Diversity of Coxiella-like and Francisella-like endosymbionts, and Rickettsia spp., Coxiella burnetii as pathogens in the tick populations of Slovakia, Central Europe

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Abstract

Ticks are important vectors of pathogens affecting humans and animals worldwide. They do not only carry pathogens but diverse commensal and symbiotic microorganisms are also present in ticks. A molecular screening for tick-borne pathogens and endosymbionts was carried out in Ixodes ricinus, Dermacentor reticulatus and Haemaphysalis inermis questing ticks collected in Slovakia. The presence of Rickettsia spp., Coxiella burnetii, Coxiella-like and Francisella-like microorganisms was evaluated by PCR in 605 individuals and by randomly sequencing 66 samples. Four species of rickettsiae (R. raoultii, R. slovaca, R. helvetica and R. monacensis) were identified and reported with an overall prevalence range between 0.4 and 50.3% (±8.0) depending on tick species, sex and locality. Partial sequencing of the gltA gene of 5 chosen samples in H. inermis showed 99% identity with Candidatus Rickettsia hungarica. The total prevalence of C. burnetii in ticks was 2.2±1.7%; bacteria were confirmed in I. ricinus and D. reticultaus ticks. The sequences from 2 D. reticulatus males and 1 I. ricinus female ticks were compared to GenBank submissions and a 99.8% match was obtained with the pathogenic C. burnetii. Coxiella–like endosymbionts were registered in all three species of ticks from all studied sites with an average prevalence of 32.7±3.7%. A phylogenetic analysis of this Coxiella sp. showed that it does not group with the pathogenic C. burnetii. The prevalence of Francisella-like microorganisms in questing ticks was 47.9±3.9%, however H. inermis (n = 108) were not infested. Obtained sequences were 98% identical with previously identified Francisella-like endosymbionts in D. reticulatus and I. ricinus. Coxiella-like and Francisella-like microorganisms were identified for the first time in Slovakia, they might be considered as a non-pathogenic endosymbiont of I. ricinus, D. reticulatus and H. inermis, and future investigations could aim to assess their role in these ticks. However, this work provided further data and broadened our knowledge on bacterial pathogens and endosymbionts present in ticks in Slovakia to help understanding co-infestations, combined treatments and public health issues linked to tick bites.

Keywords	tick; pathogen; endosymbiont; Slovakia

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Diversity of *Coxiella*-like and *Francisella*-like endosymbionts, and *Rickettsia* spp., *Coxiella burnetii* as pathogens in the tick populations of Slovakia, Central Europe

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ABSTRACT

Ticks are important vectors of pathogens affecting humans and animals worldwide. They do not only carry pathogens but diverse commensal and symbiotic microorganisms are also present in ticks. A molecular screening for tick-borne pathogens and endosymbionts was carried out in *Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis inermis* questing ticks collected in Slovakia. The presence of *Rickettsia* spp., *Coxiella burnetii*, *Coxiella*-like and *Francisella*-like microorganisms was evaluated by PCR in 605 individuals and by randomly sequencing 66 samples. Four species of rickettsiae (*R. raoultii*, *R. slovaca*, *R. helvetica* and *R. monacensis)* were identified and reported with an overall prevalence range between 0.4 and 50.3% (±8.0) depending on tick species, sex and locality. Partial sequencing of the *gltA* gene of 5 chosen samples in *H. inermis* showed 99% identity with *Candidatus* Rickettsia hungarica. The total prevalence of *C. burnetii* in ticks was 2.2±1.7%; bacteria were confirmed in *I. ricinus* and *D. reticultaus* ticks. The sequences from 2 *D. reticulatus* males and 1 *I. ricinus* female ticks were compared to GenBank submissions and a 99.8% match was obtained with the pathogenic *C. burnetii*. *Coxiella*–like endosymbionts were registered in all three species of ticks from all studied sites with an average prevalence of 32.7±3.7%. A phylogenetic analysis of this *Coxiella* sp. showed that it does not group with the pathogenic *C. burnetii*. The prevalence of *Francisella*-like microorganisms in questing ticks was 47.9±3.9%, however *H. inermis* (n = 108) were not infested. Obtained sequences were 98% identical with previously identified *Francisella*-like endosymbionts in *D. reticulatus* and *I. ricinus*. *Coxiella*-like and *Francisella*-like microorganisms were identified for the first time in Slovakia, they might be considered as a non-pathogenic endosymbiont of *I. ricinus*, *D. reticulatus* and *H. inermis*, and future investigations could aim to assess their role in these ticks. However, this work provided further data and broadened our knowledge on bacterial pathogens and endosymbionts present in ticks in Slovakia to help understanding co-infestations, combined treatments and public health issues linked to tick bites.

Keywords: tick, pathogen, endosymbiont, Slovakia

1. Introduction

Ticks are obligate blood sucking ectoparasites of vertebrate animals. Microbial communities hosted by ticks include tick-borne pathogens (viruses, bacteria, protozoa) and non-pathogenic microorganisms such as commensal and mutualistic microbes abundant in ticks (Andreotti et al., 2011; Carpi et al., 2011; Williams-Newkirk et al., 2014; Duron et al., 2015a, 2017). Diversity within microbial communities could be correlated to tick species, different tissues and organs, season, geographical regions, tick life stage, and feeding statuses (Carpi et al., 2011; Lalzar et al., 2012; Menchaca et al., 2013; Zhang et al., 2014; Egyed and Makrai, 2014; Budachetri et al., 2014; Qiu et al., 2014; Williams-Newkirk et al., 2014; Zolnik et al., 2016;).

