5 min, followed by 45 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 70°C for 30 s, and the final extension at 72°C for 5 min [61].

Sequencing of PCR products

A total of 30 randomly selected PCR amplicons of the *Anaplasmataceae* family (n=7), *Babesia* spp. (n=4), *Bartonella* spp. (n=4), *Borrelia* spp. (n=9), and *Rickettsia* spp. (n=6) were purified with the QIAquick PCR purification kit (Qiagen, Germany) and then sequenced in both directions by using the primers, which were used for the respective PCRs (Table 3). Sequencing was carried out at the Eurofins Genomics (Germany) and Genomed (Poland). The analyses of assembled sequences were performed with BLASTn via GenBank and some sequences were deposited in the GenBank database under the following accessions: MK721201, MK721202, MK721203, MK721204 for *Bartonella bovis*; MK775117, MK775122, MK775120, MK775119, MK775123, MK775121, MK760255 for *Ca. N. mikurensis*; and MK721210, MK721205, MK721206, MK721207, MK721208, MK721209 for *Rickettsia raoultii*.

Statistical analyses

Frequencies and percentages of ticks were calculated. Fisher exact tests were used to compare the frequencies between different origins of the ticks. Exact logistic regression models were fitted to determine whether the number of ticks is different across the five studied regions due to that the number of ticks in some of the categories is too small and/or when some of the numbers are zero. Firth correction method was also applied when the quasi-complete separation of the data was present. All the statistical analysis was performed in SAS version 9.4 (SAS Institute, Cary, NC, USA).

Results

Out of 522 adults and 2 nymphs collected, 3, 99 and 422 ticks were identified as *Ixodes hexagonus* (1 and 2 engorged female ticks were collected from a cat and vegetation, respectively), *Ixodes ricinus* (including 2 questing nymphs), and *Dermacentor reticulatus*. Thus, a total of 354 and 170 of engorged and questing ticks, respectively, were collected from wild and domestic animals (1 from bear, 1 from racoon, 1 from red fox, 2 from lynx, 18 from cats, 104 from cattle, and 227 from dogs); and vegetation, respectively (Tables 1 and 2; and Supplemental Table 1) in Chernivtsi, Khmelnytskyi, Kyiv, Ternopil, and Vinnytsia oblasts (referred to here as regions). *D. reticulatus* represented the most dominant engorged tick species (79.4%; 149 females and 132 males) followed by *I. ricinus* (20.3%; 65 females and 7 males) and *I. hexagonus* (0.3%; 1 female) ticks. Similarly, out of 170 questing ticks, 82.9% (82 females and 59 males), 15.9% (24 females, 1 male, and 2 nymphs), and 1.2% (2 females) ticks were *D. reticulatus*, *I. ricinus*, and *I. hexagonus*, respectively (Tables 1 and 2).

To investigate and compare the prevalence rates of the tick-borne pathogens in the ixodid ticks collected from the five studied regions, a total of 522 adult ticks of *D. reticulatus*, *I. ricinus*, and *I. hexagonus* were individually PCR-analyzed for *A. phagocytophilum*, the *Anaplasmataceae* family, *Babesia* spp., *Bartonella*

spp., and *Rickettsia* spp. In addition, *I. ricinus* and *I. hexagonus* ticks were also examined for the presence of *B. burgdorferi* s.l. DNA.

First, the results showed that, in contrast to *I. ricinus* and *D. reticulatus* ticks, all three adults of *I. hexagonus* tested negative for the six pathogens (data not shown). Furthermore, the present study demonstrated that, between the five cities, the prevalence rates of *A. phagocytophilum* for both engorged and questing adults (referred to here as the combined prevalence) of *I. ricinus* and *D. reticulatus* varied from 4.8% and 2.1% to 21.7% and 4.2%, respectively (Tables 4 and 5). Interestingly, all the questing adults of *I. ricinus* were PCR-negative for *A. phagocytophilum* DNA (PCR-positive ticks/total ticks analyzed [0/25]). For each tick species, no statistical difference in the respective prevalence rates of *A. phagocytophilum* was detected between the five cities ($p>0.05$; Tables 4 and 5). However, the mean prevalence rate of *A. phagocytophilum* in all the *I. ricinus* ticks combined for the five cities (10.3%; 10/97) was significantly higher than that in *D. reticulatus* ticks (2.8%; 12/422; $p<0.01$).

