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Original article

Isolated populations of *Ixodes lividus* ticks in the Czech Republic and Belgium host genetically homogeneous *Rickettsia vini*

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ABSTRACT

In the last two decades, the advent of molecular methods has revealed a remarkable diversity of rickettsiae (*Rickettsiales*: *Rickettsiaceae*) in invertebrates. Several species of these obligate intracellular bacteria are known to cause human infections, hence more attention has been directed towards human-biting ectoparasites. A spotted fever group *Rickettsia* sp. was previously detected in *Ixodes lividus* ticks (Ixodidae) associated with sand martins (Hirundinidae: *Riparia riparia*). In order to identify whether this rickettsia varies among isolated tick populations, a total of 1758 *I. lividus* ticks and five *Ixodes ricinus* ticks (Ixodidae) were collected in the Czech Republic and 148 *I. lividus* ticks were collected in Belgium, from nests of sand martins, European bee-eaters (Meropidae: *Merops apiaster*), Eurasian tree sparrows (Passeridae: *Passer montanus*), and from captured sand martins. We screened 165 and 78 *I. lividus* ticks (from the Czech Republic and Belgium, respectively) and all five *I. ricinus* ticks for the presence of rickettsial DNA. Only *I. lividus* samples were positive for *Rickettsia vini*, a spotted fever group rickettsia that commonly infects the tree-hole tick *Ixodes arboricola* (Ixodidae). Maximum likelihood analysis of the rickettsial sequences showed that the most closely related organism to *R. vini* corresponds to an uncharacterized rickettsia detected in *Argas lagenoplastis* (Argasidae), a nidicolous soft tick of the fairy martin (Hirundinidae: *Petrochelidon ariel*) in Australia. The observed variability of *R. vini* sequences from isolated tick populations was low; all 85 sequenced samples were identical to each other in five out of six partial rickettsial genes, except for the *sca4* sequence (99.9% identity, 808/809 nt) that differed in *I. lividus* ticks from two sampling sites in the Czech Republic.

1. Introduction

Rickettsiae are obligate intracellular bacteria of invertebrates; some rickettsial species can propagate in vertebrates and are transmitted via blood-sucking arthropods (Parola et al., 2013). Based on whole-genome analyses, a single topology of the genus *Rickettsia* has been proposed (Murray et al., 2016). The members of the well-sampled spotted fever group are mainly associated with ixodid ticks (Parola et al., 2013). While many ticks are typically exophilous, some ancestral groups exhibit a nidicolous behavior, parasitizing their hosts in caves, burrows, and nests. These nidicolous ticks include representatives of the family Argasidae and many species of the genus *Ixodes* (Ixodidae), e.g., *Ixodes arboricola* and *Ixodes lividus* (Sonenshine and Roe, 2014). Recently, a new rickettsial organism, *Rickettsia vini*, was detected with a prevalence of 94–100% (Palomar et al., 2012a; Palomar et al., 2015) in *I. arboricola*, a tick that feeds strictly on birds roosting and breeding inside tree cavities in Palearctic ecozone (Heylen et al., 2012; Heylen et al., 2014a). Occasionally, this rickettsial organism has also been detected in the immature stages of the field-dwelling *Ixodes ricinus* collected from passerine birds (Keskin et al., 2014; Nováková et al., 2015,2016;

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Palomar et al., 2012a, 2012b; Špitalská et al., 2011). *Ixodes lividus*, a nidicolous tick both morphologically and genetically closely related to *I. arboricola* with a Palearctic distribution (Guglielmone et al., 2014), has been found to harbor an uncharacterized rickettsial DNA with 100% prevalence (Elfving et al., 2010; Graham et al., 2010).

While a large proportion of *I. arboricola* hosts are birds with limited migration, the main host of *I. lividus* ticks is the sand martin (Hirundinidae: *Riparia riparia*), a passerine bird that breeds in Holarctic ecozone during the warm season and migrates to southern latitudes in winter (Svensson et al., 2010). During the breeding season, *R. riparia* colonizes river banks in high densities and nests in sand holes (burrows) that are up to 1 m long. This bird is also able to nest at anthropogenic mining places (Heneberg, 2013). Potential hosts of nidicolous ticks are the Eurasian tree sparrow (Passeridae: *Passer montanus*), the species that commonly occupies old *R. riparia* burrows, and the European bee-eater (Meropidae: *Merops apiaster*), which also roost in burrows.

Based on overlapping geographical distributions and the remarkably similar prevalences of the rickettsial agents of the above mentioned nidicolous hard tick species, we conducted this study in order to provide further characterization of the *I. lividus* rickettsial agent. We expected that the two rickettsiae should be closely related, hence we reconstructed a phylogenetic tree with an emphasis on rickettsiae associated with nidicolous ticks. To see whether specific differences among rickettsial agents exist in tick populations with geographically distinct distributions, we included ticks from 24 areas in the Czech Republic and one area in Belgium.

