Rickettsia spp. in rodent-attached ticks and first evidence of Spotted fever Group Rickettsia species Candidatus Rickettsia uralica in Europe.

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Abstract

BACKGROUND Rickettsia spp. are human pathogens that cause a number of diseases and are transmitted by arthropods, including ixodid ticks. Estonia contributes a region, where the distribution area of two exophilic tick species of known medical importance, Ixodes persulcatus and I. ricinus, overlap. The presence of the nidicolous rodent-associated I. trianguliceps has recently been shown for Estonia. Although there is no Estonian data available on human disease caused by tick-borne Rickettsia spp., the presence of three Rickettsia species in non-nidicolous ticks, albiet at very dissimilar rates, was also previously reported. The aim of this study was to screen, identify and characterize Rickettsia species in nidicolous and non-nidicolous ticks attached to rodents.

RESULTS Nymphs and larvae of I. ricinus (n = 1004), I. persulcatus (n = 75) and I. trianguliceps (n = 117) attached to rodents and shrews caught in different parts of Estonia were studied for the presence of Rickettsia spp. by nested PCR. Ticks were removed from 314 small animals of 5 species (bank voles Myodes glareolus, yellow necked mice Apodemus flavicollis, striped field mice A. agrarius, pine voles M. subterranius and common shrews S. araneus). Rickettsial DNA was detected in 8.7% (103/1186) studied ticks. In addition to R. helvetica, previously found in questing ticks, this study reports the first identification of the recently described I. trianguliceps-associated Candidatus R. uralica in west of the Ural.

Background

Rickettsia is a genus of small, obligate intracellular gram-negative bacteria. Based on genomic analyses they are classified into four groups: the spotted fever group (SFG), the typhus group, the ancestral group and the transitional group, respectively [1]. Most SFG rickettsiae are transmitted by ticks of the Ixodidae family [2], and transmission may occur
transovarially as well as transstadially [3, 4]. Several agents of tick-borne rickettsioses are known to circulate in Europe, including R. conorii, R. massilae, R. slovaca, R. raoultii, R. monacensis and R. helvetica [5, 6], of which the latter is the most frequently detected species in numerous Ixodidae ticks including I. ricinus, I. persulcatus, I. trianguliceps, and Dermacentor reticulatus [5]. Although R. helvetica is not believed to be highly pathogenic to humans, there had been several reports from Sweden [7, 8], Netherlands [9], France and Italy [10] which describe rash, mild fever, febrile illness, meningitis and other clinical symptoms, associated with this agent in patients. As for Estonia, the wide distribution of R. helvetica, as well as the presence of R. monacensis and Candidatus R. tarasevichiae in questing ticks was shown by the study of Katargina et al, but no human cases due to R. helvetica infection nor to the other two species have been reported to date [11].

Recent studies show that Ixodes ticks could serve not only as vectors but also as reservoir hosts of R. helvetica. At the moment there is no clear understanding on whether any mammal species is the host of the R. helvetica, while on the other hand rickettsial DNA was found in the blood of wild animals such as rodents, roe deer and wild boars [12] and domestic animals like dogs and cats [13]. It can be only be assumed that mammals can be potential hosts, and that they may affect the natural transmission and distribution of Rickettsiae.

In the present study, our aim was to investigate the presence of Rickettsiae in ticks collected from small mammals.

Methods

Sample collection, species identification and DNA extraction.

The study was performed retrospectively on 1186 ticks, removed from small mammals, collected at five sites in Estonia, located in four mainland counties - Järvamaa (Päälasvere – 58.7443, 25.7015 and Retla – 58.746940, 25.650310), Pärnumaa (Viisireiu – 58.0813,
24.8497 and Kalita – 58.06873, 24.84333; performed only in 2012), Lääne-Virumaa (Piisupi – 59.22381, 26.13076 and Saksi – 59.22877, 26.13450), Tartumaa (Järvselja 58.248799, 27.301058; study years 2013–2014) – and one island county – Saaremaa (Järise – 58.506091, 22.414334 and Paatsa – 58.515070, 22.376764). Live-trappings of mice, voles and shrews was carried out once a month during April-November 2012–2014 in natural habitats using Sherman LFA perforated live-traps (Ethical Committee Permission No. 124 by Estonian Ministry of Agriculture). All procedures with animals were performed by an experienced authorized person as described in detail in Värv et al, in-press [14]. Each animal was individually investigated for the presence of ectoparasites, which were removed, fixed in ethanol and stored at -20 in separate tubes until use.