The present study demonstrated that the ranges of combined prevalence rates for the *Anaplasmataceae* family in adults of *I. ricinus* and *D. reticulatus* were, respectively, 60.0%-85.0% and 46.1%-55.6% with no statistical significance identified between the five cities for each tick species (Tables 4 and 5). In contrast, the combined prevalence rate of the *Anaplasmataceae* family in the *I. ricinus* ticks (69.1%; 67/97) was significantly higher than that in the *D. reticulatus* ticks (52.1%; 220/422; $p<0.01$). Interestingly, sequencing results of 7 random *Anaplasmataceae* family PCR-positive amplicons demonstrated over 99% identity with a partial sequence of 16S rRNA gene of *Ca. N. mikurensis* for all the 7 PCR products.

Consistently, no statistical difference in the prevalence rates of *Rickettsia* spp. was observed for the *I. ricinus* or *D. reticulatus* ticks between the five cities ($p>0.05$). The combined prevalence rates of *Rickettsia* spp. varied, between the five cities, from 15.4% and 23.1% to 30.0% and 31.0% for all the *I. ricinus* and *D. reticulatus* ticks, respectively. Finally, the sequencing analysis showed that 6 randomly selected *Rickettsia* PCR-positive amplicons were 99-100% identical to a fragment of *sca4* gene of *Rickettsia raoultii*.

Interestingly, *Babesia* DNA was not detected in any of the questing *I. ricinus* ticks (0/25) (Table 4). Similarly, the engorged adults of *I. ricinus* from Chernivtsi (0/23), Kyiv (0/20), and Ternopil (0/13) were also PCR-negative. Moreover, *Babesia* DNA was not detected in any of the engorged adults of *D. reticulatus* collected from Ternopil (0/52) and Vinnytsia (0/97). In contrast, 9.5% and 5.0% of the engorged adults of *I. ricinus* from Khmelnytskyi and Vinnytsia, respectively, tested positive for *Babesia* DNA (Table 4). In comparison, the lower prevalence rates of *Babesia* spp. were observed in *D. reticulatus* ticks collected from Chernivtsi (2.3%), Khmelnytskyi (2.6%), and Kyiv (1.4%). Sequencing of 4 random *Babesia*-positive amplicons revealed that the 18S rRNA sequences belonged to *Babesia canis* with >99% identity. Overall, there was no significant difference in the prevalence rates of *Babesia* spp. between the engorged and/or questing *I. ricinus* and *D. reticulatus* ticks or between the five cities for each tick species ($p>0.05$).

The combined prevalence rates of *Bartonella* spp. for the *I. ricinus* and *D. reticulatus* ticks varied from 0.9% and 3.4% to 15.4% and 7.7%, respectively (Tables 4 and 5). A total of 9.3% and 4.7% *I. ricinus* and *D. reticulatus* ticks, respectively, collected from all the five cities tested positive for *Bartonella* DNA. The sequence analysis of 4 randomly chosen *Bartonella* PCR-positive amplicons showed 99-100% identity with a *tmRNA* gene fragment of *Bartonella bovis*.

Finally, the current data also showed that *B. burgdorferi* s.l. DNA was detected in *I. ricinus* ticks from all five cities. The prevalence rates for the engorged and questing ticks varied from 26.7% and 0% to 44.4% and 14.3%, respectively, with no statistical significance identified for any of the five studied regions ($p>0.05$). The mean combined prevalence of *B. burgdorferi* s.l. in the *I. ricinus* adults for the five cities was 25.8% (Table 4). Sequencing results of 9 random *B. burgdorferi* s.l.-specific PCR amplicons suggested that 7 out of 9 (77.8%) amplicons belonged to *B. afzelii* (the identity values ranged from 95.8% to 99.6%). The other two amplicons were each 99.6% identical to the 16S rRNA genes of *B. burgdorferi* s.s. or *B. spielmanii*.

