Letter to the Editor

A Nested-Polymerase Chain Reaction Assay to Identify and Genotype *Brucella*[*]

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Brucella is gram-negative, facultative, intracellular bacteria implicated in infectious zoonosis diseases, particularly among domestic animals, that are also transmittable to humans. The genus *Brucella* is classified based on the primary host preferences, pathogenicity, host preference, and phenotypic characteristics of its species[1]. The clinical features of brucellosis overlap with those of an extensive range of infectious and non-infectious diseases; therefore, laboratory testing is deemed the most reliable approach to diagnose this infection[2]. Microbiological culturing and serological examinations are the most common methods for the detection of *Brucella*. Although the isolation of these bacteria is the 'gold standard' approach, the microbial culture often gives false negative results and dependent on the culture medium, the quantity of the circulating bacteria, and the species of *Brucella*[3]. Therefore, serological tests such as the standard serum agglutination test (SAT) seem to be more effective for diagnosis, although an occasional case of cross-reaction or false-positive reaction in the samples from areas with subclinical prevalence of brucellosis has been promoted[4]. The present study is a comprehensive analyses of the genomic nucleotide sequences of *Brucella* spp. that also developed a nested-polymerase chain reaction (PCR) assay for the identification and genotyping of *Brucella*, especially for those contained in blood specimens.

A total of 36 reference strains (*B. abortus* biovars[1-7], *B. melitensis* biovars[1-3], *B. suis* biovars[1-5], *B. canis*; *B. ovis*; *B. neotomae*, *B. pinnipedialis*, and *B. microti*) were used. All strains of *B. abortus*, *B. melitensis, B. suis*, *B. canis*; *B. ovis*; and *B. neotomae* were preserved in Brucellosis Laboratory, CDC, China. The information on the DNA of *B. ceti*, *B. microti*, and *B. pinnipedialis* strains were sourced from the US National Center for Biotechnology Information (NCBI) website. The *Rev1, M5,* and *M28* belonged to the *B. melitensis* vaccine strains; *104M* and *S19* to the *B. abortus* vaccine strains; *VBI22* and *VacciS2* to the *B. suis* vaccine strains; and *45/20* and *B1119* to the *B. abortus* rough strains.

A total of 89 clinical strains isolated from the patient's blood samples were typed through the conventional biological methods. A total of 7 blood and sera samples were collected from the patients and sheep. The B114, B115, and lanzhou-26 were clinical human anticoagulant whole blood samples. The serum antibody titer was > 1:100. However, no *Brucella* species was isolated. The B243, B251, and B252 samples were collected from the sera of sheep from a farm. The *B. melitensis* biovars 3 strains was isolated from B252 sample. *B. canis* strain was isolated from BJ10 samples collected from anticoagulant whole blood samples from dogs at the farm. The samples were positive for *Brucella* antibodies (Table 1)[5].

A total of 7 non-*Brucella* DNA were used to verify the specificity of the nested-PCR assay, which included *Bacillus cereus*, *Bacillus anthracis*, *Escherichia coli* o:157, *Salmonella*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica* o:9, and *Vibrio cholerae*. *E. coli* o:157 strain was treated as a negative quality control in this study.

The Bacterial Genomic DNA Extraction Kit (Spin column; Tiangen Biotechnology [Beijing] Co., Ltd, China) was used to extract the nucleic acid DNA from the reference strains and the clinical isolates. Blood and serum samples were extracted by using this kit.