DNA extraction from ticks was performed with ammonium hydroxide solution according to Moran-Cadenas et al. [15]. Tick species were identified by ITS2 based multiplex PCR assay as previously described by Värv et al. [16]. Only ticks identified at the species level by the ITS2 multiplex PCR were included in this study, and ticks that remained undetermined were omitted.

Rickettsia spp. screening and detection of Rickettsia genospecies

All ticks identified at the species level were screened individually by a nested PCR targeting a 667 bp fragment of Rickettsia sp. citrate synthase gltA gene using primers glt1-4 as described by Igolkina et al. [17] with subsequent sequencing of all positive samples. For samples identified as Ca. R. uralica and randomly selected samples identified as R. helvetica by initial screening, additional PCR amplification of ~ 770 bp fragment of outer membrane protein B (ompB) gene was performed with primers 120-2788F and 120-3599R and under conditions described previously [18]. Additionally, a subset of the latter samples was amplified by nested PCR of 834 bp fragment of cell surface antigen 4 (sca4) gene with primers sc4-1 and Rj2837r for the primary reaction, and sc4-3 and sc4-4 for the
nested reaction, as described by Igolkina et al. [17]. PCR products of all positive samples were sent for direct sequencing to the core laboratory of the Estonian Biocentre (Tartu, Estonia), followed by nucleotide sequence alignment using BioEdit v7.2.5 (Ibis Biosciences, USA) and genospecies identification with BLASTN® tools (http://www.ncbi.nlm.nih.gov/BLAST.cgi).

Statistical analysis

The statistical significance between the proportions among sample groups was estimated with two-tailed Fisher’s exact test (differences considered to be statistically significant with P values < 0.05). A modified Wald method was used for the calculation of 95% confidence intervals (CI) [19].

Results

Rickettsia screening and Rickettsia species

A total of 993 I. ricinus (69 nymphs and 924 larvae), 117 I. trianguliceps (24 nymphs and 93 larvae) and 76 I. persulcatus presence of Rickettsia.

Rickettsial DNA was detected in 8.7% (103/1183) of studied ticks, with statistically significant differences of prevalence rates between I. ricinus and I. trianguliceps – 10.0% (99/993) and 3.4% (4/117), respectively (P = 0.0177) (Table 1). Similarly, a statistically significant difference in the prevalence rate was shown between I. ricinus and I. trianguliceps larval stages (P = 0.0374). However, the prevalence of Rickettsia spp. between larval and nymphal stages showed similar rates without any significant differences within both I. ricinus and I. trianguliceps: 10.0% in larva vs 10.1% in nymphs for I. ricinus and 3.2% vs 4.2% for I. trianguliceps larvae and nymphs, respectively (Table 1). Rickettsia spp. was not detected in I. persulcatus.
Table 1
Rickettsia spp. detection in ticks collected from small mammals and prevalence (%).