Discussion

Previous investigations showed that only 3.6% and 5.2% of questing *I. ricinus* ticks collected from Eastern Ukraine (Spring, 2006) and Kyiv (Fall, 2013 and Spring, 2014) were positive for *A. phagocytophilum* [53, 62]. Consistently, the more recent prevalence rates of *A. phagocytophilum* in questing adults of *I. ricinus* concurrently collected from Kyiv and the Chernobyl Exclusion Zone (CEZ) in Spring 2016 were also low: 2.7% and 0.4%, respectively [51, 63]. In contrast, in the present study, the prevalence rates of *A. phagocytophilum* in the engorged adults of *I. ricinus* varied from 4.8% to 31.2%; whereas all the questing *I. ricinus* ticks tested negative. In comparison, the prevalence rates of *A. phagocytophilum* in questing adults of *I. ricinus* were also shown to be relatively low in the neighboring countries: 4.2% for Belarus; 2.9% for Lithuania; 5.1–9.0 % for Moldova; 10.7% for Poland; 3.1% for Russia; and 5.5% for Slovakia [6, 62, 64-69]. Furthermore, the present study showed that the prevalence of *A. phagocytophilum* in the *D. reticulatus* ticks combined from the five studied regions (2.8%) was lower than that in the *I. ricinus* ticks (10.3%). Such a low prevalence in *D. reticulatus* ticks is consistent with the respective rate recently reported for questing adults of *D. reticulatus* collected from Kyiv (1.0%) [51, 63]. Interestingly, high variability in the prevalence rates of *A. phagocytophilum* was previously observed for adult ticks of *D. reticulatus* from the CEZ (1.0%-25.4%) [49, 52].

The present report is the first to demonstrate the detection of *Ca. N. mikurensis* in ixodid ticks from Ukraine. *Ca. N. mikurensis* is an emerging zoonotic pathogen that has been primarily detected in *I. ricinus* ticks collected from 20 European countries with a wide variation in its prevalence (0.1%-24.3%) [17]. *Ca. N. mikurensis* can also be identified in *D. reticulatus* ticks, however, in general, the prevalence in this tick species is much lower. As an example, only 0.08% (1/1,237) of questing *D. reticulatus* ticks collected from Germany tested positive for this pathogen [61]. Unfortunately, due to a very limited funding, only seven randomly selected *Anaplasmataceae* family-positive PCR amplicons were sequenced and, as a result, the presence of *Ca. N. mikurensis* was identified in 7 ixodid ticks.

Despite a few recent studies, the knowledge on the prevalence of *Rickettsia* spp. in ixodid ticks from various regions of Ukraine is still missing [51-53]. To fill this gap, the current study has identified the respective rates for the five geographically scattered cities of Ukraine. The results showed that the mean prevalence of *Rickettsia* spp. in *I. ricinus* adults (24.7%) is almost twice as high as previously reported rates in the respective ticks concurrently collected from Kyiv (12.6%) and the CEZ (11.5%) in Spring 2016 [51, 52]. In contrast, the mean prevalence of *Rickettsia* spp. (27.7%) in *D. reticulatus* adults is lower than the respective rates identified for Kyiv (35.7%) and the CEZ (53.0% and 72.6%) [50-52]. Interestingly, the other study that examined ixodid ticks from Kyiv (Fall 2013 and Spring 2014) demonstrated that only 10.1% of *D. reticulatus* adults tested positive for *Rickettsia* spp. [53]. Thus, similar to the other tick-borne pathogens, in Ukraine, the prevalence of *Rickettsia* spp. in the ixodid ticks could vary greatly. In general, this is consistent with high variability in the respective prevalence rates observed for other European countries. For example, the prevalence rates of *Rickettsia* spp. in *D. reticulatus* ticks in the neighboring countries, Belarus, Poland, and Slovakia are 22.6%, 41.8%-56.7%, and 22.3%-27.0%, respectively [6, 29, 70, 71]. In Western Europe, particularly, Germany, the Netherlands, and the United Kingdom, the respective rates for *D. reticulatus* ticks are also highly variable: 64.0%, 14.0 %, and 5.0 %, respectively [72-74].

