BRIEF COMMUNICATION

Bartonella rochalimae, B. grahamii, B. elizabethae, and Wolbachia spp. in Fleas from Wild Rodents near the China-Kazakhstan Border

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Abstract: The Alataw Pass, near the Ebinur Lake Wetland (northwest of China) and Taldykorgan (east of Kazakhstan), is a natural habitat for wild rodents. To date, little has been done on the surveillance of Bartonella spp. and Wolbachia spp. from fleas in the region. Here we molecularly detected Bartonella spp. and Wolbachia spp. in wild rodent fleas during January and October of 2016 along the Alataw Pass-Kazakhstan border. A total of 1,706 fleas belonging to 10 species were collected from 6 rodent species. Among the 10 flea species, 4 were found to be positive for Wolbachia, and 5 flea species were positive for Bartonella. Molecular analysis indicated that i) B. rochalimae was firstly identified in Xenopsylla gerbilli minax and X. conforms conforms, ii) B. grahamii was firstly identified in X. gerbilli minax, and iii) B. elizabethae was firstly detected in Coptopsylla lamellifer ardua, Paradoxopsyllus repandus, and Nosopsyllus laeviceps laeviceps. Additionally, 3 Wolbachia endosymbionts were firstly found in X. gerbilli minax, X. conforms conforms, P. repandus, and N. laeviceps laeviceps. BLASTn analysis indicated 3 Bartonella species showed genotypic variation. Phylogenetic analysis revealed 3 Wolbachia endosymbionts were clustered into the non-Siphonaptera Wolbachia group. These findings extend our knowledge of the geographical distribution and carriers of B. rochalimae, B. grahamii, B. elizabethae, and Wolbachia spp. In the future, there is a need for China-Kazakhstan cooperation to strengthen the surveillance of flea-borne pathogens in wildlife.

Key words: Bartonella spp., Wolbachia spp., flea, China-Kazakhstan border

Fleas (Insecta: Siphonaptera) are obligate hematophagous insects that infest birds and mammals worldwide. In China, 655 species and subspecies belonging to 10 families and 74 genera have been reported, which corresponds to approximately 25% of the species known worldwide. Some flea species have only been presented in the Xinjiang Uygur Autonomous Region and Central Asia, including Xenopsylla gerbilli minax and Paradoxopsyllus repandus [1-3]. The Alataw Pass, near the Ebinur Lake Wetland and Taldykorgan (east of Kazakhstan), is the second largest land passage in China. Twenty-two flea species have been reported in the region from wild rodents [4,5].

Fleas are vectors of several important zoonotic pathogens, including Yersinia pestis, Rickettsia typhi, R. felis, and Candidatus Rickettsia barbaraie [1,5]. Recently, Bartonella spp. and Wolbachia spp. lineages have been detected in several flea species [6]. Further studies indicated that rodent-associated fleas are naturally infected with zoonotic Bartonella species, including B. elizabethae, B. rochalimae, B. grahamii, B. henselae, B. clarridgeiae, and B. koehlerae [7-9]. In 1924, Herting and Wolbach found that a Wolbachia species was found in Culex pipiens, and therefore, the organism was named as Wolbachia pipientis. Wolbachia is a bacterial endosymbiont with a wide distribution and is found in 20-75% of arthropods, including Ctenocephalides felis felis, X. cheopis, Oropsylla hirsuta, and Plocopsylla achillea [10,11].

In a previous work, B. henselae, B. clarridgeiae and a new Bartonella genotype were detected in the Thai-Myanmar border [12]. Meanwhile, Bartonella melophagi and Wolbachia supergroup F from sheep keds (Melophagus orinus) in southern Xinjiang Uygur Autonomous Region, China [13]. To the best of our knowledge, little has been done on the surveillance of Bartonella spp. and Wolbachia spp. from fleas in this region. Herein we carried out molecular detection of Bartonella spp. and Wolbachia spp. in fleas collected from wild rats in the Alataw Pass,
northwest of China.

