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**Molecular investigation of hard ticks (Acari: Ixodidae) and fleas
(Siphonaptera: Pulicidae) as potential vectors of
rickettsial and mycoplasmal agents**

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ABSTRACT

The aim of the present study was twofold. First, in general, to reveal new aspects of the potential vector role of ixodid ticks and fleas by screening large numbers of specimens with recently developed molecular biological methods. Second, to evaluate the occurrence of vector-borne infectious agents in a geographical context. Altogether 3,442 unfed hard ticks (*Ixodes ricinus*, *Dermacentor marginatus*, *D. reticulatus*, *Haemaphysalis inermis*, *H.*

concinna, *H. punctata*) and 939 fleas of cats and dogs (*Ctenocephalides felis*, *C. canis*, *Pulex irritans*) were collected in Hungary. DNA was extracted and analyzed in pools for representatives of the orders Rickettsiales and Mycoplasmatales.

H. inermis was newly identified as the most important potential vector for *Rickettsia helvetica* in the study region. A novel *Rickettsia* genotype (designated '*Candidatus* R. hungarica') was also detected in the same tick species, with a maximum of 95.8% *gltA* gene sequence identity to known rickettsiae. In addition, *P. irritans* tested positive for *Rickettsia* sp. RF2125, which has not been previously described in Europe. The human pathogen *R. felis* and the feline pathogen '*Candidatus* Mycoplasma turicensis' were shown for the first time to occur in Central-Eastern Europe. Further novel findings include the presence of *Spiroplasma* spp. in *D. marginatus* and fleas.

In conclusion, this molecular study extends the geographic range and vector spectrum of several arthropod-borne agents, some of which have zoonotic potential.

Keywords: rickettsia; *Ehrlichia canis*; haemotropic mycoplasma; spiroplasma; tick; flea

1. Introduction

Blood-sucking arthropod vectors, exemplified by ticks and fleas, are able to transmit infectious agents between animals and/or humans. Most of the pathogenic bacteria belonging to the order Rickettsiales are vector-borne (Dumler et al., 2001), and this transmission route has also been suggested for several representatives of the order Mycoplasmatales (Neimark et al., 2001).

Rickettsia spp. (family Rickettsiaceae, order Rickettsiales) are Gram-negative, obligate intracellular bacteria (Dumler et al., 2001). Although they have been long known for their public health importance, recently an emerging veterinary significance of various species was

also recognized (Hechemy et al., 2006). Similarly, *Ehrlichia* spp. (family Anaplasmataceae, order Rickettsiales) include both human and animal pathogens (Dumler et al., 2001).

Haemotropic mycoplasmas (also known as haemoplasmas; order Mycoplasmatales), formerly assigned to the genera *Haemobartonella* and *Eperythrozoon* (Neimark et al., 2001), attach to red blood cells. This may lead to haemolytic anaemia in infected domestic and wild animals. Vectors of haemoplasmas may include fleas, hard ticks, and mosquitoes (Neimark et al., 2001).

Spiroplasma spp. are helical mycoplasmas. Although they have been implicated in spongiform encephalopathies (Bastian et al., 2007), unambiguous information on their exact role in diseases is still lacking. Various categories of insects and arachnids, including certain hard ticks, such as *I. ricinus*, have been shown to harbor these bacteria (Carle et al., 1995).

In Hungary *Rickettsia slovaca*-like organisms have been found in questing (unfed) *Dermacentor* spp. (Reháček et al., 1979). More recently engorged ticks were investigated: *R. helvetica* and *R. monacensis* have been detected in *I. ricinus*, *R. raoultii* (formerly *Rickettsia* sp. RpA4) in *D. reticulatus*, and a distinct rickettsia genotype in *Haemaphysalis concinna* (Sréter-Lancz et al., 2006). However, no other tick species – particularly not questing ones – or flea species have been evaluated. Similarly, no data have been published to date on the occurrence of *Ehrlichia canis*, haemotropic *Mycoplasma*, or *Spiroplasma* spp. in ticks or fleas of this region in Europe. Therefore, the present study was undertaken to obtain relevant and updated information on these important vector-borne agents.

