Infectious Diseases of Poverty

SHORT REPORT

Open Access

Molecular characteristics of *Brucella melitensis* isolates from humans in Qinghai Province, China

Zhi-Jun Zhao[1], Ji-Quan Li[3], Li Ma[1], Hong-Mei Xue[1], Xu-Xin Yang[1], Yuan-Bo Zhao[1], Yu-Min Qin[1], Xiao-Wen Yang[2], Dong-Ri Piao[2], Hong-Yan Zhao[2], Guo-Zhong Tian[2], Qiang Li[1], Jian-Ling Wang[1], Guang Tian[1], Hai Jiang[1,2*] and Li-Qing Xu[1*]

Abstract

Background: The prevalence of human brucellosis in Qinghai Province of China has been increasing rapidly, with confirmed cases distributed across 31 counties. However, the epidemiology of brucellosis transmission has not been fully elucidated. To characterize the infecting strains isolated from humans, multiple-locus variable-number tandem repeats analysis (MLVA) and whole-genome single-nucleotide polymorphism (SNP)-based approaches were employed.

Methods: Strains were isolated from two males blood cultures that were confirmed *Brucella melitensis* positive following biotyping and MLVA. Genomic DNA was extracted from these two strains, and whole-genome sequencing was performed. Next, SNP-based phylogenetic analysis was performed to compare the two strains to 94 *B. melitensis* strains (complete genome and draft genome) retrieved from online databases.

Results: The two *Brucella* isolates were identified as *B. melitensis* biovar 3 (QH2019001 and QH2019005) following conventional biotyping and were found to have differences in their variable number tandem repeats (VNTRs) using MLVA-16. Phylogenetic examination assigned the 96 strains to five genotype groups, with QH2019001 and QH2019005 assigned to the same group, but different subgroups. Moreover, the QH2019005 strain was assigned to a new subgenotype, IIj, within genotype II. These findings were then combined to determine the geographic origin of the two *Brucella* strains.

Conclusions: Utilizing a whole-genome SNP-based approach enabled differences between the two *B. melitensis* strains to be more clearly resolved, and facilitated the elucidation of their different evolutionary histories. This approach also revealed that QH2019005 is a member of a new subgenotype (IIj) with an ancient origin in the eastern Mediterranean Sea.

Keywords: *Brucella melitensis*, Multiple-locus variable-number tandem repeats analysis, Whole-genome sequencing, Single-nucleotide polymorphism

*Correspondence: jianghai@icdc.cn; qhxlq2006@163.com
†Zhi-Jun Zhao and Ji-Quan Li contributed equally to this work
[1] Qinghai Institute for Endemic Disease Prevention and Control, The department of brucellosis prevention and control, Xining 810021, Qinghai, China
Full list of author information is available at the end of the article

Background

Brucellosis, which is caused by bacteria in the *Brucella* genus, is one of the most important zoonoses worldwide and is considered a "forgotten, neglected zoonosis" by the World Health Organization [1]. This disease is endemic in regions within Africa, Asia, Latin America and other

Zhao *et al. Infect Dis Poverty*   (2021) 10:42

Page 2 of 6

countries along the Mediterranean Sea [2, 3]. Human infections can occur due to the consumption of contaminated non-pasteurized milk or cheese, or by occupational exposure to infected animals or their carcasses, uterine secretions, or aborted fetuses [4]. While the mortality rate of brucellosis is low, the morbidity rate is much higher. Worldwide, incidence of human brucellosis varies widely, from < 0.01 to > 200 per 100 000 people in endemic disease areas [5]. In the mainland of China, the total incidence rate of human brucellosis increased from 0.92 per 100 000 people in 2004 to 4.2 per 100 000 people in 2014 [6]. Recently, in Qinghai Province, the incidence rate has increased from 0.04 per 100 000 people in 2011 to 1.96 per 100 000 people in 2018, with confirmed cases distributed across 31 counties within the entire province. Thus, brucellosis is becoming a major public health problem that impacts human physical and mental health.

Brucella is a gram-negative, facultative, intracellular pathogen that causes brucellosis in both humans and animals. Currently, the *Brucella* genus contains 12 accepted species, with four of those, namely, *B. melitensis*, *B. abortus*, *B. suis and B. canis* are considered zoonotic as they regularly cause humans infection [7]. While various species differ in host preference, virulence and/or zoonotic potential, most *Brucella* maintain a 97–99% genomic sequence identity [8, 9].