Comparative analysis of the whole genome sequences of *Brucella* spp. from the NCBI databases revealed that the nucleotide sequences in transposase IS711 *orfA* and *orfB* of *Brucella* spp. possess nucleotide polymorphisms and contain

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Brucella species and biovars specificity. The oligonucleotide primers were designed based on *orfA* and *orfB* (Figure 1). A nested-PCR assay was established to identify and genotype the strains of *Brucella* spp. The primers used included F1: 5′-AACGTAACCATACATAGCGCATG-3′ and R1: 5′-ACAGATGAGCAATGGAACCGGAT-3′ and F2: 5′-GAATGGGTGCAATTTCTCGC-3′ and R2: 5′-ATATC-TTCCGGGGCGAGTTGGTA-3′. The first PCR was conducted with the primer pairs F1 and R1 using the

Table 1. The 36 reference strains, 89 clinical isolates, and 7 blood and sera samples used in this study

NO.	Strain ID	Biovars	Note	NO.	Strain D	Biovars	Note	NO.	Strain D	Biovars	Note	NO.	Strain D	Biovars	Note
1	A13334	A1	REF	34	63/290	Ovis	REF	67	13106	M3	Isolate	100	78037	M2	Isolate
2	2308	A1	REF	35	25840	Ovis	REF	68	13107	M3	Isolate	101	79029	M3	Isolate
3	9-941	A1	REF	36	B2-94	Pinni	REF	69	13108	M3	Isolate	102	79054	M3	Isolate
4	86/8/59	A2	REF	37	208	S1	Isolate	70	13138	S1	Isolate	103	79077	M3	Isolate
5	Tulya	A3	REF	38	226	S3	Isolate	71	13139	S2	Isolate	104	79104	M3	Isolate
6	292	A4	REF	39	1048	A3	Isolate	72	13147	S1	Isolate	105	79130	M3	Isolate
7	B3196	A5	REF	40	1057	A1	Isolate	73	13160	A1	Isolate	106	80345	M3	Isolate
8	870	A6	REF	41	1148	A3	Isolate	74	13195	M3	Isolate	107	80347	M3	Isolate
9	63/75	A7	REF	42	3001	M3	Isolate	75	13248	M2	Isolate	108	80355	M2	Isolate
10	C86	A9	REF	43	5017	M3	Isolate	76	13331	S2	Isolate	109	80356	M2	Isolate
11	104M	A	Vacci	44	7033	M3	Isolate	77	13332	S1	Isolate	110	80359	A3	Isolate
12	S19	A	Vacci	45	8257	A1	Isolate	78	13341	M3	Isolate	111	80392	A9	Isolate
13	45/20	A	REF	46	8416	A9	Isolate	79	23385	Canis	Isolate	112	81006	M3	Isolate
14	B1119	A	REF	47	9057	S3	Isolate	80	52141	Canis	Isolate	113	81011	A7	Isolate
15	16M	M1	REF	48	10009	S3	Isolate	81	57002	A3	Isolate	114	81033	M3	Isolate
16	63/9	M2	REF	49	10036	Canis	Isolate	82	60033	A1	Isolate	115	84001	M3	Isolate
17	Eher	M3	REF	50	10062	S3	Isolate	83	61005	M3	Isolate	116	86028	M1	Isolate
18	NI	M	REF	51	11038	Canis	Isolate	84	63006	M2	Isolate	117	86028	S1	Isolate
19	M5	M	Vacci	52	11055	A3	Isolate	85	63031	A1	Isolate	118	88040	S1	Isolate
20	M28	M	Vacci	53	11059	M3	Isolate	86	64008	S3	Isolate	119	90001	A3	Isolate
21	Rev1	M	Vacci	54	12088	M3	Isolate	87	64027	M2	Isolate	120	92008	M3	Isolate
22	1330	S1	REF	55	12099	M3	Isolate	88	64029	A1	Isolate	121	B257	A1	Isolate
23	Thomsen	S2	REF	56	12110	M3	Isolate	89	65050	M2	Isolate	122	HN-13	M1	Isolate
24	686	S3	REF	57	13031	M3	Isolate	90	65066	M2	Isolate	123	HNLG	M3	Isolate
25	40	S4	REF	58	13034	M3	Isolate	91	65079	A1	Isolate	124	HNLR	M3	Isolate
26	513	S5	REF	59	13051	M3	Isolate	92	66148	M2	Isolate	125	HNMB	M3	Isolate
27	VacciS2	S	Vacci	60	13067	M3	Isolate	93	72003	M2	Isolate	126	B114	/	Blood
28	VBI22	S	Vacci	61	13080	M3	Isolate	94	73011	M3	Isolate	127	B115	A1	Blood
29	RM6/66	Canis	REF	62	13081	M3	Isolate	95	73096	S3	Isolate	128	BJ10	Canis	Blood
30	10759	Ceti	REF	63	13083	M3	Isolate	96	73099	M3	Isolate	129	Lanzhou-26	A1	Blood
31	28753	Ceti	REF	64	13096	S3	Isolate	97	73101	M3	Isolate	130	B243	S3	Serum
32	4915	Micro	REF	65	13102	M3	Isolate	98	73162	M3	Isolate	131	B251	/	Serum
33	5K33	Neoto	REF	66	13105	M3	Isolate	99	78014	M3	Isolate	132	B252	M3	Serum

