Ticks and tick-borne pathogens in popular recreational areas in Tallinn, Estonia: an underestimated risk of tick-borne diseases

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Research

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Abstract

BACKGROUND

For over several decades ticks are noticed to be widely present in the green spaces of urban environments. Rodents that provide essential blood-meals for subadult ticks and serve as natural reservoirs for many known tick-borne diseases, such as Lyme borreliosis and tick-borne encephalitis, might be quite abundant in green areas within urban settlements. In that way, the improvement of green infrastructures within cities possess not only to better human well-being but might also increase the risk of being bitten by a tick and having a tick-borne disease. This study aimed to provide a first insight into ticks and tick-borne pathogen presence and prevalence in popular recreational green areas in Tallinn, Estonia.

METHODS

Ticks were collected by flagging in May-June, 2018. Tick species identification was performed on morphological criteria. Tick-borne pathogens detection was performed by pathogen-specific PCR and qPCR reactions.

RESULTS

855 *Ixodes* ticks were collected from a total area of 12 000 m^2. An estimated tick abundance were up to 18.5 ticks per 100 m^2. 34.3% of all ticks were revealed positive for at least one tick-borne pathogen. *Borrelia burgdorferi* s.l. was found in 17.5%, *Rickettsia* sp in 13.8%, *Neoehrlichia mikurensis* on 5.4%, *B. miyamotoi* in 2.5%, *Anaplasma phagocytophilum* in 0.6% and tick-borne encephalitis virus in 0.5% of ticks.

CONCLUSION

This study reports the occurrence of *Ixodes* tick species in popular recreational, outdoor sports and leisure areas in the largest city of Estonia, with abundance and prevalence rates compatible or even exceeding those detected previously in the most endemic foci in the natural environments. Taking into account increasing Lyme borreliosis incidence, the risk of acquiring a tick bite and being infected with a tick-borne disease in urban recreational sites should not be ignored and proper information about the precautions might be considered.

Backround

Ticks, especially those belonging to *Ixodes ricinus* complex, are among the most medically important vectors for various diseases in the Northern Hemisphere. Traditionally the risk of a tick bite followed by Lyme borreliosis (LB) or tick-borne encephalitis (TBE) has been associated with deciduous or mixed boreal forests, pastures and meadows [1]. The development of green infrastructures such as parks, recreational and leisure zones, outdoor sports facilities and health trails within the cities improve citizen attraction towards outdoor activities but also increases the risk for various zoonotic and vector-borne diseases.

Ticks are widely present in the green spaces of the urban environments even despite a low local floral and faunal diversity. Many rodent species, that are essential blood-meal providers for larval and nymphal ticks and reservoirs for tick-borne pathogens (TBPs) such as *Borrelia burgdorferi* sensu lato (BBSL) and tick-borne encephalitis virus (TBEV), are highly established in urban areas, well adapted to anthropogenic pressure and may thus play a role in the increasing incidence of tick-borne diseases (TBDs) in cities. Moreover, urban heat island effect may reflect positively on arthropods sensitive to cold temperatures by promoting their survival and shortening developmental diapause periods [2]. Several studies from Europe report the detection of various TBPs in ticks found within cities: TBEV, BBSL, *B. miyamotoi*, *Neoehrlichia mikurensis*, *Anaplasma phagocytophilum* and *Rickettsia helvetica* [3, 4, 5]. Therefore, urban green areas introduce a potential risk not only for acquiring a tick bite but also for TBDs.

Estonia has been an endemic area for TBE and LB for decades, reaching oppositely record incidence rates values by 2020. While the incidence of TBE fell almost threefold to its lowest of the last 20 years rate of 5.1 cases per 100 000 population, the number of LB cases was a record high comparing the same period – 182.1 cases per 100 000 population, which is three times higher for compared to 2013 levels [6]. Still, about 15% of confirmed TBD-infected individuals with assumable geographical origin of a tick bite, were reported within urban areas [7]. Two non-nidicolous *Ixodes* tick species of medical importance, *I. ricinus* and *I. persulcatus* have been shown to maintain the circulation of various TBPs in Estonia. In addition to European (TBEV-Eu) and Siberian (TBEV-Sib) TBEV subtypes, at least five species of BBSL, as well as various TBPs including tick-borne *Rickettsiales* (*A. phagocytophilum*, *Ehrlichia muris*, *N. mikurensis*, *R. helvetica, R. monacensis*, *Ca*. R. tarasevichae), relapsing fever group *B. miyamotoi* and several *Babesia* species have been detected in questing ticks in their natural habitats [8, 9, 10, 11, 12, 13, 14].

This study provides the first insight into ticks and TBPs distribution within urban areas in the city of Tallinn by investigating the presence and abundance of ticks and analysis of the taxonomic content of tick-borne pathogens in the popular recreational areas and leisure sites located

within the city and in the nearest peri-urban surroundings.

Materials And Methods

Study areas selection and tick collection sites

Two main aspects were considered when choosing study areas: suitable habitats for ticks and popularity among visitors. Satellite imagery from Google Earth and Estonian Land Board Web Map application (xgis.maaamet.ee/maps), as well as personal observations and data on recreational areas from the official Tallinn webpage (www.tallinn.ee) were used for initial identification of potentially suitable study areas which were then visited to determine specific flagging sites. Urban areas were selected according to the presence of bushes, broad-leaf or temperate forested area and a litter layer. Peri-urban collection sites were situated mostly in deciduous forests or their edges, with known recreational popularity among visitors. According to observations, 17 urban and 3 peri-urban areas were selected in collaboration with the respective authorities where applicable (Fig. 1). Each transect was located along trails or their closest proximity to imitate visitors´ behavior as closely as possible. The observations and variables recorded at each site included date, time, transect length passed and air temperature. Habitat and vegetation type as well signs of any host presence by visualizing an actual animal or any direct evidence (e.g. tracks, birdsongs, nests, faeces) were also noted.

