

SHORT REPORT

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# First detection of *Candidatus Rickettsia barbariae* in the flea *Vermipsylla alakurt* from north-western China

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## Abstract

**Background:** *Vermipsylla* is a genus of the family Vermipsyllidae within the order Siphonaptera of fleas. *Vermipsylla alakurt* is mainly distributed in alpine pastoral areas of Kazakhstan, Mongolia, China and Nepal, and infests sheep, yaks and horses, causing irritation, poor condition, anaemia and even death. However, to date, no rickettsial agents have been reported in *V. alakurt*.

**Findings:** A total of 133 fleas were collected directly from the tails of three sheep flocks ( $n = 335$ ) in Minfeng County, Xinjiang Uygur Autonomous Region, north-western China. Of these, 55 fleas were identified by morphological examination and molecular analysis of four loci (the ribosomal 18S and 28S rDNA genes and the mitochondrial genes cytochrome *c* oxidase subunit II and elongation factor 1-alpha). Eight *Rickettsia*-specific gene fragments originated from seven genes: the 17-kilodalton antigen gene (*17-kDa*), citrate synthase gene (*gltA*), 16S rRNA gene (*rrs*), outer membrane protein A gene (*ompA*), surface cell antigen 1 gene (*sca1*), PS120 protein gene (*gene D*), and outer membrane protein B gene (*ompB*, two fragments), were used to identify the species of *Rickettsia* in 53 fleas. The amplified products were sequenced and included in a phylogenetic analysis to verify the taxonomic identification of the rickettsial agents. Based on morphological and molecular evidence, the flea was identified as *Vermipsylla alakurt*. Nine samples were positive (16.98 %, 9/53) for *Rickettsia* spp. The phylogenetic tree revealed that the rickettsial agents found in *V. alakurt* cluster with *Candidatus Rickettsia barbariae*.

**Conclusions:** Our study suggests that: (i) *V. alakurt* may serve as a carrier for *Candidatus R. barbariae*; and (ii) *Candidatus R. barbariae*, previously reported in Israel, is the eighth newly discovered validated *Rickettsia* species in China. This finding extends our knowledge of the distribution of *Candidatus R. barbariae* and the profile of carriers, which not only comprise ticks but also fleas.

**Keywords:** *Candidatus Rickettsia barbariae*, *Vermipsylla alakurt*, North-western China

## Findings

### Background

Fleas (Insecta: Siphonaptera) are small, laterally flattened, wingless, and highly specialised insects [1]. About 2575 species belonging to 16 families and 246 genera have been described, but only a minority is closely associated with humans and other animals [1, 2]. *Vermipsylla* is a genus of the family Vermipsyllidae within the Siphonaptera [3–5]. At least eight species, i.e.

*Vermipsylla alakurt* (Kazakhstan, Mongolia, China), *V. asymmetrica* (China), *V. ibexa* (China), *V. minuta* (China), *V. parallela* (China), *V. perplexa* (China, Nepal), *V. quilianensis* (China) and *V. yae* (China), have been described [6, 7]. *Vermipsylla alakurt* was first identified in China in 1965, in the southern region of Xinjiang Uygur Autonomous Region (XUAR, north-western China) [6]. During December to January, the adult flea is mainly endemic in alpine pastoral areas in XUAR and Qinghai Province (northern China). It infests sheep, yaks and horses, and causes irritation, poor condition, anemia and even death [8–10].

Fleas are mainly blood vessel feeders [11]. The effect of concern of this dietary preference is that fleas

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themselves are hosts to pathogens, and thus provide a natural avenue for pathogen dispersal [12, 13]. Members of the Rickettsiaceae, such as *Rickettsia typhi* and *R. felis*, are well known as flea-borne pathogens [14]. To the best of our knowledge, little is known about rickettsial agents in *V. alakurt*. In the present study, a molecular investigation was carried out to identify *Rickettsia* spp. in *V. alakurt*.

