Letter to the Editor

Bartonella Species Investigated among Rodents from Shaanxi Province of China*

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Bartonella spp. are rod-shaped, Gram-negative, aerobic, fastidious, slow bacteria which cause diseases in humans and animals by parasitizing the endothelial and red blood cells of their hosts[1,2]. Rodents are the most important natural reservoir hosts of *Bartonella*[3]. In 57 countries, epidemiological studies of rat-borne *Bartonella* have been carried out. Information on the *Bartonella* infection rate in rodents has been obtained from most of these countries. The infection rate of rodents in Portugal, Egypt, Japan, Canada, and the United States is more than 90%, the infection rate in Thailand and Russia is about 80%, and the infection rate in China is about 67%. To date, 22 species of rodent-borne *Bartonella* have been described. Of these, *B. grahamii, B. vinsonii* subsp. *arupensis*, *B. vinsonii* subsp. *Berkhoffii*, and *B. elizabethae* have been found to be associated with human illness. People become infected with rodent-borne *Bartonella* incidentally, especially when they are exposed to the habitats of wild rodents harboring various *Bartonella* species. *Bartonella* infection can affect multiple organs and poses a risk to public health[4].

In China, 16 species of *Bartonella* have been isolated from rodents. The investigation was carried out in various provinces including Heilongjiang, Fujian, Zhejiang, Yunnan, Hainan, Qinghai, Inner Mongolia, and Taiwan China[4]. No investigation has been reported in Shaanxi province.

From north to south in Shaanxi province, the North Mountains and Qinling Mountains divide Shaanxi province into three natural regions: Shanbei Plateau, Guanzhong Plain, and Qinba Mountain. The province spans the northern subtropics, warm temperate zone, and temperate zone. Because of the variety of landscape types, 55 species of rodents are found in Shaanxi province[5].

In order to investigate *Bartonella* species in rodents in Shaanxi Province, rodents were captured using mouse snap traps in 9 counties; three on the Shanbei Plateau (Dingbian, Yuyang, and Wuqi counties), two on the Guanzhong Plain (Dali and Chang'an counties), and four in the Qinba Mountains (Zhenping, Nanzheng, Ningshan, and Zhenba counties) (Table 1 and Supplementary Figure S1, available in www.besjournal.com), between 2014 and 2017. Following the capture of each animal, we recorded collection time, site, habitat, species, gender, weight, head-body length, and tail length, and collected spleen samples under sterile conditions, which were transported back to the laboratory in liquid nitrogen and stored at −80 °C until use.

Tweenty five mg spleen was homogenized within 200 μL sterilized trypsin soy broth, plated onto trypsin soy agar containing 5% (vol/vol) defibrinated sheep blood, incubated at 37 °C with 5% CO_2, and later checked for growth of *Bartonella* species on alternate days for up to 20 d. To obtain pure colonies, suspected colonies were selected and separately subcultured twice on fresh agar plates[6]. Small, round, gray-white colonies were morphologically identified as *Bartonella*, transferred onto fresh plates, and then stored in 30% glycerol in a freezer at −80 °C for further analysis.

DNA templates were prepared directly from bacterial colonies by the boiling method. The sample

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was added to 100 μL sterile deionized water, heated for 10 min at 100 °C and centrifuged at 6,000 rpm for 5 min at 4 °C. The supernatant was used as a source of DNA template for PCR to detect the *Bartonella* citrate synthase (*gltA*) gene. The primers used for the amplification of the 379-base pair fragment were forward BhCS781.p (5′-GGGGACCAGCTCATGGTGG-3′) and reverse BhCS1137.n (5′-AATGCAAAAAGAA