| Small mammal catching location | Number of ticks infected/tested (prevalence, %) [95% CI] | Rickettsia spp. genospecies (prevalence comparatively all positives samples) |
|-------------------------------|--------------------------------------------------------|--------------------------------------------------------------------------|
|                               | R. ricinus | I. persulcatus | I. trianguliceps |
|                               | Nymphs     | Larvae         | Total          | Nymphs | Larvae | Total | CaRu | Rh |
| Järvamaa                      | 7/69       | 92/924         | 100/10100      | 99/993 | 64/0.05 | 0.08 | 103/11000 | 100/10000 |
| (10.1%)                       | (4,7–-19.8%) | (10.0%)* | [8.2–12.1%] | (10.0%)** | [8.3–12.0%] | [0.01–21.9%] | (3,2%) ** [0.7–9.5%] | (1.1–8.8%) | (2.9%) |
| Pärnumaa                      | 4/15       | 39/186         | 43/201         | 0/1    | -      | 0/1  | 2/22 | 2/25 |
| (26.7%)                       | (21.0%)    | (21.4%)        | (21.4%)        | -      | -      | -    | 19.4% | (7.7%) |
| Saaremaa                      | 0/24       | 13/247         | 13/271         | -      | -      | -    | 0/2  | 0/13 |
| (5.2%)                        | (5.2%)     | (4.8%)         | (4.8%)         | -      | -      | -    | -    | (4.8%) |
| Tartumaa                      | 0/4        | 4/52           | 4/56           | 0/11   | 0/62  | 0/6  | -    | 4/152 |
| (7.7%)                        | (7.7%)     | (7.1%)         | (7.1%)         | (7.7%) |
| TOTAL                         | 7/69       | 92/924         | 100/10100      | 99/993 | 64/0.05 | 0.08 | 103/11000 | 100/10000 |
| (10.1%)                       | (4,7–-19.8%) | (10.0%)* | [8.2–12.1%] | (10.0%)** | [8.3–12.0%] | [0.01–21.9%] | (3,2%) ** [0.7–9.5%] | (1.1–8.8%) | (2.9%) |
| Nymphs                        | 8/105      | 95/1081        | 103/11000      | 100/10000 |
| (7.62%)                       | (7,2–14,5%) | (9.44%) | (7,2–10,6%) |
| Larvae                        | 95/1081    | 95/1081        | 100/10000      | 100/10000 |
| (9.44%)                       | (7,2–10,6%) | (9.44%) | (7,2–10,6%) |

Fisher’s exact and Poisson probability test: *- statistically significant difference, P = 0.0374; ** - statistically significant difference, P = 0.0177.
CI – Confidence Interval of a Proportion, Wald method
Rh – Rickettsia helvetica; CaRu – Candidatus Rickettsia uralica

Rickettsial DNA was detected in ticks from all study sites, with the lowest prevalence rates in Tartumaa and Saaremaa (2,6% and 4,8%, respectively) and the highest rate at 19,4% in Pärnumaa. Interestingly, that the Saaremaa county prevalence rate did not statistically differ when compared to results from other counties, while the Tartumaa results were statistically lower (Järvamaa and Lääne-Virumaa P < 0,04; Pärnumaa P < 0,0001). The Pärnumaa results showed statistically significant differences (P < 0,001) compared to all other study sites results.

Regarding mammal species, Rickettsia spp. DNA was detected in ticks collected from 56 out of 314 animals of 3 species - M. glareolus (n = 36), A. flavicollis (n = 19) and S. araneus (n = 1) (Table 2); Thus 21,8% (36/165) and 13,5% (19/141) of all bank voles and
yellow-necked mice, respectively, had at least one Rickettsia positive tick attached. There was a statistically significant difference observed between the prevalence rates of Rickettsia spp. in ticks removed from these rodent species: 5.8% (27/463) for yellow-necked mice vs 10.3% (69/670) for bank voles (P = 0.0166, two-tailed Fisher Exact test) (Table 2). The highest prevalence of rickettsial DNA was observed in ticks from M. glareolus from Pärnumaa county (23.8%) (Table 2). The number of analyzed ticks collected from a single animal varied from 1 to 32, while the rates of Rickettsia-positive ticks varied from 4.8% – 100%.