Based on climate and weather observations from previous years, the month of May was predicted to be the most suitable time for the survey. Sites were surveyed for ticks from 9th May to 1st June, 2018, preferably during morning hours from 9 to 11 am, as ticks are active and dew is not heavy on the vegetation, which might influence tick attachment to the collection cloth. Each site was surveyed once.

Tick collection and species identification

Tick collections were performed using the flagging technique with a 1 m^2 light-colored flannel cotton cloth, attached to a wooden T-shaped handle, which was dragged over the vegetation and observed for tick presence at every 5 meters passed. The minimum transect was 300 m^2 per collection. However, no unique transect sizes nor standardized flagging was used as the terrain, weather and vegetation conditions at every chosen area varied and no specific focus on tick density evaluation had been planned. Ticks were removed from the cloth with tweezers, placed into separate glass vials according to stage and sex, and stored at + 4°C prior to species identification. The presence of larvae was noted but these were not collected, counted nor included in any analysis of the study. Adults and nymphal ticks were identified individually using a stereomicroscope according to morphological keys [15]. Ambiguous specimens were additionally identified by molecular keys using PCR based on internal transcribed spacer 2 (ITS2) and partial 16S rRNA gene as described previously [10, 16]. Mean abundance (number of nymphal and adult ticks per 100 m), as well as index of abundance were calculated as described [17].

Nucleic acid isolation and TBPs screening

All ticks were individually processed for DNA and RNA isolation by blackPREP Tick DNA/RNA kit (Analytik Jena, Germany). The initial tick lysis step was increased twice in time and the homogenization step was performed twice according to manufacturers' recommendations. Homogenization was performed using MixerMill MM301 (Retsch, Haan, Germany). Mixing mill cassettes with vials, containing tick homogenate, were flipped over between homogenization steps to assure better milling performance. DNA and RNA solutions were then stored at -20C and −70C, respectively, prior to further individual screening for the presence of TBPs.

All ticks' nucleic acids extracts were analyzed individually. Positive ticks' samples and deionized PCR-grade water were used as positive and negative controls, respectively, at every amplification step. To reduce contamination risk, every procedure, including tick pre-extraction washes, RNA/DNA extraction, PCR reaction mix preparation, DNA/RNA adding step, PCR reaction and gel-electrophoresis were performed in separate rooms.

The initial screening of TBEV RNA, based on the amplification of 3´ non-coding region, was performed with primers F-TBE1, R-TBE1 and probe TBE-W [18]. For further sequencing and genotyping, all positive samples were amplified additionally by nested RT-PCR for the partial E protein gene with SuperScript III Reverse Transcriptase kit (ThermoFisher Scientific, USA) and primers described elsewhere [13, 19].

Nested PCR reactions were used for screening and genotyping of BBSL, *B. miyamotoi*, Anaplasmataceae as well as *Rickettsia* species identification prior initial screening.

The detection of BBSL and *B. miyamotoi* DNA was performed by amplification of 245–256 bp long 5S-23S transcribed internal spacer region and 532 bp fragment of *p66* gene, respectively [20, 21]. To confirm *B. miyamotoi* species, p66-positive samples were additionally amplified for 379 bp long *glpQ* gene fragment.

Identification of *Anaplasmataceae* DNA in the tick samples was performed by amplifying 524 bp gene fragment of *16S rRNA* gene [10]. For further sequencing analysis, all positive samples were subjected to amplification of 1350 bp long *Anaplasmataceae 16S rRNA* gene [10] under modified cycling conditions (Supplementary Table 1). Samples positive after initial screening, but negative for 1350 bp long 16S rRNA fragment,

were additionally amplified for a 1300 bp long fragment of heat shock operon *groESL* gene with primers described elsewhere [22], under modified cycling conditions (Supplementary Table 1).

Rickettsia spp. screening in tick DNA samples was performed with qPCR amplifying 74 bp region of *gltA* gene [11]. Genotyping of *Rickettsia* species in positive qPCR samples was performed by sequencing of 667 bp long region of *gltA* gene, amplified by nested PCR [23]. Samples positive for qPCR, but negative for *gltA* region, were additionally subjected to PCR amplification of a 769 bp long *ompB* gene region as described by Roux and Raoult [24] and an 843 bp long region of *sca4* gene as described by Igolkina *et al.* [23].

Detailed information on all PCR reactions, including targets, product size, primer/probe sets and amplification conditions used in the current study are presented in Supplementary Table 1.

All final nested PCR products were visualized by 1% agarose gel electrophoresis, stained with ethidium bromide.

For genotyping, all final PCR products were subjected to direct Sanger sequencing, performed at Core Facility, Institute of Genomics, University of Tartu.

Genotyping analyses of retrieved sequences were performed using BioEdit and MEGA 7.0 software BLASTN® tools (http://www.ncbi.nlm.nih.gov/BLAST.cgi).

Results

Tick sampling

From the 20 sites visited during the study, ticks were found at 13 sites within the city, and at all 3 peri-urban sites. There were no ticks found in the city central parks Hirvepark and Toompark, von Glehni park, Järve health trails and Sanatooriumi park (sites 5, 6, 14 and 16, respectively) (Figure 1). As tick sampling was not standardized for the collection area and time, the collection results could not be extrapolated to questing tick density in the surveyed regions. However, mean tick abundance and abundance index were calculated for all study sites.