## Methods

### Collection of fleas and morphological identification

In December 2013, fleas (133 in total) were collected directly from the tails of three sheep flocks ( $n = 335$ ) at Yeyike Town (3300 m above sea level; 36°74' 93" N, 83° 00' 26" E), Minfeng County, near the Taklimakan Desert, in the southern region of XUAR. The fleas were first identified morphologically. According to an agreement between the Veterinary Research Institute, Xinjiang Academy of Animal Sciences (XAAS) and the School of Medicine, Shihezi University (SU), the fleas were divided into three samples on the basis of their number of morphological differences at the species level. One sample ( $n = 78$ ), belonging to XAAS, was used for full-length mitochondrion sequencing (these data have not been published). The second sample ( $n = 53$ ), belonging to SU, was used for the molecular study of fleas and the detection of flea-borne pathogens. The last sample ( $n = 2$ , a male and a female) was for morphological identification by the two cooperating units [6, 15].

### Molecular studies on fleas

Total genomic DNA of 53 fleas was extracted from individual specimens using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). The DNA of six randomly selected fleas was employed for multi-locus sequence analysis using four genes [18S ribosomal DNA (18S rDNA), 28S ribosomal DNA (28S rDNA), cytochrome *c* oxidase subunit II (*COII*) and elongation 1-alpha (*EF-1a*)] to examine the phylogenetic relationships within the Siphonaptera. The primers in this study were shown in Table 1. The PCR cycling condition consisted of a pre-PCR step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 40 s, annealing for 50 s at 59.9 °C for amplifying 18S rDNA and 28S rDNA, 52.6 °C for *EF-1a* gene, and an extension of 72 °C for 1 min, with a final extension of 72 °C for 10 min. The PCR products were purified using the TIANgel Midi Purification Kit (TIANGEN) and sequenced by Sangon Biotech Co., Ltd (Shanghai, China).

### Detection of rickettsial agents and sequence analysis

For genetic detection of *Rickettsia* spp., six PCR targets were assessed within each sample to determine the presence of *Rickettsiae*: a 434 bp product of the gene

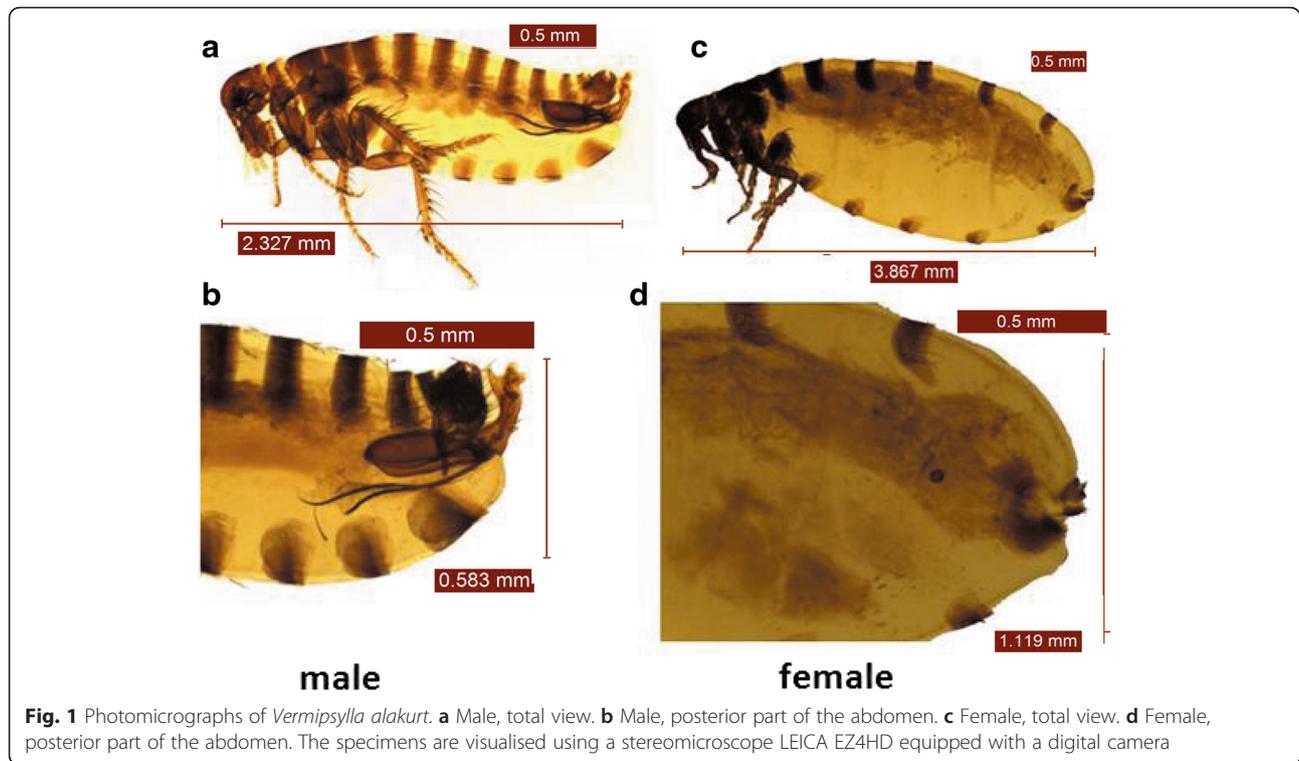
**Table 1** List of the primers used in the study