Ixodes ricinus, *Dermacentor reticulatus*, *Dermacentor marginatus*, *Haemaphysalis concinna*, *Haemaphysalis inermis* and *Haemaphysalis punctata* tick species are common and widespread in Slovakia. *Ixodes ricinus* ticks, considered as vectors and reservoir hosts, were collected from different localities in Slovakia, where it had been previously found to be infected with *Rickettsia helvetica* and *Rickettsia monacensis*, while *Dermacentor* spp. ticks were found infected with *Rickettsia slovaca* and *Rickettsia raoultii* (Špitalská et al., 2012, 2014, 2016; Minichová et al., 2017). Although these rickettsial species (Proteobacteria: Rickettsiales) are known to be pathogenic to humans they are usually linked to mild clinical symptoms (Uchiyama, 2012; Oteo and Portillo, 2012). Rickettsial species and the role of *Haemaphysalis* ticks as vectors in Slovakia have not been revealed to this day.

Coxiella burnetii (Proteobacteria: Legionellales) is the etiological agent of human Q fever, a zoonotic disease distributed worldwide and causing a disease with symptoms including fever, hepatitis, and respiratory complications (Raoult, 1993). Ticks play an important role in the circulation of *C. burnetii* in natural foci and are responsible for the dissemination of the infection among animals. The presence of *C. burnetii* was previously isolated from *I. ricinus*, *D. reticulatus*, *D. marginatus*, *H. concinna* and *H. inermis* ticks in Slovakia (Řeháček et al., 1991, Špitalská and Kocianová, 2003). *Coxiella*-like endosymbionts (CLEs), similar to *C. burnetii* are present in different tick species such as *Ornithodoros muesebecki*, *Rhipicephalus sanguineus*, *Haemaphysalis longicornis*, *Ixodes woodi*, *I. ricinus*, *Amblyomma americanum* (Zhong, 2012; Al-Deeb et al., 2016), without specific tissue location. The prevalence of CLEs varies among different species of ticks. As summarised by Zhong (2012) it is ranging from 5 to 100%. CLEs have not been studied in arthropods in Slovakia so far.

Francisella tularensis (Proteobacteria: Thiotrichales) is the etiological agent of the tularemia (Chu and Weyant, 2003). *Francisella tularensis* naturally occurs in vertebrates, invertebrates, and in contaminated soil, water, and vegetation (Mörner, 1992). The clinical presentation of tularemia varies depending upon the route of infection. The principal tick vectors include species of the genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes* and *Ornithodoros* (Gordon et al., 1983). Many tick species are also hosts of *Francisella*-like endosymbionts (FLEs), bacteria closely related to *F. tularensis*

(Dergousoff and Chilton, 2012). The pathogenic potential of FLEs remains unknown. FLEs appear to replicate intracellularly, and they are transmitted transovarially. To date, there is no evidence of horizontal transmission through tick bites (Ivanov et al., 2011). FLEs are widely distributed in Europe and were identified in *D. reticulatus*, *Hyalomma marginatum*, *Hyalomma aegyptium* and *Rhipicephalus sanguineus* sensu lato in Hungary, Portugal, France, Germany and Bulgaria (Sréter-Lancz et al., 2009; Ivanov et al., 2011; Kreizinger et al., 2013; De Carvalho et al., 2011; Michelet et al., 2013; Gehringer et al., 2013;). No data are known for the occurrence of FLEs in ticks of Slovakia.

No recent reports are available on the occurrence of rickettsial species in *Haemaphysalis* ticks, *Coxiella*-like and *Francisella*-like endosymbionts in ticks, and simultaneous occurrence of pathogenic *Rickettsia* species and *C. burnetii* with CLEs and FLEs in potential arthropod vectors in Slovakia. To understand better the circulation in Slovakia of these pathogens and symbionts we collected questing *D. reticulatus*, *I. ricinus* and *H. inermis* ticks.

2. Material and methods

2.1. Collection of ticks

A total of 605 questing ticks of following species *D. reticulatus*, *I. ricinus*, and *H. inermis* were collected in March and April 2012, during year 2016 and in May 2017. Ticks were collected by dragging a woollen flag over the lower vegetation and along the paths in mixed forests in four localities Gabčíkovo, Zohor, Stará Lesná, and Hrhov. Gabčíkovo (47°54 N, 17°34.983 E) is situated in southwest Slovakia, 110 m above sea level (asl), alluvial habitat near river Danube. Zohor (48°20.374 N, 16°56.791 E) is situated in west Slovakia, 144 m asl, with mixed deciduous forest of oak, hornbeam and hazel near river Morava. Ticks were collected on the edge of forests near the Zohor, Láb and Vysoká pri Morave villages. Stará Lesná (49°08.166 N, 20°18.575 E), High Tatras, 770 m a.s.l is located in north Slovakia, with deciduous forest of birch, rowan and spruce. Ticks were collected across the woods along the forest path, while the last site was a typical mixed forest with a predominance of beech, oak and hornbeam. The last sampling site was located in the Slovak Karst National Park, near the village Hrhov (200–220 m a.s.l., 48°34.899 N, 20°46.743 E). Ticks were collected on the edges of the forests and pastures in this area.

2.2. DNA extraction from ticks

Ticks were washed with sterile water, dried, transferred to individual tubes and crushed with a sterile carbon steel surgical scalpel blade (Surgeon, JAI Surgicals Ltd., India). Total DNA was isolated from ticks separately using the method of alkaline hydrolysis (Rijpkema et al., 1996). The concentration and purity of DNA were measured by NanoPhotometer Pearl (Implen, Germany). DNA samples were stored at −20 °C and later used as templates for the PCR amplifications.