In humans, *B. melitensis* is the most virulent species and has been the predominant species associated with human brucellosis in China from 1953 to 2013 [10]. To ensure accurate epidemiological surveillance and to distinguish infected and vaccinated individuals, species identification and subtyping is essential [11]. Multiple loci variable-number tandem repeat analysis (MLVA) can provide the epidemiological relatedness among *Brucella* strains [12]. In a study examining *Brucella* in the Qinghai-Tibet Plateau region, none of the genotypes matched any of the sequences in the *Brucella* MLVA database (2012), possibly due to these strains having unique geographical characteristics or due to *B. melitensis* being the predominant species in that area [13]. Moreover, *Brucella* studies have also utilized whole-genome sequencing (WGS), which provides excellent genetic resolution, to resolve differences in closely related species [14]. In this study, we performed an MLVA typing of a collection of strains from the Qinghai province, 2018–2019. We selected two strains showing very distinct MLVA profiles for analysis using whole-genome single nucleotide polymorphism (SNP)-based approaches to elucidate the characteristics and homologies for epidemiological purposes. These findings will provide further insight into *Brucella* epidemiology and enable improved control and prevention of brucellosis in Qinghai Province.

Methods

Bacterial strains

Positive blood cultures were examined for brucellosis at the Qinghai Institute for Endemic Disease Prevention and Control in Qinghai Province, China, and deemed *Brucella*-positive between 2018 and 2019. The strains were identified as *B. melitensis* based on morphology and conventional identification methods according to standard biotyping procedures, including CO_2 requirement, inhibition of growth by basic fuchsin and thionin, agglutination with monospecific antisera (A, M) and phage typing (Bk_2, Tb).

Total genomic DNA was extracted from the two *B. melitensis* strains, QH2019001 and QH2019005, using a Wizard Genomic DNA Purification Kit (Promega, city, USA) according to the manufacturer's instructions. The obtained DNA was then assessed via agarose gel electrophoresis and quantified using a Qubit 2.0 Fluorometer (Thermo Scientific, city, USA).

Brucella MLVA-16 genotyping scheme

MLVA-16 was performed as previously described [14]. All data were analyzed using BioNumerics (version 5.1; Applied Maths, Sint-Martens-Latem, Belgium). Clustering analysis was based on the categorical coefficient and the unweighted pair group method using the arithmetic averages (UPGMA) method [15].

Whole-genome sequencing and single-nucleotide polymorphism analyses

Sequencing libraries were generated using a NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, city, USA) following the manufacturer's recommendations. WGS for the QH2019001 and QH2019005 strains was performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) using an Illumina NovaSeq 6000 (PE 150 bp). The raw data generated using the Illumina sequencing platform were assembled using SOAPdenovo (version 2.04). The obtained genomic sequences were available in GenBank under accession numbers of GCA 016411965.1 and GCA 016806105.1.

Genomic alignments between a sample genome and reference genome (or among more than two sample genomes) were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) using the MUMmer [16] and LASTZ [17, 18] tools. Additionally, 94 *B. melitensis* genomes (Additional file 1: Table S1) were retrieved from GenBank and used for comparison and preliminary phylogenetic analyses. A phylogenetic tree was constructed using TreeBeST and PHYML (maximum-likelihood based, with 1000 bootstrap replicates utilized) [19, 20].

Zhao *et al. Infect Dis Poverty* (2021) 10:42

Page 3 of 6

Results

Identification of *Brucella* strains

The *Brucella* strains QH2019001 and QH2019001 were identified as *B. melitensis* biovar 3 using a conventional biotyping method (Table 1).

MLVA-16 genotyping results

The MLVA-16 assay was used to determine the genotypes for 40 *B. melitensis* strains from human patients in Qinghai province, in 2018–2019. Two strains, QH2019001 (1–4-3–13-2–2-3–2-4–40-8–6-4–3-18–5) and QH2019005 (1–5-3–13-120 3–2-3–2-4–38-8–5-4–7-16–5) stand alone in the clustering analysis shown in Fig. 1, and define new MLVA-11 genotypes. For the two *B. melitensis* strains, variable number tandem repeat (VNTR) differences were

present in bruce08 and bruce42 (panel 1) and bruce19, bruce04, bruce09 and bruce16 (panels 2A and 2B). When combining these findings with epidemiological data, *B. melitensis* strain QH2019001 appears to have been imported from Anhui Province, while the QH2019005 strain appears to be native to Qinghai Province (Fig. 1).