Note. A: *B. abortus* biovar, M: *B. melitensis* biovar, S: *B. suis* biovar, Canis: *B. canis,* Ceti: *B. ceti,* Pinni: *B. pinnipedialis,* Neoto: *B. neotomae*, Ovis: *B. ovis,* Micro: *B. microti*, REF: Reference strain, Isolate: Clinical isolate. /: *Brucella* was not detected in the blood samples by culturing or nested-PCR assay. REF, reference.

extracted DNA as the template. The second PCR was conducted with the primer pairs F2 and R2 using the first PCR amplicons as the template.

The first PCR reaction system included 2×Taq mastermix (12.5 μL, primer F1, and primer R1 [10 μmol/L]; Kangwei Century Biotechnology Co., Ltd, China) 0.6 μL each and DNA template 2 μL, to which 9.3–25.0 μL of distilled water was added. The first PCR reaction conditions were as follows: 94 °C for 4 min; 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 60 s, 30 cycles. The final extension was performed at 72 °C for 5 min. The second PCR reaction system and reaction conditions were the same as for the first PCR system, except for the template DNA from the first PCR amplicons (nested-PCR). A bright electrophoresis band of approximately 666 bp was amplified for the strains of *Brucella* spp. by using the nested-PCR assay. A total of 7 non-*Brucella* DNA could not be amplified by nested-PCR assay. The nested-PCR amplicons were purified and sequenced. Multiple sequences alignments were performed using the Clustal W, and a schematic representation of each locus was generated using the MEGA 5.1 by using the unweighted group average method (UPGMA), Neighbor-Joining tree construction, and Tamura-Nei algorithm with 1,000 bootstraps.

The sensitivity of the nested-PCR assay was determined by using 36 reference strains. All strains could amplify the positive bands. The minimum detection limit was tested by using 2-fold decreasing dilutions of *B. suis* 1330 DNA. The initial DNA (56.2 ng/μL) was diluted to 0.03 fg/μL. After several testing, the sensitivity of the nested-PCR was 3.35 fg, which was equivalent to 1 copy number of *Brucella* DNA in a 25-μL reaction system, considering that 24-fg nucleic acid DNA equals to approximately 7 copies of *Brucella* DNA[6].

A total of 36 *Brucella* reference strains, 89 clinical isolates, and 7 blood samples were examined by nested-PCR assay. The nested-PCR products were sequenced, and the sequences were clustered (Figure 2). The cluster analysis was performed as follows:

1) Three strains isolated from marine animals were assigned to 1 group, which included *B. ceti* 10759, *B. cet*i 28753, and *B. pinnipedialis* B2–94.

2) All *B. melitensis* biovar 2 and *B. melitensis* biovar 3 strains were clustered together.

3) All *B. abortus* biovar 1 strains were clustered together, along with 1 *B. neotomae* 5K33 strain.

