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Distinctive phenotypic and molecular characteristics of *Brucella abortus* strains isolated from Mongolia

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Abstract: Recently, some *Brucella abortus* were isolated from human and various livestock in Mongolia, and the classical biotyping and molecular detection methods (PCR assays, 16S rRNA sequencing analysis, and multilocus variable number tandem repeat analysis (MLVA)) were applied to investigate them. Of the 16 isolates, nine and seven were confirmed as biovar (bv.) 3 and untyped by the classical biotyping assay, respectively. The phenotypic characteristics of the untyped isolates were consistent with those of the former *B. abortus* bv. 7, which is not included in the current *Brucella* taxonomy. Additionally, genus- and species-specific PCR supported that the untyped Mongolian isolates belonged to *B. abortus*. The untyped isolates were clustered into a peculiar genotype in the results of 16S rRNA sequencing and MLVA patterns using 16 loci. Taken all together, the untyped *B. abortus* isolates, which are some of the most predominant *B. abortus* biovars in Mongolia, were estimated to be bv. 7. Moreover, the Mongolian *B. abortus* isolates were closely associated with Chinese isolates according to MLVA. Therefore, animal quarantine and control measures should be strengthened to prevent the spillover of *Brucella* species into adjacent countries.

Key words: *Brucella abortus*, phenotyping, molecular detection, bv. 3 and 7, Mongolia

1. Introduction

Brucellosis is a major zoonosis that occurs in domestic animals and wildlife as well as humans worldwide (1). The genus *Brucella* consists of 11 species, and their classifications are mainly based on biological and biochemical characteristics and host preferences (2).

In general, classical biotyping methods are used to identify and differentiate *Brucella* species and biovars. Based on them, three species of *Brucella* are currently divided into several biovars: *B. abortus* (bvs. 1–6 and 9), *B. melitensis* (bvs. 1–3), and *B. suis* (bvs. 1–5). *B. abortus* biovars have been changed. Specifically, *B. abortus* bv. 8 disappeared after 1978, and *B. abortus* bv. 7 was also deleted by the International Subcommittee on Taxonomy of *Brucella* in 1986 because its reference strain (63/75) consisted of a mixture of bv. 3 and 5 (3). However, the debate about the existence of *B. abortus* bv. 7 is ongoing (4,5). Recently, Garin-Bastuji et al. (3) suggested that *B. abortus* bv. 7 should be reintroduced into *Brucella* classification, using the oldest Mongolian isolate (99-9971-135, 1988) as a potential reference strain for bv. 7.

In addition, many molecular detection methods are also available for discriminating some *Brucella* species and biovars. For example, *B. abortus* species-specific PCR (BaSS-PCR) and enhanced AMOS-ERY PCR were able to differentiate *B. abortus* bvs. 1, 2, and 4 from the other biovars (6,7). *Omp*2a-PCR is capable of distinguishing *B. abortus* biovars based on the size differences of the *omp*2a genes between bvs. 1, 2, and 4 and the other biovars. 16S rRNA sequencing analysis is also helpful for identifying *Brucella* at the species level (8). Additionally, several multiplex PCR techniques can discriminate between all *Brucella* species, including vaccine strains that have been developed previously (9–11).

To date, studies examining brucellosis in Mongolia have mainly focused on serological monitoring and prevalence

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in humans and domestic animals (1,12,13). Previous analysis of seminomadic herders in central Mongolia also demonstrated that human brucellosis was related to animal brucellosis (14). Now, many *Brucella* isolates have been obtained from humans and various animals in Mongolia. Therefore, the aims of this study were to characterize and investigate the strains by using the classical biotyping assay and molecular detection methods.

2. Materials and methods

2.1. Bacterial strains and classical biotyping

From 2012 to 2014, the Institute of Veterinary Medicine in Mongolia provided *Brucella* strains isolated from humans and aborted domestic animals along with epidemiological data from 2 hospitals and 10 farms. After primary bacterial growth, pure colonies were obtained after more than 3 serial passages. A total of 16 *B. abortus* isolates and 16 *Brucella* reference strains (*B. abortus* bvs. 1–6 and 9, and nine other *Brucella* species) were tested according to classical typing, as described previously (3).