A total of 186 adults and 669 nymphs were collected from over 12 000 m^2 of vegetation screened at 17 urban and 3 peri-urban sites (Table 1). All ticks were identified by morphological criteria and by ITS2 based PCR as *I. ricinus* except one *I. persulcatus* collected in Sütiste park.

Among all visited sites, significantly higher numbers of ticks were collected at Estonian Open Air Museum, Tallinn Zoo and Pirita forest park that accounted for 26.4%, 24.7%, and 14.3% of the total number of collected ticks, respectively. Concordantly, the estimated mean abundance of ticks at the urban sites was also the greatest at Estonian Open Air Museum and Tallinn Zoo (18.8 and 17.6, respectively), followed by Pirita forest park (9.8). Among the peri-urban sites, the highest number of collected ticks, as well as the highest abundance rate was observed in the Männiku forest (Table 1).

Pathogen prevalence

All adults (n=186) and nymphal (n=669) ticks were individually screened for the presence of followed tick-borne pathogens: TBEV, *B. burgdorferi* s.l., *B. miyamotoi*, *Anaplasmataceae* (*Anaplasma, Ehrlichia* and *Neoehrlichia*) and *Rickettsia spp*. Overall, TBPs were detected at every site where ticks were found, except Harku-Nõmme, although their taxonomic composition and prevalence varied.

The total prevalence of ticks with at least one pathogen was 34.3% (293/855) (Table 2). Due to non-standardized collections, estimated prevalence rates were calculated only for sites with over 50 adult and nymphal ticks collected and analyzed, that are Pirita forest park, Estonian Open Air Museum, Tallinn Zoo and Männiku. Among these, the site-specific prevalence of TBP-positive ticks was the highest at Estonian Open Air Museum and Tallinn Zoo – 43.8% and 42.2%, respectively, followed by Pirita forest park (31.1%) and peri-urban Männiku forest (18.9%) (Table 2). For other sites, only the presence of TBPs and the number of ticks tested positive have been noted.

Borrelia burgdorferi (sensu lato)

B. burgdorferi s.l. was the most prevalent detected TBP. In total, 150 ticks tested positive for the presence of BBSL DNA by 5S-23S based PCR, indicating a 17.5% prevalence among all analyzed ticks, and 51.2% among ticks with at least one TBP. BBSL was found in ticks collected at almost every collection site, except Pirita river valley, and Harku-Nõmme. The highest site-specific prevalence was observed at Estonian Open-Air Museum and Tallinn's Zoo – 25.2% and 22.7%, respectively. Surprisingly, a significantly lower rate was detected in ticks collected in the peri-urban Männiku forest.

Sequence analysis of the 5S-23S intergenic spacer region revealed the presence of three *B. burgdorferi* s.l. genospecies: *B. afzelii* (127/150; 84.7%), *B. garinii* (11/150; 7.3%), *B. valaisiana* (7/150; 4.7%) and *B. bavariensis* (1/150; 0.7%), while four samples remained unspecified at the

genospecies level.

Borrelia afzelii was detected in 11 of 13 urban and all sub-urban sites. It was the most prevalent genospecies with rates up to 24.8% and 21.8% at Open Air Museum and Tallinn's Zoo, respectively, followed by Pirita forest park (4.9%) and Männiku (4.1%) (Table 2). According to the phylogenetic analysis, all *B. afzelii* 5S-23S spacer region sequences obtained in this study had nucleotide similarity rates within 77.4% to 99.5% between each other and were 100% identical to those previously found to be circulating in Estonian questing and passerine-attached ticks (GenBank accession no. KX418639, KX418638, KX418640), and to other sequences reported from France (acc. no. KY273112, KY273113), Italy (acc. no. MT038899), Slovakia (acc. no. KX906933, KX906945), Taiwan (acc. no. JX649207) and Russia (acc. no. MK118750, AB178349).

The second most prevalent BBSL genospecies detected in urban and peri-urban ticks, although at significantly lower rates, was *B. garinii,* which was detected in 11 ticks, collected at 5 urban sites, with the majority from Pirita forest park (6/122), followed by Tallinn's Zoo (2/211). Single *B. garinii* -positive ticks were detected also at Kadrioru, Ilmarise health trails, and Estonian Open Air Museum (Table 2). The *B. garinii* 5S-23S IGS sequences of this study showed similarity rates from 79.0% to 99.5% between each other and clustered with sequences reported from Estonia (acc. no. KX418634 and KX418637) as well as from Taiwan (acc. no. JX649205), Italy (acc. no. MT038900) Belarus (acc. no. AY772205), Sweden (acc. no. JX909934), Czech (acc. no. AF497993) and Russia (acc. no. MK118761).

Borrelia valaisiana and *B. bavariensis* genospecies were also found, albeit at overall prevalence rates of <1%. *B. valaisiana* was detected at overall prevalence of 0.8% (7/855) in ticks collected at Pirita forest park (2/122), Ilmarise health trails (1/37), Stroomi (1/38), Nõmme-Mustamäe (1/30) and Sütiste (TalTech) park (2/21). *Borrelia bavariensis* was detected in a single tick from the suburban site Jägala (Table 2). According to the phylogenetic analysis of 5S-23S sequences of *B. valaisiana* obtained in this study, they were identical to each other and to *B. valaisiana* isolate 122 (acc. no. KX418641) previously detected in Estonian *I. ricinus*, removed from the Common Blackbird [25], and also to *B. valaisiana* strains reported from Spain (acc. No. MG245790), Czech Republic (acc.no AF497989) and Italy (acc.No MT038902). The *B. bavariensis* 5S-23S rRNA sequence retrieved from *I. ricinus* collected in Jägala was identical to that found in *I. ricinus* from the Estonian county Läänemaa [8] and also to strain Ir-4370, reported from Stavropol, Russia (acc.no KU672534) and *B. bavariensis* prototype strain PBi (acc. no FJ546494).