Table 1. Prevalence of *Bartonella* spp. in rodents

Taxonomic family of host	Host species	Shanbei Plateau			Guanzhong Plain			Qinba Mountain			Overall		
		n	(+)	%	*n*	(+)	%	*n*	(+)	%	*n*	(+)	%
Cricetidae	*Cricetulus barabensis*	4	1	25.00	–	–	–	–	–	–	4	1	25.00
	Meriones unguiculatus	216	75	34.72	–	–	–	–	–	–	216	75	34.72
	Eothenomys melanogaster	–	–	–	–	–	–	3	1	33.33	3	1	33.33
	Meriones meridianus	5	2	40.00	–	–	–	–	–	–	5	2	40.00
	Phodopus roborovskii	7	1	14.28	–	–	–	–	–	–	7	1	14.28
	Spermophilus alashanicus	1	0	0.00	26	2	7.69	–	–	–	27	2	7.41
	Tscherskia	2	0	0.00	1	0	0.00	–	–	–	3	0	0.00
	Allocricetulus	–	–	–		–	–	2	0	0.00	2	0	0.00
	Eothenomys Inez	–	–	–	1	0	0.00	1	0	0.00	2	0	0.00
	Cricetulus longicaudatus	9	0	–	–	–	–	–	–	–	9	0	0.00
Muridae	*Niviventer confucianus*	–	–	–	–		–	64	25	39.06	64	25	39.06
	Mus musculus	5	2	40.00	–	–		1	0	0.00	6	2	33.33
	Apodemus chevreri	–		–	2	0	0.00	25	5	20.00	27	5	18.52
	Apodemus draco	–		–	1	0	0.00	26	5	19.23	27	5	18.52
	Apodemus peninsulae	–		–	1	0	0.00	16	3	18.75	17	3	17.65
	Rattus norvegicus	4	0	0.00	10	0	0.00	17	1	5.88	31	1	3.22
	Niviventer andersoni	–	–	–	–	–	–	1	0	0.00	1	0	0.00
	Micromys minutus	–	–	–	–	–	–	1	0	0.00	1	0	0.00
	Apodemus peninsulae	–	–	–	–	–	–	5	0	0.00	5	0	0.00
	Rattus nitidus	–	–	–	–	–	–	1	0	0.00	1	0	0.00
	Apodemus agrarius	–	–	–	3	0	0.00	–	–	–	3	0	0.00
	Rattus tanezumi	–	–	–	–	–	–	2	0	0.00	2	0	0.00
	Vernaya fulva	–	–	–	–	–	–	2	0	0.00	2	0	0.00
	Niviventer fulvescens	–	–	–	–	–	–	1	0	0.00	1	0	0.00
	Platacanthomyidae	–	–	–	–	–	–	1	0	0.00	1	0	0.00
Dipodidae	*Allactaga sibirica*	5	1	20.00	–	–	–	–	–	–	5	1	20.00
Ochotonidae	*Ochotona daurica*	15	3	20.00	–	–	–	–	–	–	15	3	20.00
Total		273	85	31.14	45	2	4.44	169	40	23.67	487	127	26.08

CAGTAAACA-3′)[7]. PCR was performed in 50 μL mixtures containing 25 μL 2X TaqPCR Master Mix, 22 μL double-distilled H_2O, 1 μL (10 mol/L) of each primer, and 1 μL of DNA template.

The PCR amplification conditions were as follows: an initial step of 94 °C for 2 min; 30 amplification cycles, each consisting of 94 °C for 30 s and 48 °C for 30 s; an elongation step of 72 °C for 1 min, and a final incubation at 72 °C for 5 min. Amplified products were electrophoretically analyzed on 1% agarose gels supplemented with 0.005% of GoldView and visualized under UV light. PCR products of the expected length were then purified and sequenced on both strands by Tsingke Biotechnology (Xi'an, China).

The nucleic acid sequence homology was blasted against reported *Bartonella* species sequences in GenBank using the BLAST program at the National Center for Biotechnology Information Website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Individual gene sequences and concatenated sequences assembled for *gltA* were aligned using ClustalW, and Neighbor-joining (NJ) phylogenetic tree analysis was performed using MEGA6.06[8] with the Kimura 2-parameter model and with *Brucella abortus* as the outlier group. Bootstrap calculations were carried out for 1,000 replicates[9]. A criterion of ≥ 96% homology to *gltA* was used to define phylogroups[10].

Chi-Square tests of difference in the prevalence of *Bartonella* strains in rodents were conducted with $P < 0.05$ considered the threshold for statistical significance using the Statistical Package for the Social Sciences (SPSS 19, Chicago, IL).