2. Materials and methods

2.1. Origin of the samples

Between March and July 2007, a total of 3,442 hard ticks (Acari: Ixodidae) were collected from vegetation at 37 locations in Hungary using the cloth-dragging and flagging method. The species, stage, and sex of the ticks were determined by microscopic examination. Subsequently, ticks were allotted into pools (Table 1) taking into account their size, i.e. for optimal DNA extraction. The last pool of each species contained the remainder of specimens.

In addition, 939 fleas (Siphonaptera: Pulicidae) were collected – 420 from cats and 519 from dogs – at 14 small animal clinics in various parts of the country. All fleas of one species removed from one animal were pooled (Table 1). *Ctenocephalides felis* were obtained from cats, *C. canis* from cats and dogs, and *Pulex irritans* from dogs. Both tick and flea samples were stored in 70% ethanol until molecular biological analysis.

2.2. DNA extraction

Pools of ticks and fleas were air-dried, washed twice in phosphate-buffered saline without $MgCl_2$ or $CaCl_2$ (DPBS; Invitrogen, Basel, Switzerland) and minced with scissors in 100 μ l DPBS at the bottom of 2 ml Eppendorf tubes. Between the preparations of pools, the scissors were decontaminated by washing in tap water, burning, and cooling in distilled water.

From all tick pools and from those of *C. canis* and *P. irritans*, DNA was extracted using the MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics, Rotkreuz, Switzerland). Briefly, 150 μ l of DPBS and 375 μ l of lysis buffer containing guanidinium thiocyanate and Triton X-100 were added to each pool, together with a 5 mm stainless steel bead (Schieritz and Hauenstein AG, Arlesheim, Switzerland). The samples were homogenized in a Mixer Mill device (Retsch GmbH, Haan, Germany) for 2 min at 30,000 Hz. After a short centrifugation at 5,000 g, 500 μ l of the supernatant from each sample was loaded onto the MagNA Pure LC Instrument (Roche Diagnostics). Two negative extraction controls

containing only DPBS were included per 30 samples in order to monitor for cross-contamination. DNA from the 187 pools of *C. felis* was extracted using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany). In both procedures the elution volume was 100 µl.

The presence of amplifiable DNA was confirmed for each pool using a real-time PCR assay to detect the 18S rRNA gene (Applied Biosystems, Rotkreuz, Switzerland). The concentration of amplifiable DNA was determined as sufficient if the threshold cycle (Ct) value was less than 25. Samples were stored at -20 °C until analysis.

2.3. Real-time PCRs

All samples were first screened with real-time PCR. Cycling conditions were: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Ninety three samples (including three negative extraction controls) were analyzed in one assay, together with two – external and internal – negative and one positive PCR controls. The tests were performed using either the ABI Prism 7500 (for *Rickettsia* spp.) or 7700 (for all others) Sequence Detection Systems (Applied Biosystems).

2.3.1. TaqMan PCRs for *Rickettsia* spp.

First an assay that amplifies a 65-bp portion of the 23S rRNA gene of *R. helvetica* was performed. Then all samples negative in the 23S rRNA PCR (or chosen for confirmatory analysis) were tested with TaqMan PCR for a 74-bp fragment of the *gltA* gene in spotted fever and typhus group rickettsiae (Boretti et al., in press).

2.3.2. *TaqMan PCR for Ehrlichia canis*

This method to detect a portion of the 16S rRNA gene of *E. canis* was performed as described previously (Foley et al., 2007).

2.3.3. *Universal SYBR Green PCR for haemotropic mycoplasmas*

The assay served to screen all DNA samples for the presence of haemotropic mycoplasmas (Willi et al., submitted for publication). In brief, the reaction volume was 25 µl, consisting of 12.5 µl of SYBR Green Mastermix (Applied Biosystems), a final concentration of 300 nM of forward primer (5'-AGC AAT RCC ATG TGA ACG ATG AA- 3') and an equimolar mixture of two reverse primers (5'-TGG CAC ATA GTT TGC TGT CAC TT-3' and 5'-GCT GGC ACA TAG TTA GCT GTC ACT-3'), and 5 µl of template DNA. After 40 cycles a dissociation run was performed: 95 °C for 15 s, 60 °C for 20 s, a temperature increase from 60 °C to 95 °C in 20 min, and finally 95 °C for 15 s.