Flea specimens were collected from wild rodents from January to October 2016 along the Chinese Alataw Pass-Kazakhstan border. Briefly, the wild rodents were captured in Sherman traps (H.B. Sherman Traps, Tallahassee, Florida, USA), which were placed at the entrances of occupied burrows [14]. This study was approved by the Animal Research Ethics Committee of Shihezi University (Approval No. AECSU2013-18). The sampling sites included Hejiaoke Border Station and wetlands around Ebinur Lake, in which the wild rodents were abundant, and there was a potential risk to introduce the flea-borne diseases vectored by wild rodents. Fleas were collected from individual rodents by brushing the animal’s fur [15]. Each wild rodent was identified to the species level [16], and capture location was recorded. The fleas were morphologically identified [3] and stored in 70% ethanol. All flea samples were then grouped into pools ranging from 2 to 15 specimens on the basis of species, and host species, as previously reported [15,17]. Before DNA extraction, fleas were washed with purified sterile water for 3 min [17]. The total genomic DNA of each pool was extracted using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China).

To ensure absence of contamination, negative control reactions contained double distilled water instead of positive DNA were included in each PCR run [7]. Each pool was screened for 3 different runs using 3 target genes (citrate synthase [gltA, 380 bp], 16S-23S rDNA intergenic spacer [ITS, 100-300 bp] and riboflavin synthase [ribC, 588 bp]) to confirm the identification of Bartonella spp. [17,18]. Moreover, 2 primer pairs were used to amplify the 16S rDNA (936 bp) and the outer surface antigen (wsp, 602 bp) fragment of Wolbachia spp. in each sample, according to the method of Robertson et al. [19].

### Table 1. Information of sampled fleas including captured numbers, flea pools and their hosts

| Flea species                        | No. of captured (pool No.) | Host species (no.)                                        |
|-------------------------------------|---------------------------|----------------------------------------------------------|
| Echidnophaga oschanini              | 36 (5)                    | Rhombomys opimus (4), Meriones libycus (2)               |
| Xenopsylla conformis conformis      | 41 (4)                    | Rhombomys opimus (2), Meriones libycus (5), Meriones tamariscinus (2) |
| Xenopsylla gerbilli minax           | 1,073 (101)               | Rhombomys opimus (33), Meriones libycus (2), Meriones tamariscinus (2), Meriones meridianus (1) |
| Coptopsylla Lamellifer ardua        | 36 (11)                   | Rhombomys opimus (9)                                     |
| Ctenophthalmus dolichus dolichus    | 8 (1)                     | Meriones tamariscinus (4)                                |
| Rhadinopsylla cedestis              | 8 (2)                     | Meriones tamariscinus (3)                                |
| Leptopsylla nemorosica              | 2 (1)                     | Mus musculus Linnaeus (1)                               |
| Mesopsylla eucta shikho             | 4 (1)                     | Allactaga sibirica (2)                                  |
| Paradoxopsyllus repandus            | 281 (45)                  | Rhombomys opimus (24), Meriones libycus (1), Meriones tamariscinus (1), Meriones meridianus (1) |
| Nosopsyllus laeviceps laeviceps     | 217 (38)                  | Rhombomys opimus (32), Meriones libycus (9), Meriones tamariscinus (3), Meriones meridianus (4) |
| **Total**                           | 1,706 (209)               | Rhombomys opimus (104), Meriones libycus (19), Meriones tamariscinus (16), Meriones meridianus (6), Allactaga sibirica (2), Mus musculus Linnaeus (1) |