2. Materials and methods

2.1. Collection of ticks

Nest materials were collected from 20 *M. apiaster* burrows, 8 burrows made by *R. riparia* but inhabited by *P. montanus,* and 325 *R. riparia* burrows, in May 2013 (during the pre-breeding season; materials were from nests built in 2012) and September 2013 (during the post-breeding season; materials from 2013 nests); materials were gather from 24 sampling sites in the Czech Republic (Table 1). Nest material was retrieved quantitatively from each burrow using a metal spoon attached to a 2 m pole. Nesting bird species was identified by characteristic appearance of the nest (among other features, by the presence of hymenopteran exoskeletons for *M. apiaster*, long grass straws located at the burrow entrance for *P. montanus*, duck feathers present in the nests for *R. riparia*). Material from each nest was placed into a separate zip-lock plastic bag, brought to the laboratory and stored at 8 °C until analyzed. The nest material was spread on a white plastic plate and observed for the presence of ticks (each nest was examined for 15 min). In addition to the above specimens, bird-derived *I. lividus* ticks were collected from a subset of 10 out of 49 infested birds, from a total of 135 *R. riparia* that were caught with mist-nets in August 2013, in Kieldrecht, Antwerp, Belgium (Table 2). All ticks were stored in 70% ethanol and identified according to Nosek and Sixl (1972). Specific identification of larvae, which were also tested for the presence of rickettsial DNA, was supported by amplification of a partial sequence of the *16S rDNA* mitochondrial gene, a control for extraction-positive samples (Table S1).

2.2. PCR amplification and DNA sequencing

DNA was extracted according to Sangioni et al. (2005) from selected *I. lividus* ticks. A maximum of 10 immature ticks from each sampling site, with each individual from a different nest hole (if applicable), has been chosen. In contrast, all adult ticks and all *I. ricinus* ticks were used (Tables 1 and 2). Additionally, rickettsial DNA from 3 females *I. arboricola,* which were collected in Břeclav, the Czech Republic, as a part of a previous study (Nováková et al., 2015) were used.

Isolated DNA was subjected to polymerase chain reaction (PCR) under the following conditions: 25 µL of PCR reaction was composed of 12.5 µL Combi PPP Master Mix (Top Bio, Vestec, Czech Republic), 1.5 µL of each primer at 20 µM, 7.0 µL of PCR H_2O, and 2.5 µL DNA (10–24 ng/µl). Samples were tested using a battery of primers targeting fragments of six rickettsial genes (Table S1). When the amplification of the partial sequence of the *gltA* gene was negative, additional PCR targeting the tick mitochondrial *16S rRNA* gene was performed in order to verify that DNA extraction was successful. For each reaction, negative (PCR H_2O) and positive controls (DNA from *Rickettsia rickettsii* strain I12B-infected Vero cells, 15 ng/µl) were used. PCR products were visualized on 1.5% agarose gels (Roche Diagnostics, Basel, Switzerland) and samples were purified using a Gel/DNA Fragments Extraction Kit (Geneaid, Taipei City, Taiwan). Sanger dideoxy sequencing was performed by Macrogen (Amsterdam, Netherlands).

2.3. Phylogenetic analysis

Sequences were analyzed by BLAST (Basic Local Alignment Tool) to assign similarities to other *Rickettsia* sequences available in the Nucleotide Database of National Center for Biotechnology Information (NCBI). Sequences were aligned via ClustalW version 2.1 (Larkin et al., 2007), using gap opening penalty 8, gap extension penalty 1.5 for pairwise as well as multiple alignments, DNA weight matrix IUB and transition weight 0.4. Bayesian inference in MrBayes version 3.2.6 (Ronquist and Huelsenbeck, 2003) was performed in order to infer the phylogenetic tree topologies, using following parameters: mixed model of nucleotide substitution, gamma-distributed rates among sites, four Monte Carlo Markov chains for 2,000,000 cycles, chains were sampled every 1,000th generation, first 25% of the samples were discarded as a burn-in, and *R. felis* was used as outgroup. The final tree topologies were consequently generated employing 50% majority-rule consensus. We obtained summary statistics of Bayesian analyses as follows: average standard deviation of split frequencies 0.0049–0.0078, maximum standard deviation of split frequencies 0.019–0.043, average potential scale reduction factor 1.000–1.001 for all the trees, and maximum potential scale reduction factor 1.002–1.012. In order to include as many rickettsial species as possible, only DNA sequences of the *gltA*, *ompA*, and *ompB* genes were aligned. The *gltA*, *ompA*, and *ompB* partial sequences were analyzed separately, and also concatenated.

3. Results

In total, 1758 unfed *I. lividus* ticks (1707 larvae, 15 nymphs, 10 males, 26 females) and 5 unfed *I. ricinus* ticks (one larva, four nymphs) were found in *R. riparia* nests. Ticks were found at 17 out of the 24 examined sampling sites in the Czech Republic (Table 1). From the total captured 135 *R. riparia* in Belgium, the infestation prevalence with ticks was 23.0% (14/61) for adult birds and 47.3% (35/74) for fledglings. Ticks were collected from four adults and six juveniles. In total, 148 ticks (72 larvae and 76 nymphs) were derived from *R. riparia* in Belgium (Table 2).

DNA was successfully isolated (i.e., sample was positive for rickettsial DNA or tick mitochondrial DNA) from 178 of 243 *I. lividus* ticks (136/165 ticks from the Czech Republic and 42/78 ticks from Belgium), and 5/5 *I. ricinus* ticks. Rickettsial DNA was detected in all 178 *I. lividus*, but none in *I. ricinus* ticks. From each locality in the Czech Republic (if applicable), three DNA-containing samples from *I. lividus* ticks were sequenced. In total, 43 samples from the Czech Republic and 42 samples from Belgium were sequenced.

The rickettsial agent in all the samples was determined to be *R. vini*. The identification was supported by 100% nucleotide sequence identity of all 5 loci previously sequenced from *R. vini* (Table S2). All 85 sequenced samples of *I. lividus* ticks tested in this study were identical to each other and to previously amplified sequences of *R. vini* isolated from *I. arboricola* ticks, except for the *sca4* sequence (99.9% identity, 808/809 nt) in *I. lividus* ticks from two sampling sites in the Czech Republic (Table S2).