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SHORT COMMUNICATION

Rickettsia species in fleas collected from small mammals in Slovakia

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Abstract Epidemiological and epizootiological studies of *Rickettsia felis* and other *Rickettsia* spp. are very important, because their natural cycle has not yet been established completely. In total, 315 fleas (Siphonaptera) of 11 species of Ceratophyllidae, Hystrichopsyllidae and Leptopsyllidae families were tested for the presence of *Rickettsia* species and *Coxiella burnetii* with conventional and specific quantitative real-time PCR assays. Fleas were collected from five rodent hosts (*Myodes glareolus*, *Apodemus flavicollis*, *Apodemus agrarius*, *Microtus subterraneus*, *Microtus arvalis*) and three shrew species (*Sorex araneus*, *Neomys fodiens*, *Crocidura suaveolens*) captured in Eastern and Southern Slovakia. Overall, *Rickettsia* spp. was found in 10.8 % (34/315) of the tested fleas of *Ctenophthalmus agyrtes*, *Ctenophthalmus solutus*, *Ctenophthalmus uncinatus* and *Nosopsyllus fasciatus* species. Infected fleas were coming from *A. flavicollis*, *A. agrarius*, and *M. glareolus* captured in Eastern Slovakia. *C. burnetii* was not found in any fleas. *R. felis*, *Rickettsia helvetica*, unidentified *Rickettsia*, and rickettsial endosymbionts were identified in fleas infesting small mammals in the Košice region, Eastern Slovakia. This study is the first report of *R. felis* infection in *C. solutus* male flea collected from *A. agrarius* in Slovakia.

Keywords *Rickettsia felis* · Fleas · Small mammals · Slovakia

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Introduction

Rickettsiae are obligate intracellular Gram-negative bacteria that are associated with arthropod vectors and are responsible for mild to severe diseases in humans, with worldwide distribution (Raoult and Roux 1997). They are classically transmitted to humans via arthropod vectors such as ticks, mites, fleas, and lice. The arthropod association of various rickettsiae is diverse and varied.

Ixodes ricinus, *Dermacentor marginatus*, *Dermacentor reticulatus*, *Haemaphysalis concinna*, *Haemaphysalis punctata* and *Haemaphysalis inermis* (the family Ixodidae, the order Acari) are exophilic tick species occurring in Slovakia (Slovák 2010). The presence of six rickettsial species/strains has been confirmed (*Rickettsia slovaca*, *Rickettsia raoultii*, *Rickettsia monacensis* strains IRS3 and IRS4, *Rickettsia helvetica* and *Rickettsia africae*), mostly in localities of the southern part of Slovakia. The prevalence of *R. helvetica* in questing *I. ricinus* ticks is usually up to 20 % depending on the sea level, locality, and sex of ticks (Spitalská et al. 2014, 2015; Švehlová et al. 2014). *Dermacentor* species are vectors for *R. raoultii* and *R. slovaca* with the prevalence of *R. raoultii* 8.1–8.6 % and 22.3–27 % in *D. marginatus* and *D. reticulatus* from vegetation and hosts, respectively. The prevalence of *R. slovaca* is 20.6–24.3 % in *D. marginatus* and 1.7–3.4 % in *D. reticulatus* (Špitalská et al. 2012). *R. africae* usually transmitted by ticks was also identified in *Ceratophyllus garei* flea collected from reed warblers

(*Acrocephalus scirpaceus*) migrated from Africa (Sekeyová et al. 2012). *Rickettsia felis* is a rickettsial species primarily associated with the cat flea, *Ctenocephalides felis*. The pathogen is maintained by transovarial and transstadial transmission with no lethal effect. It is the etiological agent of flea-borne spotted fever with clinical symptoms similar to malaria and dengue. However, it appears to be milder than other rickettsioses. The bacteria can also infect humans (Raoult and Parola 2007). The host is usually infected by flea faeces coming into contact with scratched or broken skin (Azad and Beard 1998). The expansion of *R. felis* hosts and potential vectors can include mites, lice and ticks (Abdad et al. 2011). To our knowledge, this rickettsial species has never been identified in Slovakia to date.