Table 2
Small mammals with Rickettsia positive ticks.

| Animal species | Animal collection county | № of infested rodents/ № rodents with at least one Rickettsia positive tick (% of rodents infested by pos ticks) | Total № ticks removed from infested rodents | № of Rickettsia positive ticks (total № ticks removed from the same rodent) | % positive ticks from removed ticks |
|----------------|--------------------------|---------------------------------------------------------------|------------------------------------------|----------------------------------------------------------------|----------------------------------|
| Ap. flavicollis | Järvamaa                | 23/2 (8.7%)                                                    | 81                                       | 2 (3)                                                          | 2.5%                             |
|                | Laâne-Virumaa            | 52/6 (11.5%)                                                   | 107                                      | 9 (19)                                                         | 8.4%                             |
|                | Saaremaa                 | 31/2 (6.5%)                                                    | 141                                      | 5 (26)                                                         | 3.5%                             |
|                | Pärnumaa*                | 17/6 (35.3%)                                                   | 84                                       | 8 (37)                                                         | 9.5%                             |
|                | Tartumaa**               | 18/3 (16.7%)                                                   | 50                                       | 3 (6)                                                          | 6.0%                             |
| TOTAL          |                          | 141/19 (13.5%)                                                 | 463                                      | 27 (91)                                                        | 5.8% #                           |
| My. glareolus  | Järvamaa                | 49/6 (12.2%)                                                   | 234                                      | 23 (42)                                                        | 9.8% *                           |
|                | Laâne-Virumaa            | 38/6 (15.8%)                                                   | 102                                      | 8 (35)                                                         | 7.8% *                           |
|                | Saaremaa                 | 13/5 (38.5%)                                                   | 112                                      | 8 (58)                                                         | 7.1% *                           |
|                | Pärnumaa*                | 37/18 (48.6%)                                                  | 122                                      | 29 (93)                                                        | 23.8% *                          |
|                | Tartumaa**               | 28/1 (3.6%)                                                    | 100                                      | 1 (6)                                                          | 1.0% *                           |
| TOTAL          |                          | 165/36 (21.8%)                                                 | 670                                      | 69 (234)                                                       | 10.3% #                          |
| Ap. agrarius   | Saaremaa                 | 3/0 (0.0%)                                                     | 20                                       | 0 (0)                                                          | 0.0                              |
|                | TOTAL                    | 3/0 (0.0%)                                                     | 20                                       | 0 (0)                                                          | 0.0                              |
| S. araneus     | Järvamaa                | 2/0 (0.0%)                                                     | 10                                       | 0 (0)                                                          | 0.0                              |
|                | Pärnumaa*                | 1/1 (100%)                                                     | 21                                       | 7 (21)                                                         | 33.3%                            |
| TOTAL          |                          | 3/1 (33.3%)                                                    | 31                                       | 7 (21)                                                         | 22.6%                            |
| M. subterranius| Tartumaa**               | 2/0 (0.0%)                                                     | 2                                        | 0 (0)                                                          | 0.0                              |
| TOTAL          |                          | 2/0 (0.0%)                                                     | 2                                        | 0 (0)                                                          | 0.0                              |
| TOTAL          |                          | 314/56 (17.8%)                                                 | 1186                                     | 103 (346)                                                      | 8.68%                            |

* - 2012 only, ** - 2013 and 2014
 الإسلامي - P < 0.002; # - P = 0.0089
Ap. - Apodemus; My. - Myodes; S. - Sorex; M. - Microtus

According to the partial gltaA gene sequencing results there were two Rickettsia detected, R. helvetica and Ca. R. uralica. R. helvetica DNA was present in the majority of Rickettsia
positive samples – 97,1% (100/103) and was detected in 9,97% (99/993) of I. ricinus ticks but was also found in 0,85% (1/117) of I. trianguliceps ticks. R. helvetica DNA was detected in ticks removed from yellow-necked mice, bank voles and common shrews from all study locations.

Another Rickettsia species was identified as Ca. R. uralica. It was detected in three I. trianguliceps ticks removed from two bank voles collected in Pärnumaa and Järvamaa, respectively. Thus, prevalence of Ca. R. uralica in I. trianguliceps amounted to 2,9% (3/117; CI: 0,55 – 7,6%). Ca. R. uralica was not detected in I. ricinus (Table 1).