2.3.4. *Species-specific TaqMan PCR for haemotropic mycoplasmas*

Samples that tested positive or gave inconclusive results in the SYBR Green assay were further analyzed. Real-time TaqMan PCR assays specific for the 16S rRNA gene of feline (*M. haemofelis*, '*Candidatus M. haemominutum*', and '*Candidatus M. turicensis*'), canine (*M. haemocanis*, '*Candidatus M. haematoparvum*'), and murine haemotropic mycoplasmas (*M. coccoides*) were performed as previously described (Wengi et al., 2008; Willi et al., 2006; Willi et al., 2007). In addition, for bovine/ovine haemoplasmas (*M. wenyonii* and *M. ovis*) a 119-bp long fragment of the 16S rRNA gene was amplified (Meli et al., submitted for

publication), involving the forward primer 5'-CCA CGT GAA CGA TGA AGG TCT T-3', the reverse primer 5'-GGC ACA TAG TTA GCT GTC ACT TAT TCA A-3', and the probe 5'-6FAM-AGT ACC ATC AAC GCG CGC TGA TTT CCT AG-MGB-3'. The 25 µl PCR reaction mixture contained 900 nM of each primer, 0.25 µM of probe, 12.5 µl of 2× qPCR MasterMix (Eurogentec, Seraing, Belgium), and 5 µl of template DNA.

2.4.1. Conventional PCRs for *Rickettsia* spp.

Selected *gltA* TaqMan PCR-positive samples (n = 19, with the lowest Ct values) were further analyzed by a conventional PCR. A 381-bp portion of the *gltA* gene was amplified using the primers Rp877p and Rp1258n, as previously described (Boretto et al., in press). Additionally, in the case of one sample that gave a sequence different from those deposited in GenBank, a longer *gltA* gene fragment (796 bp) was amplified with the primers CS477f and CS1273r. Part of the PCR products were stained with ethidium bromide and visualized in agarose gel.

2.4.2. Conventional PCRs for *Mycoplasmatales*

Samples positive in the species-specific TaqMan real-time PCR were further analyzed for the nearly complete 16S rRNA gene as described earlier (Willi et al., 2006). Samples that yielded inconclusive SYBR Green real-time PCR results, but were negative in all species-specific TaqMan assays, were subsequently evaluated with a conventional PCR for a shorter, approximately 200 bp region in the 16S rRNA gene of haemoplasmas (Jensen et al., 2001).

2.5. Sequencing

After purification of the conventional PCR product with the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) and determination of the DNA concentration with the NanoDrop ND-1000 spectrophotometer (Witec Ag, Littau, Switzerland), cycle sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems). The cycling conditions were as follows: 1 min at 96 °C, then 25 cycles of 96 °C for 30 sec and 50 °C for 15 sec, and finally incubation at 60 °C for 4 min. Products were purified with SigmaSpin™ Post-Reaction Purification Columns (Sigma-Aldrich), and evaluated on the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

2.6. Statistical analysis

Pool infection prevalence was calculated from the number of positive pools, expressed as the percentage of the number of evaluated pools. Given that there must have been at least one infected individual in each positive pool, the minimum prevalence was determined from the number of positive pools, expressed as the percentage of the number of evaluated arthropod individuals of the species. Confidence intervals (CI) for the prevalence rates at the 95% level were calculated according to Sterne's method (Reiczigel, 2003).

2.7. Phylogenetic analyses and nucleotide sequence accession numbers

Obtained sequences were edited, aligned and compared to reference GenBank sequences as described previously (Hornok et al., 2009). New sequences were submitted to the GenBank, with the accession numbers EU170604 for '*Candidatus* Mycoplasma haemominutum', EU170605-15 for *Spiroplasma* spp., and EU853830-5 plus EU853837-8 for *Rickettsia* spp.

3. Results

All extraction and PCR negative controls were negative.

3.1. *Rickettsia* spp.