Genomic characteristic and phylogenetic analysis based on whole-genome SNPs

The total contig sequence lengths were 3 291 786 bp for QH2019001 and 3 289 996 bp for QH2019005, with both having a GC content of 57.2%. To gain insight into the geographical distributions of the 96 examined *B. melitensis* strains (including standard strains and *B. melitensis* strains around the world), a phylogenetic

Table 1 Biotyping identification results for the two *Brucella melitensis* strain

Strain IDs	CO$_2$ Requirement	Sensitivity to dyes		Serum agglutination		RTD	
		Fuchin 1:25 000	Thionin1:50 000	A	M	BK2	Tb
QH2019001	-	+	+	+	+	+	+
QH2019005	-	+	+	+	+	+	+

RTD Routine test dilution, *BK2* Berkeley, *Tb* Tbilisi
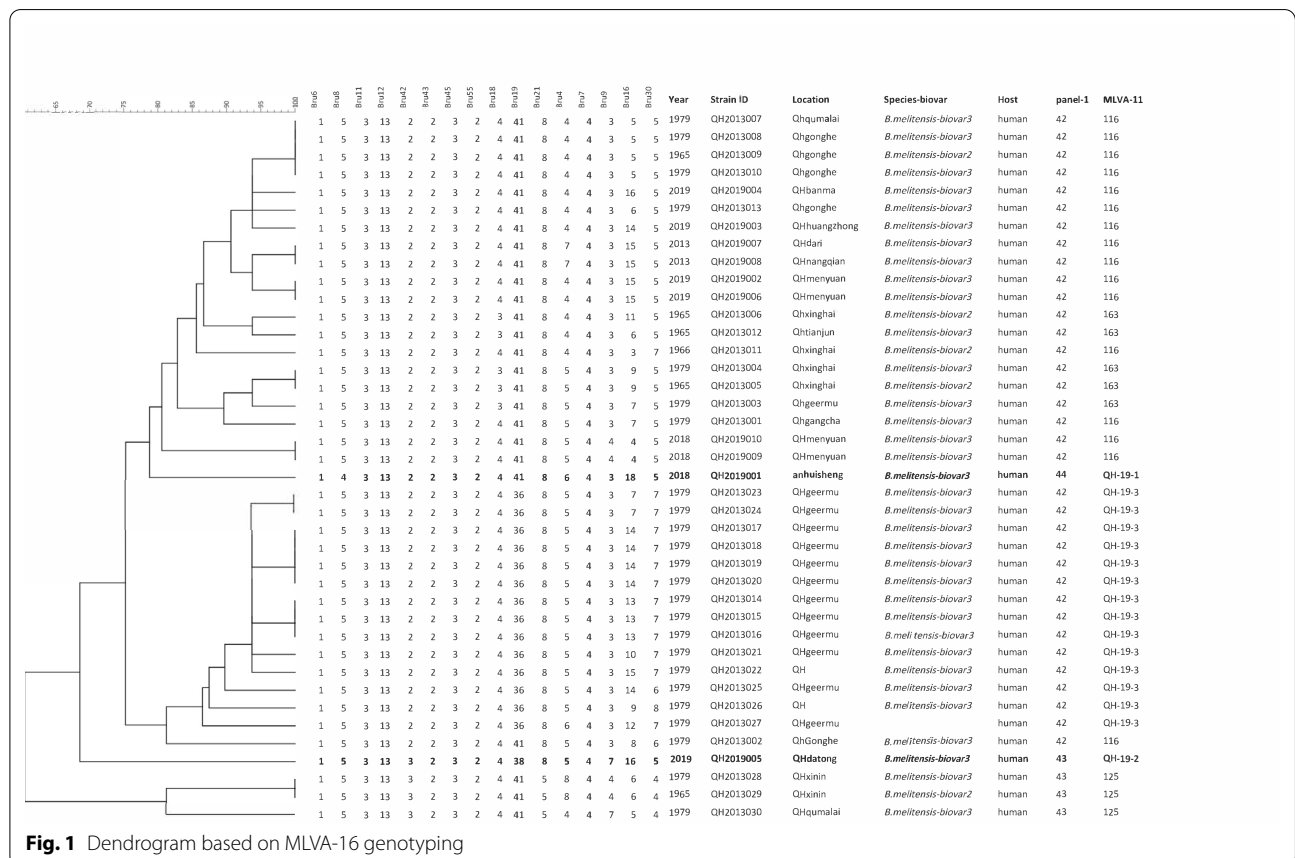
Fig. 1 Dendrogram based on MLVA-16 genotyping