Data on the prevalence of *Babesia* spp. in ixodid ticks from Ukraine are very scarce and limited only to Kyiv [51, 53]. The most recent study showed that only 0.5% and 5.1% of *I. ricinus* and *D. reticulatus* adults, respectively, collected from Kyiv were PCR-positive for *Babesia* DNA [51]. In contrast, none of *I. ricinus* (n=260) and *D. reticulatus* (n=100) adults collected from the CEZ tested positive [52]. Consistently, the current report showed that *Babesia*-positive ixodid ticks were detected in 3 out of 5 cities and that the overall prevalence was only 1.4%-2.6%. In comparison, 4.2% of questing *D. reticulatus* adults collected from Poland carried *Babesia* DNA [29]. In Russia, the prevalence rates of *Babesia* spp. for questing adults of *I. ricinus* and *D. reticulatus* were, respectively, 3.9% and 20.3% [67].

The first and only studies that have demonstrated the detection of *Bartonella* spp. in questing ixodid ticks from Ukraine were concurrently performed in Kyiv and the CEZ in Spring 2016 [51, 52]. It was demonstrated that the respective rates in questing adults of *I. ricinus* and *D. reticulatus* collected from Kyiv were 2.7% and 1.0%, respectively [51]. Moreover, somewhat higher prevalence was observed in questing adults of *I. ricinus* (8.1%) and *D. reticulatus* (3.0%) from the CEZ [52]. The present study showed that 16.0% and 3.5% of questing adults of *I. ricinus* and *D. reticulatus*, respectively, collected from the five cities were *Bartonella* DNA-positive. In European countries, the prevalence rates of *Bartonella* spp. in questing adults of *I. ricinus* may vary greatly and, in general, are similar to those calculated for Ukraine. As an example, 0%, 1.6%, 1.7%, 2.9%, and 12.0% of questing adults of *I. ricinus* collected from Portugal, Czech Republic, Poland, Germany, and France, respectively, tested positive for *Bartonella* DNA [75-77]. Interestingly, as opposed to *B. bovis* identified in the present study, *Bartonella henselae* was the only *Bartonella* species consistently detected in the respective ixodid ticks from Kyiv and the CEZ by the two previous studies [51, 52]. In comparison, *Bartonella doshiae* was the most dominant *Bartonella* species detected in questing *I. ricinus* ticks from Austria (53.8%), followed by *Bartonella henselae* (38.5%) and *Bartonella grahamii* (7.7%) [78].

Similar to Western Europe and the United States, LB is one of the most dominant tick-borne diseases in Ukraine [11, 36, 37, 79]. However, despite the incidence of human LD has been steadily increasing in Ukraine [80], the studies on the prevalence of *B. burgdorferi* s.l. in *I. ricinus* ticks from different regions of Ukraine are very limited. There are only a few studies that have utilized molecular approaches to detect the LB pathogen in the ixodid ticks from Ukraine. Two recent independent investigations demonstrated that 3.9%-10.4% *I. ricinus* adults collected from various recreational parks of Kyiv (Central Ukraine) carried *B. burgdorferi* s.l. DNA [51, 53]. In the CEZ, just two hours north from Kyiv, the respective prevalence was marginally higher (13.5%) [52]. In Western Ukraine, specifically Lviv and Volyn regions, the prevalence of PCR-positive pools for *B. burgdorferi* s.l. in *I. ricinus* ticks was 29.3% [48]. In contrast, a much lower rate (4.4%) was recently noted for *I. ricinus* ticks collected from the Ternopil region (Western Ukraine) [54]. Thus, collectively, the data showed that the prevalence of *B. burgdorferi* s.l. in *I. ricinus* ticks may vary considerably between and within different parts of Ukraine. This is well consistent with the latest meta-analysis of surveillance data, which demonstrated that, in Europe, variation in the reported prevalence rates of *B. burgdorferi* s.l. in questing *I. ricinus* ticks may be as low as <1% to as high as >46% [43].