2.2. DNA extraction and molecular identification

The genomic DNA of 16 Mongolian *B. abortus* isolates and 16 reference *Brucella* strains were prepared using the QIAamp DNA extraction kit (QIAGEN Korea Ltd., Seoul, Korea) according to the manufacturer's protocols. The 16 reference strains were as follows: *B. abortus* bv. 1 (544), *B. abortus* bv. 2 (86/8/59), *B. abortus* bv. 3 (Tulya), *B. abortus* bv. 4 (292), *B. abortus* bv. 5 (B3196), *B. abortus* bv. 6 (870), *B. abortus* bv. 9 (C68), *B. canis* (RM6/66), *B. suis* bv. 1 (1330), *B. ovis* (63/290), *B. neotomae* (5K33), *B. melitensis* bv. 1 (16M), *B. ceti* (B1/94), *B. pinnipedialis* (B2/94), *B. microti* (CCM4915), and *B. inopinata* (B01). BaSS-PCR assay and differential multiplex PCR were performed using the extracted *Brucella* DNA samples (6,11). In addition, *omp*2a-PCR was used as a complementary method for reconfirmation of the *Brucella* growth on thionine dye (3).

2.3. 16S rRNA sequencing analysis

The genomic DNAs of *Brucella* strains were extracted using the InstaGene Matrix (Bio-Rad, Hercules, CA, USA). The 16S rRNA genes were amplified and sequenced using universal primers (27F: 5' - AGAGTTTGATCMTGGCTCAG - 3', 1492R: 5' - TACGGYTACCTTGTTACGACTT - 3', 518F: 5' - CCAGCAGCCGCGGTAATACG - 3', and 800R: 5' - TACCAGGGTATCTAATCC - 3') (15). This sequence alignment was analyzed using CLC Main Workbench software version 6.0 (QIAGEN Corp., Copenhagen, Denmark).

2.4. MLVA-16 assay

Multiple locus variable number of tandem repeats analysis (MLVA) with 16 loci was used for the comparison of genetic diversity among the *B. abortus* Mongolian isolates (16,17). Data were analyzed by the BioNumerics program version 5.1.0 (Applied Maths NV, Sint-Martens-Latem, Belgium). Forty *B. abortus* strains from a Web database (*Brucella*2012 and *Brucella*2013, http://mlva.u-psud.fr/mlva4/genotyping/) and 4 *B. abortus* bv. 7 (3) were added to compare the MLVA genotypes. A clustering analysis was performed on the basis of the categorical coefficient and the unweighted pair group method using the arithmetic averages method.

3. Results

3.1. Classical biotyping assays

Of the 16 *B. abortus*, 9 exhibited characteristics of *B. abortus* bv. 3, and the remaining 7 isolates had particular biotyping profiles (Table 1). These 7 isolates showed typical characteristics of *B. abortus* species, but they did not correspond to any current biovars; they were analyzed by agglutination test with monospecific sera (A +, M +, R -) and growth on dyes (thionine +, basic fuchsin +) (Table 1). Their unique characteristics were identical to former *B. abortus* bv. 7.

3.2. Molecular identification

In the multiplex PCR, all 16 isolates were confirmed as *B. abortus* with four amplicons (data not shown). All isolates showed only two amplicons of 180 and 800 bp in BaSS-PCR, but no 500-bp amplicon was detected to differentiate among *B. abortus* bvs. 1, 2, and 4. In addition, all of the isolates generated the same PCR product of 1216 bp in *omp2a*-PCR (Figure 1). These results revealed a thionine resistance phenotype that is not characteristic of bv. 1, 2, or 4 (3).

3.3. 16S rRNA sequence analysis

A comparative sequence analysis among the untyped *B. abortus* isolates and other *Brucella* species or other *B. abortus* biovars revealed a 1-bp difference in the 16S rRNA region. This site was a C-to-T transition at position 926 of the partial 16S rRNA sequence (1454 bp) on the chromosome II regions of the reference *B. abortus* (NC_006933.1).

