Preplanned Studies

Identification of Novel *Bartonella washoensis* Sequence Type 22 in *Marmota himalayana* — Jiuquan City, Gansu Province, China, 2021–2022

Ran Duan[1,&]; Xiaojin Zheng[2,&]; Qun Duan[1]; Asaiti Bukai[2]; Peng Zhang[1]; Shuai Qin[1]; Xinmin Lu[2]; Dongyue Lyu[1]; Haonan Han[1]; Dan Zhang[1]; Zhaokai He[1]; Junrong Liang[1]; Deming Tang[1]; Jinxiao Xi[3]; Huaiqi Jing[1]; Xin Wang[1,#]

Summary

What is already known about this topic?

The prevalence of rodent-adapted *Bartonella* species has been increasing significantly. However, the specific *Bartonella* species carried by *Marmota himalayana* (*M. himalayana*), a large rodent species, and the potential risk it poses to human populations remain unknown.

What is added by this report?

Bartonella washoensis (*B. washoensis*), associated with human endocarditis, was initially identified in *M. himalayana*, exhibiting a detection rate of approximately one-third and demonstrating a predilection for the heart and lungs. The discovery of the novel Sequence Type 22 has expanded both the isolation source and genetic lineage of *B. washoensis*.

What are the implications for public health practice?

Individuals residing within the *M. himalayana* plague focus are at an elevated risk for *B. washoensis* infection. Consequently, there is a pressing need for public health warnings and efficient clinical case identification in this population.

The emergence of *Bartonella* species has been identified as a cause of blood-culture-negative endocarditis. While the list of *Bartonella* species carried by rodents has rapidly expanded, species carried by *Marmota himalayana* (*M. himalayana*) remain unknown. To investigate this, *Bartonella washoensis* (*B. washoensis*) screening was conducted using samples obtained from both deceased and captured marmots during plague surveillance in Jiuquan City, Gansu Province from 2021 to 2022. The *B. washoensis* species were identified through 16s rRNA gene and multi-locus sequence typing (MLST), with phylogenetic trees constructed using the neighbor-joining method.

The detection rate of *B. washoensis* in captured marmots (29.58%, 21/71) was found to be significantly higher than that of deceased marmots (10.28%, 11/107), with relatively high rates observed in marmot heart and lung samples. The new sequence type, Sequence Type 22 (ST22), discovered in marmots, possessed five loci of novel sequences and clustered between *B. washoensis* from *Spermophilus dauricus* in China and *Spermophilus columbianus* in the United States. Importantly, human endocarditis-associated *B. washoensis* was identified for the first time and demonstrated a high prevalence in *M. himalayana*.

Our findings suggest that *B. washoensis* in marmots may have a preference for heart and lung tissue, and individuals in specific areas may be at risk of *B. washoensis* infection. Consequently, there is a pressing need for continued surveillance of *B. washoensis* and identification of clinical cases.

Overall, 13 of the 45 known *Bartonella* species are documented to infect humans. Species such as *Bartonella quintana* (*B. quintana*), *Bartonella henselae* (*B. henselae*), *B. washoensis*, *Bartonella koehlerae* (*B. koehlerae*), *Bartonella alsatica*, *Bartonella elizabethae*, and others have emerged as causes of blood-culture-negative endocarditis (*1*). Among these species, *B. henselae* and *B. quintana* are the most frequent causes of infectious endocarditis in humans (*2*–*3*). Recently, the list of *Bartonella* species carried by rodents has rapidly expanded. *M. himalayana* is the primary host of *Yersinia pestis* (*Y. pestis*) and is also known to carry a variety of bacteria and parasites pathogenic to humans (*4*–*5*). Although *Bartonella* has been reported in *M. himalayana* in China, the specific infecting species of *Bartonella* remains unidentified (*6*). In this study, human endocarditis-associated *B. washoensis* was initially identified in *M. himalayana* during the plague surveillance conducted from 2021 to 2022, and its public health risks were analyzed.