Zhao *et al. Infect Dis Poverty*   (2021) 10:42

Page 4 of 6

analysis was performed using the whole-genome SNPs. The strains were divided into five major genotypes as follows: Genotype I corresponds to the West Mediterranean clade, Genotype II to the East Mediterranean clade, and genotypes III to V belong to the Americas group. Genotype III was recently designated as the African clade (strain 65/112) by Foster et al. [21] (Fig. 2). The 16M_2 strain was isolated from an infected mouse that
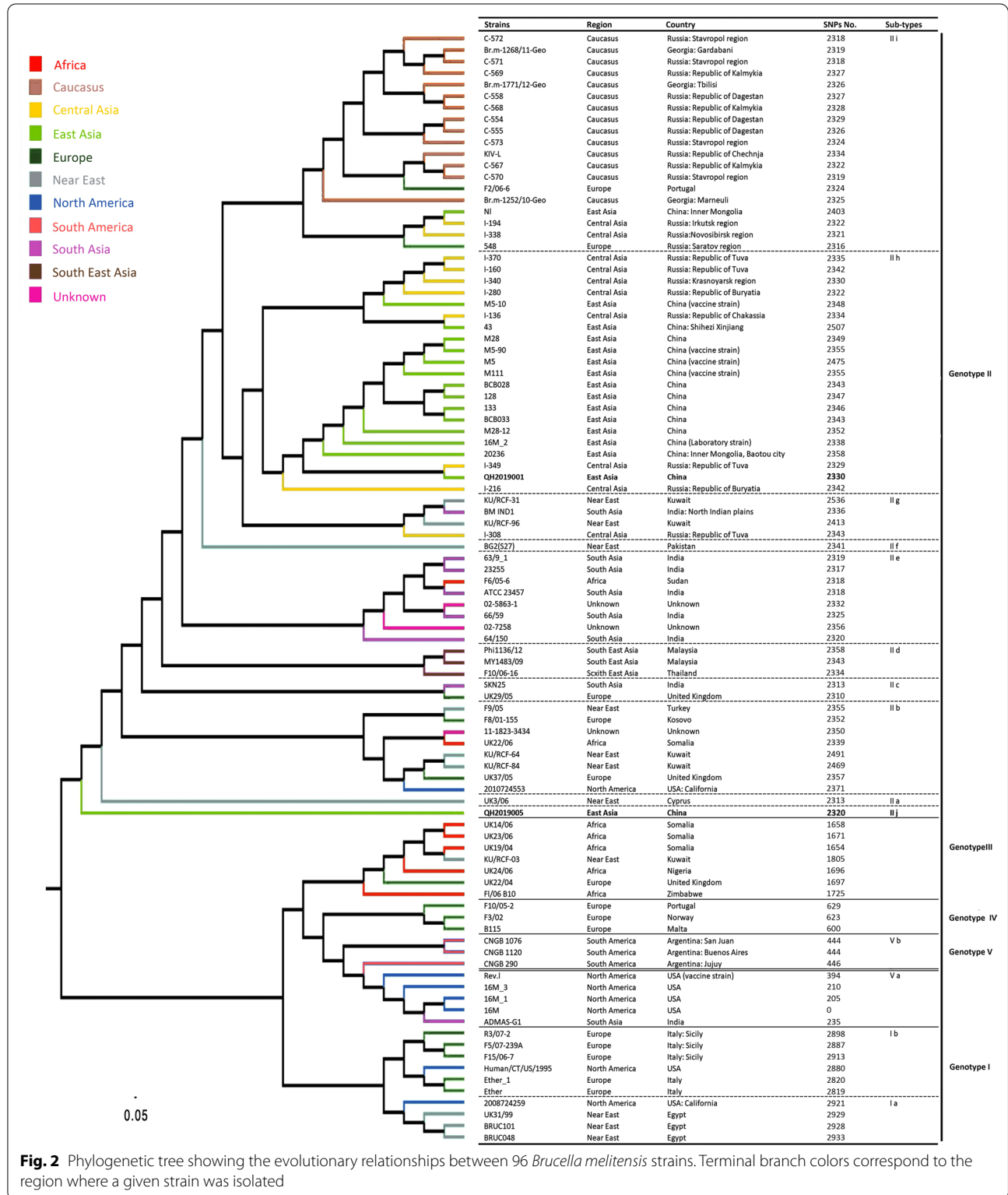