Using real-time TaqMan PCR for the 23S rRNA gene, *R. helvetica* was detected in 98.5% (95% CI: 91.6-100%) of the *H. inermis* pools (minimum prevalence 19.7%; 95% CI: 15.5-24.5%) (Table 2). A total of 92.6% (95% CI: 86.7-96.4%) of the *I. ricinus* pools were also shown to be infected (minimum prevalence 7.2%; 95% CI: 6-8.5%). Further two tick species and one flea species were found to harbor *R. helvetica* (Table 2). The minimum prevalence of *Rickettsia* spp. in *D. marginatus* and in *D. reticulatus* was 17.5% (95% CI: 13.2-22.5%) and 16% (95% CI: 12.3-20.3%), respectively (Table 2).

One pool of *H. inermis* collected in northern Hungary contained a *gltA* sequence, a 712-bp region of which showed only 95.8% maximal identity to that of any other known *Rickettsia* spp. available in the GenBank. Hence the name '*Candidatus R. hungarica*' is proposed for this new isolate. In addition, *R. monacensis* was detected in *I. ricinus*, *R. slovaca* was found in *D. marginatus*, and *R. raoultii* was identified in *D. reticulatus* (Table 2). Moreover, '*Candidatus R. kotlanii*' was shown in an *H. concinna* pool, and *R. felis* as well as *Rickettsia* sp. RF2125 were found in flea pools (Table 2). The phylogenetic relationships of these specimens are depicted in Fig. 1.

3.2. *Ehrlichia canis*

Tick pools were evaluated for the presence of *E. canis*. Two pools of female *I. ricinus*, both collected in southwestern Hungary, were found to be PCR-positive.

3.3. *Haemotropic Mycoplasma spp.*

All tick pools and the *C. canis* and *P. irritans* pools were PCR-negative for haemotropic *Mycoplasma* spp. Two *C. felis* pools were positive in the assay specific for '*Candidatus M. haemominutum*'; one of them was also found to contain '*Candidatus M. turicensis*'. The presence of '*Candidatus M. haemominutum*' was also confirmed by sequencing of the 16S rRNA gene (EU170604). This isolate showed 100% identity to a feline isolate recently reported in Hungary (EU128752).

3.4. *Spiroplasma spp.*

Fifteen tick pools and four flea pools gave inconclusive results in the real-time SYBR Green assay and were negative in all TaqMan assays designed to detect individual haemoplasma species. Five of the 15 tick pools and three of the four flea pools were found to be negative by conventional PCR. In the remaining pools, representatives of the genus *Spiroplasma* were found. These included six *I. ricinus* pools (five female, one nymph), four *D. marginatus* pools (three male, one female), and one *C. felis* pool. Sequencing revealed 95-98% identity to *Spiroplasma* spp. deposited in GenBank (AJ132412).

4. Discussion

This is the first molecular study of the occurrence of *Rickettsia* spp., *E. canis*, haemotropic mycoplasmas and *Spiroplasma* spp. in questing ticks and in fleas obtained from cats and dogs in Hungary. Since fed (engorged) ticks may harbour infectious agents that were acquired during the blood meal but are irrelevant to their vector potential, only ticks collected from the vegetation were included in the present survey.

Many rickettsiae cause emerging diseases (Hechemy et al., 2006; Brouqui et al., 2007). The geographical distribution of *Rickettsia* spp. depends on their association with particular tick vectors and animal reservoirs. *R. helvetica*, a potential human pathogen (Brouqui et al., 2007), is transmitted by *I. ricinus* in Europe (Parola, 2004) and by other *Ixodes* spp. in Asia (Fournier et al., 2002). Results of the present study introduce *H. inermis* as a new and significant potential vector of this rickettsia. *H. inermis* is an emerging species (Hornok and Farkas, 2009), occurring sporadically in most European countries, as well as in the Middle East and South Asia. In addition, *H. concinna*, *H. punctata*, and the cat flea *C. felis* were identified for the first time in this study as possible vectors of *R. helvetica*. However, based on the low number of infected pools, they appear to be of only minor importance.

Remarkably, a novel *Rickettsia* genotype has been found in *H. inermis*. The difference of at least 4.2% between the *gltA* sequences of the new genotype and the sequences of other rickettsiae, argue for its status as a candidate new species ('*Candidatus R. hungarica*'). For comparison, previous work has estimated the *gltA* sequence to diverge by up to 2.3% between members of the SFG group (Regnery et al., 1991). Furthermore, characterization of a shorter, 381-bp portion of the *gltA* gene has previously been used to describe other new species of the genus (Higgins et al., 1996), and here it showed a minimum of 4.8% sequence dissimilarity between the new isolate and relevant GenBank sequences.