Peripheral blood mononuclear cells (PBMCs) were isolated from *M. himalayana* blood samples collected during plague surveillance in the Altun Mountains,

which is part of the *M. himalayana* plague focus in the Qinghai-Tibet Plateau (*7*). DNA was extracted from the samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The 16S rRNA gene (27F, 1492R) was amplified, sequenced, and aligned using BLASTn. Additionally, six housekeeping loci of *B. washoensis* (16S rRNA gene, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*) were amplified and sequenced (*8*). Allele numbers and sequence type (ST) assignments were determined through pubMLST (*9*).

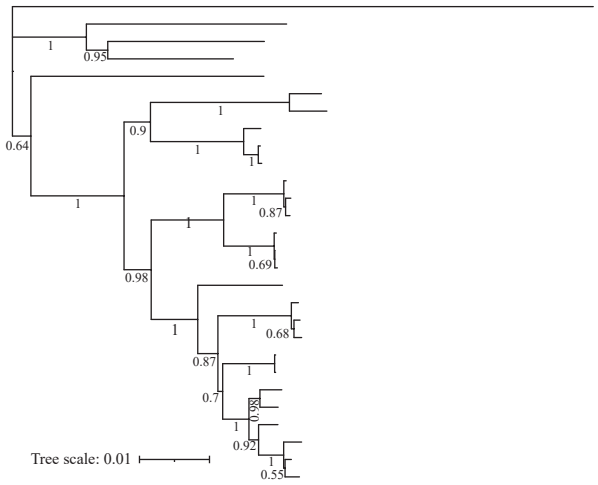
Sequences were extracted from the reference genome of 40 *Bartonella* species by aligning with six housekeeping genes from *B. washoensis*. A phylogenetic tree was constructed using the neighbor-joining (NJ) method based on the concatenated sequence of these six housekeeping genes. NJ trees were generated for both the *Bartonella* genus and the *B. washoensis* species. In the *B. washoensis* species tree, four *Bartonella* species closely related to *B. washoensis* (*B. quintana, Bartonella senegalensis*, *B. henselae*, and *B. koehlerae*) were also incorporated.

During the 2021–2022 plague surveillance in the Altun Mountains, samples were collected from six organs (heart, liver, spleen, lung, kidney, and bone) of 107 *M. himalayana* found dead in the environment and 71 live-captured marmots. These findings were within the *M. himalayana* plague focus of Qinghai-Tibet Plateau. The *B. washoensis gltA* gene was screened, sequenced, and aligned according to a previously published method (*10*). Detection rates were compared between deceased and live marmots, among the six sampled organs, and between *B. washoensis* and *Y. pestis*.

Chi-squared tests were used to compare the differences between groups, with *P*-values<0.05 considered statistically significant. Fisher's exact test was employed if the theoretical frequency ranged between 1<T<5. Statistical analyses were conducted using the SPSS software (version 19.0, IBM Corp., NY, USA).

The highest BLASTn match for the 16S rRNA gene from marmots in our study was *B. washoensis* (GenBank: AB519060.1), exhibiting 100% coverage and 100% identity. Other *Bartonella* species, such as *Bartonella volans* (GenBank: EU294521.1), were identified with 100% coverage and 99.77% identity, surpassing the species identification threshold (*10*). Based on the NJ tree of 40 *Bartonella* species, which was constructed using concatenated sequences of housekeeping genes, the sequences derived from marmots formed a cluster on the same branch as *B. washoensis*. Neighboring branches included *B. quintana*, *B. senegalensis*, *B. henselae*, and *B. koehlerae* (Supplementary Figure S1, available in http://weekly.chinacdc.cn).