Conclusions

In summary, the current study has examined the prevalence rates of selected zoonotic pathogens in ixodid ticks collected from animals and vegetation in the five regions of Ukraine. As a result, the prevalence rates of *A. phagocytophilum*, the *Anaplasmataceae* family, *Rickettsia* spp., *Babesia* spp., *Bartonella* spp., and *B. burgdorferi* s.l. were identified for Chernivtsi, Khmelnytskyi, Kyiv, Ternopil, and Vinnytsia. In addition to reporting the most updated data for Kyiv and Ternopil; this study is the first to provide the prevalence rates of the important tick-borne pathogens for the other three major cities of Ukraine (Chernivtsi, Khmelnytskyi, and Vinnytsia) and their respective administrative regions. Moreover, this investigation is the first to detect *Ca. N. mikurensis* in ixodid ticks from Ukraine. Overall, the present data should be useful not only for medical and veterinary practitioners, but also the public health officials, especially, when assessing the risks associated with tick-borne zoonoses in Ukraine. In addition, the authors hope that this report will also contribute, via dissemination of translated version of this article, to increasing the overall awareness of tick-borne diseases among the population of Ukraine.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

V.A.L. conceived the experiments, performed some experiments, analyzed, and interpreted the data, and wrote the manuscript.

A.B.M. performed some experiments and was involved in the data collection.

B.V. was involved in critical revision of the article.

D.C. and L.B. performed some experiments.

K.A.S. and Y.Y. were involved in the data collection and critical revision of the manuscript.

E.D. performed some experiments and was involved in conception and design of this study.

Z.G. and S.G. were involved in conception and design of this study.

S.L. and L.Z. statistically analyzed the data.

A.S.R. was the major contributor in the data analyses and interpretation, and writing the manuscript.

All authors read and approved the final manuscript.

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Tables

Table 1. Total numbers of ixodid ticks collected from five studied regions of Ukraine.

Regions/their respective cities	<i>Ixodes ricinus</i>						<i>Dermacentor reticulatus</i>						<i>Ixodes hexagonus</i>					
	From animals			From vegetation			From animals			From vegetation			From animals			From vegetation		
	F*	M*	N*	F	M	N	F	M	N	F	M	N	F	M	N	F	M	N
Chernivtsi	13	3	0	6	1	0	37	34	0	9	5	0	0	0	0	0	0	0
Khmelnyskyi	19	2	0	0	0	0	40	37	0	21	19	0	1	0	0	0	0	0
Kyiv	15	0	0	5	0	1	32	22	0	11	6	0	0	0	0	2	0	0
Ternopil	7	2	0	4	0	0	21	15	0	11	5	0	0	0	0	0	0	0
Vinnytsia	11	0	0	9	0	1	19	24	0	30	24	0	0	0	0	0	0	0
Total	65	7	0	24	1	2	149	132	0	82	59	0	1	0	0	2	0	0

*F, M, and N denote female, male, and nymphal ticks, respectively.

Table 2. Total numbers of *Ixodes ricinus* and *Dermacentor reticulatus* ticks collected from animals.

Regions/their respective cities	Ixodid ticks [^]											
	<i>Ixodes ricinus</i>						<i>Dermacentor reticulatus</i>					
	Cats		Dogs		Other animals		Cats		Dogs		Other animals	
	F%	M%	F	M	F	M	F	M	F	M	F	M
Chernivtsi	2	0	11	3	0	0	2	0	9	11	26*	23*
Khmelnyskyi	2	0	17	2	0	0	2	7	23	30	15*	0
Kyiv	0	0	14	0	1 [#]	0	0	0	28	22	4 ^{&}	0
Ternopil	0	0	5	2	2*	0	0	0	14	8	7*	7*
Vinnytsia	2	0	7	0	2*	0	0	0	8	13	11*	11*
Total	6	0	54	7	5	0	4	7	82	84	63	41

[^] Only one female tick of *Ixodes hexagonus* was collected from a cat in Khmelnytskyi.

% F and M denote female and male ticks, respectively.

[#] the respective ixodid tick was collected from bear.