Rodents (487 individuals) were sampled from nine counties within three regions of Shaanxi Province to detect the presence of *Bartonella* infection. The tested rodents represented 27 species belonging to four families (Table 1). The overall prevalence of *Bartonella* species in rodents was observed to be 26.08% (127/487). Statistical analysis showed that the prevalence of *Bartonella* infection varied significantly among regions ($\chi^2 = 15.056$, $P = 0.001$), counties ($\chi^2 = 36.964$, $P = 0.000$), and species ($\chi^2 = 42.758$, $P = 0.000$).

Bartonella infection was found in 14 species of rodents in eight counties. The highest *Bartonella* prevalence were in *Meriones meridianus* (40.00%, 2/5) and *Mus musculus* (40.00%, 2/5), followed by *Niviventer confucianus* (39.06%, 25/64) and *Meriones unguiculatus* (34.72%, 75/216) (Table 1). A high *Bartonella* prevalence was found in rodents collected from Zhenping county (40.38%, 21/52) and Dingbian county (34.98%, 78/223) (Table 2).

Sequencing and phylogenetic analysis of the *gltA* fragment was performed among representative isolates (Figure 1 and Supplementary Table S1, available in www.besjournal.com). A criterion of ≥ 96% homology to *gltA* was used to define phylogroups, and the sequences were divided into 10 phylogroups. The homology of GP1, Gp4, Gp5, Gp8, and Gp10 was above 97% with *B. queenslandensis*, *B. sylvatica*, *B. taylorii*, *B. jaculi*, and *B. japonica*, respectively. GP2, GP3, and Gp9 were above 97% with *B. elizabethae*, *B. grahami*, and *B. washoensis*, respectively. The latter strains are pathogenic to humans. We did not find sequences of ≥ 96% homology clustered with Gp6 and Gp7 in the GenBank. We used the NJ and maximum likelihood (ML) methods to construct phylogenetic trees and obtained the same results. Thus, NJ method was used for further analysis.

In this study, *B. elizabethae* was detected from *Meriones unguiculatus* in Dingbian county in the Shanbei Plateau region. In this county, *Meriones unguiculatus* is the dominant species, accounting for 90% of rodent communities, and thrives in the desert and semi-desert steppe, as well as sandy or salinized farmland, ridges, wasteland, thickets and so on. In China, *Meriones unguiculatus* is distributed in Inner Mongolia, Jilin, Liaoning, Hebei, Shanxi, Shaanxi, Gansu, and Ningxia provinces.

B. washoensis was detected from *Spermophilus alashanicus* in Dali county in the Guanzhong Plain region. *Spermophilus alashanicus* is very common in this area, mainly living in dry grassland and desert steppe. It is a solitary species, each individual inhabiting a single hole in a habitat of low and sparse plants, roadsides, ridges, fallow land and so on. This animal is distributed in Inner Mongolia, Shanxi,

Table 2. Prevalence of *Bartonella* spp. in counties

Regions	County	No. examined	No. (%) positive
Shanbei Plateau	Dingbian	223	78 (34.98)
	Yuyang	35	4 (11.43)
	Wuqi	16	3 (18.75)
Guanzhong Plain	Dali	36	2 (5.56)
	Chang'an	8	0 (0.00)
Qinba Mountain	Zhenping	52	21 (40.38)
	Nanzheng	29	3 (10.34)
	Zhenba	30	7 (23.33)
	Ningshan	58	9 (15.52)
Total		487	127 (26.08)

Shaanxi, Gansu, Ningxia provinces.

B. grahamii was detected in Ningshan county from *Rattus norvegicus* and in Zhenba county from *Rattus confucianus* in Qinba Mountain. *Rattus norvegicus* is distributed all over the world. It is very adaptable to different environments and can inhabit and breed in cities and villages in every season. *Rattus confucianus* is a common species in Qinba Mountain and it is widely distributed especially in the provinces located south of the Yangtze River. This species lives in vegetation-rich mountain forests, thickets, thatched grass, brook grass, weeds near farmland, rock seams, wasteland, and vegetable gardens, etc.

B. elizabethae was detected from *Meriones unguiculatus; B. washoensis* was detected from *Spermophilus alashanicus; B. grahamii* was detected from *Rattus norvegicus* and *Rattus confucianus* and so on. This indicates that *Bartonella* has host specificity.