The housekeeping gene allele numbers of *B. washoensis* in marmots were identified as 2-15-18-20-20-18 for the 16S rRNA gene, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*, which collectively formed the novel ST 22. Excluding the 16S rRNA gene, the other five genes displayed at least 5, 8, 18, 14, and 26 base differences from known sequences. The NJ trees for *B. washoensis* (Figure 1) revealed that *B. washoensis* isolates from marmots clustered together with those from ground squirrels (*Spermophilus*), showing the greatest similarity to *B. washoensis* from *S. columbianus*. The other four



	Country	Year	Host	Source
B. bacilliformis				
B. senegalensis				
B. koehlerae				
B. henselae				
B. quintana				
1487_18	Germany	2018	*Homo sapiens*	Mitral valve
ER14-3	China	2007	*Sciurus vulgaris orientis*	Blood
SR22-1	China	2007	*Tamias sibiricus*	Blood
SR25-1	China	2007	*Tamias sibiricus*	Blood
SR24-1	China	2007	*Tamias sibiricus*	Blood
AR4-1	USA	2007	*Tamiasciurus hudsonicus*	Blood
AR15-2	USA	2007	*Tamiasciurus hudsonicus*	Blood
AR2-2	USA	2007	*Tamiasciurus hudsonicus*	Blood
AM9-1	USA	2007	*Glaucomys volans*	Blood
AM5-1	USA	2007	*Glaucomys volans*	Blood
AM2-1	USA	2007	*Glaucomys volans*	Blood
Sb944nv	USA	2000	*Spermophilus beecheyi*	Blood
DR10-1	China	2007	*Spermophilus dauricus*	Blood
DR3-1	China	2007	*Spermophilus dauricus*	Blood
DR1-1	China	2007	*Spermophilus dauricus*	Blood
A22-731	China	2022	*Marmota Himalayana*	Blood
A22-727	China	2022	*Marmota Himalayana*	Blood
CJ25-1	USA	2007	*Spermophilus columbianus*	Blood
CJ23-2	USA	2007	*Spermophilus columbianus*	Blood
CJ22-1	USA	2007	*Spermophilus columbianus*	Blood
RJ21-1	USA	2007	*Spermophilus richardsonii*	Blood
RJ33-1	USA	2007	*Spermophilus richardsonii*	Blood
RJ30-1	USA	2007	*Spermophilus richardsonii*	Blood

FIGURE 1. Phylogenetic trees of *B. washoensis* based on concatenated sequences of housekeeping genes.
Note: Red means in this study.

Bartonella species (*B. quintana, B. senegalensis, B. henselae,* and *B. koehlerae*) formed separate clusters outside the *B. washoensis* strains.

The detection rate of *B. washoensis* in captured marmots (29.58%, 21/71) was found to be significantly higher than that in self-dead marmots (10.28%, 11/107, χ^2=10.778; *P*=0.001). In the captured marmots, the top four positive organs were bone (29.27%, 12/41), spleen (15.52%, 9/58), lung (13.56%, 8/59), and heart (9.30%, 4/43) (Table 1). Statistical differences were observed between the highest rate in the bone (29.27%) and the lowest in the liver (1.72%, χ^2=19.159; *P*<0.002). For self-dead marmots, the top four positive organs included spleen (17.50%, 7/40), lung (9.76%, 4/41), heart (7.69%, 3/39), and bone (4.00%, 4/100), with no significant differences between different organs (Fisher exact test, χ^2=9.573; *P*=0.059). The detection rate was also lowest in the liver (2.38%, 1/42) in self-dead marmots. Moreover, *B. washoensis* positive rates showed no differences between marmots with or without *Y. pestis* (Supplementary Table S1, available in https://weekly.chinacdc.cn/) (χ^2=0.628; *P*=0.428). *B. washoensis* was detected in 7.84% (4/51) of *Y. pestis*-negative self-dead marmots, and in 12.5% (7/56) of *Y. pestis*-positive self-dead marmots. The 77 *B. washoensis*-positive samples revealed three different *gltA* sequence types, displaying one to three single nucleotide polymorphisms (SNPs), all of which were synonymous mutations.