Gene	Primer	Sequence (5'–3')	Reference
18S rDNA	18Sai	CCTGAGAAACGGCTACCACATC	[26]
	18S7R	GCATCACAGACCTGTTATTGC	[26]
28S rDNA	28SrD3.2a	AGTACGTGAAACCGTTTCASGGGT	[26]
	28SrD5b	CCACAGCGCCAGTCTGCTTAC	[26]
<i>COII</i>	A-tLEU	ATGGCAGATTAGTGCAATGG	[27]
	B-tLYS	GTTTAAGAGACCAGTACTTG	[27]
<i>EF-1a</i>	EF-1a-F	GGACACAGAGATTTTCATCAAGAACA	This study
	EF-1a-R	GCAATGTGRGCHGTGTGGCA	This study
<i>ompB</i>	ompB-F	ATTTACAAGCAGGTGGTG	This study
	ompB-R	GCAGTGTACCAGGGATTG	This study
<i>geneD</i>	geneD-F	CGGTAACCTAGATACAAGTGA	This study
	geneD-R	TATAAGCTATTGCGTCATCTC	This study

encoding the 17 kilodalton antigen (17-kDa), 1332 bp of 16S rRNA (*rrs*), 1060 bp of citrate synthase (*gltA*), 488 bp of cell surface antigen 1 (*sca1*), 491 bp of outer membrane protein A (*ompA*), and 812 bp of *ompB*, according to a previous description [16]. To confirm further the presence of rickettsial DNA in *V. alakurt*, two new pairs of primers were designed, based on another region of *ompB* (526 bp) and the PS120-protein-encoding gene (*gene D*; 920 bp) fragment sequences (accession no. GU353186 and EU272188) (see Table 1). PCR conditions consisted of a pre-PCR step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 40 s, annealing for 30 s at 55 °C for amplifying *ompB* and *gene D*, and an extension of 72 °C for 1 min, with a final extension of 72 °C for 10 min. Each PCR assay included a negative control (distilled water instead of flea DNA template) and a positive control (with DNA from *R. raoultii* obtained from wetlands of Ebinur Lake in XUAR) [17]. Purification and sequencing of the positive PCR products were as described above. A phylogenetic tree was constructed using the maximum-likelihood and neighbor-joining algorithms implemented in MEGA 6 software [18].

## Results

The collected fleas were identified primarily as *V. alakurt* by morphological identification. Their foreheads were smooth and curved without outgrowths. The head of the intromittent organ of the male fleas looks like winter gloves (with the back four fingers held together) (Fig. 1a, b). The head of the spermathecae is ellipsoid, and the tail part is thin and long, with a sausage-like shape (Fig. 1c, d). Data on the four nucleotide sequences (18S rDNA, 28S rDNA, *COII* and *EF-1a*) from the six fleas indicated that the fleas obtained from the sheep had similarity values of 99.22, 98.28, 87.62 and 91.29 %, respectively, with *Chaetopsylla* (*Vermipsyllidae*) (no *V.*