Meriones unguiculatus, *Spermophilus alashanicus*, *Rattus norvegicus*, and *Rattus confucianus* are widely distributed in the above-mentioned habitats. People working or living in these places who come into close contact with rodents are vulnerable to pathogenic *Bartonella* infection. Ningshan county is located in the Qinling Mountain area and Zhenba county is located in the Bashan area. Qinling Mountain and Bashan Mountain are the main tourist areas of Shaanxi province, and visitors to this area are vulnerable to infection by pathogenic bacteria if they do not pay attention to personal hygiene. Therefore, the risk of *Bartonella* infection should be assessed.

Small mammals, as the largest host group of *Bartonella*, have great regional differences.

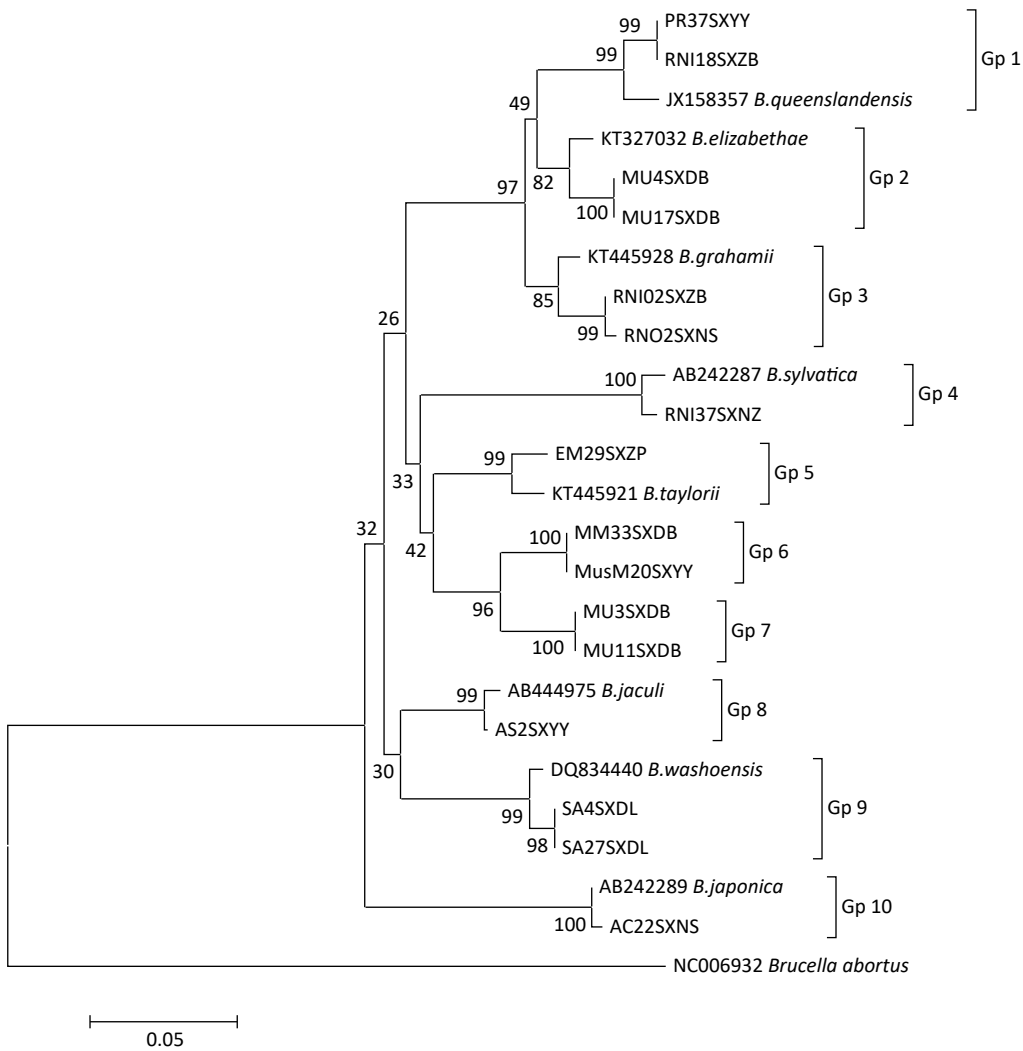

Figure 1. Phylogenetic tree analysis based on gene sequences of *gltA*.