DISCUSSION

In the current study, the first identification of human endocarditis-associated *B. washoensis* was observed with a relatively high detection rate in *M. himalayana* (approximately 1/3 in captured marmots and 1/10 in self-dead marmots). An increasing number of *Bartonella* species have been found to contribute to blood culture-negative endocarditis, with *B. henselae* and *B. quintana* being the most common causes of human cases (*2–3*) (Supplementary Figure S1, available in http://weekly.chinacdc.cn) (double asterisk). *B. washoensis* has recently been associated with human and dog endocarditis (*10–11*) and exhibits a close relationship with *B. quintana* and *B. henselae* in the NJ tree, based on housekeeping gene sequences. *B. washoensis* was detected in six types of marmot organs, suggesting a systemic distribution in its carriers. The spleen, heart, lung, and bone displayed the highest detection rates. The order of rates in captured and self-dead marmots varies, which may be attributable to sample freshness or other causes of death. Nonetheless, higher rates were detected in the heart and lung of marmots, implying that *B. washoensis* may exhibit a preference for these organs. In light of its phylogenetic position in the NJ tree, it is suggested that *B. washoensis* from *M. himalayana* closely resembles human-endocarditis-related *Bartonella*. Moreover, the presence of *Y. pestis* does not impact *B. washoensis*, and *B. washoensis* may serve as a cause of marmot mortality independent of *Y. pestis*. Lastly, the detection rate of *B. washoensis* is lowest in the liver of marmots, rendering the spleen the recommended site for detection.

The detection of *B. washoensis* in *M. himalayana* has expanded the known range of rodent species that carry this bacterium, while the discovery of the novel ST22 has enhanced its genetic lineage by adding a marmot-origin branch between *S. columbianus* and *S. dauricus*. *B. washoensis* has been previously identified in squirrels from the provinces of Hebei and Zhejiang in China (*12*). However, the housekeeping gene sequences of *B. washoensis* in marmots and squirrels exhibit significant differences. Five of the six housekeeping genes, excluding the 16S rRNA gene, have 5–26 SNPs when

TABLE 1. Detection rates of *B. washoensis* in six organs of marmots.

Sample	Captured marmot			Self-dead marmot		
	Positive	Total	Positive rate (%)	Positive	Total	Positive rate (%)
Bone	12	41	29.27*	4	100	4.00
Spleen	9	58	15.52	7	40	17.50
Lung	8	59	13.56	4	41	9.76
Heart	4	43	9.30	3	39	7.69
Kidney	3	43	6.98	1	37	2.70
Liver	1	58	1.72*	1	42	2.38

* means statistical significance between positive rates of bone and liver in captured marmots.

compared with the closest known sequences. Most *gltA* genes of *B. washoensis* observed in marmots differ by two bases, suggesting their close relationship with the ST22 type. The geographical isolation of the Qinghai-Tibet Plateau may contribute to the divergence of pathogens in *M. himalayana*, such as the new bacterial species *Streptococcus respiraculi* (*13*), *Helicobacter himalayensis* (*14*), and the novel parasite species *Enterocytozoon bieneusi* (*5*). Our research team will continue efforts to isolate and purify *B. washoensis* from marmots in order to further elucidate its biological and genomic characteristics.

In this study, we identified human endocarditis-associated *B. washoensis* for the first time, with a relatively high detection rate in *M. himalayana* and even higher in the heart and lungs of marmots. The new sequence type, ST22, of *M. himalayana*-derived *B. washoensis* is considerably different from the previously reported housekeeping gene sequence, expanding the isolation source and genetic lineage of *B. washoensis. Bartonella* has become an emerging cause of human endocarditis worldwide; however, related monitoring, detection, and diagnosis are insufficient in our country. Our findings suggest that individuals in high-risk areas are susceptible to *B. washoensis* infection, necessitating public health warnings and enhanced clinical case identification.

Conflicts of interest: No conflicts of interest.

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[#] Corresponding author: Xin Wang, wangxin@icdc.cn.