Borrelia miyamotoi

Borrelia miyamotoi, belonging to the relapsing-fever group *Borrelia*, was detected in 2.5% of all analyzed ticks (21/855). This genospecies was found mostly in Estonian Open Air Museum (10/226, 4.4%) and Tallinn Zoo (8/211, 3.8%), followed by peri-urban Männiku forest (2/74, 2.7%). A single *B. miyamotoi*-positive *I. ricinus* was also collected from the surroundings of Tallinn Zoo (Table 2). Analysis of the *B. miyamotoi* partial *p66* gene showed that nucleotide sequences of this study are identical to each other and sequences revealed previously in the Estonian tick population [9].

Rickettsiales

Rickettsia sp. were the second most prevalent bacterial TBP after BBSL: its presence was detected in 13.8% (118/855) of analyzed tick samples. Prevalence in the study sites ranged between 10.8% (8/74) in peri-urban Männiku and 18% (22/122) at Pirita forest park (Table 2). According to phylogenetic analysis of partial *gltA* gene nucleotide sequences, all *Rickettsia* positive samples belonged to the *R. helvetica* species. Sequences were identical to each other and sequences previously reported in Estonian *Ixodes* ticks Katargina *et al.* [11]. Samples that were sequenced for partial *sca4* and *ompB* genes were also classified as *R. helvetica* species and were identical to each other within each gene fragment.

A total of 6.0 % (51/ 855) single analyzed ticks collected at 4 urban and 2 peri-urban sites tested positive for the presence of *Anaplasmataceae* DNA according to partial 16S rRNA PCR results. The highest prevalence was observed among ticks collected at Tallinn Zoo (24/211, 11.4%) and Estonian Open Air Museum (18/226, 8.0%), followed by Pirita forest park with a 4.9% prevalence (6/122). Single *Anaplasmataceae*-positive ticks were also collected in the Tallinn's Zoo surrounding area and sub-urban Vääna-Jõesuu and Jägala areas. The analysis of *Anaplasmataceae* 16S rRNA sequences revealed the presence of two species: *A. phagocytophilum* (0.6%, 5/855) and *N. mikurensis* (5.4%, 46/855) (Table 2). At Pirita forest park two ticks out of 122 tested positive for the presence of *A. phagocytophilum* DNA, as did single ticks from three other locations: Open Air Museum, Tallinn Zoo and the sub-urban area of Jägala. 16S rRNA partial nucleotide sequences of *A. phagocytophilum* obtained in this study were 99.7% - 99.9% similar to each other. The comparison to previously reported sequences from Estonian questing ticks (acc.no HQ629920, HQ629922, HQ629920) and sequences reported from Russia (acc.no HQ629911), Sweden (acc.no AY527213) and Austria (acc.no JX173652) showed 99.7% - 100% similarity

The highest prevalence of *N. mikurensis* was observed in questing ticks collected from Tallinn Zoo (10.9%, 23/211), followed by Estonian Open Air Museum (7.5%, 17/226) and Pirita forest park (3.3%, 4/122). Single *N. mikurensis*-positive ticks were also found in the surrounding area of Tallinn Zoo and peri-urban Vääna-Jõesuu. Sequences of *N. mikurensis* partial *16S rRNA*, retrieved in this study, showed 98.2% - 99.6% similarity to GenBank sequences reported previously from Estonian ticks (acc. no KU535862) and 98.1% - 99.4% similarity to sequence from Germany (acc. no KU865475) and Russian Western Siberia (acc.no MN736126).

TBEV

According to qRT-PCR results, TBEV was the least common of detected TBPs, detected with in 4 *I. ricinus* nymphs of all 855 examined individual ticks found at Pirita river valley, Ilmarise health trails, Estonian Open Air Museum and Männiku forest (total prevalence of 0.5%). Two samples were successfully sequenced and genotyped (Table 2).

According to the analysis of the partial E gene sequence obtained from an *I. ricinus* tick sample from the Estonian Open Air Museum, it clustered with TBEV-Sib sequences previously detected in Estonian *I. persulcatus* ticks collected in Eastern Estonia (TBEV isolates Est222 and Est221, accession numbers KT748749 and KT748748, respectively) at an identity rate of 99.8%, and belonging to the Baltic lineage within TBEV-Sib [13, 26]. Another TBEV partial E gene sequence, retrieved from an *I. ricinus* sample collected at Ilmarise health trails, clustered within the TBEV-Eu subtype with 98.7% similarity to previously reported Estonian strain Est3476 (acc.no GU183383) and 99.6% similarity to TBEV strain Latvia-8110 (acc.no. AJ319583).

Mixed infections

In all, 15.0 % (44/293) of all TBP-positive ticks contained double infections and 4 tick samples tested positive for three tick-borne pathogens (4/293, 1.4%). The most frequently detected TBP combination in double infected ticks was *B. afzelii* with *N. mikurensis* (18/44) or *R. helvetica* (14/44) and these originated mainly from Open Air Museum, Tallinn Zoo and Pirita-Pirita forest park. It is noteworthy that of the 4 TBEV-positive tick samples, 2 were co-infected with bacterial TBP: one tick sample from Estonian Open Air Museum was positive for TBEV-Sib and *N. mikurensis*, and the TBEV-Eu – positive sample from Männiku was also positive for unspecified BBSL. Of the four tick samples with triple infections, three tested positive for the presence of *B. afzelii*, *R. helvetica* and *N. mikurensis*, and one for *B. afzelii, R. helvetica* and *B. miyamotoi*.