Bartonella has a wide adaptability to different geographical environments and its geographical distribution characteristics are associated with its host animals[4]. In this study, the highest infection rate of *Bartonella* was found in Zhenping county (40.38%). Zhenping county is located in the southernmost end of Shaanxi province, at the northern foot of the Daba Mountain range, and at the junction of Sichuan, Shaanxi, and Hubei provinces. The second highest infection rate was found in Dingbian county (34.98%), which is located in the northwest corner of Shaanxi province, at the transition zone between the Loess Plateau and the desert steppe, and at the junction of Shaanxi, Gansu, Ningxia, and Inner Mongolia provinces. The sampling area of Zhenba county was located at the junction of Shaanxi, Sichuan, and Chongqing provinces, and Yuyang area is located at the border between Shaanxi and Inner Mongolia. The epidemiological significance of the detection of *Bartonella* in these four districts is also useful for reference to neighboring provinces. Of the nine counties surveyed, *Bartonella* was detected in all except Chang'an county, which indicates that *Bartonella* is widely distributed in Shaanxi province. *Bartonella* was isolated from 14 rodent species belonging to 12 genera, indicating that *Bartonella* is distributed in many host species. In the Chang'an area, no *Bartonella* was detected in 15 rat species, which may be related to the sample size and requires further investigation.

In conclusion, our study identified eight genotypes of *Bartonella* in Shaanxi province. Three of these genotypes, *B. elizabethae*, *B. grahamii*, and *B. washoensis*, are associated with human illness. There is therefore a risk infection of the human population, so population monitoring and assessment of the infection risk should be carried out. However, two genotypes could not be determined, and further studies are required to identify these genotypes through detection of related genes such as *rpoB* and *ftsZ*.

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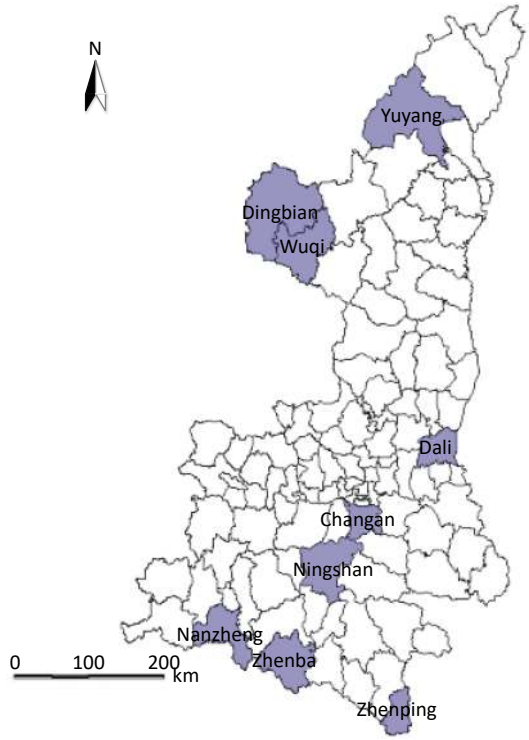

Supplementary Figure S1. Sampling sites in nine counties of Shaanxi province, China.

Supplementary Table S1. The alignment of *gltA* gene sequences with sequences retrieved from GenBank database

Sample sequence name	Species name	GenBank accession number	Identity (%)	Group
PR37SXYY, NC18SXZB	*B. queenslandensis*	JX158357	97.7	1
MU4SXDB, MU17SXDB	*B. elizabethae*	KT327032	97.7	2
NC02SXZB, RN2SXNS	*B. grahamii*	KT445928	97.3–97.7	3
NC37SXNZ	*B. sylvatica*	AB242287	99.7	4
EM29SXZP	*B. taylorii*	AT445921	97.7	5
MM33SXDB, MuM20SXYY	*B. vinsonii* subsp. *arupensis*	FJ946842	92.0	6
MU3SXDB, MU11SXDB	*B. vinsonii* subsp. *arupensis*	FJ946842	92.9	7
AS2SXYY	*B. jaculi*	AB444975	99.0	8
SA4SXDL, SA27SXDL	*B. washoensis*	DQ834440	99.0	9
AC22SXNS	*B. japonica*	AB242289	99.0	10