The objective of this work was the molecular detection of *Rickettsia* species and *Coxiella burnetii* in fleas collected from rodents and shrews in Eastern and Southern Slovakia.

Materials and methods

Origin of the samples

In total, 315 fleas (Siphonaptera) were removed from *Myodes glareolus* (*n*=41), *Apodemus flavicollis* (*n*=43), *Apodemus agrarius* (*n*=35), *Microtus subterraneus* (*n*=3), *Microtus arvalis* (*n*=1), *Sorex araneus* (*n*=2), *Neomys fodiens* (*n*=1) and *Crocidura suaveolens* (*n*=1) captured in Eastern Slovakia, in Košice region (Botanical garden, Košice—Čermeľ and Hýľov) and in Southern Slovakia, in vicinity of Lučenec town (Bušince, Veľká nad Ipľom). *M. glareolus*, *Mi. subterraneus* and *Mi. arvalis* are members of the family Cricetidae and *A. flavicollis* and *A. agrarius* of the family Muridae of the order Rodentia. *S. araneus*, *N. fodiens* and *C. suaveolens* are members of the family Soricidae, of the order Soricomorpha, and shrews were released after deparasitation in nature. Hosts and ectoparasites were collected four to five times at the locality between June 2009 and September 2012. Study sites were located in sylvatic mixed forest (Hýľov; 500–750 m asl.; 48° 44′ N, 21° 4′ E and Čermeľ; 208–600 m asl.; 48° 45′ N, 21° 8′ E), in suburban deciduous forest (Botanical garden, Košice; 208 m asl.; 48° 44′ N, 21° 14′ E), as well as in alluvial habitats near river Ipeľ (Veľká nad Ipľom; 165–170 m asl; 48° 14′ N, 19° 35′ E and Bušince; 160–170 m asl; 48° 11′ N, 19° 30′ 59.58″ E). Rodents were trapped alive using Swedish bridge metal traps following the protocol of Stanko (1994a) and Stanko and Miklisova (1995). Rodent trapping was carried out using 100–150 traps per site for two-night trapping. Small mammals were euthanized according to the laws of the Slovak Republic under the licences of the Ministry of Environment of the Slovak Republic No. 297/108/06-3.1 and No. 6743/2008-2.1. Fleas with different engorgements were collected from infested animals using forceps. The species and sex of the fleas were determined by microscopic examination according to Rosický (1957). Flea samples were stored in 70 % ethanol until molecular analysis.

DNA extraction and molecular analysis

Fleas 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). DNA from fleas was extracted using DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's recommendations. The quantity and quality of DNA were assessed by NanoPhotometer Pearl (Implen, Germany). DNA samples were stored at −20 °C and later used as templates for the PCR amplifications. Flea samples were screened by PCR-based methods for the presence of tick-borne pathogens, *Rickettsia* spp. and *C. burnetii*.

For bacterial infection in flea samples, primer pair fD1-800r targeted fragment of the 16S rRNA of eubacteria was used (Weisburg et al. 1991). Identification of *Rickettsia* spp. was performed using the TaqMan PCR based on the genus-specific variable region of the *rrs* gene and PCR based on the genus-specific variable regions of the *gltA* and *sca4* genes using primer sets RpCS.877p–RpCS.1258n and D767f–D1390r targeting 381- and 623-bp fragments, respectively (Regnery et al. 1991; Sekeyová et al. 2001; Melničáková et al. 2013). The primer pairs CBCOS and CBCOE targeting the *com1* gene encoding an approximately 27-kDa outer membrane-associated immunoreactive protein of *C. burnetii* was employed for the detection of *C. burnetii* in flea samples (Špitalská and Kocianova 2003). The DNA from *Rickettsia*-uninfected ticks and nuclease-free water were used as negative controls in each reaction. DNAs from *R. helvetica* and *R. slovaca* originating from ticks and *Rickettsia typhi* and *C. burnetii* originating from the collection of Institute of Virology were used as positive controls. PCR amplifications were conducted using DyNAzyme™ PCR Master Mix (Finnzymes, Finland) and HOT FIREPol® DNA Polymerase (Solis BioDyne, Estonia) as recommended by the manufacturer on thermocycler PTC-200 Peltier Thermal Cycler or Labcycler (SensoQuest, Germany). PCR products were analysed by electrophoresis in a 1 % agarose gel, stained with GelRed™ (Biotium, Hayward, California) and visualized with a UV transilluminator.

