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▣ BRIEF COMMUNICATION

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

Xiaoping Yin[1,2,†], Shanshan Zhao[2,†], Bin Yan[3,†], Yanhe Tian[1], Teer Ba[1], Jiangguo Zhang[1], Yuanzhi Wang[2,*]

[1]*Alataw Customs, Alashankou, Xinjiang Uygur Autonomous Region 833418, China;* [2]*School of Medicine, Shihezi University, Shihezi, Xinjiang Uygur Autonomous Region 832002, China;* [3]*Clinical Laboratory Center, Zaozhuang Municipal Hospital, Zaozhuang, Shandong 277100, China*

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 barbariae* [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 ovinus*) in southern Xinjiang Uygur Autonomous Region, China [13]. To the best of our knowledge, little has been done on the surveillace 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,

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* Corresponding author (wangyuanzhi621@126.com)
† These authors contributed equally to this work.

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]. The PCR

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

Flea species	No. of captured (pool No.)	Host species (no.)
Echidnopgaga 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 nemorosa	2 (1)	*Mus musculus* Linnaeus (1)
Mesopsylla eucta shikno	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 nemorosa	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 data bases 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-

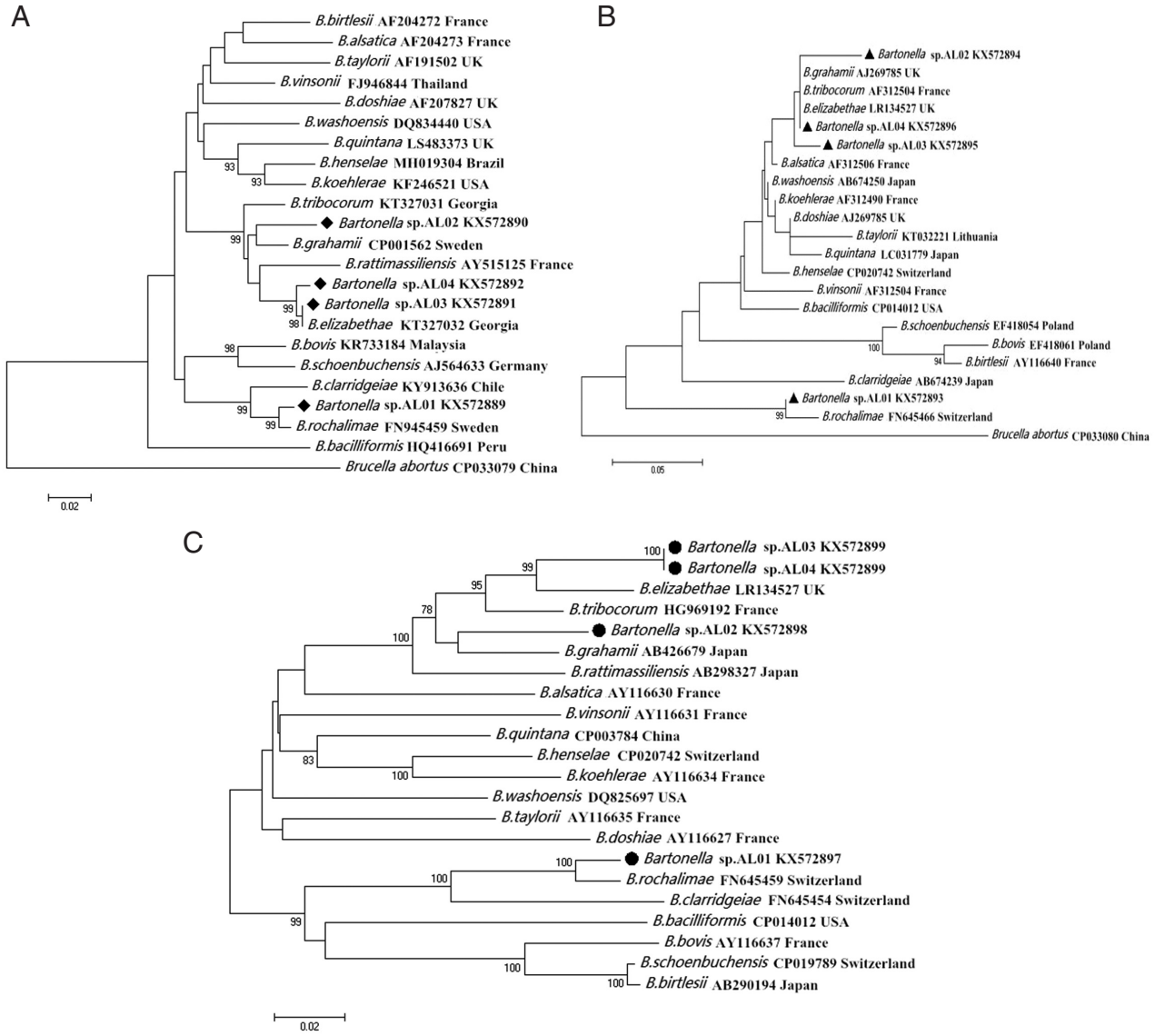

Fig. 1. Maximum likelihood (ML; 1,000 bootstrap replicates) and neighbor joining (NJ; 500 bootstrap replicates) phylogenetic tree of the (A) *gltA*, (B) *ITS*, and (C) *ribC* genes. *Bartonella* sp. sequences AL01-04 (◆, ▲, ●) obtained in this study.