Discussion

More than 30 years have passed since the urbanization of arthropod vectors and the occurrence of tick-borne pathogens within cities and industrial regions were first reported [27, 28]. Since then, continuous ecologic and climate changes along with socio-demographic drivers have altered tick-associated natural environments resulting in reports of an increase in tick abundance and various tick-borne pathogens in cities, parks, outdoor leisure areas and other urbanized regions across Europe [4, 5, 29, 30, 31]. Furthermore, the northward expansion of *I. ricinus* and the occurrence of *I. persulcatus* to the west and north of its main distribution area may also drive the spread of tick-borne pathogens into new areas, giving rise to new foci as well as affecting public health [32, 33].

This study confirms the occurrence of *Ixodes* tick species in popular recreational, outdoor sports and leisure areas in the capital and largest city of Estonia, with abundance rates compatible or even exceeding those detected previously in the most endemic foci in the natural environments [34]. The number of ticks collected at different urban and peri-urban locations during this study might not emulate the actual abundance as this study was not focused on tick density and flagging was not highly standardized. It is, however, noteworthy, that a large number of ticks was found in larger, less fragmented forest-type parks with needle- and broad-leaved trees, and underwood with rich litter, as well as signs of the presence of an ample variety of urbanized small and medium-sized mammals such as *Apodemus* and *Myodes* rodents, shrews, hedgehogs, ground nesting and feeding birds, foxes and roe deer. Such an environment is a prerequisite for tick survival, development and maintenance. As seen in this study, Zoo, Open Air Museum and Pirita forest park, which are situated in large urban parks with areas similar to natural environments with a variety of small- and medium-sized animal species living there – showed significantly higher tick abundance rates compared to those, similar in vegetation but poorer in animal species and more fragmented in size (Sanatooriumi park, Järve forest health trails, Harku-Nõmme and von Glehni park). As a contrast, the carefully managed Kadriorg park, the largest and most popular park in Tallinn with many mowed open areas, but also rich in landscapes and plant communities with oak and chestnut trees, where rodents, hedgehogs, squirrels and various migratory bird species are abundant, as well as small, well-maintained parks with regularly mowed open grass, such as Hirve and Toompark located in the city center, were extremely poor habitats for *Ixodes* ticks. These are highly fragmented areas with a high anthropogenic habitat disturbance which might negatively affect tick presence and maintenance. While not focusing on tick density and lacking statistical analysis, our study results are generally in agreement with other European studies, pointing out the negative correlation of tick density towards urbanization rather than in relation to natural hosts [35]. As seen in Zoo and Open Air Museum, and also in the peri-urban Männiku forest, the presence of urbanized synanthropic carnivores, i.e. foxes, or roe-deer together with small animals, suitable environment and vegetation is essential for the maintenance of tick populations.

It is well known that the circulation of TBEV in natural foci is maintained by small rodents, which are competent reservoir hosts, and ticks, that are both hosts and vectors [36]. As many rodent species are well-adapted to a human-affected and urbanized environment, the presence of TBEV foci and therefore, the occurrence of autochthonous human TBE cases even within large cities is possible [37]. According to our earlier studies, TBEV was found in questing ticks at prevalence rates varying from 0.2 to 0.8% in the *I. ricinus* allopatric area and up to 4.9% in the areas of *I. persulcatus* co-circulation [13] which is in line with the results of this study as well as with prevalence rates of TBEV in *I. ricinus* in European foci [38]. According to epidemiological data of Estonian Health Board in about 18% of TBE patients in Harju county had a tick bite history from Tallinn [7]. The results of this study not only confirm the presence of TBEV foci in green areas within the city but also indicate circulation of European and Siberian subtypes of TBEV in *I. ricinus* ticks within urban and peri-urban areas. The presence of TBEV-Sib in *I. ricinus* ticks in locations with

no *I. persulcatus* co-circulation had also been previously shown [13]; thus, it may be assumed that TBEV-Sib might be potentially spread into *I. ricinus* distribution areas without the presence of its principal vector, *I. persulcatus*.

The epidemiological reports of the Estonian Health Board showed that in 2014-2018 up to 19% of all confirmed Lyme borreliosis patients in Harju county with the known geographical origin of a tick bite, had been bitten by ticks within Tallinn [7]. The presence of four Lyme borreliosis associated species – *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. bavariensis* – is in correspondence with previous results conducted in Estonia, however positivity rates differed significantly, being two to three times higher than detected earlier [8]. The results of this study are compatible with BBSL overall prevalence rates calculated for Scandinavia, the Balkan peninsula, and Central Europe (15.5%, 18.5% and 19.3%, respectively) [39]. Similar results have also been shown in urban and suburban areas in Switzerland (18%) and the urban Lazienki Park in Warsaw, Poland (17.3%) [35, 40]. As seen in Europe and our previous studies, *B. afzelii* and *B. garinii* were the most prominent species found in *I. ricinus* ticks. Noteworthy, that in natural sylvatic areas *B. afzelii* and *B. garinii* constituted 56.1% and 20.3%, respectively, of all *Borrelia* sp. -positive *I. ricinus* ticks [8], while in urban and peri-urban ticks of this study the same indicators constituted 84.7% and 7.3%, respectively. A similar disproportion has also been observed in Poland [35], Switzerland [40] and Belgium [41]. Such a divergence might be explained by pathogen dilution-amplification effects in natural vs fragmented urban environments as well as by differences in host availability. Natural forests and other sylvatic areas with little anthropogenic disturbance, fragmentation and transformation are inhabited or visited during migration stops by large amounts and varieties of avian species which might serve as sources of avian-associated TBPs, such as *B. garinii* and *B. valaisiana [42]*. Thus, in the terms of anthropopressure, the contact of ticks with birds and a prevalence of avian-associated TBPs is significantly decreased in comparison to natural areas and may lead to a lower presence of *B. garinii* and *B. valaisiana* in urban ticks. In contrast, rodents, which are highly adapted to an urbanized environment, smaller agglomeration-surrounded areas and human interruption, promote sub-adult tick population maintenance, facilitate the frequency of tick-host contacts and trigger an increase and amplification of rodent-associated *Borrelia*, such as *B. afzelii* [41].