* the respective ixodid ticks were collected from cattle.

[&] 2, 1, and 1 ixodid ticks were collected from lynx, racoon, and red fox, respectively.

Table 3. Primers used for PCR amplification and sequencing.

Pathogens	The original primer names (their sequences)	Target genes	Expected length of amplicon, base pairs	References
<i>A. phagocytophilum</i>	MSP2-3f (5'-CCAGCGTTTAGCAAGATAAGAG-3') MSP2-3r (5'-GCCCAGTAACAACATCATAAGC-3')	<i>msh2</i>	334	[60]
The family of <i>Anaplasmataceae</i>	ApNeo F (5'-GGGGATGATGTCAARTCAGCAY-3') ApNeo R (5'-CACCAGCTTCGAGTTAAGCCAAT-3')	<i>16S rRNA</i>	257	[61, 81]
<i>Rickettsia</i> spp.	D767f (5'-CGATGGTAGCATTAAAAGCT-3') D1390r (5'-CTTGCTTTTCAGCAATATCAC-3')	<i>sca4</i>	590-653 bp	[59]
<i>Babesia</i> spp.	BabCOX1R (5'-ACTCTATAGGTATTTGACGTAATT-3') BabCOX1F (5'-TGTTAAAAAACTTTATA-3')	<i>18S rRNA</i>	340	[57]
<i>Bartonella</i> spp.	ssrA-F (5'-GCTATGGTAATAAATGGACAATGAAATAA-3') ssrA-R (5'-GCTTCTGTTGCCAGGTG-3')	<i>tmRNA</i>	301	[82]
<i>B. burgdorferi</i> s.l.	SC1 1F (5'-GCTGGCAGTGCGTCTTAA-3') SC1 1R (5'-CTTAGCTGCTGCCTCCCGTA-3')	<i>16S rRNA</i>	325 bp	[58]

Table 4. Prevalence of tick-borne pathogens in adult ticks of *Ixodes ricinus* from five studied regions of Ukraine.

Origin of ticks	<i>A. phagocytophilum</i>	The family of <i>Anaplasmataceae</i>	<i>Rickettsia</i> spp.	<i>Babesia</i> spp.	<i>Bartonella</i> spp.	<i>B. burgdorferi</i> s.l.
Chernivtsy						
From animals	5/16 (31.2%)*	13/16 (81.2%)	5/16 (31.2%)	0/16 (0%)	1/16 (6.2%)	5/16 (31.2%)
From vegetation	0/7 (0%)	2/7 (28.6%)	1/7 (14.3%)	0/7 (0%)	1/7 (14.3%)	1/7 (14.3%)
Combined	5/23 (21.7%)	15/23 (65.2%)	6/23 (26.1%)	0/23 (0%)	2/23 (0.9%)	6/23 (26.1%)
Khmelnyskyi						
From animals	1/21 (4.8%)	14/21 (66.7%)	6/21 (28.6%)	2/21 (9.5%)	1/21 (4.8%)	6/21 (28.6%)
From vegetation	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
Combined	1/21 (4.8%)	14/21 (66.7%)	6/21 (28.6%)	2/21 (9.5%)	1/21 (4.8%)	6/21 (28.6%)
Kyiv						
From animals	2/15 (13.3%)	8/15 (53.3%)	2/15 (13.3%)	0/15 (0%)	1/15 (6.7%)	4/15 (26.7%)
From vegetation	0/5 (0%)	4/5 (80.0%)	2/5 (40.0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Combined	2/20 (10.0%)	12/20 (60.0%)	4/20 (20.0%)	0/20 (0%)	1/20 (5.0%)	4/20 (20.0%)
Ternopil						
From animals	1/9 (11.1%)	7/9 (77.8%)	2/9 (22.2%)	0/9 (0%)	1/9 (11.1%)	4/9 (44.4%)
From vegetation	0/4 (0%)	2/4 (50.0%)	0/4 (0%)	0/4 (0%)	1/4 (25.0%)	0/4 (0%)
Combined	1/13 (7.7%)	9/13 (69.0%)	2/13 (15.4%)	0/13 (0%)	2/13 (15.4%)	4/13 (30.8%)
Vinnytsya						
From animals	1/11 (9.1%)	10/11 (90.9%)	3/11 (27.3%)	1/11 (9.1%)	1/11 (9.1%)	4/11 (36.4%)
From vegetation	0/9 (0%)	7/9 (77.8%)	3/9 (33.3%)	0/9 (0%)	2/9 (22.2%)	1/9 (11.1%)
Combined	1/20 (5.0%)	17/20 (85.0%)	6/20 (30.0%)	1/20 (5.0%)	3/20 (15.0%)	5/20 (25.0%)