### Table 2. Positivity of flea pools for Bartonella and Wolbachia

| Flea species                        | No. of tested flea | No. of tested flea pool | No. of Wolbachia spp. -positive pools (MFIR, %) | No. of Bartonella spp. -positive pools (MFIR, %) | No. of co-infection (MFIR, %) |
|-------------------------------------|--------------------|-------------------------|-----------------------------------------------|-------------------------------------------------|-------------------------------|
| Echidnophaga oschanini              | 36                 | 5                       | 0 (0)                                         | 0 (0)                                           | 0 (0)                         |
| Xenopsylla gerbilli minax           | 1,073              | 101                     | 45 (4.19)                                     | 98 (9.13)                                       | 43 (4.01)                     |
| Xenopsylla conformis conformis      | 41                 | 4                       | 2 (4.88)                                      | 1 (2.44)                                        | 0 (0)                         |
| Coptopsylla Lamellifer ardua        | 36                 | 11                      | 0 (0)                                         | 7 (19.44)                                       | 0 (0)                         |
| Rhadinopsylla cedestis              | 8                  | 2                       | 0 (0)                                         | 0 (0)                                           | 0 (0)                         |
| Ctenophthalmus dolichus dolichus    | 8                  | 1                       | 0 (0)                                         | 0 (0)                                           | 0 (0)                         |
| Leptopsylla nemorosica              | 2                  | 1                       | 0 (0)                                         | 0 (0)                                           | 0 (0)                         |
| Mesopsylla eucta shikho             | 4                  | 1                       | 0 (0)                                         | 0 (0)                                           | 0 (0)                         |
| Paradoxopsyllus repandus            | 281                | 45                      | 19 (6.76)                                     | 34 (12.10)                                      | 16 (5.69)                     |
| Nosopsyllus laeviceps laeviceps     | 217                | 38                      | 9 (4.15)                                      | 29 (13.36)                                      | 7 (3.23)                      |
| **Total**                           | 1,706              | 209                     | 75 (4.40)                                     | 169 (9.91)                                      | 66 (3.87)                     |

MFIR, minimum field infection rates.
products were purified using the TIANgel Midi Purification Kit (TIANGEN), cloned into the pBS-T vector, and used for the transformation of One-shot TOP 10 chemically competent Escherichia coli cells. Three to five positive clones were selected for sequencing by Sangon Biotech Co., Ltd. (Shanghai, China). The resulting sequences were compared with sequences from centralized databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was constructed using the maximum likelihood (ML) and neighbor joining (NJ) algorithms present in MEGA 6 software [20].

A total of 1,706 fleas, belonging to 10 species, were collected from 6 rodent species and grouped into 209 flea pools. The average flea index was 11.53 (148/1,706). The detailed data on the hosts, flea pools, and flea species are shown in Table 1. Among the 10 flea species, 4 were found to be positive for Wolbachia and 5 flea species were positive for Bartonella (Table 2). The negative controls yielded no PCR products, and all positive flea samples were positive for the different target genes.

All the amplicons of the Bartonella gltA gene were firstly sequenced. After alignment and clustering, the other 2 genetic markers, Bartonella ITS and ribC genes, were sequenced in the representative samples. BLAST analysis indicated genetic varia-

![Phylogenetic tree](image)
tions in the 3 species of *Bartonella* sp. found in this study on the basis of *gltA*, *ITS*, and *ribC* genes. Phylogenetic analysis indicated that i) *Bartonella* sp. AL01, claded into *B. rochalimae*, was found in *X. gerbilli minax* and *X. conformis conformis*; ii) *Bartonella* sp. AL02, claded into *B. grahamii*, was detected in *X. gerbilli minax*; and iii) *Bartonella* sp. AL03 and AL04, were both claded into *B. elizabethae*. The former was detected in *N. laeviceps laeviceps* and the latter was detected in *Coptopsylla lamellifer ardua* and *P. repandus* (Fig. 1).

The analysis of the Wolbachia 16S rDNA fragment indicated

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**Fig. 2.** Wolbachia (◆, ▲) collected from *Nosopsyllus laeviceps laeviceps*, *Xenopsylla* sp., *Paradoxopsyllus repandus*. Phylogenic trees of (A) 16S rRNA and (B) *wsp* sequences Maximum-likelihood (1,000 bootstrap replicates) and neighbour-joining (500 bootstrap replicates) were applied.
that Wolbachia from Xenopsylla genus (X. gerbilli minax and X. conormis conformis), P. repandus, and N. laeviceps laeviceps were 99.77% (869/871), 100% (871/871), and 99.54% (867/871) to Candidatus Wolbachia brouqui (DQ402520). Wolbachia endosymbiont in Ectopis griseescens from China tea garden (KT862198), and Wolbachia endosymbiont in Aegealista alni (AM180550), respectively. Similarly, the analysis of the wsp fragment indicated that the degree of identity of Wolbachia endosymbionts were 98.9% (554/560), 92.5% (520/562), and 95.1% (527/554) to Wolbachia endosymbiont in Tribolium confusum (KC305360), Wolbachia tonolepta hieroglyphica (IF747227), and Wolbachia endosymbiont in Cybaeus penedentatus (GQ480746), respectively. Moreover, phylogenetic analysis of the 16S rDNA sequence indicated that 3 Wolbachia endosymbionts in this study were clustered into the non-Siphonaptera Wolbachia group (Fig. 2).