[1] State Key Laboratory of Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China; [2] Akesai Kazak Autonomous County Center for Disease Control and Prevention, Jiuquan, China; [3] Gansu Provincial Center for Disease Control and Prevention, Lanzhou, China.
[&] Joint first authors.

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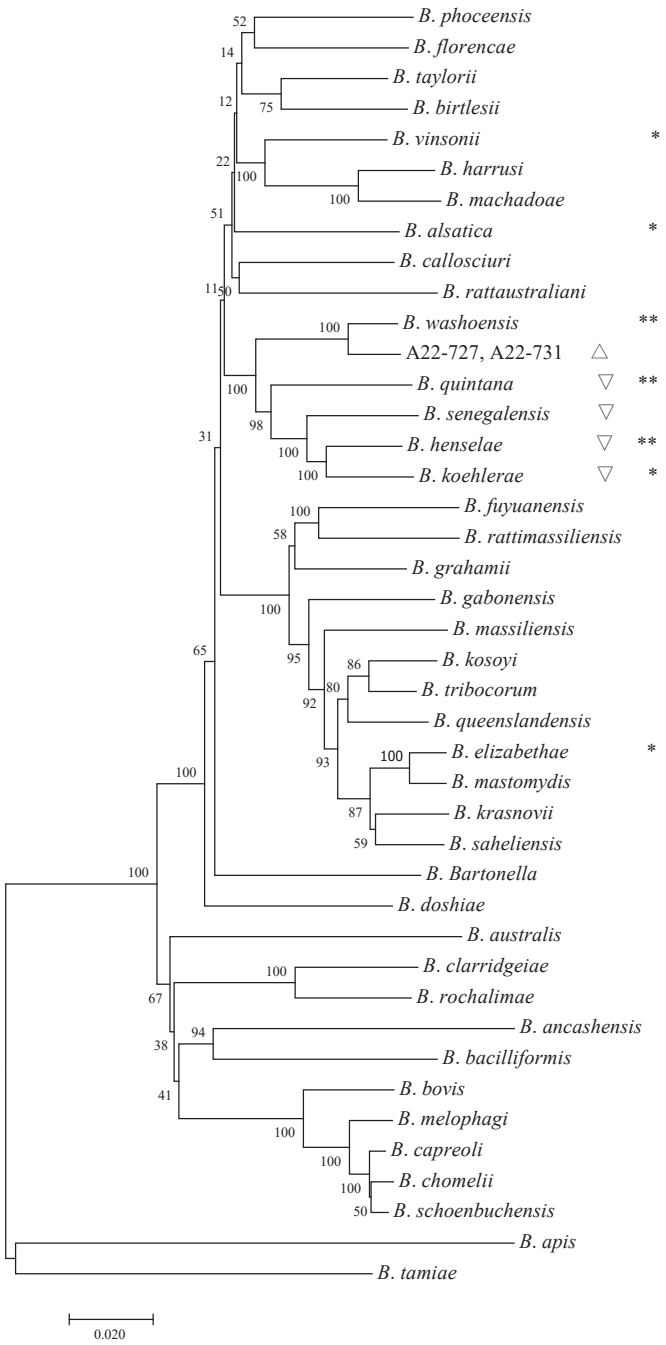

SUPPLEMENTARY FIGURE S1. *Bartonella* phylogenetic trees based on the concatenated sequence of *B. washoensis* housekeeping genes.
Note: △ means in this study; ▽ means four *Bartonella* species close to *B. washoensis*; * means animal endocarditis-related species; ** means human endocarditis-related species.

SUPPLEMENTARY TABLE S1. Prevalence of *Bartonella washoensis* in deceased marmots with and without *Yersinia pestis* infection.

Yersinia pestis	***Bartonella washoensis***		
	+	**−**	**Positive rates (%)**
−	4	47	7.84
+	7	49	12.50

Note: "+" means positive; "−" means negative.