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Original Article

Multiple-locus variable-number tandem-repeat analysis of *Brucella* isolates from patients in Xinjiang China

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Abstract: Objective: This study is to characterize and identify the human Brucella strains in Xinjiang, China with multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) scheme. Methods: Brucella strains were isolated and cultured from 62 brucellosis patients. The bacteria strains were subjected to the oxidase, catalase, rapid urease, and nitrate reduction tests, and the species identification was performed using the VITEK-2 Compact system. These Brucella strains were further identified and characterized using the 16 VNTR loci in a MLVA-16 methodology. Results: Twelve Brucella strains had been identified out of 62 patients, which were all recognized as Brucella melitensis (B. melitensis) according to the results from the VITEK-2 Compact system. Based on panel 1 (MLVA-8), these 12 Brucella isolates were clustered into three known genotypes and two new genotypes, in which 7 strains were clustered into genotype 45 (1-5-3-12-2-2-3-2), 1 strain was classified as genotype 42 (1-5-3-13-2-2-3-2), 1 stain was with genotype 62 (1-3-3-13-2-2-3-2), and the other 3 trains revealed two new genotypes, i.e., (1-5-3-12-2-3-3-2) and (1-5-3-11-2-3-3-2). Using panel 2A+2B (MLVA-16), we found that no genotypes of these strains were identical to the known genotypes, generally with differences in 2-4 loci. However, three strains shared the same genotype. Conclusion: Brucella strains in 62 brucellosis patients from Xinjiang are all identified as B. melitensis. Based on MLVA-8, two new genotypes have been discovered. These findings might contribute to the understanding of the pathogenesis and epidemiology of brucellosis in Xinjiang, China.

Keywords: *Brucella*, brucellosis, multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA), genotyping, Xinjiang

Introduction

Brucellosis is recognized as one of the most common zoonotic diseases worldwide [1], which is caused by bacteria of the genus *Brucella*. At present, approximately 0.5 million new cases are reported each year, primarily in the Mediterranean region, the Middle East, Africa, South and Central America, and Asia [2, 3]. Particularly, in Central Asia, the incidence of brucellosis has been rapidly increasing [4, 5], with continuous disease reports over the past decade [6-8].

In China, brucellosis has been a persistent public health problem. Since 1999, an increasing trend of the incidence of brucellosis has been observed in China, with specific geographical features [9]. Brucellosis is prevalent in northwestern China, including Xinjiang area, where living is mainly dependent on ruminant livestock [9, 10]. Therefore, it is important to develop rapid and accurate genotyping methods for epidemiological investigation and disease control.

Traditional detection and identification of *Brucella* are mainly based on bacteriological and serological techniques. Bacteriological procedures are always tedious and time-consuming, with unsatisfactory positive results. Even though serological techniques are often fast, the frequencies of false-positive and false-negative results are rather high. Moreover, genotyping cannot be achieved. As an alternative, multiple-locus variable-number tandem-repeat

Table 1. Basic information of the *Brucella* isolates

Strains	Isolation time	Specimen source	Patient age	Gender	Ethnicity	Symptoms	Residence
A	2010	Blood	36	Male	Kazak	Fever, aortic insufficiency	Urumqi
B	2010	Blood	45	Male	Han	Fever, oscheocele	Aletai
C	2010	Blood	3	Female	Kazak	Fever, anaemia, arthralgia	Aletai
D	2010	Blood	14	Male	Uighur	Fever, arthralgia	Kuqa
E	2010	Blood	9	Female	Hui	Fever, arthralgia	Dabancheng
F	2010	Blood	50	Female	Han	Fever, lumbago	Urumqi
G	2010	Blood	46	Male	Uighur	Fever	Keping County
H	2010	Blood	47	Male	Uighur	Fever, anaemia	Kuqa
I	2010	Blood	3	Male	Uighur	Fever, anaemia, arthralgia	Kuqa
J	2011	Blood	56	Male	Uighur	Fever	Turfan
K	2011	Blood	47	Female	Uighur	Fever	Urumqi
L	2011	Blood	17	Female	Kazak	Fever, anaemia	Aletai

(VNTR) analysis (MLVA) has been introduced and developed in recent years, which greatly facilitates genotyping and epidemiological studies [11-14]. MLVA-16 contain eight minisatellite (panel 1: Bruce 06, Bruce 08, Bruce 11, Bruce 12, Bruce 42, Bruce 43, Bruce 45, Bruce 55) and eight microsatellite markers (panel 2A: Bruce 18, Bruce 19, Bruce 21; panel 2B: Bruce 04, Bruce 07, Bruce 09, Bruce 16, Bruce 30) [15]. In this study, MLVA-16 scheme was used to genotype a collection of 12 human *Brucella* strains isolated from patients in Xinjiang, a geographical area with high incidence of brucellosis in China. Results from this study might contribute to the understanding of the pathogenesis and epidemiology of brucellosis in this area.