The presence of *Borrelia miyamotoi* in questing ticks has also been shown for several European countries, including Estonia [9, 43]. In this study, the prevalence rate of 2.5% in all analyzed *I. ricinus* ticks and a site-specific prevalence from 2.7% to 4.4% is in line with prevalence described for suburban France [44], Switzerland [40] and also with our previous results for Valgamaa and Võrumaa counties, where *I. ricinus* co-circulates with *I. persulcatus* and the highest positivity rates of *B. miyamotoi* (2.8%) in ticks were reported so far [9]. Rodents, especially *Myodes* and *Apodemus*, are known reservoirs for *B. miyamotoi* in Europe and a study of Jahfari et al. [29] also indicated European hedgehogs (*Erinaceus europaeus*) as reservoirs. As the population density of peridomestic mice and voles is even higher in the urban and peri-urban regions due to favorable breeding and survival factors [45] and as *B. miyamotoi*, being related to relapsing fever *Borrelia*, is transovarially transmitted, the higher infection rates in the urban areas versus natural wooded sites may be due to higher amplification rates of this pathogen within urban landscape fragments compared to larger natural woodlands and pastures.

Although Anaplasmataceae species are known to cause a variety of human diseases, only single sporadic cases of human infections have been reported in Estonia so far. Our previous studies indicated the circulation of at least four medically important species within the Anaplasmataceae family in Estonian *I. ricinus* populations, i.e. *A. phagocytophilum* (average 1.7%-2.6%) [9], *N. mikurensis* (1.3%) [10], followed by *R. helvetica* and *R. monacensis* [11]. The detection of *Rickettsia* spp. in urban and suburban *I. ricinus* ticks has been reported from Germany, the Czech Republic, Poland and Slovakia at rates from 1 to 47% [46, 47, 48, 49, 50]. The results of this study showed almost three times higher *R. helvetica* overall positivity rate compared to those observed in questing ticks in natural environments (6.6%) [11] ; however, the prevalence rate in sub-urban site Männiku corresponds to that of natural tick habitats in Harjumaa county, as reported by Katargina *et al.* [11]. It is generally agreed upon that *I. ricinus* is the main vector and natural host of *R. helvetica* [30] and as a spotted-fever group *Rickettsia*, it is transmitted transstadially and transovarially among ticks. Thus, these factors along with high tick abundance in urban green areas may contribute to higher infection rates of this pathogen. Also, urban populations of European hedgehogs, as proposed by a study by Jahfari *et al.* [29], may play role in a pathogen maintenance in natural cycles in areas influenced by anthropogenic pressure within cities.

Neoehrlichia mikurensis appeared to be also slightly more prevalent in urban *I. ricinus* ticks compared to those found in the natural *I. ricinus* allopatric areas with a site-specific prevalence of 3.3%-10.9% (average 5.4%) versus 1.0% - 9.1% (average 0.9%), respectively. The data of this study are in line with those reported from urban and sylvatic areas in Austria, Sweden and Germany [51, 52, 53] with prevalence rates from 4.2% to 6.4%. Such a widespread of the pathogen may be connected not only to arthropod vectors but also to reservoir hosts – bank voles and yellow-necked mice – which are largely spread and have well-established populations within city shrubbery parks, cemeteries, outdoor activity and recreational places. Some studies also claim that non-rodent species such as hedgehogs, but not insectivores, may also contribute to *N. mikurensis* maintenance in urban and peri-urban green zones and human dwellings [29, 54]. Since *N. mikurensis* has been associated with human clinical cases with symptoms including fever, malaise, septicemia and weight loss in immunocompromised as well as in healthy persons [55, 56, 57], the possible emerging status of this pathogen should be considered as a potential risk for public health in sylvatic and urban habitats.

In vector-borne diseases, an infection may only occur if human activity coincides with the activities of animal reservoirs and vectors. Climate change and urbanization, which both lead to environmental and microclimatic landscape composition and land-use changes, may impact every pathogen-host-vector system stage in different ways, thus affecting the whole system. Green infrastructure improvements within the cities

support human population welfare. However, even patchy urban green areas may provide suitable environmental and microclimatic conditions for ticks, tick-borne pathogens, and their hosts, which in turn, may lead to an increased incidence of tick-borne diseases within the cities. On the other hand, urban heat island effects have a negative impact on tick survival and activity periods, as well as dense fragmentation and human disturbance lead to reduced biodiversity. Although the risk of acquiring a tick bite and being infected with a tick-borne disease in urban recreational sites may vary significantly between locations, it should not be ignored and proper information about the precautions might be considered at least in the most popular outdoor locations.

Conclusion

The risk of getting a tick bite, bacterial or viral tick-borne disease must not be underestimated even in urban environments as this study showed. Proper precaution measures might be taken into consideration by local authorities as well as by citizens and tourists as those might get a tick-borne disease even in green urban recreational zones without going far in the woods.

Abbreviations

BBSL: Borrelia burgdorferi sensu lato; TBP(s): tick-borne pathogen(s); TBEV: tick-borne encephalitis virus; LB: Lyme borreliosis; TBE: tick-borne encephalitis; TBD: tick-borne disease; ITS2: internal transcribed spacer 2.