PCR positivity for *R. slovaca*, another clinically important species (Brouqui et al., 2007), has already been detected in two engorged *D. marginatus* individuals in Hungary (Raoult et al., 2002). The present study extends these findings to unfed ticks. Although only five PCR-positive *D. marginatus* samples were sequenced, *R. slovaca* was identified in all cases. If we assume that the majority of the PCR-positive *D. marginatus* pools had been infected with *R. slovaca*, the observed minimum prevalence of 17.5% is higher than the prevalence of 7.2% reported previously in this region (Reháček et al., 1979). Similarly, the sequencing of all five *D. reticulatus* pools detected *R. raoultii* (formerly *Rickettsia* sp. RpA4). If we extrapolate this to the remainder of the PCR-positive samples, the high rate of infection in this species is comparable to previous reports evaluating engorged ticks in Hungary (Sréter-Lancz et al., 2006) and Germany (Dautel et al., 2006).

The identification of *R. monacensis* in questing *I. ricinus* and of '*Candidatus R. kotlanii*' in *H. concinna* in the present study corroborates their earlier detection in engorged specimens (Sréter-Lancz et al., 2006). On the other hand, although the human pathogen *R. felis* (Higgins et al., 1996) had already been found in Western Europe, both in Germany and France (Rolain et al., 2003), this is the first report of a highly similar sequence from Central-Eastern Europe. Furthermore, *Rickettsia* sp. RF2125 was shown for the first time in Europe and in the human flea, *Pulex irritans*; as formerly this species had been identified in Asia, North America, and Africa in *C. canis* (AF516333), *C. felis* (AY953289), and *Echidnophaga gallinacea* (Loftis et al., 2006), respectively.

In Europe, *E. canis*, the etiological agent of canine monocytic ehrlichiosis, is transmitted by *Rh. sanguineus* (Groves et al., 1975). This restricts its endemicity to the Mediterranean and Balkanian countries, with prevalences declining towards the north (Solano-Gallego et al., 2006). Recently, however, *E. canis* was also detected in *I. ricinus* collected from vegetation in the Netherlands (Wielinga et al., 2006). Since the method used in the present study is highly

specific for *E. canis* (Foley et al., 2007) and cross-contamination of the samples is very unlikely, our results raise the possibility of the indigenous status of *E. canis* in Hungary. However, confirmation of this would require sequencing, which could not have been carried out from the two positive pools due to the low bacterial load.

Vectors of haemotropic mycoplasmas, the causative agents of haemolytic anaemia, may include fleas and hard ticks, as suggested by previous transmission experiments (Seneviratna et al., 1973; Woods et al., 2005). This is further substantiated by the fact that unfed ixodid ticks contained '*Candidatus M. haemominutum*' (Taroura et al., 2005). Nevertheless, although haemoplasma infections have been reported in Hungary (Hornok et al., 2008; Hornok et al., 2009), the current study failed to detect PCR positivity in tick pools. This may suggest that ticks do not play a significant role in the transmission of haemotropic mycoplasmas in Hungary, confirming previous results from Switzerland (Willi et al., 2007). On the other hand, feline haemoplasmas are frequently detected in fleas (Willi et al., 2007). Accordingly, '*Candidatus M. haemominutum*' and '*Candidatus M. turicensis*' were found in *C. felis*, the latter for the first time in Central-Eastern Europe. The 100% identity of the 16S rRNA gene sequence of the flea isolate with that of one Hungarian cat (EU128752), two Swiss cats (DQ157141, DQ157147), and one UK cat (AF 271154) is consistent with the small genetic differences between haemoplasmas from geographically distant places (Tasker et al., 2003).