2.3. Molecular analysis

Ticks samples were screened by PCR-based methods for the presence of *Rickettsia* spp. and *C. burnetii* tick-borne pathogens, CLEs and FLEs tick endosymbionts. *Rickettsia* species were identified based on the amplification of the *gltA*, *ompA* and *sca4* genes, *C. burnetii* and FLEs based on the 16S rRNA, and CLEs based on the *GroEl* gene (Forsman et al., 1994; Roux et al., 1996; Sekeyová et al., 2001; Melničáková et al., 2003; Boretti et al., 2009; Duron et al., 2014). Rickettsial species were identified by species-specific real-time PCR, *Rickettsia helvetica* identification was based on the 23S rRNA gene, *Rickettsia slovaca* and *R. raoultii* identification were based on the *ompB* gene (Boretti et al., 2009; Jiang et al., 2012). PCR amplifications were performed on a TPersonal thermocycler (Biometra, Germany) or a Labcycler (SensoQuest, Germany). PCR products were analysed by electrophoresis in a 1% agarose gel stained with GelRed™ (Biotium, Hayward, California, USA) and visualized under a UV transilluminator. The real-time PCR assays were performed using a Bio-Rad CFX96™ Real-Time System. Negative and positive controls were included in each PCR-based assays.

2.4. DNA sequencing and phylogenetic analysis

In total, 66 randomly selected amplicons from *gltA*, *ompA*, 16S rRNA and *GroEl* genes were purified and both strands were sequenced by Macrogen Inc. (Amsterdam, The Netherlands). Obtained sequences were compared with available sequences listed in the GenBank nucleotide sequence database. The phylogenetic trees were produced according to the Neighbor-Joining method using bootstrap analyses with 1,000 replicates using MEGA 5 software (Felsenstein, 1985; Saitou and Nie, 1987; Tamura et al., 2011).

2.5. Statistical analysis

Statistical analyses to test for differences in the prevalence of microorganisms in questing ticks between tick species, tick sex and sites were carried out using Fisher's exact test with an online calculator (http://www.socscistatistics.com). A p value < 0.05 was considered significant. Ninety-five percent confidence intervals (CI) were calculated using an online calculator (http://epitools.ausvet.com.au).

3. Results

A total of 605 ticks of three species, 334 *D.reticulatus* (154 females and 180 males), 163 *I. ricinus* (93 females, 48 males and 22 nymphs), and 108 *H. inermis* (75 females, 33 males) were collected from vegetation of natural sites Zohor, Gabčíkovo, Stará Lesná and Hrhov, in Slovakia (Table 1).

Rickettsia spp. DNA was confirmed in 215 (35.5±3.8%) ticks of all three species in all studied sites. DNA of *R. raoultii* and *R. slovaca* were identified in *D. reticulatus* ticks (78 females and 90 males), *R. raoultii* was dominant species. DNA of *R. helvetica* and *R. monacensis* were found in *I. ricinus* ticks (9 females, 6 males and 3 nymphs), *R. helvetica* was dominant species (Table 1). No significant difference was found in the prevalence of *R. raoultii* (49.4±7.9% versus 49.4±7.3%) or *R. helvetica* (9.7±6% versus 12.5±9.4%) between female and male ticks, respectively. No significant difference was also observed for *R. helvetica* between adult and nymphal stages (9.7±6%, 12.5±9.4%). 12 positive samples for Rickettsia in *H. inermis* were randomly selected for further sequencing. Partial sequencing of *gltA* gene of 5 samples (MG821159) showed 99% identity with *Candidatus* Rickettsia hungarica isolate Hu5-2007 (EU853834) identified in *H. inermis* collected in Hungary (Hornok et al., 2010). Unfortunately, partial sequencing of the *gltA* gene of 7 samples (MG821160) did not match any rickettsial species identified in this study. They showed 99% identity with uncultured *Bartonella* sp. (KJ663731) previously identified in *R. sanguineus* collected in Sicily, Italy (Otranto et al., 2014). Appendix 1 shows a phylogenetic tree constructed on the basis of the *gltA* sequences. Partial sequencing of *ompA* gene of all randomly chosen 12 samples did not show any identity with known rickettsial species and fragments of *sca4* gene were not successfully amplified.

Coxiella burnetii DNA was found in 7 *D. reticulatus* (4 females and 3 males) and in 8 *I. ricinus* (3 females, 3 males and 2 nymphs) ticks.. *H. inermis* ticks were *C. burnetii* negative (Table 1). However, the overall prevalence of CLEs in this study was 32.7±3.7%. The highest prevalence was recorded in *H. inermis* (58 females, 33 males) with significant difference between the prevalences in males and females. However, the differences between prevalence in males and females of *D. reticulatus* (36 females, 56 males) and *I. ricinus* (13 females, 4 males and 6 nymphs) were not statistically significant. The presence of CLEs in *I. ricinus* collected in Zohor and Stará Lesná was statistically significantly different. Totally 33 randomly chosen *Coxiella* spp.-positive samples were analysed by sequencing of the *groEL* gene fragments (Appendix 2). Sequences from 2 *D. reticulatus* males and 1 *I. ricinus* female samples (MG860513) were 99% identical with sequences of *C. burnetii* (CP014557, CP020616, LK937696). A phylogenetic analysis of 30 *Coxiella* spp. from this study showed that they do not group with the pathogenic *C. burnetii*. Eighteen sequences, 7 from *H. inermis* females and 11 from *H. inermis* males (MG860512) were 80% identical to *Coxiella* endosymbiont of *Rhipicephalus geigyi* isolate Rgei1 (KP985514) identified in *R. geigyi* from Benin (Duron et al., 2015a). Two sequences from *D. reticulatus* females (MG860511) were 87% identical to *Coxiella* endosymbiont of *Ornithodoros sonrai* isolate Oson1 (KP985474) identified in *O. sonrai* collected in Senegal (Duron et al., 2015a). Next two sequences, from *D. reticulatus* female and *I. ricinus* male (MG860510) were 99% identical to *Rickettsiella* endosymbiont of *Ixodes ventalloi* isolate ixoventa6 (KY678006) identified in *I. ventalloi* tick tissues (Duron et al., 2017). Two sequences derived from 1 female and 1 male *I. ricinus* (MG860509)

were 99% identical to *Rickettsiella* endosymbiont of *Ixodes arboricola* isolate ixoarbo827 (KY677998) identified in *I. arboricola* (Duron et al., 2017). And the last six sequences (MG860514) derived from 3 *I. ricinus* males, 2 *D. reticulatus* males and 1 *D. reticulatus* female were 99% identical to *Serratia proteamaculans* (CP000826).

