First detection of *Rickettsia helvetica* in small mammals in Lithuania

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**Abstract**

A total of 489 small mammals belonging to seven species captured in Lithuania during 2013–2014 were investigated for *Rickettsia* pathogens. The overall prevalence of *Rickettsia* spp. was 27.6%, with a higher prevalence detected in *Micromys minutus* (45.9%), followed by *Apodemus flavicolis* (29.4%), *Sorex araneus* (25%) and *Myodes glareolus* (23.7%). Sequence analysis of the *gltA* gene and the 17 kDa protein coding gene revealed the presence *Rickettsia helvetica*. This study demonstrates not only the first reported presence of *R. helvetica* in small mammals in Lithuania but also the first report of *R. helvetica* in *M. minutus* more generally.

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**Introduction**

*Rickettsiae* are intracellular vector-borne bacteria with complex life cycles that are transmitted to vertebrates, including humans, by a variety of haematophagous arthropod vectors such as ticks, fleas and mites [1]. Members of the genus *Rickettsia* are divided into the spotted fever group *Rickettsioses*, typhus group *Rickettsioses* and an ancestral group (including *Rickettsia bellii* and *Rickettsia canadensis*) [2]. There are currently 30 *Rickettsia* species with standing in nomenclature and a growing number of ‘*Candidatus*’ species, whose taxonomic position is still unclear. In Europe, rickettsioses are mainly caused by spotted fever group *Rickettsia*, which is transmitted by hard ticks (such as *Ixodes ricinus*, *Dermacentor reticulatus* and *Rhipicephalus* spp.). At least eight *Rickettsia* species that are transmitted by ticks (including *Rickettsia aeschlimannii*, *Rickettsia conori*, *Rickettsia massiliae*, *Rickettsia helvetica*, *Rickettsia monacensis*, *Rickettsia sibirica*, *Rickettsia slovaca* and *Rickettsia raoultii*) are characterized as human pathogens in Europe [2,3]. In Lithuania, four *Rickettsia* species (*R. sibirica*, *R. slovaca*, *R. raoultii* and *R. helvetica*) have been reported. The presence of *R. sibirica* and *R. slovaca* has been confirmed in human and cattle serum samples [4]. With prevalences of 17% and 4.9%, respectively, *R. helvetica* has recently been detected in *I. ricinus* ticks and *R. raoultii* in *D. reticulatus* ticks [5].

In Europe, investigations on the prevalence of *Rickettsia* have mainly focused on arthropod vectors, and several studies (conducted in the Netherlands, Germany, Slovakia, Hungary, Italy, Poland, Austria and Croatia) have investigated the presence of *Rickettsia* spp. in wild-living small mammals [6–8]. As reviewed by Cull et al. [9], small mammals are important hosts for the immature stages of ixodid ticks and are considered reservoir hosts of the tick-borne encephalitis virus, *Borrelia burgdorferi* (sensu lato), *Borrelia miyamotoi*, *Babesia microti*, *Anaplasma phagocytophilum* and ‘*Candidatus* Neoehrlichia mikurensis.’ However, there is still a lack of data on the prevalence of rickettsioses in different species of small mammals, and the role of these small mammals in the maintenance of these pathogens is poorly understood.

The aims of the present study were to investigate the presence of *Rickettsia* spp. in different species of small mammals in Lithuania, to characterize the detected rickettsioses using partial sequencing of the *gltA* gene and 17 kDa protein coding gene and to determine the prevalence of *Rickettsia* spp. in small mammals from different locations in two regions of the country.
Materials and methods

Small mammal trapping

Small mammals were captured by live traps or snap traps in 11 locations situated in the coastal area (Curonian Spit; sites 1–8) and central part (sites 9–11) of Lithuania during 2013–2014 (Table 1). Permission to trap wild small mammals was provided according to regulations 1 (2013-04-10) and 15 (2014-03-31) of the Ministry of the Environment of the Republic of Lithuania. All trapped animals were marked and identified to species level, and age and sex were recorded.