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, cladded into *B. rochalimae*, was found in *X. gerbilli minax* and *X. conforms conforms*; ii) *Bartonella* sp. AL02, cladded into *B. grahamii*, was detected in *X. gerbilli minax*; and iii) *Bartonella* sp. AL03 and AL04, were both cladded 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

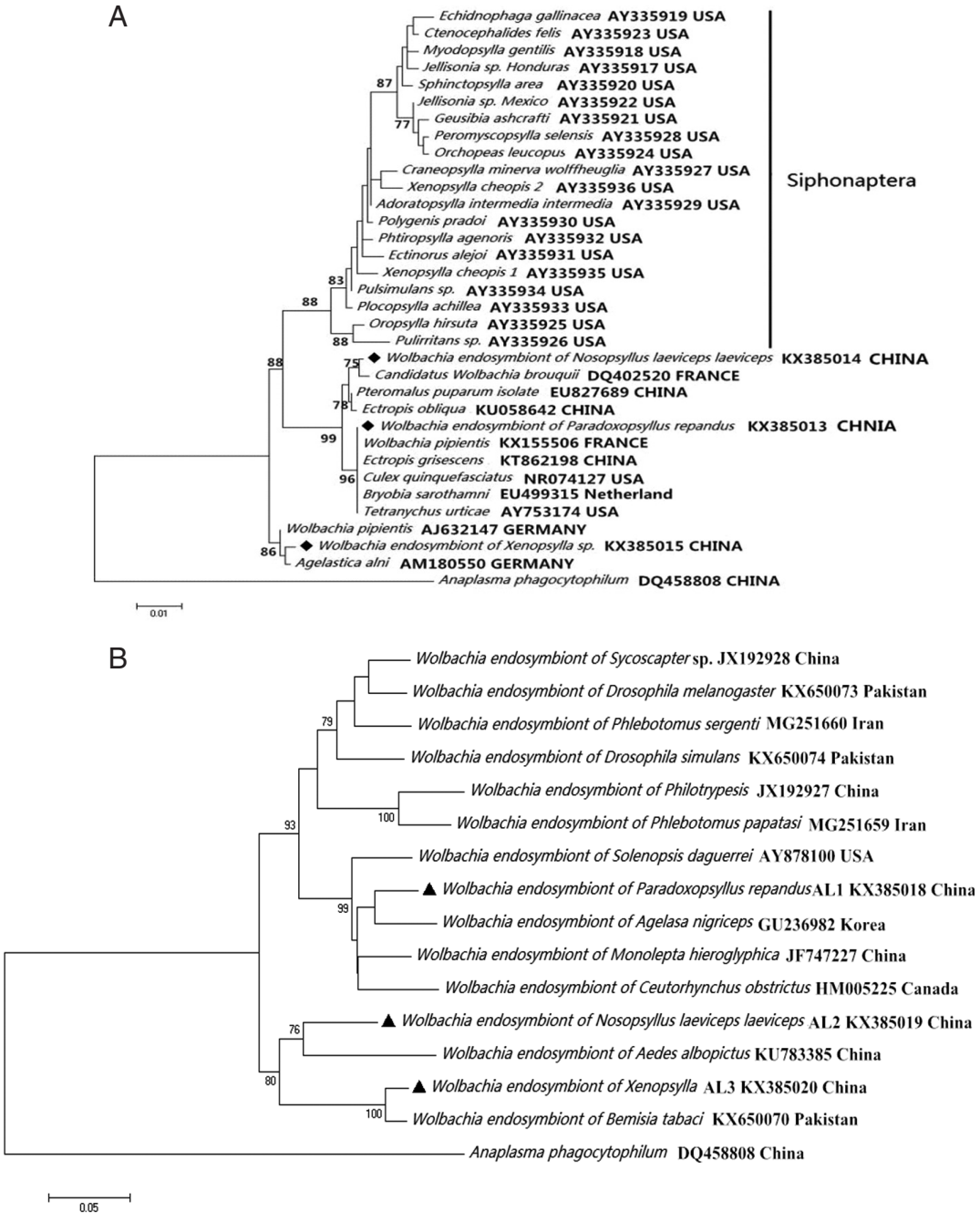

Fig. 2. *Wolbachia* (◆, ▲) collected from *Nosopsyllus laeviceps laeviceps*, *Xenopsylla* sp., *Paradoxopsyllus repandus*. Phylogemic trees of (A) 16S rRNA and (B) *wsp* sequences Maximum-likelihood (1,000 bootstrap replicates) and neighbour-joining (500 bootstrap replicates) were applied.

that *Wolbachia* from *Xenopsylla* genus (*X. gerbilli minax* and *X. conformis conformis*), *P. repandus*, and *N. laeviceps laeviceps* were 99.77% (869/871), 100% (871/871), and 99.54% (867/871) to *Candidatus* Wolbachia brouquii (DQ402520), *Wolbachia* endosymbiont in *Ectropis grisescens* from China tea garden (KT862198), and *Wolbachia* endosymbiont in *Agelastica 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* (JF747227), 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*: KX572889, KX572890, KX572891, and KX572892; *ITS*: KX572893, KX572894, KX572895, and KX572896; *ribC*: KX572897, 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. conforms conforms* and *C. lamellifer 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, Peru and United States [22-25]. In this study, *B. rochalimae* was found in 2 flea species (*X. gerbilli minax* and *X. conforms conforms*), and this finding is consistent with that of the study of Sofer and Nasereddin, 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. lamellifer 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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