*Values correspond to number of PCR-positive ticks/total number of ticks tested and the respective percentage of PCR-positive ticks are indicated in parentheses.

Table 5. Prevalence of tick-borne pathogens in adult ticks of *Dermacentor reticulatus* from five studied regions of Ukraine.

Origin of ticks	<i>A. phagocytophilum</i>	The family of <i>Anaplasmataceae</i>	<i>Rickettsia</i> spp.	<i>Babesia</i> spp.	<i>Bartonella</i> spp.
Chernivtsy					
From animals	0/71 (0%)*	35/71(49.3%)	15/71(21.1%)	2/71(2.8%)	4/71(5.6%)
From vegetation	2/14 (14.3%)	9/14 (64.3%)	7/14 (50.0%)	0/14 (0%)	1/14 (7.1%)
Combined	2/85 (2.3 %)	44/85 (51.8%)	22/85 (25.9%)	2/85 (2.3%)	5/85 (5.9 %)
Khmelnyskyi					
From animals	2/77 (2.6%)	52/77 (67.5%)	21/77 (27.3%)	3/77 (3.9%)	3/77 (3.9%)
From vegetation	1/40 (2.5%)	13/40 (32.5%)	14/40 (35.0%)	0/40 (0%)	1/40 (2.5%)
Combined	3/117 (2.6%)	65/117 (55.6%)	35/117 (29.9%)	3/117 (2.6%)	4/117 (3.4%)
Kyiv					
From animals	2/54 (3.7%)	29/54 (53.7%)	13/54 (24.1%)	1/54 (1.8%)	3/54 (5.6%)
From vegetation	1/17 (5.9%)	8/17 (47.1%)	9/17 (52.9%)	0/17 (0%)	0/17 (0%)
Combined	3/71 (4.2%)	37/71 (52.1%)	22/71 (31.0%)	1/71 (1.4%)	3/71 (4.2%)
Ternopil					
From animals	2/36 (5.6%)	19/36 (52.8%)	7/36 (19.4%)	0/36 (0%)	3/36 (8.3%)
From vegetation	0/16 (0%)	5/16 (31.2%)	5/16 (31.2%)	0/16 (0%)	1/16 (6.2%)
Combined	2/52 (3.8%)	24/52 (46.1%)	12/52 (23.1%)	0/52 (0%)	4/52 (7.7%)
Vinnytsya					
From animals	1/43 (2.3%)	24/43 (55.8%)	15/43 (34.9%)	0/43 (0%)	2/43 (4.6%)
From vegetation	1/54 (1.8%)	26/54 (48.1%)	11/54 (20.3%)	0/54 (0%)	2/54 (3.7%)
Combined	2/97 (2.1%)	50/97 (51.5%)	26/97 (26.8%)	0/97 (0%)	4/97 (4.1%)

*Values correspond to number of PCR-positive ticks/total number of ticks tested and the respective percentage of PCR-positive ticks are indicated in parentheses.

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Figures

Figure 2

The map of Ukraine# with indicated regions of Ukraine (Chernivtsi, Khmelnytskyi, Kyiv, Ternopil and Vinnytsia) where ixodid ticks were collected. # The map (without the indication of the tick collection sites) was adapted from www.wikipedia.org and the map license link is as follows: https://commons.wikimedia.org/w/index.php?title=File:Ukraine_topo_en.jpg&oldid=368772447. * Indicated by red circles are the cities, whose administrative regions included the tick collection sites.

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