The 15 nucleotide sequences from Bartonella (gltA: KX-572889, KX572890, KX572891, and KX572892; ITS: KX-572893, KX572894, KX572895, and KX572896; ribC: KX-572897, KX572898, KX572899, and KX572900) and Wolbachia (16S rDNA: KX385013, KX385014, and KX385015; wsp: KX385018, KX385019 and KX385020) were deposited in the GenBank database.

Bartonella infections occur worldwide and cause severe diseases in humans. Herein, we report the presence of 3 human pathogens of the genus Bartonella (B. rochalimae, B. grahamii, and B. elizabethae). The finding conveys us that X. gerbilli minax, X. conormis conforms and C. lamelifer ardua are novel carriers for these 3 Bartonella species. This information increases our knowledge of the carriers and geographical distribution of Bartonella.

B. rochalimae was recently isolated from the blood of a human patient who presented with fever, rash, and splenomegaly after receiving multiple insect bites in 2007 [21]. A previous study identified B. rochalimae in Chile, China, France, Greece, Israel, and United States [22-25]. In this study, B. rochalimae was found in 2 flea species (X. gerbilli minax and X. conormis conforms), and this finding is consistent with that of the study of Sofer and Nasereeddin, wherein B. rochalimae was detected in the fleas species Ctenocephalides sp., Xenopsylla sp., and Pulex sp. [24,26]. B. grahamii, primarily isolated from Clethrionomys glareolus, was involved in a case of neuroretinitis in a human subject in 1999 [27]. B. grahamii has been found in various countries, including Canada, China, Denmark, Finland, France, Germany, Japan, North Korea, Poland, Russia, Slovenia, South Korea, Spain, Sweden, Thailand, United Kingdom, and United States [25,28,29]. Furthermore, a previous study found that B. grahamii was transmitted by Ctenophthalmus nobilis in the United Kingdom [29]. Of note, in this study, we detected B. grahamii and B. rochalimae in X. gerbilli minax, which is also the vector of Y. pestis [4]. Therefore, X. gerbilli minax bites in humans should be paid more attention. B. elizabethae, the causative agent of endocarditis [30], has been detected in Canada, China, France, Israel, Japan, Thailand, Tunisia, United States and Vietnam [23,30-32]. In our study, B. elizabethae was detected in 3 flea species (C. lamelifer ardua, P. repandus, and N. laeviceps laeviceps). Although there is no evidence to prove these flea species are vectors for transmitting bartonellosis, the infestations of these flea species should not be underestimated.

Wolbachia is a bacterial genus that infects several arthropods, including fleas (Siphonaptera), lice (Phthiraptera), and other ectoparasites of mammals and birds. Wolbachia is a highly diverse genus and has been subdivided into 11 phylogenetic supergroups (A to K) on the basis of a multi-locus sequence typing [10,11]. In the present study, 3 Wolbachia endosymbionts were found in N. laeviceps laeviceps, Xenopsylla sp., and P. repandus. This result extends the geographical distribution and number of carrier hosts of Wolbachia. Additionally, there is an interesting phenomenon that why Wolbachia-positive flea species and Bartonella-positive flea species are limited in some several flea species, and highly co-infected in this study. In the future, there should be more efforts to explore it.

The habitats on both sides of the China-Kazakhstan border are similar, without natural geographical barriers, and this allows wild rodents to traverse the border freely. Some of the flea species found in China are also found in Kazakhstan [5]. However, to the best of our knowledge, few studies have investigated the flea species and their pathogens near China-Kazakhstan border. These results suggest that Kazakhstan should strengthen the investigation of fleas and flea-borne diseases in border regions.

B. rochalimae, B. grahamii, B. elizabethae, and 3 Wolbachia endosymbionts were firstly detected in indigenous flea species near the China-Kazakhstan border. The findings extend our knowledge of the geographical distribution and reservoir hosts of Bartonella spp. and Wolbachia spp., increase the awareness of physicians and public health workers on flea-borne diseases and highlight the importance of flea control in transboundary regions.
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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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