Declarations

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Author's contributions

MV: Methodology, investigation, resources, original draft writing, supervision. OB: validation, investigation, review and editing. JG: conceptualization, methodology, investigation, resources, original draft writing, visualization, supervision, project administration, funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

All additional data associated with this study can be obtained from the corresponding author on reasonable request. Unique nucleotide sequences obtained during this study were submitted to GenBank database under the following accession numbers: MW916612 – MW916613 for TBEV, MW924118 – MW924135 for *B. burgdorferi* s.l. species, MW924974 – MW924983 for *B. miyamotoi*, MW924984 – MW925050 for *R. helvetica*, MW922752 – MW922756 for *A. phagocytophilum* and MW922757– MW922793 for *Neoehrlichia mikurensis*. Due to number of identical sequences, especially within *B. burgdorferi* s.l. and *R. helvetica,* only unique sequences were deposited and duplicate sequences were omitted from submission. Samples which nucleotide sequences allowed to perform pathogen genotyping but with partially poor chromatogram or with possible mixed infections of several pathogen species strains were also excluded from depositing.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Tick collection sites and tick collection results.

site No	Name	Description	Latitude/longitude	m2 flagged	No. of ticks collected						
					L*	N	M	F	Total	mean abundance**	IA***
1	Pirita river valley	riverside with rich herbal lower vegetation and bushes;	59.4574, 24.9023	450	-	16	1	1	**18**	4.0	6.0
2	Pirita forest park	large urban mixed forest, with hills and swamp areas and rich litter;	59.4604, 24.8593	1250	+	107	7	8	**122**	9.8	40.7
3	Kadrioru	large urban park with mainly broadleaved trees;	59.4415, 24.7982	300	-	4	1	1	**6**	2.0	2.0
4	Ilmarise health trails	large natural-like urban mixed forest with swamps	59.3659, 24.6666	1400	+	30	5	2	**37**	2.6	12.3
5	*Hirve/Toompark*	*central city park, some bushes with a litter;*	*59.4336, 24.7374*	*600*	-	-	-	-	**-**	-	-
6	*von Glehni park*	*a park in the large urban mixed forest*	*59.3925, 24.6577*	*300*	-	-	-	-	**-**	-	-
7	Stroomi	urban broadleaved natural-like forest at the seaside;	59.4372, 24.6921	1200	+	27	6	5	**38**	3.2	12.7
8	Estonian Open Air Museum	broadleaved and mixed type urban semi-forested area at the seaside;	59.4323, 24.6395	1200	++	175	18	33	**226**	18.8	75.3
9	Zoo surroundings	bushy area, rich in a litter;	59.4207, 24.653	300	++	7	7	9	**23**	7.7	7.7
10	Sütiste forested park	urban mixed-type forest	59.3944, 24.6899	600	-	5	3	2	**10**	1.7	10.0
11	Nõmme-Mustamäe	urban mixed type forested area	59.38952, 24.6745	600	-	26	3	1	**30**	5.0	15.0
12	Hiiu grove	mixed semi-forest	59.3780, 24.6778	150	-	4	3	2	**9**	60	9.0
13	Harku-Nõmme	large forest area, mixed-type trees but mainly coniferous; mossy and needle litter	59.3874, 24.6106	100	-	2	-	2	**4**	4.0	4.0
14	Järve health trails	semi-forested area, mainly with pine trees and herbal lower layer	59.3997, 24.7299	600	-	-	-	-	**-**	-	-
15	Tallinn Zoo	natural-like broadleaved forested areas with a rich lower layer	59.4208, 24.6616	1200	+++	158	23	30	**211**	17.6	70.3
16	*Sanatooriumi park*	semi-forested area, mainly with pine trees and herbal or mossy lower layer	*59.3762, 24.6638*	*600*	-	-	-	-	**-**	-	-
17	Sütiste park (TalTech)	urban mixed-type semi-forest	59.3931, 24.6811	200	-	20	-	1	**21**	10.5	21.0
	URBAN total					**581**	**77**	**97**	**923**		
1su	Männiku	mixed and coniferous forest with a mainly herbal or mossy lower layer	59.3273, 24.6797	400	-	63	6	5	**74**	18.5	37.0

2su	Vääna-Jõesuu	mixed and coniferous forest with a mainly herbal or mossy lower layer	59.4202, 24.354	250	-	20	-	1	**21**	8.4	21.0
3su	Jägala	mixed forest with a rich lower layer of bushes and grass	59.4234, 25.2110	400	-	5	-	-	**5**	1.3	5.0
	PERI-URBAN total				**-**	**88**	**6**	**6**	**100**		
	TOTAL			12100		**669**	**83**	**103**	**855**		

* - L - larvae, N - nymphs, M - male, F – female; the presence of larvae has been noted without exact count

** number of ticks per 100 m^2;

*** index of abundance = no. of ticks / all minutes of collection by all collectors x 60 (one-hour reduction index).

Table 2. Tick-borne pathogens detected in urban and peri-urban questing ticks.