Strains	Region	Country	SNPs No.	Sub-types	Genotype
C-572	Caucasus	Russia: Stavropol region	2318	II i	
Br.m-1268/11-Geo	Caucasus	Georgia: Gardabani	2319		
C-571	Caucasus	Russia: Stavropol region	2318		
C-569	Caucasus	Russia: Republic of Kalmykia	2327		
Br.m-1771/12-Geo	Caucasus	Georgia: Tbilisi	2326		
C-558	Caucasus	Russia: Republic of Dagestan	2327		
C-568	Caucasus	Russia: Republic of Kalmykia	2328		
C-554	Caucasus	Russia: Republic of Dagestan	2329		
C-555	Caucasus	Russia: Republic of Dagestan	2326		
C-573	Caucasus	Russia: Stavropol region	2324		
KIV-L	Caucasus	Russia: Republic of Chechnja	2334		
C-567	Caucasus	Russia: Republic of Kalmykia	2322		
C-570	Caucasus	Russia: Stavropol region	2319		
F2/06-6	Europe	Portugal	2324		
Br.m-1252/10-Geo	Caucasus	Georgia: Marneuli	2325		
NI	East Asia	China: Inner Mongolia	2403		
I-194	Central Asia	Russia: Irkutsk region	2322		
I-338	Central Asia	Russia:Novosibirsk region	2321		
548	Europe	Russia: Saratov region	2316		
I-370	Central Asia	Russia: Republic of Tuva	2335	II h	
I-160	Central Asia	Russia: Republic of Tuva	2342		
I-340	Central Asia	Russia: Krasnoyarsk region	2330		
I-280	Central Asia	Russia: Republic of Buryatia	2322		
M5-10	East Asia	China (vaccine strain)	2348		
I-136	Central Asia	Russia: Republic of Chakassia	2334		
43	East Asia	China: Shihezi Xinjiang	2507		
M28	East Asia	China	2349		Genotype II
M5-90	East Asia	China (vaccine strain)	2355		
M5	East Asia	China (vaccine strain)	2475		
M111	East Asia	China (vaccine strain)	2355		
BCB028	East Asia	China	2343		
128	East Asia	China	2347		
133	East Asia	China	2346		
BCB033	East Asia	China	2343		
M28-12	East Asia	China	2352		
16M_2	East Asia	China (Laboratory strain)	2338		
20236	East Asia	China: Inner Mongolia, Baotou city	2358		
I-349	Central Asia	Russia: Republic of Tuva	2329		
QH2019001	**East Asia**	**China**	**2330**		
I-216	Central Asia	Russia: Republic of Buryatia	2342		
KU/RCF-31	Near East	Kuwait	2536	II g	
BM IND1	South Asia	India: North Indian plains	2336		
KU/RCF-96	Near East	Kuwait	2413		
I-308	Central Asia	Russia: Republic of Tuva	2343		
BG2(S27)	Near East	Pakistan	2341	II f	
63/9_1	South Asia	India	2319	II e	
23255	South Asia	India	2317		
F6/05-6	Africa	Sudan	2318		
ATCC 23457	South Asia	India	2318		
02-5863-1	Unknown	Unknown	2332		
66/59	South Asia	India	2325		
02-7258	Unknown	Unknown	2356		
64/150	South Asia	India	2320		
Phi1136/12	South East Asia	Malaysia	2358	II d	
MY1483/09	South East Asia	Malaysia	2343		
F10/06-16	Scith East Asia	Thailand	2334		
SKN25	South Asia	India	2313	II c	
UK29/05	Europe	United Kingdom	2310		
F9/05	Near East	Turkey	2355	II b	
F8/01-155	Europe	Kosovo	2352		
11-1823-3434	Unknown	Unknown	2350		
UK22/06	Africa	Somalia	2339		
KU/RCF-64	Near East	Kuwait	2491		
KU/RCF-84	Near East	Kuwait	2469		
UK37/05	Europe	United Kingdom	2357		
2010724553	North America	USA: California	2371		
UK3/06	Near East	Cyprus	2313	II a	
QH2019005	**East Asia**	**China**	**2320**	II j	
UK14/06	Africa	Somalia	1658		Genotype III
UK23/06	Africa	Somalia	1671		
UK19/04	Africa	Somalia	1654		
KU/RCF-03	Near East	Kuwait	1805		
UK24/06	Africa	Nigeria	1696		
UK22/04	Europe	United Kingdom	1697		
FI/06 B10	Africa	Zimbabwe	1725		
F10/05-2	Europe	Portugal	629		Genotype IV
F3/02	Europe	Norway	623		
B115	Europe	Malta	600		
CNGB 1076	South America	Argentina: San Juan	444	V b	Genotype V
CNGB 1120	South America	Argentina: Buenos Aires	444		
CNGB 290	South America	Argentina: Jujuy	446		
Rev.l	North America	USA (vaccine strain)	394	V a	
16M_3	North America	USA	210		
16M_1	North America	USA	205		
16M	North America	USA	0		
ADMAS-G1	South Asia	India	235		
R3/07-2	Europe	Italy: Sicily	2898	I b	
F5/07-239A	Europe	Italy: Sicily	2887		
F15/06-7	Europe	Italy: Sicily	2913		
Human/CT/US/1995	North America	USA	2880		Genotype I
Ether_1	Europe	Italy	2820		
Ether	Europe	Italy	2819		
2008724259	North America	USA: California	2921	I a	
UK31/99	Near East	Egypt	2929		
BRUC101	Near East	Egypt	2928		
BRUC048	Near East	Egypt	2933		