Materials and methods

Bacterial strains and culture

This study included 62 brucellosis patients from the Xinjiang Uygur Autonomous Region People's Hospital and the First Affiliated Hospital of Xinjiang Medical University (**Table 1**). The Brucella strains were isolated from their blood samples. Signed informed consent was obtained from every patient and the study was approved by the ethics review board of the First Affiliated Hospital of Xinjiang Medical University Blood samples were cultured in the Bact/Alert 3D automatic blood culture system (BioMerieux, Marcy-L'Etoile, France) for 7-15 d, and then streaked onto blood plates. After 48-72-h incubation, tiny gray colonies were observed, which were Gram-negative cocci. These isolates were subjected to chemical tests, and the species identification was performed with the VITEK-2 Compact system (BioMerieux).

Oxidase test

For the oxidase test, bacterial smear was added to filter paper containing 1% N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD; Bellancom, Beijing, China). Color development was observed within 10 s. Deep purple was considered as positive.

Catalase test

For the catalase test, the bacterial cultures were scraped onto a clean slide, and a drop of 3% H_2O_2 was added. Plenty of bubbles indicated a positive reaction.

Rapid urease test

For the rapid urease test, bacteria were inoculated into medium containing urease. Color change from yellow to red was considered as a positive result.

Nitrate reduction test

For the nitrate reduction test, bacterial cultures were inoculated into nitrate broth prepared in-house. After incubation at 35°C for 24 h, the nitrate reagents were added. Appearance of red color in the culture medium was considered as positive results. Strains of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as the quality control.

DNA preparation and MLVA genotyping scheme

Total genomic DNA was extracted using a MiniBEST Bacterial Genomic DNA Extraction kit

Table 2. Primer sets for MLVA

Loci	Forward primer	Reward primer
Bruce 06	5'-ATGGGATGTGGTA GGGTAATCG-3'	5'-ATGGGATGTGGTA GGGTAATCG-3'
Bruce 08	5'-ATTATTCGCAGGCTCGTGATTC-3'	5'-ATTATTCGCAGGCTCGTGATTC-3'
Bruce 11	5'-CTGTTGATCTGACCTTGCAACC-3'	5'-CTGTTGATCTGACCTTGCAACC-3'
Bruce 12	5'-CGGTAAATCAATTGTCCCATGA-3'	5'-CGGTAAATCAATTGTCCCATGA-3'
Bruce 42	5'-CATCGCCTCAACTATACCGTCA-3'	5'-CATCGCCTCAACTATACCGTCA-3'
Bruce 43	5'-TCTCAA GCCCGATATGGA GAAT-3'	5'-TCTCAA GCCCGATATGGA GAAT-3'
Bruce 45	5'-ATCCTTGCCTCTCCCTACCAG-3'	5'-ATCCTTGCCTCTCCCTACCAG-3'
Bruce 55	5'-TCA GGCTGTTTCGTCATGTCTT-3'	5'-TCA GGCTGTTTCGTCATGTCTT-3'
Bruce 04	5'-CTGACGAAGGGAAGGCAATAAG-3'	5'-CGATCTGGAGATTATCGGGAAG-3'
Bruce 07	5'-GCTGACGGGGAAGAACATCTAT-3'	5'-ACCCTTTTTCAGTCAAGGCAAA-3'
Bruce 09	5'-GCGGATTCGTTCTTCAGTTATC-3'	5'-GGGAGTATGTTTTGGTTGTACATAG-3'
Bruce 16	5'-ACGGGAGTTTTTGTTGCTCAAT-3'	5'-GGCCATGTTTCCGTTGATTTAT-3'
Bruce 18	5'-TATGTTAGGGCAATA GGGCAGT-3'	5'-GATGGTTGAGAGCATTGTGAAG-3'
Bruce 19	5'-GACGACCCGGACCATGTCT-3'	5'-ACTTCACCGTAACGTCGTGGAT-3'
Bruce 21	5'-CTCATGCGCAACCAAAACA-3'	5'-GATCTCGTGGTCGATAATCTCATT-3'
Bruce 30	5'-TGACCGCAAAACCATATCCTTC-3'	5'-TATGTGCAGAGCTTCATGTTCG-3'

Figure 1. *Brucella* culture and Gram staining. Representative pictures of bacterial cultures at 48 h (A) and 72 h (B), as well as Gram staining (C) for isolate A were shown.

