Anaplasma phagocytophilum in ticks in Slovenia
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Abstract
Ticks act as vectors of many pathogens of domestic animals and humans. Anaplasma phagocytophilum in Europe is transmitted by the ixodid tick vector *Ixodes ricinus*. *A. phagocytophilum* causes a disease with diverse clinical signs in various hosts. The diversity of the *groESL* operon of *A. phagocytophilum* has been found in ticks elsewhere. In Slovenia, the variety of the *groESL* operon was conducted only on deer samples. This study, the prevalence of infected ticks was estimated and the diversity of *A. phagocytophilum* was evaluated. On 8 locations in Slovenia, 1924 *I. ricinus* ticks were collected from vegetation in the years 2005 and 2006, respectively. All three feeding stages of the tick’s life cycle were examined. The prevalence of ticks infected with *A. phagocytophilum* in the year 2005 and in the year 2006 was 0.31% and 0.63%, respectively, and it did not differ considerably between locations. The similarity among the sequences of *groESL* ranged from 95.6% to 99.8%. They clustered in two genetic lineages along with *A. phagocytophilum* from Slovenian deer. One sequence formed a separate cluster. According to our study, the prevalence of *A. phagocytophilum* in ticks is comparable to the findings in other studies in Europe, and it does not vary considerably between locations and tick stages. According to *groESL* operon analysis, two genetic lineages have been confirmed and one proposed. Further studies on other genes would be useful to obtain more information on genetic diversity of *A. phagocytophilum* in ticks in Slovenia.

Findings
Ticks and tick-borne diseases affect animal and human health worldwide. A vector of many diseases in Europe and Slovenia is *Ixodes ricinus* [1]. It can be found in the forest, in shrubby or wooded pastures and on surfaces with low vegetation [2]. Ticks’ feeding cycle includes three stages: larva, nymph and adult. *I. ricinus* feeds on livestock, deer, dogs and a wide variety of other species, including humans [2]. *I. ricinus* is a confirmed vector of the bacterium *Anaplasma phagocytophilum* [3]. The tick becomes infected as it feeds on an infected host. Anaplasmata are transmitted from stage to stage as the tick molts (trans-stadially), but not transovarially. No anaplasmata have been detected in unfed larvae so far [4]. *A. phagocytophilum*, the agent of granulocytic anaplasmosis, was formerly known as human granulocytic ehrlichiosis agent (HGE agent), *Ehrlichia phagocytophila* and *E. equi* [5]. *A. phagocytophilum* causes a disease with diverse clinical signs in various hosts from asymptomatic to life-threatening [4]. No fatal infection in humans has been documented in Europe so far. On the contrary, in the USA, the fatality rate in humans is 1% [4]. Important reservoir hosts of the bacterium are small mammals and deer. Humans, dogs, horses represent accidental hosts [4]. The wild boar is suggested as a reservoir host for a variant that infects humans [6]. The prevalence of infected ticks in Europe ranges from 0.4% - 66.7% [7]. To describe the diversity of *A. phagocytophilum*, the *groESL* operon is widely used as the 16 S rRNA gene is too conservative [8]. It has been shown that, based on this operon, anaplasmata among deer in Slovenia cluster in two genetic lineages [9]. An immense diversity of *groESL* sequences of *A. phagocytophilum* in ticks has also been described in Germany [10]. The variants matched to the sequences found in a German and a Swedish horse and in a Slovenian patient [10]. In a previous study in Slovenia, the estimated prevalence of infected ticks from one location was 3.2% [11]. The ticks in the present study were being collected every month for two years from several locations. The prevalence of ticks infected with *A. phagocytophilum* was estimated and the diversity of the *groESL* operon of detected DNA of *A. phagocytophilum* was evaluated.

The study was performed in the years 2005 and 2006 at 8 locations in Slovenia. The criteria for selecting the locations were the tick-borne pathogens’ presence in human
patients or a higher altitude of the location compared to others. Ticks were collected at forest edges by dragging a flag with a surface of 1 m² over 100 m of vegetation [1]. Every 2.5 m, the flag was examined for ticks. The species, stage and sex of ticks were determined by a professional entomologist. Ticks were decontaminated in 70% ethanol and sterile double distilled water and pooled in groups of 30 larvae, 10 nymphs or 5 adults. Adult ticks were first cut in half and a half of each adult tick was used for pooling. The remaining half of the dissected adult tick was frozen and stored separately. Pools of ticks were stored at -20°C until further analysis. The pooled samples were used for DNA extraction. First, they were homogenized using TissuLyser (Retsch for Qiagen, Hilden, Germany). DNA was extracted with BioSprint 15 DNA Blood Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). To assess the efficiency of DNA extraction, tick mitochondrial 16 S rRNA was examined [12]. For the initial screening of samples, primer pair Ehr521 and Ehr790, specific for the 16 S rRNA of genus Anaplasma sp. and Ehrlichia sp., was used [11]. All positive samples were additionally tested for the groESL operon. A nested PCR would amplify a 1296-bp fragment of groESL operon of A. phagocytophilum variants [8]. A. phagocytophilum, grown in a HL-60 cell culture, was used as a PCR positive control. If a pool of adult ticks was positive, the stored half of each dissected tick from a pool was used for DNA extraction and further amplifications. All amplicons of groESL operon were further analyzed by sequencing on both strands with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed with computer programs of the Lasergene 1999 software package (DNASTAR, Madison, WI, USA) based on Clustal W algorithm [13]. The distance matrices were calculated using Kimura two-parameter method 1980 [14] and the Neighbor-joining method [15] was used for the construction of a phylogenetic tree with TreeCon software (Yves Van de Peer, Department of Biochemistry, University of Antwerp, Antwerpen, Belgium). The stability of inferred topology was assessed with 1000 bootstrap replicates. The prevalence of infection was calculated using the program PooledInfRate version 3.0 (a Microsoft® Excel Add-In, developed by Brad Biggerstaff, CDC, Fort Collins, CO). Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL). P values of 0.05 or less were considered statistically significant.