Rickettsia-positive flea samples were screened for the presence of *R. felis* using *R. felis*-specific nested PCR with specific primers gltA-F1–gltA-R1 and gltA-F2–gltA-R2 amplifying a partial region of *gltA* gene (Hii et al. 2011). *Rickettsia*-positive flea samples were screened for the presence of *R. helvetica* using TaqMan PCR assay targeting a 65-bp fragment of the 23S rRNA gene using DyNAmo™ Probe qPCR (Finnzymes, Finland) on Bio-Rad CFX96™ Real-Time System as

previously described by Boretti et al. (2009). Each run of TaqMan PCR reactions included a negative template control, a positive control and DNA standards containing 3×10^{0}–3×10^{6} target copies with a sensitivity of three copies of the DNA. Randomly selected amplicons of *gltA*, *sca4* and 16S rRNA genes were purified and analysed by sequencing both DNA strands. The sequencing was performed by Macrogen (http://www.macrogen.com). Obtained DNA sequences were compared to available sequences in GenBank database using the Basic Local Alignment Search Tool (BLAST) on http://blast.ncbi.nlm.nih.gov/. A phylogenetic analysis was further performed using MEGA5 software (Tamura et al. 2011).

Statistical analysis

Statistical analysis to test the differences between the prevalences in males and females was carried out with the Fisher exact test using Past version 2.17b software (Hammer et al. 2001). A p value <0.05 was considered significant.

Results

A total of 315 fleas (Siphonaptera) belonging to 11 species of three families, Ceratophyllidae, Hystrichopsyllidae and Leptopsyllidae (Table 1), were analysed individually for the presence of rickettsial species and *C. burnetii*. Fleas were removed from five rodent hosts (*M. glareolus*, *A. flavicollis*, *A. agrarius*, *Mi. subterraneus*, *Mi. arvalis*) and three shrew species (*S. araneus*, *N. fodiens*, *C. suaveolens*). In our study, we confirmed rickettsia in four flea species (Table 1) collected from *A. flavicollis*, *A. agrarius* and *M. glareolus*. Infected fleas were coming from three studied areas in Eastern Slovakia (Botanical garden, Čermeľ and Hýľov). Overall, *Rickettsia* spp. was found in 10.8 % (34/315) of the tested fleas, without statistical significance between flea males and females ($p=0.86$). *Ctenophthalmus agyrtes* and *Ctenophthalmus solutus* were the most infected species of fleas (10.4 and 16.0 %, respectively, Table 1). More than one *Rickettsia*-infected fleas were collected from two *A. flavicollis* and two *A. agrarius*. *C. burnetii* was not found in any flea samples.

Six randomly chosen *Rickettsia*-positive amplicons were sequenced. The sequence of *gltA* gene isolated from *C. solutus* male flea collected from *A. agrarius* (marked as 44) was 96 % (364/380 bp) identical to a validated bacterium *R. felis* (CP000053) and 98 % similar to three uncultured *Rickettsia* spp. detected in blood patient from Senegal (JQ674485) and in two *Anopheles gambiae* mosquitoes from Africa (JN620082, JQ354961; Socolovschi et al. 2012). Figure 1 shows a phylogenetic tree constructed on the basis of the *gltA* sequences. The amplified fragment of *sca4* gene from the same flea was 98 % (580/591 bp) identical to *R. felis* (CP000053), *Rickettsia* spp. in *C. felis* from Senegal (KF666474) and in a common household insect pest, *Liposcelis bostrychophila* (the family Liposcelidae, the order Psocoptera; Behar et al. 2010). Subsequently, this DNA of *R. felis* from *C. solutus* male was used as positive control in nested PCR to identify the presence of *R. felis* in all collected fleas. Any other positive sample was not found.