Analysis of the 16S rRNA gene revealed that FLEs were present in 47.9±3.9% ticks. The highest prevalence (79.9±4.3%) was found in *D. reticulatus* ticks (140 females, 127 males) , while *H. inermis* ticks were negative (Table 1). Statistically, significant differences were found between the prevalence in *D. reticulatus* collected in Zohor and Gabčíkovo (86.6±4.0% versus 47.4±13.0%), the prevalence in the sex of *D. reticulatus* (70.6±6.7% in males and 90.9±4.5% in females), and the prevalence in *I. ricinus* ticks in Stará Lesná and Zohor (33.3±11.9 versus 2.9±3.3). Totally, 21 randomly selected *Francisella sp.*-positive ticks (11 from females and 10 from males of *D. reticulatus*) showed identical DNA sequences to each other (MG889594) and were 98% identical with FLEs from isolate FLE D1 (JX561116) identified in *D. reticulatus* tick collected in France (Michelet et al., 2013) and with FLEs of *I. ricinus* (JQ740890) previously identified in *I. ricinus* larvae collected from birds in Hungary (Hornok et al., 2013) (see Appendix 3).

The simultaneous occurrence of endosymbionts (CLEs, FLEs) and pathogens *Rickettsia* spp., *C.* burnetii) was recorded in 74 (41.1±7.2%) *D. reticulatus* males, 90 (58.4±7.9%) *D. reticulatus* females, 1 *I. ricinus* male, 3 *I. ricinus* females, and 2 *I. ricinus* nymphs. All *H. inermis* males and 42 females carried DNA of *Rickettsia* spp. and CLEs.

4. Discussion

This study is the first survey focusing on the simultaneous occurrence of bacterial tick-borne pathogens and endosymbionts in *D. reticulatus*, *I. ricinus* and *H. inermis* ticks in Slovakia. All three species represent epidemiologically and epozootiologically important genera. The list of known tick-borne pathogens is still evolving and their presence in ticks and hosts in Slovakia have been previously studied. Non-pathogenic microorganisms, commensal and mutualistic microbes are also abundant in ticks, but their presence was not identified in ticks from Slovakia until our study. Results of PCR assays and sequences analyses revealed that *Rickettsia* spp., *Coxiella burnetii*, CLEs and FLEs co-infect *D. reticulatus*, *I. ricinus* and *H. inermis* collected in four localities in Slovakia. The prevalence range of *Rickettsia* spp. in ticks in the present study was 0.4-50.3% according to tick species as in the previous studies done in Slovakia (Špitalská et al., 2012, 2014, 2016; Švehlová et al., 2014; Minichová et al., 2017). Species identification confirmed the presence of *R. helvetica* and *R. monacensis* in *I. ricinus* ticks, and *R. raoultii*, *R. slovaca* in *D. reticulatus*. These rickettsial species were identified as well in previous studies in Slovakia (Špitalská et al., 2012, 2014, 2016; Švehlová et al., 2014; Minichová et al., 2017). In this study the occurrences of *R. raoultii* in *D. reticulatus* and *R. helvetica* in *I. ricinus* ticks were without

statistical significance for localities, adult sex or between adults and nymphs. The absence of statistically significant differences could be explanated by transovarial transmission and/or transstadially survival of these rickettsiae. However, the presence of rickettsial species in *Haemaphysalis* spp. ticks have been determined only by the haemocyte test or PCR without the further identification and so the species present in this tick species in Slovakia was not known (Špitalská et al., 2002; Špitalská and Kocianová, 2003; Boldiš et al., 2008). The sequence data analysis of the *glt*A gene suggested the presence of *Cand.* R. hungarica. The identification of the above species, in this study, expands the range of rickettsial species circulating in Slovakia.

The prevalence of *C. burnetii* in ticks in the present study was 1.7-2.9% in *D. reticulatus* and 5% in *I. ricinus*. *Coxiella burnetii* was identified by PCR in questing ticks in Slovakia in 2003 (Špitalská and Kocianová, 2003). After more than one decade, Minichová et al. (2017) did not detect any questing ticks or rodents-feeding ticks. However, this pathogen was confirmed in 2.7% of ticks feeding on birds (Berthová et al., 2016), which is similar prevalence than in this study. *Coxiella* – like bacteria are diverse and widespread in ticks and distinct from *C. burnetii*. *Coxiella* – like bacteria can be transovarially and transstadially transmitted (Duron et al., 2015a, b; Machado-Ferreira et al., 2016). *Coxiella* – like bacteria are very common in ticks, but their presence has not been studied previously in Slovakia, thus information about prevalence and molecular identification in this study are new for this region. The total prevalence of CLEs was 32.7±3.7% and CLEs were found in all three species of ticks from all studied sites with the highest prevalence in *H. inermis* (84.3%). The occurrence of CLEs in *D. reticulatus* and *I. ricinus* ticks was without statistical significance between males and females. There was also no statistical difference between localities for the prevalence of CLEs in *D. reticulatus* (contrary to what we found in *I. ricinus*), which could be due to different habitats, the low number of tested ticks, and the presence of tested nymphs in one locality while being absent in the second one. The differences in CLEs occurrence are common and can be explained by many factors but it still indicates that it is the most widespread and biologically relevant tick symbiont (Bonnet et al., 2017). Molecular analysis showed that they do not group with the pathogenic *C. burnetii*, but they group with CLEs identified in different tick species (Appendix 2). Four samples in our study were similar also to *Rickettsiella* endosymbionts, a facultative mutualist genus in aphids with unknown effect in ticks, identified in *Ixodes* ticks by Duron et al. (2017), which is also the first identification in Slovakia.