Legend: Africa, Caucasus, Central Asia, East Asia, Europe, Near East, North America, South America, South Asia, South East Asia, Unknown

Scale: 0.05

Fig. 2 Phylogenetic tree showing the evolutionary relationships between 96 *Brucella melitensis* strains. Terminal branch colors correspond to the region where a given strain was isolated

Zhao *et al. Infect Dis Poverty*   (2021) 10:42

Page 5 of 6

was reported to have accumulated mutations during the infection. The position in Fig. 2 shows clearly that it is not derived from 16 M. The QH2019001 and QH2019005 strains were both associated with the genotype II group. Furthermore, several clades and subclades were isolated within the lineages and were associated with geographic attributes. The QH2019001 strain was assigned to subgenotype IIh, while the QH2019005 strain was assigned to subgenotype IIj (a new subgroup).

Discussion

MLVA genotyping provides valuable insight when examining an epidemiological linkage or trace-back investigation during a brucellosis outbreak [22–24]. Based on the MLVA-11, the QH2019001 and QH2019005 strains formed unique genotypes and represented as single independent strain, which suggests that these two strains were not related epidemiologically. Thus the exact origins of the two strains could not be determined by MLVA. However, WGS-based analysis has been shown to distinguish very closely related *B. melitensis* strains and can discriminate intraspecies relationships [25, 26]. In this study, whole-genome SNP analysis showed that the QH2019001 and QH2019005 strains were both assigned to genotype II, but to different subgroups. Furthermore, *B. melitensis* strain QH2019005 was assigned to genotype IIj (a new subtype), which is closely associated with subgenotype IIa that contained only a single strain, UK3/06. The QH2019001 strain was assigned to subgenotype IIh. The differences between the QH2019001 and QH2019005 strains suggest that the nucleotide variation may be attributed to changes in geographic distribution. It may have been driven by the relatively isolated and more unique environment of the Qinghai-Tibet Plateau, with its extremely high altitudes. Due to the environment, it is difficult for lowland livestock breeds or wild animals to survive; hence, livestock exchange between Qinghai Province and other regions would be limited [13]. Further investigation of the movement of sheep should be performed to identify the origins of these strains.

The QH2019005 strain was the most similar to the UK3/06 strain that was isolated from the Near East, thus suggesting that this strain and its new subgenotype have ancient origins. The QH2019001 strain was the most similar to the I-349 strain that was isolated from Central Asia (Russia: Republic of Tuva) [27], thus suggesting that this strain shares a common origin. Furthermore, active trade between Russia and China could have promoted the transmission of *B. melitensis* strain QH2019001 to other regions (Anhui Province) in China.

In summary, we observed that strains in this region exhibited unique characteristics of origin and evolution. Further efforts, examination of more strains, WGS, and collection of epidemiological data from the neighboring provinces are needed to accurately outline the pattern of transmission of brucellosis in Qinghai, China.