On 8 locations in Slovenia, 1924 and 5049 (6973) I. ricinus ticks were collected by flagging vegetation in the years 2005 and 2006, respectively. Ticks were separated into pools: 252 pools in 2005 and 442 pools in 2006 (Table 1). At the location of Murska Suma, other tick species were collected, namely Dermacentor reticulatus and Haemaphysalis concinna. As A. phagocytophilum is transmitted by Ixodes spp. ticks [16], only I. ricinus was examined.

The 16 S rRNA of Anaplasma sp. was detected in 26 pools of adult and nymphal stages of ticks (Table 1). None of the pool of larvae was positive in the year 2005, and were not tested in the year 2006 as A. phagocytophilum is not transovarially transmitted in ticks (Table 1). One adult tick from each pool was positive. The prevalence of infection in the year 2005 and in the year 2006 was 0.31% and 0.63%, respectively (Table 1). No statistically significant differences were found between the prevalences at various locations and in both years (p > 0.05).

The sequences of 26 PCR amplicons of the groESL operon matched A. phagocytophilum. The similarity varied from 95.6% to 99.8%. Twenty-five sequences of the groESL operon clustered in two genetic lineages, A and B (Figure 1). In the genetic lineage A, most of the sequences gathered in two clusters, 1 and 2. Ten sequences of the groESL operon were 100% identical to each other and grouped together with a reference sequence from a Slovenian dog [GenBank:EU381151] in cluster 2. Three 100% identical sequences clustered together with a sequence from a Slovenian patient [GenBank:AF033101] in cluster 1 of the same lineage. In the lineage B, three sequences were 100% identical to each other and were identical to a reference sequence from a German tick [GenBank:AY281794] (Table 2). One sequence (tick EU341) from a pool of nymphs showed 95.6% similarity to other sequences from Slovenian ticks and 96% similarity with a reference sequence [GenBank:AY281848] from a German tick and did not cluster in any of the lineages A or B. Only the sequences that were not 100% identical were included in the phylogenetic study.

Ticks are the vectors of many disease-causing pathogens. I. ricinus, the vector of A. phagocytophilum, is distributed throughout Europe, including Slovenia [1]. Environmental factors, such as climate, vegetation type, and abundance of appropriate hosts, influence the geographical distribution of pathogen vectors and, consequently, pathogens themselves [17]. In this study, ticks were collected at 8 different locations in Slovenia in two consecutive years. The overall prevalence of A. phagocytophilum infection in ticks in 2005 and 2006 was 0.31% and 0.63%, respectively, which is in agreement with the results from elsewhere in Europe [7]. In a previous study in Slovenia, the estimated prevalence of infected ticks was 3.2%, but this was most likely due to the fact that only a small number of adult ticks was collected at a single location in central Slovenia [11]. There are also some reports of higher prevalence of infected ticks, but the likely reasons are the geographical differences and
### Table 1. A. phagocytophilum in pools of ticks and its prevalence in Slovenian I. ricinus

| Year | Location | Larvae | Nymphs | Adult | Total |
|------|----------|--------|--------|-------|--------|
|      |          |        |        | Male  | Female |
|      |          |        |        |       |        |
| Črni Kal | 3 | 13 | 2 | 2 | 20 |
| Sodražica | 1 | 14/1 (0.80) | 5 | 4 | 24/1 (0.63) |
| Murska šuma | 0 | 2 | 4 | 7 | 13 |
| Rakovnik | 2 | 15 | 12 | 10 | 39 |
| Mozirje | 3 | 23/3 (1.45) | 10 | 8/1 (3.5) | 44/4 (1.24) |
| Kamniška Bistrica | 9 | 22 | 9 | 7 | 47 |
| Štefanja Gora | 3 | 27 | 9/1 (2.97) | 6 | 45/1 (0.28) |
| Osojniki | 1 | 10 | 5 | 4 | 20 |
| **Total** | **22** | **126/4 (0.34)** | **56/1 (0.53)** | **48/1 (0.69)** | **252/6 (0.31)** |