FLEs have not been studied in Slovakia to this time. Genetic analysis of FLEs identified in our samples showed a close relationship with the FLEs of *Dermacentor* spp., *Hyalomma* spp., *Rhipicephalus* spp. and *Amblyomma* spp. previously identified and distinct from the FLEs of *Ornithodoros* spp. (Michelet et al., 2013). Effect of FLEs in tick is unknown. They probably are obligate symbionts (Duron et al., 2017).

Previous studies suggested that tick endosymbionts could have evolved from mammalian pathogens or infective ancestors (Noda et al., 1997; Scoles, 2004; Machado-Ferreira et al., 2009; 2016; Gerhart et al., 2016). The composition of microbial communities in tick is highly variable. Differences in the internal bacterial flora among ticks of three species (*I. ricinus*, *D. reticulatus*, *H. concinna*) at the same localities were confirmed by Egyed and Makrai (2014) too. Infestation of ticks can occur by ingestion from the soil environment or through the blood (or skin) of the host. Variations in the prevalence and the occurrence of tick symbionts can be linked to host preferences from the larval and nymphal tick stages. For example, *H. inermis* larvae prefer lizards and feed on hosts very rapidly, only 1 - 2 hours (e.g. Nosek, 1973). By contrast, *D. reticulatus* larvae prefer several insectivores and rodent species, feeding for several days on the hosts (Nosek, 1972; Földvari et al., 2016).

Our results showed the presence of pathogenic species of *Rickettsia* and *Coxiella burnetii* and symbiotic *Coxiella*-like and *Francisella*-like microorganisms and their sympatric occurrence in *D. reticulatus*, *I. ricinus* and *H. inermis* ticks. To know tick-borne bacteria, which could be affected by the presence of another pathogens or symbionts is essential for monitoring and diagnosis of tick-borne diseases in humans and animals.

Conflict of interest

The authors declare that they have no conflict of interest.

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Legends

Table 1 Prevalence of *Rickettsia* spp., *Coxiella burnetii*, *Coxiella*-like and *Francisella*-like microorganisms in questing ticks collected at four sites in Slovakia [no. of infected/ no. of captured (prevalence %±95% CI)]

Appendix 1 Phylogenetic tree inferred from comparison of the *Rickettsia gltA* partial sequences using Neighbor-Joining method (Saitou, Nie, 1987). GeneBank accession numbers are included. Included sequences without GeneBank accession numbers were previously published and not submitted to GenBank (Minichová et al. 2017). Bootstrap values of neighbor-joining (1,000 replicates) are shown.

Appendix 2 Phylogenetic tree inferred from comparison of the *Coxiella groEL* partial sequences using Neighbor-Joining method (Saitou and Nie, 1987). GeneBank accession numbers are included. Bootstrap values of neighbor-joining (1,000 replicates) are shown.

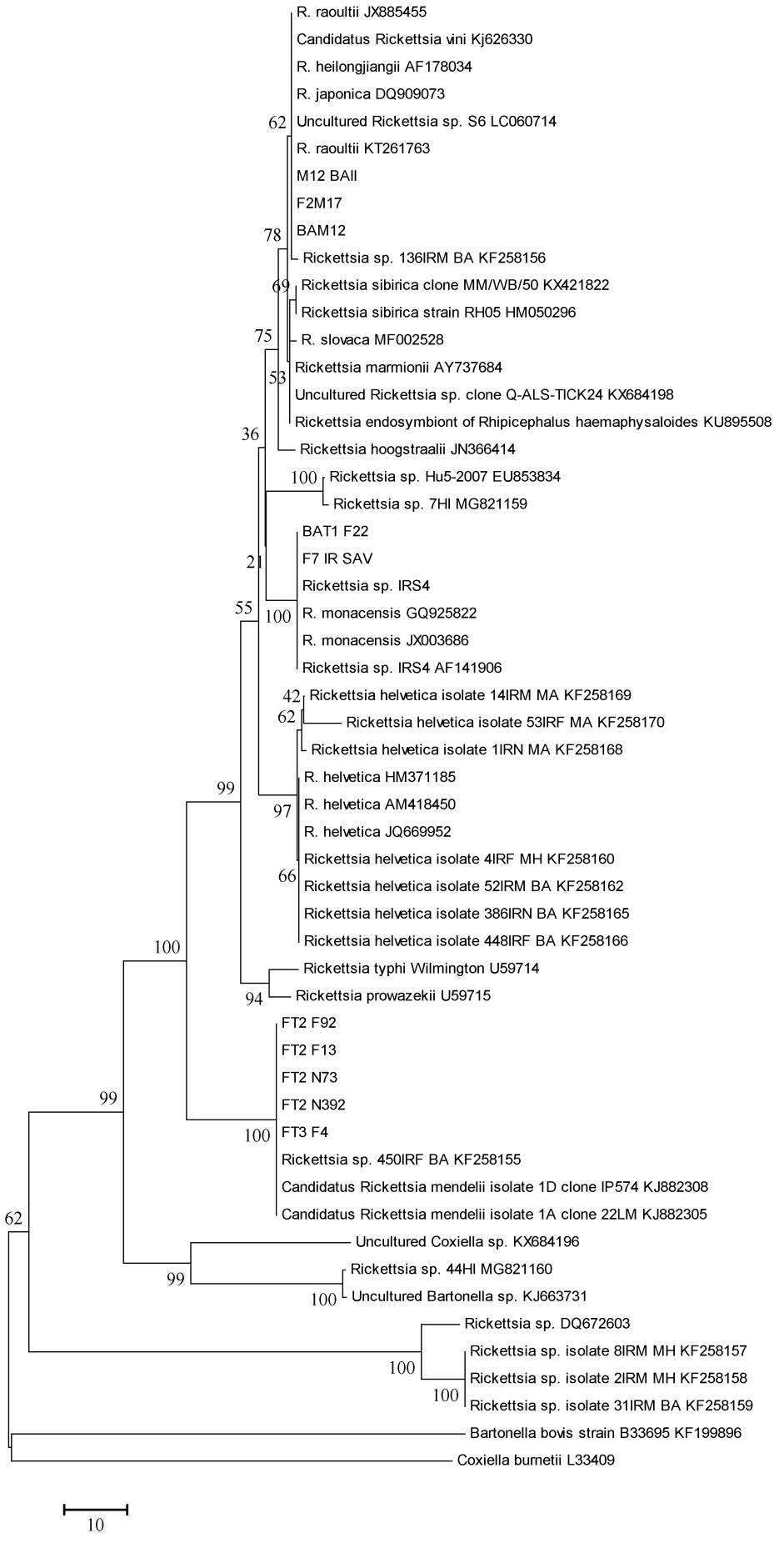
Appendix 3 Neighbor-joining phylogenetic tree showing relationships of 16S rRNA gene sequences obtained from *Francisella* species and *Francisella*-like endosymbionts (FLEs) with the novel *Francisella*-like isolate from *Dermacentor reticulatus* ticks collected in Slovakia. Bootstrap values of neighbor-joining (1,000 replicates) are shown.