3.4. MLVA-16 assay

All of the *B. abortus* strains showed the same profile patterns for 8 markers of panel 1 (4-5-3-12-2-2-3-1) and 3 markers of 2A (6-42-8). Additionally, of the 5 markers of panel 2B, bruce 16 and 30 displayed only one allelic type, whereas the bruce 04, 07, and 09 markers had two, three, and five allelic types, respectively (Table 2). The nine *B. abortus* bv. 3 isolates were divided into four different genotypes, though 7 untyped isolates were found to be in relation with only one genotype (Table 2; Figure 2). The untyped *B. abortus* isolates predicted to be bv. 7 showed an identical MLVA profile with the Mongolian strains (99-9971-135 and 99-9971-159) in a recent report (3) (Table 2).

Table 1. *B. abortus* isolates from Mongolia and reference strains, and their biochemical characteristics.

Division	Strain ID.	Biovar	CO_2	H_2S	Oxidase	Catalase	Urease	Agglutination with monospecific sera			Growth on dyes		Lysis by phages at RTD				
								A	M	R	Thionine	Basic fuchsin	Tb	Tb 10^4	Wb	Iz	RC
Mongolian isolates	A1-3452	Untyped	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
	A1-3453	Untyped	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
	A1-3470	Untyped	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
	A1-3476	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
	A1-3480	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
	A1-4111	Untyped	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
	A1-4113	Untyped	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
	A1-4141	Untyped	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
	A1-4142	Untyped	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
	A1-4164	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
	A1-4165	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
	A1-4166	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
	A1-4167	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
	A1-4168	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
	A1-4169	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
	A1-4170	3	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
Reference strains	544	1	(+)[1]	+	+	+	(+)[1]	+	-	-	-	+	+	+	+	+	-
	86/8/59	2	(+)[1]	+	+	+	+	+	-	-	-	-	+	+	+	+	-
	Tulya	3	(+)[1]	+	(+)[1]	+	+	+	-	-	+	+	+	+	+	+	-
	292	4	(+)[1]	+	+	+	+	-	+	-	-	(+)[1]	+	+	+	+	-
	B3196	5	-	-	+	+	+	-	+	-	+	+	+	+	+	+	-
	870	6	-	-	+	+	+	+	-	-	+	+	+	+	+	+	-
	99-9971-135[2]	7	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
	C68	9	+/-	+	+	+	+	-	+	-	+	+	+	+	+	+	-

[1] Most strains were positive.

[2] Garin-Bastuji et al. (3).

In the MLVA data, a total of 60 *B. abortus* strains, including ones from the Web database, were divided into 2 major clusters (A and B), and cluster B was split into 5 subgroups (B1–B5) (Figure 2). Cluster A included *B. abortus* isolates from Africa, and the B1 subgroup mainly consisted of European strains. The *B. abortus* isolates were included in two subgroups: B2 and B3. Of a total of 46 genotypes, the untyped isolates clustered to one genotype, B2, with a representative of *B. abortus* bv. 7 strain in a previous study (3). One strain (A1-4170) out of the nine *B. abortus* bv. 3 isolates belonged to the B2 subgroup and was closely related to the Chinese strains from cattle. Most of the bv. 3 isolates, including those obtained from camels, were located in the B3 subgroup and these were also grouped with the Chinese *B. abortus* bv. 3.

4. Discussion

In our research, 16 *B. abortus* isolates were identified by classical biotyping and molecular detection. In the classical biotyping, these isolates showed the same results, except in the agglutination test with M and the CO_2 requirement. The characteristics of the untyped strains did not match those of any biovars of *B. abortus* in the current taxonomy. Thus, additional molecular detection methods, such as species-specific multiplex PCR, BaSS-PCR, and *omp2a*-PCR, were performed to evaluate the biovars in greater detail.