Site No*	Location name	No of TBP positive ticks/ No. ticks analyzed (%)	*Borrelia* genus						Rickettsiales			TBEV
			No. ticks (total prevalence %) ***						No. ticks (total prevalence %) ***			
			B. burgdorferi s.l.					*B. miy*				
			BBSL total	BA**	BG	BV	Bbav		Rh	An. ph	N. mik	
1	Pirita river valley	5/18							4 (1F)			
2	Pirita forest park health trails	38/122 (31.1%)	14 (11.5%)	6 (4.9%)	6 (4.9%)	2 (1.6%)			22 (18.0%)	2 (1.6%)	4 (3.3%)	1 (unsp.)
3	Kadrioru	1/6	1		1							-
4	Ilmarise health trails	9/37	3		1	1			6			-
7	Stroomi	7/38	5	4		1			3			1 (unsp.)
8	Estonian Open Air Museum	99/226 (43.8%)	57 (25.2%)	56 (24.8%)	1 (0.4%)			10 (4.4%)	31 (13.7%)	1 (0.4%)	17 (7.5%)	-
9	Zoo surroundings	8/23	4	3				1	3		1	1 (0.4%) (TBEV-Sib)
10	Sütiste forested park	1/10	1	1								-
11	Nõmme-Mustamäe	1/30	1			1						-
12	Hiiu grove	3/9	3	3					1			-
13	Harku-Nõmme	0/4										-
15	Tallinn Zoo	89/211 (42.2%)	48 (22.9%)	46 (21.8%)	2 (0.9%)			8 (3.8%)	32 (15.2%)	1 (0.5%)	23 (10.9%)	-
17	Sütiste park (TalTech)	5/21	4	2		2			1			-
1su	Männiku	14/74 (18.9%)	4 (5.7%)	3 (4.1%)				2 (2.7%)	8 (10.8%)			-
2su	Vääna-Jõesuu	11/21	3	2					7		1	1 (1.4%) (TBEV-Eu)
3su	Jägala	2/5	2	1			1			1		-
	TOTAL	**293/855 (34.3%)**	**150/855 (17.5%)**	***127/150 (84.7%)***	***11/150 (7.3%)***	***7/150 (4.7%)***	***1/150 (0.7%)***	**21/855 (2.5%)**	**118/855 (13.8%)**	**5/855 (0.6%)**	**46/855 (5.4%)**	**4/855 (0.5%)**
				127/855 (14.9%)	**11/855 (1.3%)**	**7/855 (0.8%)**	**1/855 (0.1%)**					

* Sites with no ticks collected were excluded

** BBSL – B. burgdorferi s.l., BA – *B. afzelii*, BG- *B. garinii*, BV – *B. valaisiana*, Bbav – *B. bavariensis*, Bmiy – *B. miyamotoi*, Rh – *R. helvetica*, An. ph – *A. phagocytophilum*, N. mik - *N. mikurensis*, TBEV- tick-borne encephalitis virus

*** Due to non-standardized collections, estimated prevalence rates were calculated only for sites with a total over 50 of adult and nymphal ticks collected and analyzed

Figures

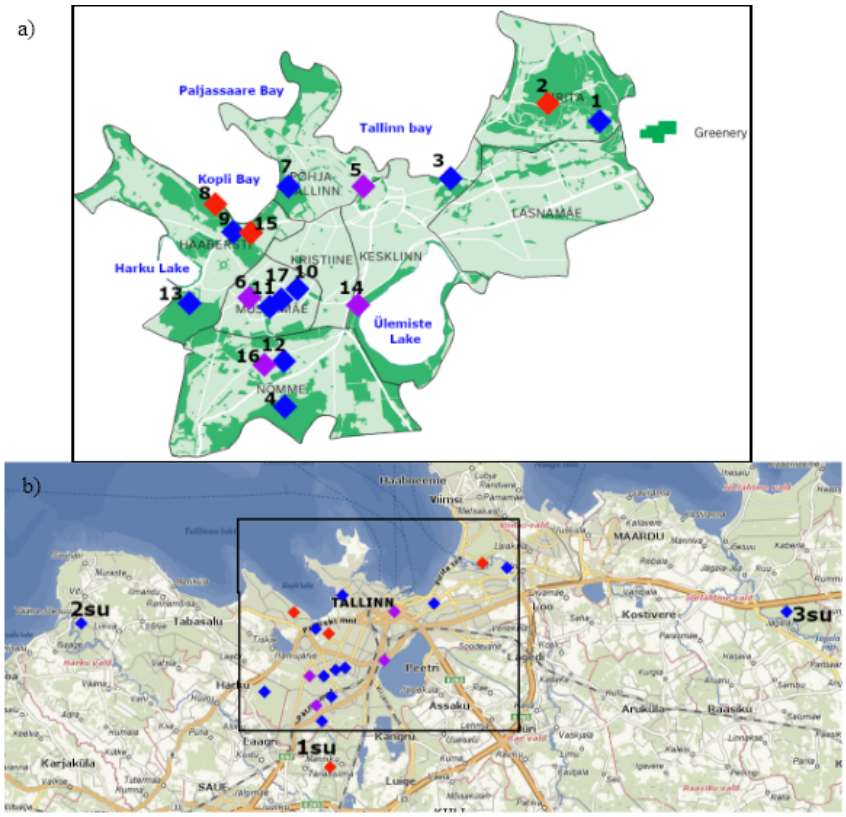

Figure 1

Tick collection sites: a) urban collection sites, located in green areas within the city (Tallinn green areas map retrieved Jan. 21, 2021, https://www.tallinn.ee/est/statistika/Tallinna-tervisestatistika) and b) all tick collection sites. Purple marks represent places with no ticks collected, red – sites with over 50 ticks collected and blue with 1 to 49 ticks collected. Site names according to numbers are as follows: 1- Pirita river valley, 2- Pirita forest park, 3 – Kadrioru park, 4- Ilmarise health trails, 5- Hirve and Toompark, 6- von Glehni park, 7- Stroomi, 8- Estonian Open Air Museum, 9- Zoo surroundings, 10 – Sütiste park, 11- Nõmme-Mustamäe, 12- Hiiu grove, 13- Harku-Nõmme, 14- Järve health trails, 15- Tallinn's Zoo, 16- Sanatooriumi park, 17- Sütiste park (TalTech), 1su- Männiku, 2su- Vääna-Jõesuu, 3su- Jägala. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area o bbnhjr of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

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