R. helvetica was identified by *R. helvetica*-specific TaqMan PCR and sequencing of *gltA* gene isolated from *C. agyrtes* male collected also from *A. agrarius* (marked as 63). Using *R. helvetica*-specific TaqMan PCR assay, all other fleas were negative. Unidentified *Rickettsia* species were found in *Ctenophthalmus uncinatus* male (marked as 108) and *C. solutus* females (marked as 109, 110) from *A. agrarius* and *A. flavicollis*. The amplified fragments of *gltA* gene in these samples were 99.3 % identical to each other. Sequences showed the greatest similarity to *Rickettsia*

Table 1 Presence of rickettsiae in fleas (Siphonaptera) collected from small mammals in Eastern and Southern Slovakia

Species	No. of *Rickettsia positive* (no. of sequenced)/no. of tested male fleas	No. of *Rickettsia* positive (no. of sequenced)/no. of tested female fleas
Ctenophthalmus agyrtes (Hystrichopsyllidae)	5 (1)/49	5/47
C. assimilis (Hystrichopsyllidae)	0/2	0/4
C. solutus (Hystrichopsyllidae)	9 (1)/68	12 (2)/63
C. uncinatus (Hystrichopsyllidae)	1 (1)/4	1 (1)/12
Doratopsylla dasycnema (Hystrichopsyllidae)	0/0	0/2
Palaeopsylla soricis (Hystrichopsyllidae)	0/1	0/1
Amalareus penicilliger (Ceratophyllidae)	0/8	0/17
Nosopsyllus fasciatus (Ceratophyllidae)	0/8	1/14
Megabothris turbidus (Ceratophyllidae)	0/6	0/6
Ceratophyllus sciurorum (Ceratophyllidae)	0/1	0/0
Peromyscopsylla bidentata (Leptopsyllidae)	0/0	0/2
Total	15 (3)/147	19 (3)/168

