

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, albeit 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. subterraneus* 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 *Rickettsia 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 *Ixodes 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 sampling sites in Estonia, located in four mainland counties - Järvamaa (58.7365°; 25.6682°), Lääne-Virumaa (59.2260°; 26.1335°), Pärnumaa (58.0687°; 24.8433°, collecting was performed only in 2012), Tartumaa (58.2493°; 27.3023°, collecting in 2013 and 2014) – and one island county – Saaremaa (58.5075°; 22.4107°). 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 Värvi *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°C 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* gene based multiplex PCR assay as previously described by Värvi *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* spp. citrate synthase A (*gltA*) gene using primers *glt1-4* as described by Igoikina *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 (*sc4*) gene with primers *sc4-1* and *Rj2837r* for the primary reaction, and *sc4-3* and *sc4-4* for the nested reaction, as described by Igoikina *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).

Results

***Rickettsia* screening and *Rickettsia* species**

A total of 993 *I. ricinus* (69 nymphs and 924 larvae), from all 5 mammal species, 117 *I. trianguliceps* (24 nymphs and 93 larvae), from *M. glareolus* and *A. flavicollis*, and 76 *I. persulcatus* (12 nymphs and 64 larvae), from *M. glareolus*, *A. flavicollis* and *Microtus subterraneus*, were studied for presence of *Rickettsia* spp.

Rickettsial DNA was detected in 8,7% (103/1183) of studied ticks, with statistically significant differences of positivity 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 positivity rate was shown between *I. ricinus* and *I. trianguliceps* larval stages (P = 0,0374). However, the positivity rate 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*. As small mammal samples were not analyzed for *Rickettsia* spp. presence, it is unknown how ticks acquired the pathogen by transstadial, transovarial transmission, co-feeding or with blood meal.

Rickettsial DNA was detected in ticks from all study sites, with the lowest positivity 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 positivity 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 positivity 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 ($P = 0,0166$) (Table 2). The highest positivity rate 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%.

According to the partial *gltA* gene sequencing results there were two *Rickettsia* detected, *R. helvetica* and *Ca. R. uralica*. *Rickettsia 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. *Rickettsia 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, positivity rate of *Ca. R. uralica* in *I. trianguliceps* amounted to 2,9% (3/117). *Candidatus Rickettsia 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 5,1%, and 6,7% for *I. ricinus*, respectively. (P value = 0,0002 for overall and P = 0,0068 for *I. ricinus* positivity and 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 positivity 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 [19]. 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 positivity 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 [20] 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 tick-borne pathogens (TBP), such as *Anaplasma phagocytophilum* [21], *Candidatus Neorhlichia mikurensis* and *Babesia microti* [22], *Francisella tularensis* [23] 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 positivity 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 [24], the Netherlands and Germany [12, 25, 26] and also in *Erithacus rubecula* (European robins) and *Prunella modularis* (dunnocks) from Hungary [27], the significance of these animals in the transmission and maintenance cycle of *Rickettsia* is still debatable [28]. 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% to 100%, most likely indicating that the ectoparasites might acquire these pathogens not only during blood meals on these animals, but also previously infected by transstadial, transovarial or horizontal transmission [29]. Similar data on varied *Rickettsia* spp. infection rates in ticks removed from the same animal have been reported from Lithuania [20].

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 [30, 31, 32], 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]. *Rickettsia helvetica* is also the most prevalent tick-borne *Rickettsiae* species as elsewhere in Europe and Asia [5, 33] 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 TBP at positivity rates compatible to those for *Borellia 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 *Myodes rutilus* (northern red-backed voles) and *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 show a higher positivity 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

DNA: deoxyribonucleic acid; ITS2: internal transcribed spacer 2; PCR: polymerase chain reaction; SFG: Spotted fever group; TBP: tick-borne pathogens; qPCR: quantitative polymerase chain reaction;

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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Tables

Table 1. *Rickettsia spp.* detection in ticks collected from small mammals and positivity rate (%).

Small mammal catching location	Number of ticks infected/tested (positivity rate, %)									<i>Rickettsia spp.</i> genospecies (positivity rate comparatively to all positive samples, %) (positivity rate comparatively to all studied samples, %)		
	Nymphs	<i>I. ricinus</i>		<i>I. persulcatus</i>			<i>I. trianguliceps</i>			Total	CaRu	Rh
		Larvae	Total	Nymphs	Larvae	Total	Nymphs	Larvae	Total			
Järvamaa	2/14 (14,3%)	21/288 (7,2%)	23/302 (7,6%)	-	0/1	0/1	0/3	2/19	2/22	25/325 (7,7%)	2 (8%) {0,6%}	23 (92%) {7,1%}
Lääne-Virumaa	1/12 (8,3%)	15/151 (9,9%)	16/163 (10,4%)	-	0/1	0/1	0/7	1/38	1/45	17/209 (8,1%)	-	17 {8,1%}
Pänumaa	4/15 (26,7%)	39/186 (21,0%)	43/201 (21,4%)	0/1	-	0/1	1/8	0/17	1/25	44/227 (19,4%)	1 (2,3%) {0,4%}	43 (97,7%) {18,9%}
Saaremaa	0/24	13/247 (5,2%)	13/271 (4,8%)	-	-	-	-	0/2	0/2	13/273 (4,8%)	-	13 {4,8%}
Tartumaa	0/4	4/52 (7,7%)	4/56 (7,1%)	0/11	0/62	0/73	0/6	-	0/6	4/152 (2,6%)	-	4 {2,6%}
TOTAL	7/69 (10,1%)	92/924 (10,0%)*	99/993 (10,0%)**	0/12	0/64	0/76	1/24 (4,2%)	3/93 (3,2%)*	4/117 (3,4%)**	103/1186 (8,7%)	3 (2,9%) {0,3%}	100 (97,1%) {8,4%}
	Nymphs	8/105 (7,62%)										
	Larvae	95/1081 (9,44%)										

Fisher's exact and Poisson probability test: *- statistically significant difference, P=0,0374; ** - statistically significant difference, P=0,0177.

Rh – *Rickettsia helvetica*; CaRu – *Candidatus Rickettsia uralica*

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%
	Lääne-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%□
	Lääne-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. subterraneus	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					
□ - P < 0,002; # - P = 0,0089					
Ap. – Apodemus; My. – Myodes; S. – Sorex; M. – Microtus					

Table 2
Small mammals with Rickettsia positive ticks.

Supplementary Files

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