4) The *B. ovis* 63/290 and *B. ovis* 25840 strains were clustered together, along with 1 *B. abortus* B1119 rough strains.

5) All *B. suis* biovar 3 strains were clustered

Figure 1. The nucleotide polymorphisms in transposase IS711 *orfA* and *orfB* of *Brucella* spp.* and vertical bar with a blue background: the base mutation site.

together, along with 1 *B. microti* 4915 strain.

6) A total of 6 *B. canis* strains were clustered into 2 groups, 1 group included 4 *B. canis* strains, while another included 2 *B. canis* strains along with 3 *B. suis* biovar 2 strains.

7) The 7 blood samples could not be amplified with a single primer pairs (F1 & R1 or F2 & R2). However, 5 blood samples showed bright electrophoresis bands through the nested-PCR assay with the primer pairs (F1 & R1 → F2 & R2). The cluster analysis revealed that B115 was *B. abortus* biovar 1, B252 was *B. melitensis* biovar 3, B243 was *B. suis* biovar 3, BJ10 was *B. canis*, and Lanzhou-26 was *B. abortus* biovar 1 (Figure 3). All 7 blood samples could not be amplified with a single primer B4 and B5[7] and could not be detected by fluorescent quantitative-PCR[8].

Recent studies have shown that PCR, either individually or multiplex reactions, can be used routinely to detect DNA from pure bacteria as they are rapid, highly specific, and sensitive. The PCR assay showed the highest sensitivity with the primer pairs B4 and B5[6], as it detected ≥ 500 CFU[7]. Due to the effects of inherent inhibitory factors in the blood and other tissues, real-time PCR assay was not deemed suitable for the detection of *Brucella* DNA[7]. Therefore, there is an urgent need to develop a simple and sensitive method for the identification of *Brucella*, especially for pathogenic bacteria in the blood and other tissue samples.

In this study, nested-PCR assay was unaffected by the inhibitory factors, and its detection sensitivity reached one copy number of *Brucella* DNA. Meanwhile, the species and biovars of infectious *Brucella* could be identified and typed. Until date, there exists no method that can completely match