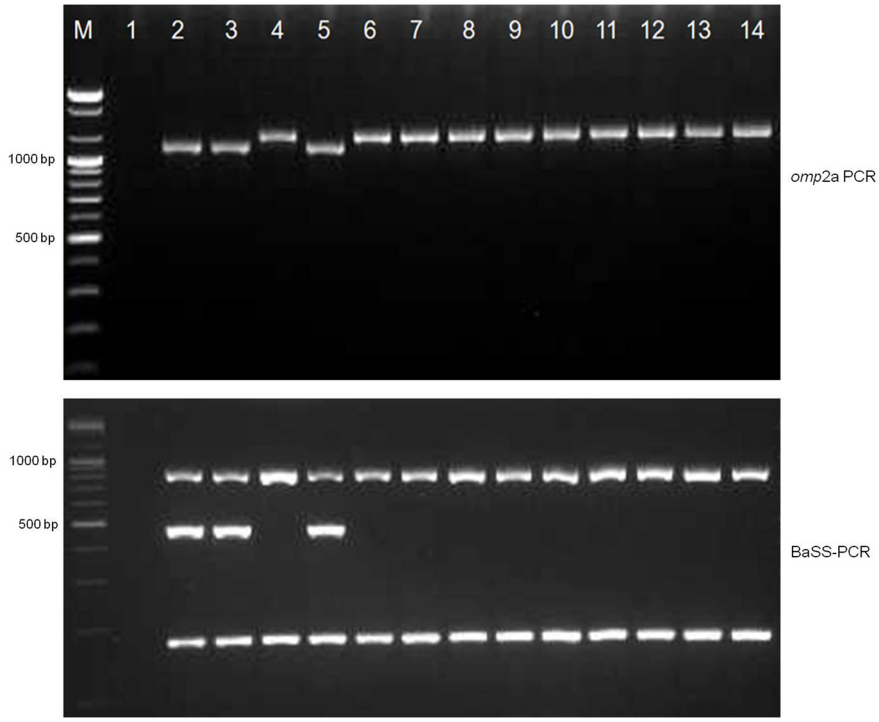

Figure 1. PCR results for the *B. abortus* reference strains and Mongolian *B. abortus* isolates.
Lane M: 100-bp DNA marker, lane 1: negative control (D. W.), lane 2: *B. abortus* bv. 1 (544), lane 3: *B. abortus* bv. 2 (86/8/59), lane 4: *B. abortus* bv. 3 (Tulya), lane 5: *B. abortus* bv. 4 (292), lane 6: *B. abortus* bv. 5 (B3196), lane 7: *B. abortus* bv. 6 (870), lane 8: *B. abortus* bv. 9 (C68), lane 9: A1-3476 (from sheep), lane 10: A1-3480 (from camels), lane 11: A1-4164 (from cattle), lane 12: A1-3453 (from sheep), lane 13: A1-3470 (from humans), lane 14: A1-4141 (from cattle).

Table 2. MLVA-16 profile patterns for the 16 Mongolian *B. abortus* isolates.

Strain	Biovar	Animals	Panel 1								Panel 2A			Panel 2B				
			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
A1-3452	Untyped	Sheep	4	5	3	12	2	2	3	1	6	42	8	5	6	4	3	3
A1-3453	Untyped	Sheep	4	5	3	12	2	2	3	1	6	42	8	5	6	4	3	3
A1-3470	Untyped	Human	4	5	3	12	2	2	3	1	6	42	8	5	6	4	3	3
A1-3476	3	Sheep	4	5	3	12	2	2	3	1	6	42	8	5	5	9	3	3
A1-3480	3	Camel	4	5	3	12	2	2	3	1	6	42	8	5	5	9	3	3
A1-4111	Untyped	Human	4	5	3	12	2	2	3	1	6	42	8	5	6	4	3	3
A1-4113	Untyped	Human	4	5	3	12	2	2	3	1	6	42	8	5	6	4	3	3
A1-4141	Untyped	Cattle	4	5	3	12	2	2	3	1	6	42	8	5	6	4	3	3
A1-4142	Untyped	Cattle	4	5	3	12	2	2	3	1	6	42	8	5	6	4	3	3
A1-4164	3	Cattle	4	5	3	12	2	2	3	1	6	42	8	4	5	8	3	3
A1-4165	3	Cattle	4	5	3	12	2	2	3	1	6	42	8	4	5	8	3	3
A1-4166	3	Cattle	4	5	3	12	2	2	3	1	6	42	8	4	5	8	3	3
A1-4167	3	Cattle	4	5	3	12	2	2	3	1	6	42	8	4	5	8	3	3
A1-4168	3	Cattle	4	5	3	12	2	2	3	1	6	42	8	4	5	8	3	3
A1-4169	3	Cattle	4	5	3	12	2	2	3	1	6	42	8	4	5	7	3	3
A1-4170	3	Cattle	4	5	3	12	2	2	3	1	6	42	8	4	7	6	3	3
99-9971-135[1]	7	Cattle	4	5	3	12	2	2	3	1	6	42	8	5	6	4	3	3