In order to confirm the species and to reveal possible nucleotide sequence variability within the detected Rickettsia species, 20 samples (all 3 samples with Ca. R. uralica and 17 samples with R. helvetica) were sequenced in the partial ompB gene and 9 samples of them (all 3 samples with Ca. R. uralica and 6 samples with R. helvetica) in the partial sca4 gene. All R. helvetica sequenced partial gene fragments were identical to each other as well as to those previously detected in questing ticks from Estonia [11]. Sequences of gltA, ompB and sca4 gene fragments amplified from all Ca. R. uralica positive samples were 100% identical to each other. Estonian sequences of gltA and sca4 gene fragments were also 100% identical to sequences reported from Siberia, except ompB gene fragment that differed in one nucleotide base, which gives 99,9% identity [17].

Discussion

In this study, ticks of the generalist species I. ricinus and I. persulcatus, as well as nidicolous I. trianguliceps, and attached to small mammals were analyzed for the presence of vector-borne Rickettsia spp. including a new species, previously unreported in Europe. Rickettsial DNA was detected in 8,7% of all investigated attached ticks, and in 10,0% of I. ricinus; rates which are significantly higher than those previously found in questing ticks in Estonia -overall prevalence rate 5,1%, and 6,7% for I. ricinus, respectively. (P value =
0,0004 for overall and P = 0,0068 for I. ricinus prevalence rates, respectively) [11]. This may be due to differences in study design and sampling: the rodent-attached ticks analyzed in this study were dominantly larvae, while previous studies were performed in questing ticks and those results reflected the prevalence in unfed nymphs and adults, which might have already had 1-2 blood-meals, undergone diapause and 1-2 moltings, thus resulting in a possible dilution of Rickettsia. As I. ricinus larvae often quest in groups, which might originate from the same hatch, a single animal could harbor a group of larvae having already acquired Rickettsia transovarially, prior their first blood-meal [20]. This might also explain why there is no difference in infection rates between rodent-attached larvae and nymphs, as shown in this study.

Up to date, I. ricinus generalist ticks represent the main vector and the natural reservoir host of tick-borne SFG Rickettsia in Europe [5]. In this study, a higher prevalence rate of rickettsial DNA was found in I. ricinus ticks compared to I. trianguliceps (10,0% vs 3,4%, respectively). High levels of rickettsial DNA detection rates in rodent-attached I. ricinus were also recently reported from Lithuania [21] where 22,6% of individually tested larvae (maximum likelihood estimation, MLE = 26,5%) were positive for Rickettsia spp.

There have been reports on the detection of several TBPs, such as Anaplasma phagocytophilum [22], Candidatus Neoehrlichia mikurensis and Babesia microti [23], Francisella tularensis [24] in nidicolous rodent-specialists I. trianguliceps ticks. As reported by Igolkina et al. [17], SFG Rickettsia was found in 41,2% (14/34) of analyzed I. trianguliceps ticks in Western Siberia, which is significantly higher than the results of the current study (3,4%, 4/117). Nevertheless, the role of I. trianguliceps in the circulation and maintenance of TBPs is still largely unknown as is its importance and participation in the transmission of pathogens between ticks and rodent hosts.

The absence of rickettsial DNA in rodent-attached I. persulcatus larvae (0/64) and nymphs
(0/12) could be explained by relatively small number of I. persulcatus covered in the current study. However, several Rickettsia species were previously reported in unfed questing I. persulcatus ticks in Estonia [11] although at significantly lower prevalence rates than in I. ricinus.

We found rickettsial DNA in ticks removed mainly from M. glareolus, A. flavicollis, but also from several S. araneus. Although there are reports on the detection of R. helvetica in various wild small- to large-sized animal samples from Lithuania [25], the Netherlands and Germany [12, 26, 27] and also in European robins (Erithacus rubecula) and Dunnocks (Prunella modularis) from Hungary [28], the significance of these animals in the transmission and maintenance cycle of Rickettsia is still debatable [29]. Although animal samples were not analyzed in this study, the Rickettsia spp. infection rates in ticks removed from the same animal varied from 4.8–100%, most likely indicating that the ectoparasites did not acquire these pathogens during blood meals on these animals, but were previously infected by transstadial, transovarial or horizontal transmission [30].