Table 1

	Rh	Rm	Rr	Rs	Rsp.	CB	CLEs	FLEs
Zohor								
DR males			65/130	0/130		2/130	42/130	105/130
			(50)			(1.5)	(32.3)	(80.8)
DR			74/147	1/147		4/147	35/147	135/147
females			(50.3)	(0.7)		(2.7)	(23.8)	(91.8)
DR			*139/277*	*1/277*		*6/277*	*75/277*	*240/277*
Subtotal			*50.2±6.0*	*0.4±0.7*		*2.2±1.7*	*27.1±5.3*	*86.6±4.0*
Gabčíkovo								
DR males			24/50	1/50		1/50	14/50	22/50
			(48)	(2)		(2)	(28)	(44)
DR			2/7	1/7		0/7	1/7	5/7
females			(28.6)	(20)			(14.3)	(71.4)
Subtotal			*26/57*	*2/57*		*1/57*	*15/57*	*27/57*
			45.6±12.9	*3.5*±5.0		*1.8*±3.4	*26.3*±11.4	*47.4*±13.0
Total DR					***168/334***	***7/334***	***90/334***	***267/334***
					50.3±5.4	***2.1±1.5***	***26.9±4.7***	***79.9±4.3***
Stará Lesná								
IR males	5/18	0/18				0/18	1/18	3/18
	(27.8)						(5.6)	(16.7)
IR females	0/20	0/20				1/20	6/20	11/20
						(5)	(30)	(55)
IR nymphs	1/22	2/22				2/22	4/22	6/22
	(4.5)	(9.1)				(9.1)	(18.2)	(27.3)
Subtotal	*6/60*	*2/60*				*3/60*	*11/60*	*20/60*
	10±7.6	*3.3*				*5±5.5*	*18.3±9.8*	*33.3±11.9*
Zohor								
IR males	1/30					3/30	1/30	1/30
	(3.3)					(10)	(3.3)	(3.3)
IR females	9/73					2/73	5/73	2/73
	(12.3)					(2.7)	(6.8)	(2.7)
IR Subtotal	*10/103*					*5/103*	*6/103*	*3/103*
	9.7±5.7					*4.9±4.1*	*5.8±4.5*	*2.9±3.3*

Total IR	***18/163***	***8/163***	***17/163***
	11.0±4.8	***4.9±3.3***	***10.4±4.7***
Hrhov			
HI males	8/33		33/33
	(24.2±14.6)		(100)
HI females	21/75		58/75
	(28±10.2)		(77.3)
Total HI	***29/108***		***91/108***
	26.9±8.4		***84.3±6.9***