Conclusion

In the present study, the molecular characteristics of two human *Brucella* strains isolated from Qinghai Province were examined to gain a further understanding of the epidemiology of brucellosis. The two strains were found to have different origins and evolutionary histories, with the native strain, QH2019005, assigned to a new subgenotype with an ancient origin in the eastern Mediterranean region. Additionally, this study further highlighted that utilizing a whole-genome SNP-based approach can enable intraspecies relationships between *B. melitensis* to be more fully examined.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40249-021-00829-0.

> **Additional file 1: Table S1.** *B. melitensis* genome information.

Availability of data and materials
All the relevant data have been provided within the manuscript or as a supporting file.

Declarations

Ethics approval and consent to participate
This research was conducted according to the principles of the Declaration of Helsinki, and the study protocol was approved by the Ethics Committees of the National Institute for Communicable Disease Control and Prevention (Beijing, China). Informed consent was obtained from the two patients who

Zhao *et al. Infect Dis Poverty*   (2021) 10:42

Page 6 of 6

received a brucellosis diagnosis at the Qinghai Institute for Endemic Disease Prevention and Control based on the Diagnosis for Brucellosis Standards (WS 269–2019) used in China. Obtained patient history and data were anonymized for the purpose of this study. Blood samples were obtained and Brucella strains were isolated.

Author details
[1] Qinghai Institute for Endemic Disease Prevention and Control, The department of brucellosis prevention and control, Xining 810021, Qinghai, China. [2] State Key Laboratory for Infectious Disease Prevention and Control, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China. [3] Key Laboratory of Plague Prevention and Research, Qinghai Institute for Endemic Disease Prevention and Control, National Health Commission (2019PT310004) and Key Laboratory for Plague Prevention and Control of Qinghai Province, Xining 810021, Qinghai, China.

References

1. Zhou K, Wu BB, Pan H, Paudyal N, Jiang J, et al. One health approach to address zoonotic BRUCELLOSis: a spatiotemporal associations study between animals and humans. Front Vet Sci. 2020;7:521. https://doi.org/10.3389/fvets.2020.00521.
2. Tuon FF, Gondolfo RB, Cerchiari NC. Human-to-human transmission of *Brucella* - a systematic review. Trop Med Int Health. 2017;22:539–46. https://doi.org/10.1111/tmi.12856.
3. Rubin B, Band JD, Wong P, Colville J. Person-to-person transmission of *Brucella melitensis*. Lancet. 1991;337(8732):14–5. https://doi.org/10.1016/0140-6736(91)93332-4.
4. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. Lancet Infect Dis. 2006;6:91–9. https://doi.org/10.1016/S1473-3099(06)70382-6.
5. Mambres DH, Boarbi S, Michel P, Bouker N, Escobar-Calle L, Desqueper D, et al. Imported human brucellosis in Belgium: bio and molecular typing of bacterial isolates, 1996–2015. PLoS ONE. 2017;12:e0174756. https://doi.org/10.1371/journal.pone.0174756.
6. Lai S, Zhou H, Xiong WY, Gilbert M, Yu HJ, Huang ZJ, et al. Changing epidemiology of human brucellosis, China, 1955–2014. Emerg Infect Dis. 2017;23:184–94. https://doi.org/10.3201/eid2302.151710.
7. Plumb GE, Olsen SC, Buttke D. Brucellosis: 'one health' challenges and opportunities. Rev Sci Tech. 2013;32:271–8. https://doi.org/10.20506/rst.32.1.2195.
8. Delvecchio VG, Kapatral V, Redkar RJ, Patra G, Mujer C, Los T, et al. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. Proc Natl Acad Sci U S A. 2002;99:443–8. https://doi.org/10.1073/pnas.221575398.
9. Zaki NA, Salloum T, Osman M, Rafei R, Hamze M, Tokajian S. Typing and comparative genome analysis of *Brucella melitensis* isolated from Lebanon. FEMS Microbiol Lett. 2017;364:fnx199. https://doi.org/10.1093/femsle/fnx199.
10. Tian GZ, Cui BY, Piao DR, Zhao HY, Li LY, Liu X, et al. Multi-locus variable-number tandem repeat analysis of Chinese *Brucella* strains isolated from