[1] Garin-Bastuji et al. (3).

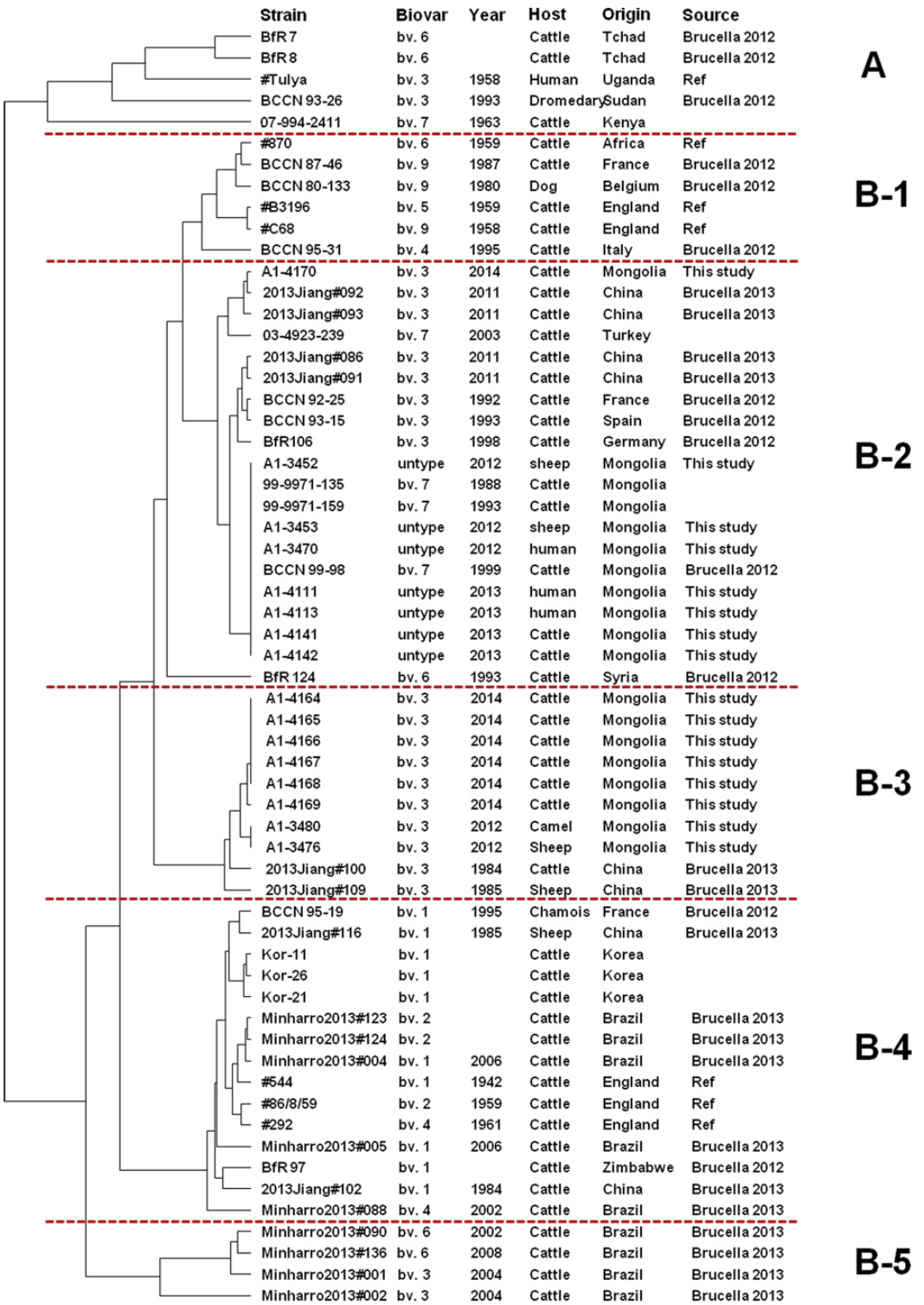

Figure 2. Clustering analysis of 60 *B. abortus* strains including 16 Mongolian strains. The MLVA data of these strains were downloaded from an MLVA database (*Brucella*2012 and *Brucella*2013). The strain name, biovar, isolation year, host, origin, and source of each strain are shown.

In agreement with the biotyping results, the molecular methods also demonstrated that the untyped isolates did not belong to bvs. 1, 2, or 4. Moreover, comparative 16S rRNA sequencing revealed that in comparison to *B. abortus* reference strains (bvs. 1–6 and 9), the untyped Mongolian strains possess a distinct genetic feature.

Taking these phenotypic and molecular typing results together, the untyped strains were suspected to be *B. abortus* bv. 7, which was deleted from the taxonomy in 1988. Garin-Bastuji et al. (3) also verified the presence of *B. abortus* bv. 7, demonstrating that the four *B. abortus* isolates from the AHVLA and ANSES harbor the same characteristics as the former bv. 7 strain. Therefore, they proposed the reintroduction of bv. 7 into the *Brucella* taxonomy. The *B. abortus* isolates in our study with the same polyphasic traits as the former bv. 7 strain might be helpful for proving the existence of *B. abortus* bv. 7.

Consistent with the above data, the MLVA profile also indicated that all Mongolian isolates are *B. abortus.* The *B. abortus* bv. 3 and untyped biovars were located very close to each other in the B2 and B3 subgroups (Figure 2). The untyped strains were identical with previous bv. 7 (3) and exhibited a consistent genotype, regardless of the animal species and province in Mongolia. These isolates might represent a linkage with the past, as they have spread to a variety of animals and humans through contact since at least the 1980s. Therefore, our study suggests the possibility of reinstating *B. abortus* bv. 7 into the *Brucella* taxonomy, and the oldest Mongolian strain (99-9971-135) would serve as a suitable reference for *B. abortus* bv. 7 strain. Furthermore, eight bv. 3 