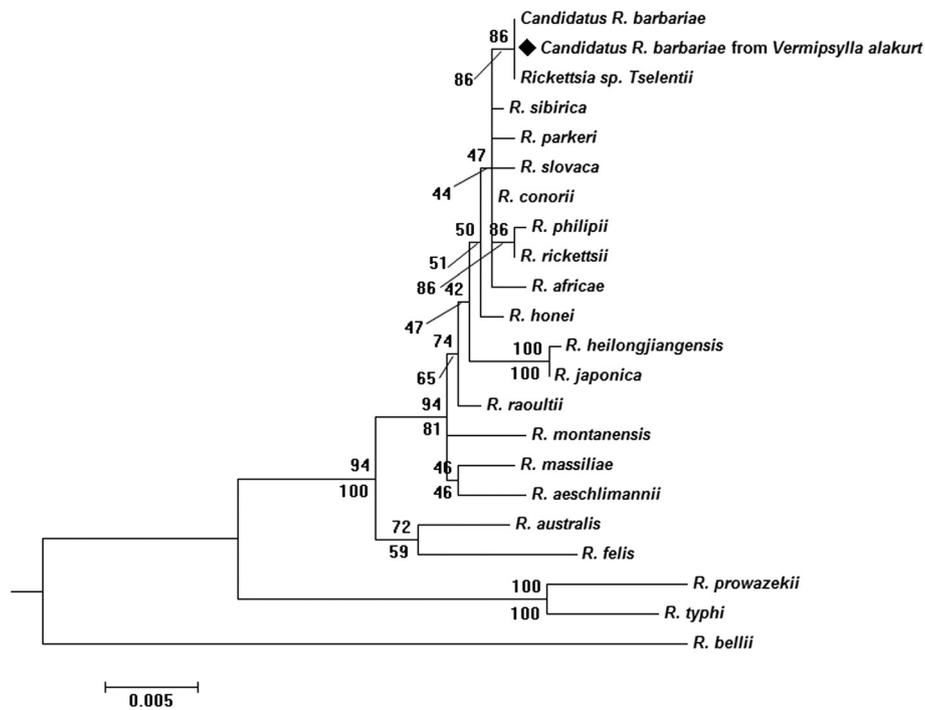
*alakurt* sequence was available in the GenBank database). Four nucleotide sequences from our study have been deposited in the GenBank database (18S rDNA: KR297206; 28S rDNA: KR297207; *COII*: KT193612; and *EF-1a*: KT193613).

Among the 53 flea samples, nine were found to be positive for six rickettsial genetic markers (*17-kDa*, *ompA*, *gltA*, *rrs*, *sca1* and *ompB*). The positive samples were from two sheep flocks. BLAST analysis showed that three of the genetic markers exhibited 99.83 to 100 % similarity with the corresponding sequences of *Candidatus R. barbariae*. The genes *gltA*, *ompB* and *sca1* were exceptions. This was attributed to: (i) the length of the *gltA* sequence from our study was 1080 bp, longer than the available sequences for *Candidatus R. barbariae* in GenBank; (ii) the partial region of *ompB* used in this study (accession no. KT284717) was different from the fragment of the *Candidatus R. barbariae* available in GenBank (accession no. GU353186); (iii) there is no *sca1* reference sequence available from *Candidatus R. barbariae*. To further identify the rickettsial agent in *V. alakurt*, another region of *ompB* (526 bp, accession no. KU645285) and *gene D*, encoding PS120-protein (920 bp, accession no. KU645286), were studied. The BLAST analysis of these sequences showed that they had, respectively, 100 % similarity with *Candidatus R. barbariae* in the loci *ompB* and *gene D*. The detailed sequence information from our study is deposited in the GenBank database (KT284715–KT284718

and KU645283–KU645286). The phylogenetic tree produced from the maximum likelihood and neighbor-joining analyses of the sequence data for five genes (*17-kDa-ompA-rrs-geneD-ompB*) revealed that the rickettsial agent in *V. alakurt* clustered with *Candidatus R. barbariae* (Fig. 2).

## Discussion

*Candidatus R. barbariae*, first identified in *Rhipicephalus bursa* ticks from Portugal in 2006 and named *Rickettsia* sp. PoTiRb169 [19], was confirmed and characterised by five genetic markers (*gltA*, *ompA*, *ompB*, *sca4* and *rrs*) in *Rh. turanicus* from Italy in 2008 [20]. Subsequently, the *Candidatus R. barbariae* genotype was respectively detected in *Rh. turanicus* from Cyprus in 2011 and in *Rh. turanicus* and *Rh. sanguineus* from Israel in 2014 [21, 22]. To confirm that the southern region of XUAR might be a natural focus for *Candidatus R. barbariae*, a total of 117 *Rh. turanicus* were collected from sheep during 2013–2014 in six counties around the Taklimakan Desert with the help of Associate Prof. Shi-Wei Wang (College of Animal Science & Technology, Tarim University). Of these, 36 samples (30.76 %) from the six counties were positive for *Candidatus R. barbariae* by seven rickettsial genetic markers (*17-kDa*, *gltA*, *rrs*, *ompA*, *sca1*, *gene D*, and *ompB*). Additionally, we concluded that *Candidatus R. barbariae* may have co-circulated with *R. massiliae* and *R. conorii*, vectored as