| Year | Location | Larvae | Nymphs | Adult | Total |
|------|----------|--------|--------|-------|--------|
|      |          |        |        | Male  | Female |
|      |          |        |        |       |        |
| Mozirje | NT | 43/5 (1.31) | 4 | 2 | 49/5 (1.24) |
| Sodražica | NT | 24/1 (0.43) | 8 | 6 | 38/1 (0.34) |
| Murska šuma | NT | 1 | 10 | 22 | 33 |
| Rakovnik | NT | 32/2 (0.65) | 17 | 14 | 63/2 (0.44) |
| **Total** | **NT** | **250/17 (0.73)** | **95/2 (0.47)** | **97/1 (0.23)** | **442/20 (0.63)** |

In parenthesis in %. NT - not tested. Ticks were sorted into pools of 10 nymphs, 30 larvae or pools of 5 halves of male or female adult ticks.

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**Figure 1** Phylogenetic relationship of anaplasmae deposited in GenBank and detected in this study in tick samples. GenBank accession numbers: Slovenian patient [GenBank:AF033101], dog1 [GenBank:EU381150], dog2 [GenBank:EU381151], wild boar [GenBank:EU184703], red deer [GenBank:AF478562], roe deer1 [GenBank:AF478558], roe deer2 [GenBank:AF478564], A. phagocytophilum [GenBank:AY529490], A. marginale [GenBank:AF414865], Ehrlichia chaffeensis [GenBank:EU10917]. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates. Only different groESL sequences from I. ricinus are shown.
the presence of appropriate hosts [7], as well as different screening methods used. The infection rate in Slovenia did not differ considerably between the tick stages (Table 1). In the year 2005, no larvae were found positive, which is in concordance with the fact that anaplasmae are not transovarially transmitted [4].

Only a few reports describe the diversity of A. phagocytophilum in infected ticks sampled from vegetation [10]. The variety of groESL operon sequences has been determined and some matched human and horse cases of anaplasmosis [10]. In this study, a high diversity of sequences of the groESL operon in ticks has been found. As discussed in a previous study of deer sequences of A. phagocytophilum [9], the sequences from the ticks in Slovenia are also delineated in two genetic lineages (Figure 1). The similarity between these sequences varied from 97.8% to 99.8%. In the lineage A (dog, human, wild boar, tick, deer samples), the similarity ranged from 99.5% to 99.9%, and in the lineage B (tick and deer samples), it varied from 99.0% to 99.8%. Different genetic lineages represent also differences in the amino acid sequence of GroESL protein (an amino acid serine (lineage A) is substituting alanine (lineage B) at the position 242). It is suggested that the strains of A. phagocytophilum that possess a variant of the protein with the serine might be pathogenic to humans [18].

A greater diversity of the groESL operon was found at the nymphal stage of ticks. For this, many reasons are possible. The groESL genetic variants other than those that cause the disease in humans and dogs in Slovenia might not be pathogenic to aforementioned hosts since they have not been found in them yet [11,19]. It is nevertheless possible that they circulate only among small mammals and deer. However, the main reason could be the number of collected ticks: more nymphs than adult ticks were collected at all locations and, consequently, higher diversity was found.

In one pool of nymphs from Mozirje, a groESL sequence showed only a 95.6% similarity with other groESL operon sequences from the ticks in Slovenia. It did not cluster within the lineages A and B. Moreover, after the translation into amino acid sequence, no difference from the lineage B was found (alanine at the position 242). Probably, a novel genetic lineage of the groESL operon of A. phagocytophilum was found. To obtain more information about genetic diversity of A. phagocytophilum in I. ricinus in Slovenia, additional genetic markers, such as ankA and msp4, should be analyzed.

I. ricinus nymphal and adult stages are responsible for the transmission of the pathogen as A. phagocytophilum was present in both stages. There was no significant
difference in the prevalences of *A. phagocytophilum* at different locations and in both years. The prevalence of infection in ticks did not differ considerably from the reports from elsewhere in Europe. With this study, we have confirmed that *I. ricinus* is a vector of a variant of *A. phagocytophilum* that causes the disease in humans and dogs. The sequencing of the groESL operon has demonstrated a great diversity of *A. phagocytophilum* in Slovenia. With phylogenetic analysis, two genetic lineages have been confirmed and another has been proposed. Further phylogenetic studies of several other genes, such as *ankA* and *msp4*, might be useful to obtain more information about genetic diversity.

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Authors’ contributions

KSS conducted the laboratory study and drafted the manuscript. MS was involved in laboratory study. DD facilitated the molecular laboratory study. NK conducted the field study and did the statistical analysis. TAZ was involved in the project design and participated in drafting the manuscript. All co-authors have read the manuscript.

Competing interests

The authors declare that they have no competing interests.

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