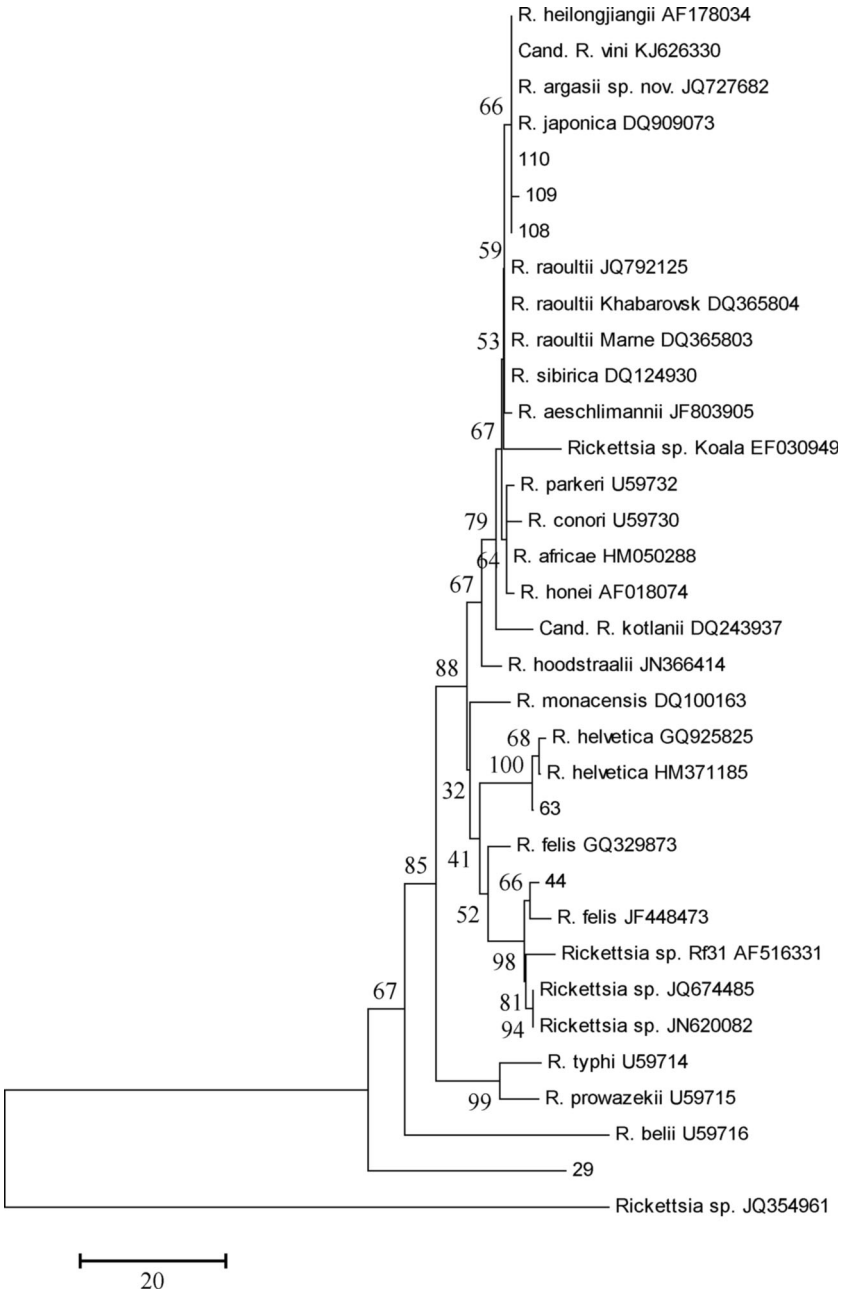

Fig. 1 Phylogenetic tree inferred from comparison of the *Rickettsia gltA* partial sequences. The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. GenBank accession numbers are included

japonica, *Rickettsia heilongjiangensis*, *Candidatus* Rickettsia vini, *Rickettsia aeschlimannii* and *R. raoultii*, all closely phylogenetically related (Fig. 1). Unfortunately, fragments of *sca4* gene were not successfully amplified.

Sequencing of *gltA* gene isolated from *C. uncinatus* female collected from *M. glareolus* (marked as 29) resulted in being unable to identify rickettsial species (Fig. 1). Sequencing of 16S rRNA showed 98 % similarity with *Rickettsia monteiroi* sp. nov. infecting the tick *Amblyomma incisum* in Brazil (FJ269037; Pacheco et al. 2011), *Rickettsia* endosymbiont of *Nesidiocoris tenuis* (the family Miridae, the order Hemiptera; KF646705), *Rickettsia* endosymbiont of the Chinese white wax scale (JQ063118), *Rickettsia* endosymbiont of *Macrolophus pygmaeus* (HE583202), *Rickettsia* endosymbiont of *Pnigalio soemius* (the family Eulophidae, the order Hymenoptera) in Italy (EU881496; Giorgini et al. 2010) and *Rickettsia bellii* strain 369L42-1 (NR_036774; Roux and Raoult 1995).

Discussion

This study highlights the presence of three *Rickettsia* species, *R. felis*, *R. helvetica* and unidentified *Rickettsia*, and rickettsial endosymbionts in fleas infesting rodents in the Košice region, Eastern Slovakia, and in vicinity of Lučenec town, Southern

Slovakia. The rickettsial infections of fleas are poorly known in Slovakia.

Fleas are holometabolous insect parasites with obligatory haematophagous imagos and non-parasitic omnivorous larvae (Krasnov 2008). Fleas are important vectors for various pathogens including viruses, bacteria and tapeworms (Hinaidy 1991; Raoult and Roux 1997; Mehlhorn 2001; Vobis et al. 2005).