Similar data on varied Rickettsia spp. infection rates in ticks removed from the same animal have been reported from Lithuania [21].

Surprisingly, 42.7% (44/103) of all Rickettsia-positive ticks were removed from rodents caught in Pärnumaa county. Although this region was not covered in the previous study on Rickettsia spp. in questing ticks in Estonia [11], our unpublished data also showed the high rate of rickettsial DNA detection in questing ticks in Pärnumaa (28%). Interestingly, this region has previously not shown such high infection rates with any TBP [31, 32, 33], and our longitudinal observations on ticks indicate that the local environment and climate of western coastal Estonia may provide favorable conditions for tick population abundance and survival.

Although spotted fever rickettsioses are known to be emerging diseases spreading across
the globe, human case reports due to R. helvetica infections are scarce. Serological or molecular tools have been used to detect R. helvetica infection in samples from patients with suspected Lyme neuroborreliosis in the Netherlands [9], with unexplained fever following a tick bite in France and Italy [10] and with rash, febrile illness and meningitis in Sweden [7, 8]. R. helvetica is also the most prevalent tick-borne Rickettsiae species as elsewhere in Europe and Asia [5, 34] as well as in the Estonian tick population, comprising over 95% of all Rickettsia species detected in questing [11] as well as in rodent-attached ticks as our current study results show. While there are no clinical reports to date of illness caused by R. helvetica in Estonia, the detection of this tick-borne pathogen at prevalence rates compatible to those for B. burgdorferi s. l. [14], R. helvetica should be considered in the surveillance of tick-borne diseases in Lyme endemic regions.

To our knowledge, this study is the first report on the detection of a newly described species, Ca. R. uralica, in Europe. In our study this genospecies was detected only in I. trianguliceps ticks, and only in those removed from voles, which is in correspondence with the initial Ca. R. uralica report from Siberia, which designates the host specificity of Ca. R. uralica to I. trianguliceps [17]. Siberian Ca. R. uralica has also been identified in I. trianguliceps removed from voles, but rickettsial DNA was not found in the mammals. The authors claim that the same Rickettsia variant was previously detected in northern red-backed voles (Myodes rutilus) and common shrews (S. araneus), which are also present in Estonia. Together with I. trianguliceps ticks these small mammals might play role in the circulation of this Rickettsia species in nature. Despite the genetic clustering of this newly described Rickettsia within the spotted-fever group, the pathogenic potential of Ca. R. uralica for domestic and wild mammals, pets or humans remains to be studied.

**Conclusion**

The results of our study indicate on/show a higher prevalence of Rickettsia spp. in ticks
from small mammals compared to those obtained previously in questing ticks. Most of rickettsial DNA were found in I. ricinus ticks which are considered as main vector and the natural reservoir host and of all samples R. helvetica was the most prevalent species. This study also provides a first report on novel Rickettsia species, Ca. R. uralica initially reported from Siberian regions in Russia, to be present in Estonian population of I. trianguliceps.

Abbreviations
qPCR
quantitative polymerase chain reaction; PCR: polymerase chain reaction;
DNA: deoxyribonucleic acid; ITS2: internal transcribed spacer 2

Declarations

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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 sequences of Candidatus Rickettsia uralica obtained during this study were submitted to GenBank database.
(https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MT063090-MT063092.

Authors’ contributions

JR and IG study initiation and experimental studies planning; JR sample collection and small mammal species identification; MV and JG DNA extraction, PCRs performing and bioinformatical analysis; MV writing the initial draft of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal experiments were approved by Estonian Ministry of Agriculture permission no. 124 (J. Remm).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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