**Fig. 2** Maximum-likelihood (ML; 1000 bootstrap replicates) and neighbour-joining (NJ; 500 bootstrap replicates) phylogenetic tree of the *17-kDa-ompA-rs-geneD-ompB* constructed with MEGA6, using the sequences of *Candidatus R. barbariae* from *Vermipsylla alakurt* (◆) in this study and sequences from *Rickettsia* species retrieved from the GenBank database. The sequences for *R. bellii* were used as an outgroup. The scale bar represents the inferred substitutions per nucleotide site. The relative support for clades in the tree produced from the ML and NJ analyses are indicated above and below the branches, respectively

*Rh. turanicus*, near the Taklimakan Desert before 2013. These findings will be reported in a separate paper.

*Candidatus R. barbariae*, an emerging member of the rickettsial spotted fever group (SFG) [21], has not been reported previously in fleas. Although the vast majority of the SFG rickettsiae are transmitted by ticks, there are exceptions. *Rickettsia africae*, a member of the SFG ordinarily transmitted by ticks, was detected in *Ceratophyllus garei* fleas from passerine birds that had migrated from Africa [23]. Herein, we report the presence of *Candidatus R. barbariae* in *V. alakurt* fleas from sheep in an alpine pastoral area in the north-west of China. This has extended our knowledge of the potential vector spectrum of *Candidatus R. barbariae*.

To date, seven validated SFG *Rickettsia* spp. have been detected in China: *R. heilongjiangii*, *R. sibirica*, *R. raoultii*, *R. slovaca*, *R. felis*, *R. aeschlimannii* and *R. massiliae* [17, 24]. *Rickettsia felis* was first confirmed in ticks (*Rh. sanguineus*), mosquitoes (*Anopheles sinensis* and *Culex pipiens pallens*), lice (*Linognathus setosus*) and fleas (*Ctenocephalides felis*) in China [25]. Here, *Candidatus R. barbariae*, as the eighth validated *Rickettsia* species, was found in China. To the best of our knowledge, this finding extends the area of occurrence for *Candidatus R. barbariae*, and is the second report in Asia.

Our findings suggest that the *V. alakurt* parasitising sheep may serve as a carrier for *Candidatus R. barbariae*. In the future, *Candidatus R. barbariae* should be genotypically explored by using genomic sequences or other genetic markers. Additionally, this rickettsial agent should be further investigated in a wider spectrum in arthropods.

### Conclusions

This is the first report of the presence of *Candidatus R. barbariae* in *V. alakurt* fleas rather than ticks, and of the occurrence of *Candidatus R. barbariae* in China. These findings extend our knowledge of the geographical distribution and reservoir hosts for *Candidatus R. barbariae*.

### Abbreviations

SFG, spotted fever group; SU, Shihezi University; XAAS, Xinjiang Academy of Animal Sciences; XUAR, Xinjiang Uygur Autonomous Region

### Acknowledgements

We would like to thank Dr Ke Zhang for the sequences submitted to the GenBank. Pro. Renfu Shao and Xinquan Zhu revised the manuscript.

### Funding

This research was supported in part by grants from the National Natural Science Foundation of China (Granted No. 81560338 and U1503283).

**Availability of data and material**

The datasets supporting the conclusions of this article are available in the GenBank (National Center for Biotechnology Information) [unique persistent identifier and hyperlink to datasets in <http://www.ncbi.nlm.nih.gov/genbank/>].

**Authors' contributions**

YZW conceived and designed the study and critically revised the manuscript. XPY, ZQL, CFC and YZW performed flea collection. SSZ, HYL and XPY performed the laboratory work. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

This study was approved by the Animal Ethics Committee of Shihezi University (Approval No. AECSU2013-17).

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Received: 24 February 2016 Accepted: 31 May 2016

Published online: 07 June 2016

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