In total, 489 small mammals representing seven species—Apodemus flavicollis, Micromys minutus, Myodes glareolus, Microtus oeconomus, Microtus agrestis, Microtus arvalis and Sorex araneus—were collected (Table 1). In 2013, a total of 256 animals were trapped: 101 A. flavicollis, 10 M. minutus, 131 M. glareolus, five M. oeconomus and nine S. araneus. In 2014, 233 small mammals were trapped: 130 A. flavicollis, 27 M. minutus, 67 M. glareolus, three M. oeconomus, three S. araneus, two M. agrestis and two M. arvalis. The dominant small mammal species trapped in the Curonian Spit was A. flavicollis, while in the central part of country, it was M. glareolus (Table 1).

PCR and sequencing

In the present study, we used the spleens of small mammals for the molecular detection of Rickettsia pathogens. DNA from the spleen samples was extracted by using a genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), according to the manufacturer’s instructions. Screenings for the presence of rickettsiae were conducted through the amplification of the 338 bp fragment of the gltA gene in nested PCR using two primer sets, RpCS.877p-RpCS.1258n and RpCS.896p-RpCS.1233n [10]. All gltA-positive samples for Rickettsia were additionally examined by seminested PCR methods using primer sets Rp17k.1p-Rp17k.539n and Rp17k.90p-Rp17k.539n by amplification of a 450 bp fragment of the 17 kDa protein coding gene [11]. A selected number of Rickettsia-positive samples for both genes were subjected to sequence analysis and were extracted from agarose gel and purified using the GenJET PCR purification kit (Thermo Fisher Scientific Baltics) according to the manufacturer’s instructions. PCR products were sequenced (Macrogen, Amsterdam, The Netherlands). The obtained sequences were analysed by the MEGA 6.05 software package and compared with the sequence data available from the GenBank using the BLAST program. A phylogenetic tree was constructed using the maximum-likelihood method. The most appropriate model of nucleotide substitution was determined according to the Bayesian information criterion using the program jModelTest2 [12,13].

Partial sequences for representative samples were submitted to GenBank under accession numbers MF491763 to MF491766 for gltA and MF491753 to MF491760 for the 17 kDa protein coding gene.

Statistical analysis

Differences in the prevalence of Rickettsia spp. between different species of small mammals, sampling locations, regions and years were assessed by Fisher’s exact test, supplemented with the Mantel-Haenszel common odds ratio estimate and 95% confidence intervals using SPSS software version 22 (IBM SPSS, Chicago, IL, USA). p < 0.05 was considered significant.

Results

Prevalence of Rickettsia spp. in small mammals

Rickettsia pathogens in small mammals were detected in seven of 11 sampling locations, with an overall prevalence of 27.6% (135/489) (Table 1). The prevalence of Rickettsia in different locations ranged from 17.6% (15/85) to 42.5% (17/40).