Rh – *Rickettsia helvetica*, Rm – *Rickettsia monacensis*, Rr – *Rickettsia raoultii*, Rs – *Rickettsia slovaca*, Rsp – *Rickettsia* species, CB – *Coxiella burnetii*, CLEs – *Coxiella*-like endosymbionts, FLEs – *Francisella*-like endosymbionts, DR – *Dermacentor reticulatus*, IR – *Ixodes ricinus*, HI – *Haemaphysalis inermis*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R. japonica DQ909073
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R. raoultii KT261763
M12 BAII
F2M17
78
BAM12
Rickettsia sp. 136IRM BA KF258156
69
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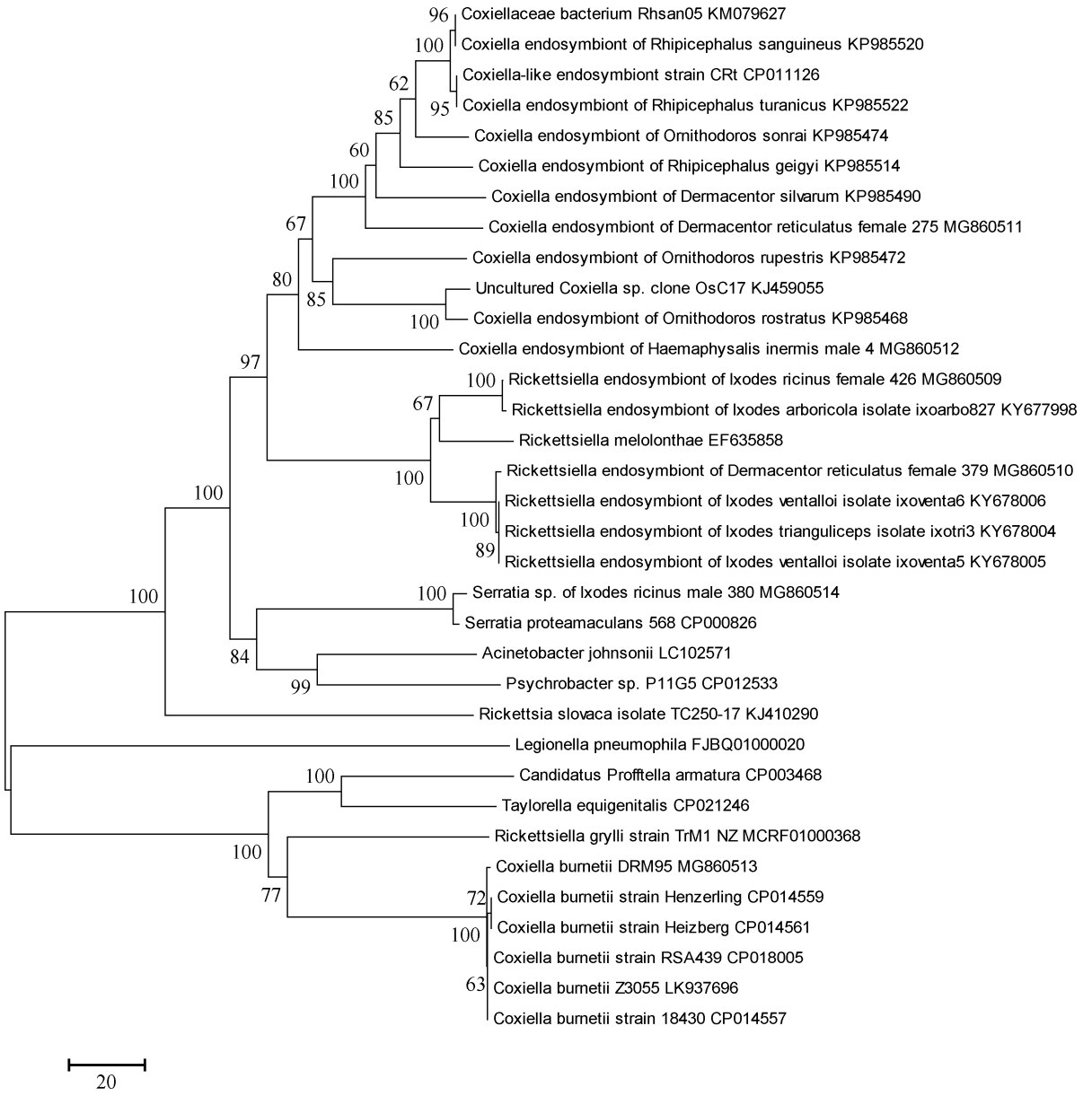
Rickettsia sibirica strain RH05 HM050296
75
R. slovaca MF002528
53
Rickettsia marmionii AY737684
Uncultured Rickettsia sp. clone Q-ALS-TICK24 KX684198
Rickettsia endosymbiont of Rhipicephalus haemaphysaloides KU895508
36
Rickettsia hoogstraalii JN366414
100
Rickettsia sp. Hu5-2007 EU853834
Rickettsia sp. 7HI MG821159
BAT1 F22
21
F7 IR SAV
Rickettsia sp. IRS4
55
100
R. monacensis GQ925822
R. monacensis JX003686
Rickettsia sp. IRS4 AF141906
42
Rickettsia helvetica isolate 14IRM MA KF258169
62
Rickettsia helvetica isolate 53IRF MA KF258170
Rickettsia helvetica isolate 1IRN MA KF258168
99
R. helvetica HM371185
97
R. helvetica AM418450
R. helvetica JQ669952
Rickettsia helvetica isolate 4IRF MH KF258160
66
Rickettsia helvetica isolate 52IRM BA KF258162
Rickettsia helvetica isolate 386IRN BA KF258165
Rickettsia helvetica isolate 448IRF BA KF258166
100
Rickettsia typhi Wilmington U59714
94
Rickettsia prowazekii U59715
FT2 F92
FT2 F13
FT2 N73
99
FT2 N392
100
FT3 F4
Rickettsia sp. 450IRF BA KF258155
Candidatus Rickettsia mendelii isolate 1D clone IP574 KJ882308
Candidatus Rickettsia mendelii isolate 1A clone 22LM KJ882305
62
Uncultured Coxiella sp. KX684196
99
Rickettsia sp. 44HI MG821160
100
Uncultured Bartonella sp. KJ663731
Rickettsia sp. DQ672603
Rickettsia sp. isolate 8IRM MH KF258157
100
Rickettsia sp. isolate 2IRM MH KF258158
100
Rickettsia sp. isolate 31IRM BA KF258159
Bartonella bovis strain B33695 KF199896
Coxiella burnetii L33409
10