1953 to 2013. Infect Dis Poverty. 2017;6:89. https://doi.org/10.1186/s40249-017-0296-0.
11. Jiang H, Wang H, Xu LQ, Hu GY, Ma JY, Xiao P, et al. MLVA genotyping of *Brucella melitensis* and *Brucella abortus* isolates from different animal species and humans and identification of *Brucella suis* vaccine strain S2 from cattle in China. PLoS ONE. 2013;8:e76332. https://doi.org/10.1371/journal.pone.0076332.
12. Flèche PL, Jacques I, Grayon M, Dahouk SA, Bouchon P, Denoeud F, et al. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiol. 2006;6:9. https://doi.org/10.1186/1471-2180-6-9.
13. Ma JY, Wang H, Zhang XF, Xu LQ, Hu GY, Jiang H, et al. MLVA and MLST typing of *Brucella* from Qinghai China. Infect Dis Poverty. 2016;4:1–8. https://doi.org/10.1186/s40249-016-0123-z.
14. Sankarasubramanian J, Vishnu US, Gunasekaran P, Rajendhran J. A genome-wide SNP based phylogenetic analysis distinguishes different biovars of *Brucella suis*. Infect Genet Evol. 2016;41:213–7. https://doi.org/10.1016/j.meegid.2016.04.012.
15. Hua GJ, Hung CL, Lin CY, Wu FC, Chen YW, et al. MGUPGMA: a fast UPGMA algorithm with multiple graphics processing units using NCCL. Evol Bioinform. 2017;3:1176934317734220. https://doi.org/10.1177/1176934317734220.
16. Marçais G, Delcher AL, Phillippy AM, Coston R, Salzberg SL, Zimin A. MUMmer4: a fast and versatile genome alignment system. PLoS Comput Biol. 2018;14:e1005944. https://doi.org/10.1371/journal.pcbi.1005944.
17. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. Bioinformatics. 2009;25(16):2078–9. https://doi.org/10.1093/bioinformatics/btp352.
18. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403–10. https://doi.org/10.1016/S0022-2836(05)80360-2.
19. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst Biol. 2010;59:307–21.
20. Vilella AJ, Severin J, Ureta-Vidal A, Heng L, Durbin R, Birney E. EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. Genome Res. 2008;19:327–35.
21. Foster JT, Walker FM, Rannals BD, Hussain MH, Drees KP, Tiller RV, et al. African lineage *Brucella melitensis* isolates from Omani livestock. Front Microbiol. 2018;8:2702. https://doi.org/10.3389/fmicb.2017.02702.
22. Liu ZH, Wang LJ, Piao DR, Wang M, Liu RH, Zhao HY, et al. Molecular investigation of the transmission pattern of *Brucella suis* 3 from Inner Mongolia China. Front Vet Sci. 2018;5:271. https://doi.org/10.3389/fvets.2018.00271.
23. Zhang FB, Li ZW, La XL, Ma XM, Zhang YX, Ji P, et al. Multiple-locus variable-number tandem-repeat analysis of *Brucella* isolates from patients in Xinjiang China. Int J Clin Exp Med. 2015;8(9):15716–23.
24. Kattar MM, Jaafar RF, Araj GF, Fleche FL, Matar GM, et al. Evaluation of a multilocus variable-number tandem-repeat analysis scheme for typing human *Brucella* isolates in a region of brucellosis endemicity. Clin Microbiol. 2008;46(12):3935–40. https://doi.org/10.1128/JCM.00464-08.
25. Georgi E, Walter MC, Pfalzgraf MT, Northoff BH, Holdt LM, et al. Whole genome sequencing of *Brucella melitensis* isolated from 57 patients in Germany reveals high diversity in strains from Middle East. PLoS ONE. 2017;12(4):e0175425. https://doi.org/10.1371/journal.pone.0175425.
26. Janowicz A, Massis FD, Ancora M, Cammà C, Patavino C, et al. Core genome multilocus sequence typing and single nucleotide polymorphism analysis in the epidemiology of *Brucella melitensis* infections. J Clin Microbiol. 2018;56(9):e00517-e518. https://doi.org/10.1128/JCM.00517-18.
27. Deniskova TE, Dotsev AV, Selionova MI, Kunz E, Medugorac L, et al. Population structure and genetic diversity of 25 Russian sheep breeds based on whole-genome genotyping. Genet Sel Evol. 2018;50:29. https://doi.org/10.1186/s12711-018-0399-5.