(Takara, Otsu, Japan), and stored at -20°C until further use.

MLVA genotyping was performed as previously described [16]. The sixteen primer sets were shown in **Table 2**, including panel 1 (Bruce 06, Bruce 08, Bruce 11, Bruce 12, Bruce 42, Bruce 43, Bruce 45, and Bruce 55), panel 2A (Bruce 18, bruce19, and Bruce 21), and panel 2B (Bruce 04, Bruce 07, Bruce 09, Bruce 16, and Bruce 30). The 50 μl PCR system contained 1 μl DNA template, 1 μl primer each, 25 μl 2× EASY TAQ PCR SM, and 22 μl ddH_2O. The reaction conditions were as follows: for Bruce 08, Bruce 11, Bruce 12, Bruce 42, Bruce 45, and Bruce 55 in panel 1, initial denaturation at 96°C for 5 min, and then 30 cycles of 96°C for 30 s, 60°C for 30 s, and 70°C for 1 min; for Bruce 06 and Bruce 43 in panel 1, initial denaturation at 96°C for 5 min, and then 34 cycles of 96°C for 30 s, 57°C for 30 s, and 70°C for 1 min. For panel 2A and panel 2B, initial denaturation at 95°C for 3 min, and then 34 cycles of 95°C for 30 s, 58°C for 30 s, and 70°C for 1 min. PCR products for panel 1 and panel 2A+2B were analyzed by 2% and 3% agarose gel electrophoresis, respectively. Moreover, the products were sequenced by Sangon Biotech (Shanghai, China).

Results

Characterization and identification of the Brucella strains

All the *Brucella* strains isolated from 62 patients in this study were subjected to the oxidase, catalase, rapid urease, and nitrate reduction tests. The VITEK-2 Compact system was used for species identification. Totally 12 *Brucella* species had been identified, which were designated as isolates A to L, respective-

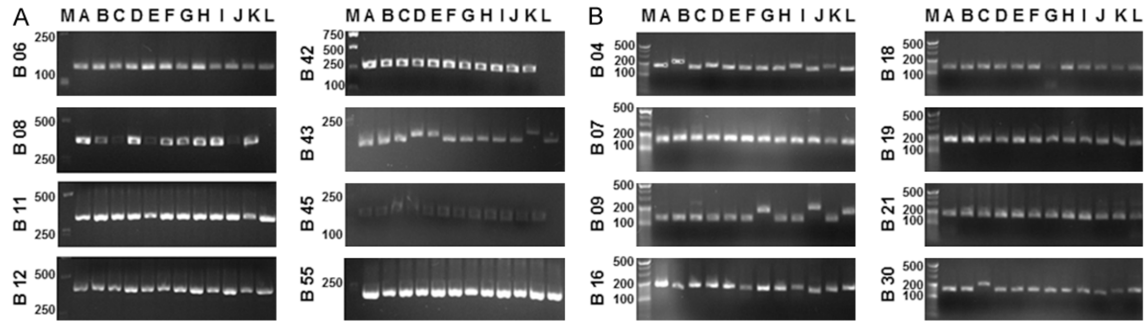

Figure 2. PCR analysis of the VNTRs for the *Brucella* strains. The 12 *Brucella* species were designated as isolates A to L, respectively. PCR analysis of the VNTR loci in panel 1 (A) and panel 2A+2B (B) for the *Brucella* strains. M, marker. Panel 1 included eight markers: Bruce 06, Bruce 08, Bruce 11, Bruce 12, Bruce 42, Bruce 43, Bruce 45, and Bruce 55. Panel 2 was composed of eight microsatellite markers: Bruce 18, bruce19, and Bruce 21 in panel 2A; and Bruce 04, Bruce 07, Bruce 09, Bruce 16, and Bruce 30 in panel 2B.