| No. | Location | Coordinates | Detection of Rickettsia spp. in different small mammal species |
|-----|----------|-------------|----------------------------------------------------------|
|     |          |             | Apodemus flavicollis | Micromys minutus | Myodes glareolus | Microtus oeconomus | Microtus agrestis | Microtus arvalis | Sorex araneus | Total         |
| 1   | Curonian  | 55°33′06.0″N | 13/44 (29.5%)       | 12/30 (40%)     | 1/7             | 0/3                | 0/1                | 0/2                | 0/1            | 15/85 (17.6) |
| 2   | Spn      | 55°16′57.1″N | 21′10″31.5″E         | 6/20 (30%)      | 1/3             | 1/6                | 0/1                | 0/2                | 0/1            | 8/32 (25)     |
| 3   | Juodkrante| 55°32′30.9″N | 20′57″30.8″E         | 14/69 (20.1%)   | 2/2             | 1/6                | 0/1                | 0/2                | 0/1            | 16/71 (22.5) |
| 4   | Pervalkos| 55°24′37.7″N | 21′05″08.4″E         | 4/10            | 1/4             | 0/1                | 0/1                | 0/2                | 0/1            | 5/15 (33.3)  |
| 5   | Nida Dump | 55°23′33.5″N | 21′02″58.4″E         | 0/5             | 0/1             | 0/5                | 0/1                | 0/2                | 0/1            | 0/2          |
| 6   | Karuvaici| 55°23′15.4″N | 15/29 (51.7%)        | 2/3             | 0/1             | 0/1                | 1/7                | 0/2                | 0/1            | 17/40 (42.5) |
| 7   | Grostas Cape | 55°32′33.6″N | 21′07″13.1″E         | 12/29 (41.4%)   | 0/2             | 0/1                | 0/2                | 1/7                | 0/2            | 12/29 (41.4) |
| 8   | Cormoranar colony | 55°31′08.4″N | 20′04″42.7″E         | 1/20 (5%)       | 0/1             | 0/1                | 0/1                | 0/2                | 0/1            | 0/2          |
| 9   | Central   | 54°47′42.4″N | 24′38″35.3″E         | 0/18            | 0/18            | 0/1                | 0/1                | 0/1                | 0/1            | 0/18         |
| 10  | Lithuania | 55°02′49.8″N | 23′10″17.4″E         | 4/20 (20%)      | 0/1             | 0/1                | 0/1                | 3/7                | 0/1            | 6/23 (25.4)  |
| 11  | Lukiškės | 55°43′06.0″N | 22′20″41.1″E         | 0/5             | 0/32            | 0/1                | 0/1                | 3/12 (25%)         | 0/37           | 3/34 (33.3)  |

Total 68/231 (29.4%) 17/37 (45.9%) 47/198 (23.7%) 0/8 0/2 0/1 3/12 (25%) 135/489 (27.6%)
Rickettsial DNA was found in three rodent species, *A. flavicollis*, *M. minutus* and *M. glareolus*, as well as in the common shrew, *S. araneus*. *Rickettsia* spp. were found in 68 (29.4%) of the 231 *A. flavicollis*, 17 (45.9%) of the 37 *M. minutus*, 47 (23.7%) of the 198 *M. glareolus* and three (25%) of the 12 *S. araneus*. Thus, significantly higher overall infection rates were detected in *M. minutus* (odds ratio, 2.3; 95% confidence interval, 1.198–4.665; p 0.013). *Rickettsia*-infected *M. minutus* were found in all locations where these rodents were captured (Table 1). The prevalence of *Rickettsia* pathogens in *A. flavicollis* and *M. glareolus* ranged in different locations from 0 to 51.7% and from 0 to 34.6%, respectively. Three *Rickettsia*-infected *S. araneus* specimens were found in one of the five sampling locations (site 10) (Table 1).

There were no significant differences between the prevalence of *Rickettsia* between sampling locations, regions and years.

### Molecular characterization of *Rickettsia* isolates derived from small mammals

Fifty PCR products of *gltA* gene (*n* = 25) and 17 kDa protein coding gene (*n* = 25) of *Rickettsia* isolates derived from 50 distinct positive specimens of *A. flavicollis*, *M. minutus*, *M. glareolus* and *S. araneus* were sequenced. The sequence analysis of the partial *gltA* gene showed that the *Rickettsia* isolates derived from *A. flavicollis*, *M. minutus*, *M. glareolus* and *S. araneus* specimens were 100% identical to each other and to the *R. helvetica* sequences deposited in GenBank. The 17 kDa protein coding gene sequences derived from *Rickettsia*-positive small mammals were 99% to 100% identical to *R. helvetica* isolates from ticks and humans. Five genotypes of the 17 kDa protein coding gene of *R. helvetica* with one to three nucleotide differences were detected in *A. flavicollis* (Fig. 1).

### Discussion

Our study is to our knowledge the first investigation on the prevalence and molecular characterization of *R. helvetica* in *A. flavicollis*, *M. minutus*, *M. glareolus* and *S. araneus* in Lithuania. To our knowledge, this is the first detection of *R. helvetica* in *M. minutus*.