From bodies of small mammals, we recorded flea species with varying degrees of host specificity. For example, *Doratopsylla dasycnema* and *Palaeopsylla soricis* are flea species with well-marked parasitism to shrews. Several tested species of fleas have a broad host range and infest so wild mice and voles too (e.g. *C. agyrtes*, *Megabothris turbidus*); another flea species are closely related to some vole species (e.g. *Amalareus penicilliger*, *Peromyscopsylla bidentata*) (Rosický 1957). Positive flea species are parasite generalist (*C. agyrtes*), or prefer only narrower host range, *C. solutus*, which prefer mice of the *Apodemus* genus in lower vegetation zones. Another infected flea species, *C. uncinatus*, has close parasite relations with *M. glareolus* in beech and fir–beech forests. *Rickettsia* sp. was confirmed by TaqMan PCR based on the genus-specific variable region of the *rrs* gene in *Nosopsyllus fasciatus* in one sample collected from *A. agrarius*, but without successful sequencing of *gltA* and *sca4* genes. *N. fasciatus* is a parasite of synantropical rodents (*Mus musculus*, *Rattus norvegicus*) which, in other habitat types, more frequently occur only on mice (*Apodemus* genus) in lowland conditions of Slovakia (Rosický 1957, Stanko 1994b).

Season influences of flea species composition. Some species have several generations during the year, e.g. *C. agyrtes*, *C. solutus* and *M. turbidus*, dominant species collected in our study. Very few species in Central Europe has only one generation in the autumn-winter season, e. g. species of *Peromyscopsylla*, *Rhadinopsylla*, *Hystrichopsylla* and *Atyphloceras* (Rosický 1957). In our group of collected fleas, we had only *P. bidentata* (two specimens); for this reason, sorting and comparison based on season influence were not significant.

This study is the first report of *R. felis* infection in *C. solutus* male flea collected from *A. agrarius* inhabiting in studied areas of Slovakia. Although *C. felis* has been designated as the main vector of *R. felis*, the competency of 24 species of fleas, ticks, mites and lice as transmission vectors has yet to be demonstrated (Abdad et al. 2011). Oliveira et al. (2008) reported *R. felis* in *Rhipicephalus sanguineus* ticks and suggested some possible horizontal transmission from fleas to ticks via a vertebrate host, presumably a dog. *R. sanguineus* has never been implicated as a vector of *R. felis*. However, due to its worldwide distribution overlapping with that of *C. felis*, it may play the role of an amplifier for horizontal transmission to the more competent vectors.

R. helvetica was first isolated from *I. ricinus* ticks in Switzerland (Burgdorfer et al. 1979). *I. ricinus* is the main vector with its distribution all around Europe with different prevalences of *R. helvetica* infections (Oteo and Portillo 2012). The scope of *R. helvetica* infection in Slovakia is known in questing *I. ricinus* ticks (Spitalská et al. 2014, 2015; Švehlová et al. 2014) and in *I. ricinus* ticks collected from free-living green lizards (Václav et al. 2011) and picked from *Prunella modularis* (Špitalská et al. 2011) and from roe deer (Štefanidesová et al. 2008). This species is recognized as the cause of spotted fever, and human cases have been reported from Sweden, France, Switzerland and Italy (Nilsson et al. 1999, 2010; Fournier et al. 2000, 2004; Baumann et al. 2003; Nilsson 2009). However, no human strain of *R. helvetica* exists worldwide. The presence of *R. helvetica* in *C. agyrtes* males collected from *A. agrarius* is the first finding of this species in fleas in Slovakia. Similarly, Sprong et al. (2009) identified *R. helvetica* in two out of 24 fleas isolated from *M. glareolus* and *Apodemus sylvaticus* in the Netherlands. Also, they identified the presence of *R. typhi* and *Rickettsia prowazekii* in *I. ricinus* ticks. The presence of *R. helvetica* and *R. monacensis* in pools of *Laelaps agilis* and *Haemogamasus nidi* (Laelapidae) and *Hirsutiella zachvatkini*, *Cheladonta costulata*, *Neotrombicula autumnalis* and *Neotrombicula vulgaris* (Trombiculidae) mites collected from small terrestrial mammals captured in south-western Slovakia was confirmed by Miťková et al. (2015). Authors supposed that mites could be infected during taking blood meal on a bacteraemic host, which was supported by the evidence of presence of the same *Rickettsia* species in blood of rodents and mites collected from rodents. All the above-mentioned findings support the hypothesis that different microorganisms can be obtained via a blood meal from an infected rodent, or by co-feeding of ectoparasites, and that cross-infections can occur under natural conditions, as different vectors share rodents as reservoir hosts.