Table 3. PCR products

Loci	bp	A	B	C	D	E	F	G	H	I	J	K	L
Bruce 06	134	143	147	146	142	143	144	144	142	145	147	141	143
Bruce 08	18	373	369	371	329	365	373	360	373	372	361	374	162
Bruce 11	63	323	312	325	321	328	328	325	313	324	314	308	326
Bruce 12	15	377	394	382	392	393	384	385	395	393	395	377	393
Bruce 42	125	295	293	300	294	300	292	293	293	293	293	293	298
Bruce 43	12	188	186	186	171	197	187	187	189	188	188	207	187
Bruce 45	18	177	156	155	152	153	153	177	158	155	154	153	162
Bruce 55	40	251	231	240	235	270	237	236	234	236	235	246	236
Bruce 18	8	142	139	140	140	140	141	143	143	140	142	141	141
Bruce 19	6	179	181	179	179	180	180	180	183	181	179	179	179
Bruce 21	8	166	166	167	169	169	166	166	167	165	166	167	168
Bruce 04	8	202	243	195	183	193	194	195	194	216	195	185	196
Bruce 07	8	153	154	151	151	150	151	153	152	154	157	152	153
Bruce 09	8	125	125	124	127	124	175	174	125	127	197	167	125
Bruce 16	8	179	163	179	181	179	175	171	179	183	162	179	194
Bruce 30	8	146	147	186	146	147	146	137	146	146	96	153	161

ly. All the 12 strains were positive for the traditional tests. Moreover, VITEK-2 Compact system indicated that these *Brucella* strains were *Brucella melitensis* (*B. melitensis*). Taken together, results from these traditional detection and identification methods suggest that, the *Brucella* strains isolated from these brucellosis patients in Xinjiang were all identified as *B. melitensis* from Malta. Representative results of bacterial culture and Gram staining for isolate A were shown in **Figure 1**.

Characterization of variable-number tandem-repeat (VNTR) loci in the Brucella strains

Results from PCR amplification of all sixteen MLVA alleles were shown in **Figure 2**. These products were subjected to forward and reverse sequencing (**Table 3**), and the complete DNA sequence was obtained by overlapping the sequences with the DNAstar software. Then the Tandem Repeats Finder program was used for the repeat sequence analysis, and the VNTRs were confirmed.

Genotyping and clustering of Brucella strains by MLVA

Results for the MLVA-16 genotyping assay were shown in **Table 4**. Resultant genotypes were compared using the *Brucella* 2010 MLVA database at http://minisatellites.u-psud.fr/MLVAnet/. According to panel 1, the 12 *Brucella* isolates were clustered into three known geno-

Table 4. MLVA genotyping of the *Brucella* strains

Strains	Bruce 06	Bruce 08	Bruce 11	Bruce 12	Bruce 42	Bruce 43	Bruce 45	Bruce 55	Bruce 18	Bruce 19	Bruce 21	Bruce 04	Bruce 07	Bruce 09	Bruce 16	Bruce 30	Panel 1 genotype
A	1	5	3	12	2	2	3	2	2	12	5	4	4	3	5	4	45
B	1	5	3	13	2	2	3	2	2	20	5	9	4	3	4	4	42
C	1	5	3	12	2	3	3	2	2	20	5	4	4	3	5	9	New
D	1	5	3	11	2	3	3	2	2	20	5	5	4	3	5	4	New
E	1	5	3	11	2	3	3	2	7	20	5	4	4	3	5	4	New
F	1	5	3	12	2	2	3	2	2	20	5	4	4	3	4	4	45
G	1	5	3	12	2	2	3	2	2	20	5	8	4	9	3	3	45
H	1	5	3	12	2	2	3	2	2	20	5	4	4	3	4	4	45
I	1	5	3	12	2	2	3	2	4	20	5	4	4	3	4	4	45
J	1	5	3	12	2	2	3	2	4	20	5	4	4	11	3	3	45
K	1	5	3	12	2	2	3	2	2	12	5	8	4	7	5	5	45
L	1	3	3	13	2	2	3	2	5	19	5	4	4	3	7	6	62
J	1	5	3	12	2	2	3	2	4	20	5	4	4	11	3	3	45

types and two new genotypes (**Figure 3**). Isolates A, F, G, H, I, J, and K were clustered into genotype 45 (1-5-3-12-2-2-3-2), isolate B was classified into genotype 42 (1-5-3-13-2-2-3-2), and isolate L was with genotype 62 (1-3-3-13-2-2-3-2). Moreover, isolates C, D, and E showed new genotypes (isolate C revealing one new genotype, and isolates D and E revealing another), with differences in 1-2 loci compared with the known genotypes.

Greater diversity in these *Brucella* isolates was observed when eight additional markers (panel 2A+2B) were included. No genotypes of these strains were found to be identical to any of the known genotypes, generally with differences in 2-4 loci. However, isolates F, H, and I shared the same genotype. Moreover, all of these 12 isolates were identified as *B. melitensis*, which was in line with the results from the traditional detections.