The genus *Spiroplasma* has an enormous diversity of often incompletely characterized or unnamed species, including non-pathogenic or symbiotic organisms that persist in insects and arachnids. These bacteria have been associated with transmissible spongiform encephalopathy in humans and ruminants (Bastian et al., 2007), but how they contribute to these diseases remains unclear. Spiroplasmas are found not only in horse flies and mosquitoes, but also in hard ticks (Carle et al., 1995). In particular, *I. ricinus* from Germany (Henning et al., 2006) and *Haemaphysalis* spp. (Carle et al., 1995) have been reported to be carriers of these agents.

However, to the best of our knowledge, this is the first report of a *Spiroplasma* infection in *D. marginatus* (for which ruminants are the most preferred hosts) and in any fleas. Since fleas are not host-specific, the spiroplasma-carrier status of some *C. felis* shown in the present study should be taken into account when assessing the risks associated with pet fleas near human beings.

In conclusion, the present study extends the geographic range of important infectious agents, reports identification of a novel rickettsia sequence, and suggests new potential vector role for some of the evaluated tick and flea species.

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483 **Figure caption:**

484

485 **Figure 1.** A bootstrap phylogenetic tree of the *gltA* gene demonstrating the relationship of
486 Hungarian isolates to other *Rickettsia* spp. using the Neighbor-Joining method. The numbers
487 at the nodes were generated from 1,000 Bootstrap resamplings. The scale bar represents the
488 mean number of differences per 50 sites.

Table 1. Pooling criteria for ticks and fleas.

	Total number	Number of adults per pool	Number of immature stages per pool¹	Total number of pools
Ticks	3442			
<i>Ixodes ricinus</i>	2116	20 ♂, 10 ♀	30 n, 24 l	41 ♂, 94 ♀, 9 n, 1 l
<i>Dermacentor marginatus</i>	369	5 ♂, 5 ♀	-	23 ♂, 34 ♀
<i>D. reticulatus</i>	361	5 ♂, 5 ♀	-	25 ♂, 46 ♀
<i>Haemaphysalis inermis</i>	315	5 ♂, 5 ♀	-	24 ♂, 40 ♀
<i>H. concinna</i>	259	3 ♂, 3 ♀	10 n, 52 l	29 ♂, 16 ♀, 7 n, 1 l
<i>H. punctata</i>	22	3 ♂, 3 ♀	1 n	5 ♂, 3 ♀, 1 n
Fleas	939			
<i>Ctenocephalides felis</i>	393	1-11	-	187
<i>C. canis</i>	526	1-12	-	195
<i>Pulex irritans</i>	20	1-4	-	15

¹Abbreviations: n, nymph; l, larva

Table 2. Real-time PCR and sequencing results of *Rickettsia* spp. in hard ticks (Ixodidae) and fleas (Ctenocephalidae, Pulicidae). Except for *Ixodes ricinus* and *Ctenocephalides felis*, only samples negative for the 23S rRNA gene were evaluated in the *gltA* gene TaqMan assay.

	Sample source	TaqMan assay to detect <i>Rickettsia helvetica</i> 23S rRNA gene (positive/tested samples)	TaqMan assay to detect <i>Rickettsia</i> spp. <i>gltA</i> gene (positive/tested samples)	Results of partial sequencing of the <i>gltA</i> gene (sequence identity: number and sex of sample source)
Ticks	<i>Ixodes ricinus</i>	126/136	9/58	<i>R. monacensis</i> (100%: 2♂)
	<i>Dermacentor marginatus</i>	0/55	48/55	<i>R. slovaca</i> (100%: 1♂, 4♀)
	<i>D. reticulatus</i>	0/70	56/70	<i>R. raoultii</i> (99.7%: 1♂, 4♀)
	<i>Haemaphysalis inermis</i>	63/64	1/1	Unknown <i>Rickettsia</i> sp. designated ' <i>Candidatus R. hungarica</i> ' (1♂)
	<i>H. concinna</i>	1/53	1/52	' <i>Candidatus R. kotlanii</i> ' (99.7%: 1♂)
	<i>H. punctata</i>	1/9	0/8	-
Fleas	<i>Ctenocephalides felis</i>	2/20	1/20	<i>R. felis</i> (99.2%: 1)
	<i>Ct. canis</i>	0/17	0/17	-
	<i>Pulex irritans</i>	0/15	1/15	<i>Rickettsia</i> sp. RF2125 (100%: 1)