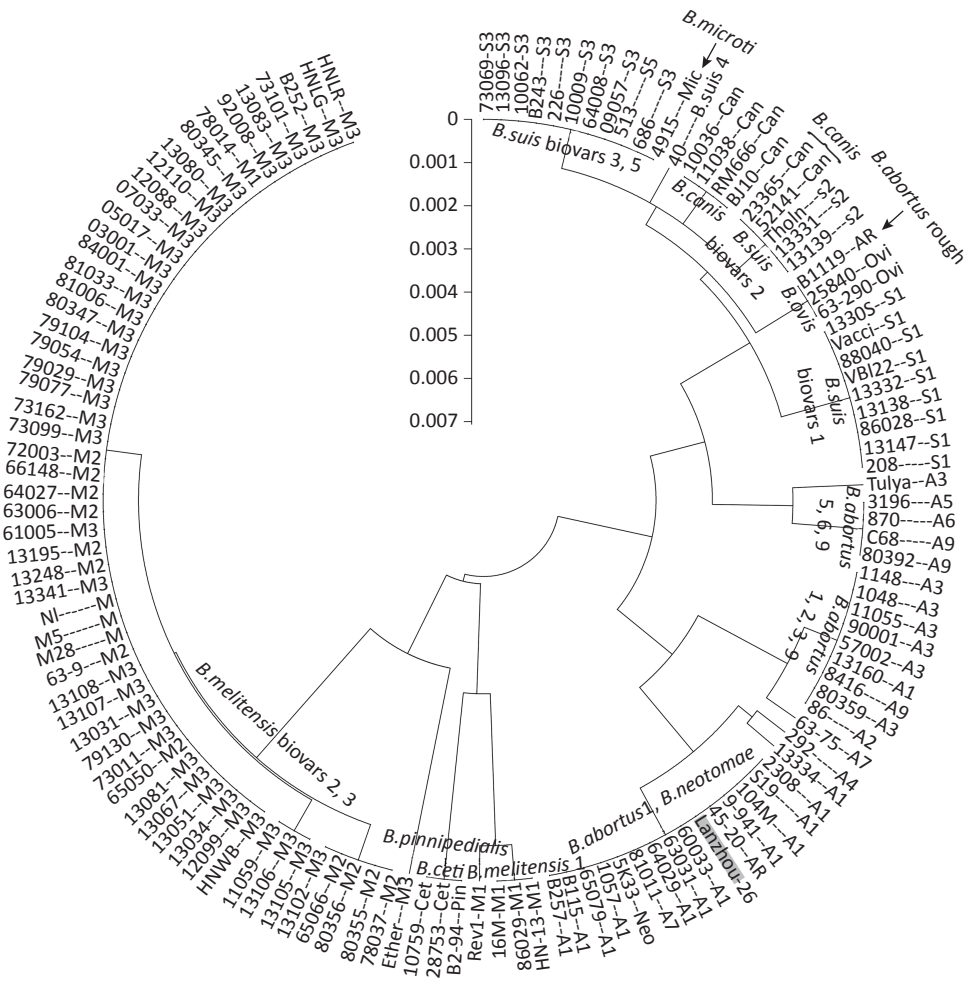

Figure 2. The cluster analysis of 36 *Brucella* reference strains, 89 clinical isolates, and 7 blood samples by nested-PCR. The black background of the code numbers indicates blood samples (B115, B243, B252, BJ10, and Lanzhou-26) in Figure 1.

the biological typing for *Brucella.* owing to the high consistency of *Brucella* DNA, such as the recently proposed and widely used multiple locus variable number of tandem repeats (MLVA) analysis[8]. Fortunately, nested-PCR assay can classify human pathogenic *Brucella*, including *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis*. The results of the present study revealed that nested-PCR can be useful for the detection and typing of *Brucella* DNA, including that of clinical strains and blood specimens.

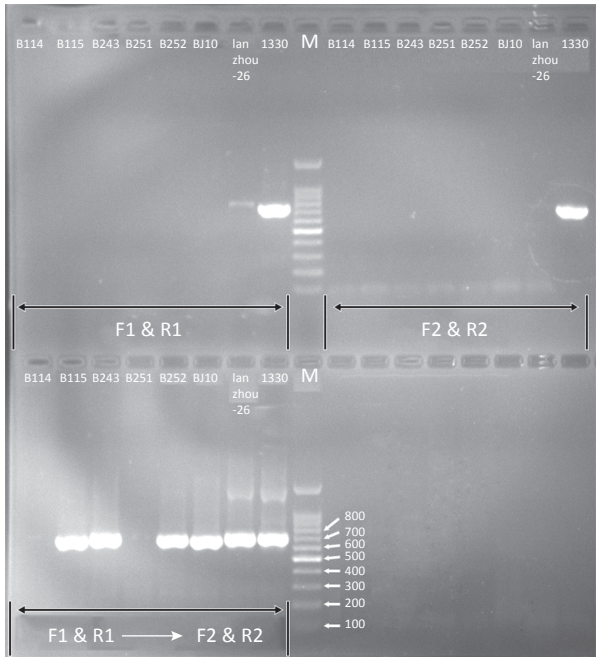

Figure 3. The nested-PCR electrophoresis of 7 extracted DNA from blood samples. The B114, B115, B243, B251, B252, BJ10, and Lanzhou-26 were the tested blood and serum samples. 1330 was *Brucella suis* biovars 1 strain. F1 & R1: PCR products with primers F1 and R1. F2 & R2: PCR products with primers F2 and R2. F1 & R→F2 & R2: nested-PCR products with primers F1 and R1 first and F2 and R2 second.

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