Coxiellaceae bacterium Rhsan05 KM079627
Coxiella endosymbiont of Rhipicephalus sanguineus KP985520
Coxiella-like endosymbiont strain CRt CP011126
Coxiella endosymbiont of Rhipicephalus turanicus KP985522
Coxiella endosymbiont of Ornithodoros sonrai KP985474
Coxiella endosymbiont of Rhipicephalus geigyi KP985514
Coxiella endosymbiont of Dermacentor silvarum KP985490
Coxiella endosymbiont of Dermacentor reticulatus female 275 MG860511
Coxiella endosymbiont of Ornithodoros rupestris KP985472
Uncultured Coxiella sp. clone OsC17 KJ459055
Coxiella endosymbiont of Ornithodoros rostratus KP985468
Coxiella endosymbiont of Haemaphysalis inermis male 4 MG860512
Rickettsiella endosymbiont of Ixodes ricinus female 426 MG860509
Rickettsiella endosymbiont of Ixodes arboricola isolate ixoarbo827 KY677998
Rickettsiella melolonthae EF635858
Rickettsiella endosymbiont of Dermacentor reticulatus female 379 MG860510
Rickettsiella endosymbiont of Ixodes ventalloi isolate ixoventa6 KY678006
Rickettsiella endosymbiont of Ixodes trianguliceps isolate ixotri3 KY678004
Rickettsiella endosymbiont of Ixodes ventalloi isolate ixoventa5 KY678005
Serratia sp. of Ixodes ricinus male 380 MG860514
Serratia proteamaculans 568 CP000826
Acinetobacter johnsonii LC102571
Psychrobacter sp. P11G5 CP012533
Rickettsia slovaca isolate TC250-17 KJ410290
Legionella pneumophila FJBQ01000020
Candidatus Profftella armatura CP003468
Taylorella equigenitalis CP021246
Rickettsiella grylli strain TrM1 NZ MCRF01000368
Coxiella burnetii DRM95 MG860513
Coxiella burnetii strain Henzerling CP014559
Coxiella burnetii strain Heizberg CP014561
Coxiella burnetii strain RSA439 CP018005
Coxiella burnetii Z3055 LK937696
Coxiella burnetii strain 18430 CP014557
96
100
62
95
85
60
100
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85
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97
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67
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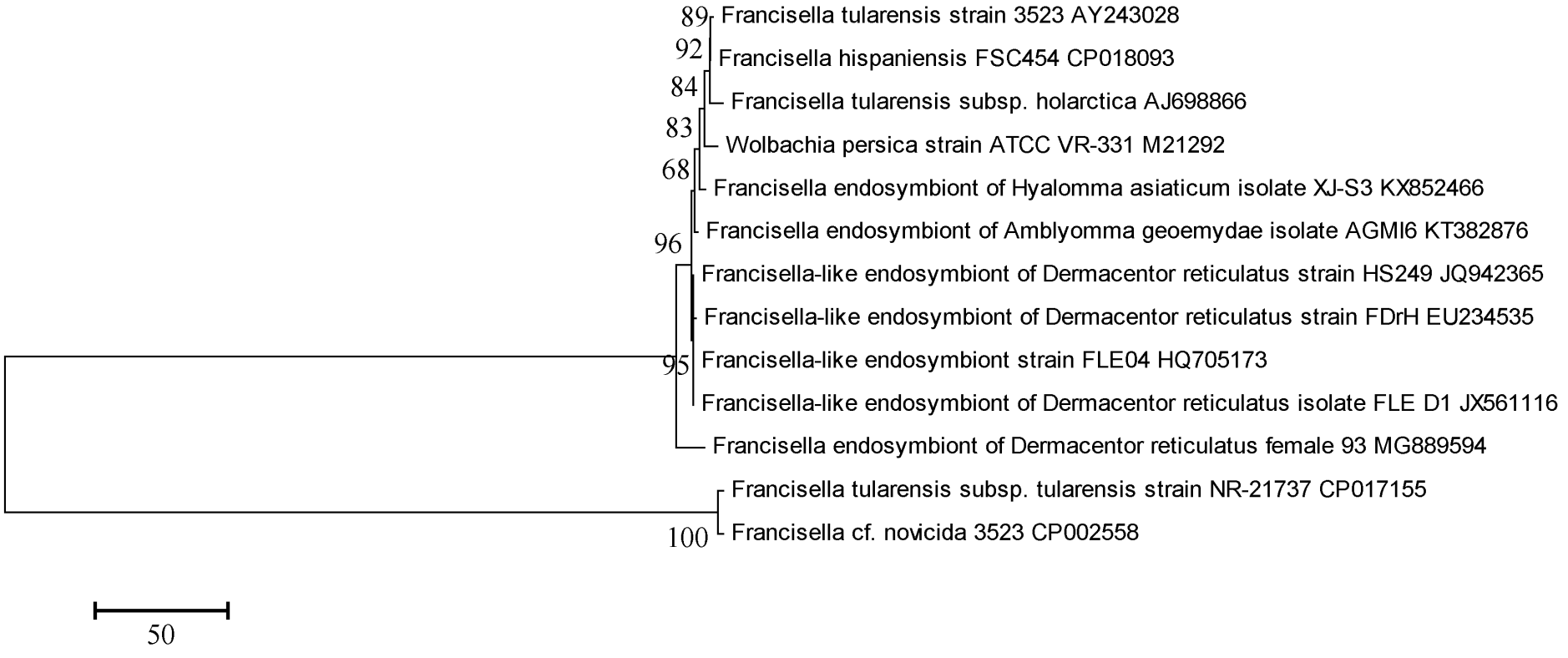
100
100
89
100
100
84
99
100
100
77
72
100
63
20


89
Francisella tularensis strain 3523 AY243028
92
Francisella hispaniensis FSC454 CP018093
84
Francisella tularensis subsp. holarctica AJ698866
83
Wolbachia persica strain ATCC VR-331 M21292
68
Francisella endosymbiont of Hyalomma asiaticum isolate XJ-S3 KX852466
96
Francisella endosymbiont of Amblyomma geoemydae isolate AGMI6 KT382876
Francisella-like endosymbiont of Dermacentor reticulatus strain HS249 JQ942365
Francisella-like endosymbiont of Dermacentor reticulatus strain FDrH EU234535
95
Francisella-like endosymbiont strain FLE04 HQ705173
Francisella-like endosymbiont of Dermacentor reticulatus isolate FLE D1 JX561116
Francisella endosymbiont of Dermacentor reticulatus female 93 MG889594
Francisella tularensis subsp. tularensis strain NR-21737 CP017155
100
Francisella cf. novicida 3523 CP002558
50