Discussion

Human brucellosis is mainly caused by direct contact with *Brucella*-infected animal reservoirs and/or consumption of raw animal products [17, 18]. *Brucella* can proliferate in phagocytic cells, and cause clinical symptoms, such as undulant fever [1, 19, 20], orchitis [21, 22], spondylitis, arthritis [23, 24], endocarditis, and fatigue. Brucellosis is a systemic infection affecting human beings regardless of age and gender, and it is difficult to diagnose due to the variable clinical symptoms [25, 26]. Moreover, no effective treatment methods are currently available for brucellosis, making the disease a huge economic and health burden for the society [27].

At present, *Brucella* can be divided into nine different species depending on the host, including *B. abortus*, *B. canis*, *B. ovis*, *B. suis*, *B. neotomae*, *B. melitensis*, *B. ceti*, *B. pinnipedialis*, and *B. microti* [28]. Different species are mainly distinguished by biochemical reactions, staining, and pathogen sensitivities. However, the traditional characterization and identification methods of *Brucella* strains are definitely cumbersome and laborious, with poor repeatability. As an alternative, emerging molecular biological techniques are more safe, rapid, specific, and sensitive. For example, the *Brucella abortus-melitensis-ovis-suis* (AMOS) PCR analysis has been developed. Accordingly, *Brucella* could be divided into four species (eight biotypes) [29]. Even more, in recent years, single nucleotide polymorphism (SNP) [30, 31] analysis has been introduced into the identification of bacteria, which could cover all the currently recognized genotypes of *Brucella* [32].

Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) genotyping is mainly based on the VNTR polymorphism inside the bacterial genome, which has been applied in the investigation of tubercle bacillus (TB), leprosy bacillus, and anthrax [15]. Le Fleche *et al.* [16] established the original MLVA genotyping method which contains 15 VNTR loci. The technique was further improved by Al Dahouk *et al.* [11] to the now well-known MLVA-16 analysis, which could detect more than 500 *Brucella* species [33-36]. Based on the MLVA-16

Figure 3. MLVA-based dendrogram and phylogenetic relationship analysis. Relationships among different *Brucella* genus were shown in the dendrogram. The length of the line represents the genetic distance.

scheme, a web-site *Brucella* database (*Brucella* 2010 MLVA database) has been built by collecting data from laboratories all over the world. In this study, the genotypes of 12 *Brucella* strains were analyzed and compared within the database. Our results showed that, according to MLVA panel 1 (MLVA-8), all the 12 isolates were *B. melitensis*, including 7 strains with genotype 45, 1 strain with genotype 42, 1 strain with genotype 62, and 3 strains with other two new genotypes. However, using MLVA panel 2A (MLVA-11) or 2A+2B (MLVA-16), neither of these two genotypes was found to be identical to the known genotypes.

Within the *Brucella* 2010 MLVA database, the "Eastern Mediterranean" genotype 42 (1-5-3-13-2-2-3-2) has been reported in Xinjiang, China [37], and the genotype 45 has been found in Turkey [35]. Unique genotypes could be reported, in comparison with the known genotypes from different regions. In this study, greater discriminations have been observed in MLVA panel 2 compared with panel 1, with only three strains identical in the genotype, indicating that distinct genotypes might be expected in the same region. However, the regional differences were limited within 1-2 repeats, suggesting their close phylogenetic relationship. All the 12 strains in this study were *B. melitensis*, without other species, which might be the dominant species in Xinjiang, in line with previous findings that *B. melitensis* is the predominant strain associated with human brucellosis outbreaks in China [38]. Particularly, in these brucellosis patients, twelve of them were engaged in pastoral livestock or with close contact with cattle. Moreover, out of the three child patients, two had a history of drinking raw milk. Of course, further in-depth studies are needed to address this issue.

In conclusion, in this study, 12 *Brucella* strains were identified out of bacterial cultures from 62 patients in Xinjiang, all were recognized as *B. melitensis*. Using panel 1 (MLVA-8), these 12 *Brucella* isolates were clustered into three known genotypes and two new genotypes.

According to panel 2A+2B (MLVA-16), none of these two genotypes were identical to the known genotypes. These findings might contribute to the understanding of the pathogenesis of brucellosis in Xinjiang, China.

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Disclosure of conflict of interest

None.

Abbreviations

VNTR, variable-number tandem-repeat; MLVA, multiple-locus variable-number tandem-repeat analysis; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; AMOS, *abortus-melitensis-ovis-suis*; SNP, single nucleotide polymorphism; TB, tubercle bacillus.

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