strains grouped into the B3 subgroup clustered with two *B. abortus* bv. 3 strains from sheep and cattle in China (Figure 2). Accordingly, the Mongolian isolates appear to, until recently, have had close genetic relationships with the strains from China. Kulakov et al. (18) performed MLVA typing for *B. abortus* bv. 3 isolates from 7 provinces in Mongolia, and Chen et al. (19) reported that *B. abortus* bv. 3 is the most prevalent type in Inner Mongolia. Because Inner Mongolia showed the highest incidence of brucellosis in China, its prevalent biovars might be related to those of Mongolia due to the geographical proximity. Such knowledge about the distribution of predominant biovars could provide key information regarding the source of infections (20). According to this work, MLVA could be a useful tool for investigating the epidemiological relatedness among *Brucella* strains of neighboring countries.

Mongolia is a large and developing country based on the livestock industry and has the second-highest incidence of human brucellosis (12). Livestock rearing is still based on a traditional nomadic system, and various animal species comingle while being raised, so brucellosis can be easily transmitted (13). Therefore, it is important to identify the characteristics of Mongolian *Brucella* strains accurately using phenotypic and molecular typing. To prevent the spread of brucellosis among animals, the Mongolian government has managed a national vaccination program for all domestic animals, except camels, since 2000 (1). However, despite its economic importance in nomadic herds, camel brucellosis appears to be overlooked (21). Camels are known to be primarily infected by *B. abortus* or *B. melitensis* and to act as silent carriers of brucellosis (22). Therefore, more strict control and prevention programs for livestock, including camels, are required to minimize economic losses in Mongolia. Animal quarantine and control measures should also be strengthened to prevent the spillover of *Brucella* species from Mongolia to China.

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