In Europe, six rickettsial pathogens—*R. helvetica*, *R. raoultii*, *R. slovaca*, *R. conorii* and *R. felis*—have been detected in rodents using molecular methods: *R. helvetica* in *A. flavicollis*, *Apodemus sylvaticus*, *A. agrarius*, *M. glareolus* and *Mus musculus* [8], *R. raoultii* in *A. flavicollis* and *M. glareolus* [14,15], *R. slovaca* in *Apodemus* spp. [16], *R. conorii* in *A. sylvaticus* and *M. glareolus* [1] and *R. felis* in *A. flavicollis* [15]. *S. araneus* has been found (based on serologic investigation) to be susceptible to *R. helvetica* infection [17].

The most common spotted fever group rickettsial pathogen, *R. helvetica*, was first isolated in 1979 from *I. ricinus* ticks in Switzerland [18]. Since then, *R. helvetica* has been reported in *I. ricinus* ticks throughout Europe [2], including the Baltic countries [5,19,20]. Cases of human infection related to *R. helvetica* have been reported in France, Italy, Switzerland, Sweden, Denmark, Austria and Slovakia [2]. Lithuania has not

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**FIG. 1.** Phylogenetic analysis of *Rickettsia* spp. in small mammals. Maximum likelihood tree for partial 17 kDa gene sequences of *Rickettsia* genus was generated with HKY model by using discrete gamma distribution (+G) and bootstrap analysis of 1000 replicates. Sequences with accession numbers were obtained from GenBank for comparison. Identification source (host) and country codes are provided after species names. Samples sequenced in present study are marked with solid circle. *Rickettsia bellii* was used as outgroup.
developed surveillance systems for rickettsioses, and so far no reported human clinical cases due to Rickettsia species infection have been registered in Lithuania.

Rickettsioses species have been identified by molecular detection from the ears, blood and spleen of small rodents. Rodent ears are the zone where ticks mostly feed; primary infection is thus localized near the tick-bite sites. After inoculation of the rickettsiae into the host, the bacteria proliferate in endothelial cells close to the inoculation site. The bacteria may be disseminated via lymph and blood to various organs, including lungs, liver, spleen, kidney and heart [21], and can be detected in blood and different internal organs of the host. In the present study, the overall prevalence of Rickettsia spp. in the spleen samples of the small mammals was high (27.7%), particularly in M. minutus (45.9%). A similar prevalence of Rickettsia spp. was detected in skin samples taken from the ears of small rodents in Germany (28.6%) by using real-time PCR, with R. helvetica identified in A. flavicollis, M. glareolus and A. agrarius, while M. glareolus also harboured R. raoultii [14]. R. helvetica was also detected in 29% of the blood samples obtained from A. sylvaticus and M. glareolus in the Netherlands [1]. However, a lower infection rate was reported in southwestern Slovakia where rickettsial DNA was detected in only 9.4% of blood samples of Apodemus spp. and M. glareolus [22]. In Croatia, Rickettsia spp. was found in 3.2% of heart samples of A. flavicollis and M. glareolus [7], while a 2× lower infection rate was reported in Hungary, where R. helvetica was found in 1.9% of spleen samples of M. musculus [23]. In a study in Poland, none of the blood samples from A. flavicollis (142) and M. glareolus (46) was found to be PCR positive for Rickettsia spp. [24].

In the present study, examined A. flavicollis, M. glareolus and M. minutus rodents were infested with I. ricinus ticks, the main vector for R. helvetica. In previous studies conducted in Lithuania, it has been shown that A. flavicollis mice and M. glareolus voles are frequently infested with immature I. ricinus ticks, with a higher prevalence of infection detected in A. flavicollis [25].

The high prevalence of R. helvetica in A. flavicollis, M. minutus and M. glareolus detected in the present study suggests that these rodents may play an important role as potential reservoir hosts and thus in the maintenance of this pathogen in nature.

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Conflict of interest

None declared.

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