Hornok et al. (2014) analysed *Ctenocephalides canis* and *Archaeopsylla erinacei* collected from hedgehogs in Hungary and identified *Rickettsia* spp. with 98–99 % sequence identity with endosymbionts and *R. helvetica* in *A. erinacei* and thus expected the role of hedgehogs in the epidemiology of rickettsiosis. *R. helvetica* was also identified in *Nycteridopsylla eusarca* bat fleas in Hungary (Hornok et al. 2012).

Similar findings about cross-infections via a blood meal or by co-feeding of ectoparasites were recorded by Colombo et al. (2011). They identified the presence of *Leishmania infantum* in fleas and ticks collected from naturally infected dogs and suggested a hypothesis of other arthropods than sandflies as a possible way of *Leishmania* transmission.

Flies are well known to carry and transmit deadly diseases such as the sandfly (leishmaniasis), the tsetse fly (trypanosomiasis), and the housefly, *Musca domestica* (anthrax). Many reports also highlighted the mechanical role of these insects to

carry pathogens on their legs and other body parts making them very successful vectors. Flies can also attack other animals (myiasis) or crops and alongside ticks and mosquitoes are responsible for huge mortality and morbidity worldwide.

However, flies such as *Drosophila* flies have been used extensively for studying human diseases such as Parkinson's disease, epilepsy or Huntington's disease showing the need to study them further to prevent them from transmitting diseases but studying them to develop new drugs against human diseases.

As known, ticks and fleas are ectoparasites of animals as well humans. They cause discomfort and health effects due to bites and ingestion of blood, and they serve as vectors for several animal and human pathogens (Kužner et al. 2013). It is important to know their exact role in the transmission of pathogens to humans and animals to avoid possible zoonotic disease. Effective prevention and treatment of tick and flea infestations in companion animals will help prevent not only disease in animals but human diseases as well (Bonneau et al. 2010). Some studies are aimed to control both parasites and persistent effectiveness. Kužner et al. (2013) confirm immediate and persistent effectiveness of the novel fipronil spot-on product against *I. ricinus* for 4 weeks and *C. felis* fleas for 5 weeks. Varloud and Hodgkins (2015) compared efficacy of three ectoparasiticides against *C. felis* fleas and *R. sanguineus* ticks and demonstrated that dogs treated with fipronil and (S)-methoprene or dinotefuran, pyriproxyfen and permethrin were protected against all stages of fleas for 2 months following four monthly applications.

The role of fleas in circulation of rickettsiae is not definitively elucidated and requires further studies aimed at monitoring rickettsiae not only in fleas but also in other ectoparasites and their hosts. Also, for validation of both co-feeding transmission and vector competence of ectoparasites of small mammals for rickettsial species, experimental studies are necessary. However, due to worldwide distribution of rickettsiae overlapping with that of ticks and fleas, incompetent vectors may play the role of an amplifier for horizontal transmission of rickettsiae to the competent vector.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Small mammals were euthanized according to the laws of the Slovak Republic under the licences of the Ministry of Environment of the Slovak Republic No. 297/108/06-3.1 and No. 6743/2008-2.1. This article does not contain any studies with human participants performed by any of the authors.

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