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RESEARCH ARTICLE

Strain-level Identification of *Brucella melitensis* Reference Strain 63/9 using Multiplex PCR Method by Targeting BMEA_B0162 and BMEA_A1238

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A B S T R A C T

Brucellosis is one of the most prevalent bacterial zoonosis worldwide. *Brucella melitensis* (*B. melitensis*), *Brucella abortus* (*B. abortus*), *Brucella canis* (*B. canis*), *Brucella suis* (*B. suis*) are common to cause disease in humans and *B. melitensis* is the most pathogenic causative agent of brucellosis in humans and animals. Fast, efficient and accurate identification of *Brucella* reference strains at the strain-level is indispensable for microbiological method quality assurance and downstream applications. *B. melitensis* 63/9 is recognized as an important reference strain for the microbiological culture collection organizations worldwide, and the identification of *B. melitensis* strain 63/9 is still lacking. The genomic sequences of *B. melitensis* 63/9 and nine other *Brucella* strains were compared. Two specific genes were selected for the multiplex PCR method. Gene BMEA_B0162 with unknown function is the key target to identify *B. melitensis* 63/9, and gene BMEA_A1238 annotated as TRAP transporter solute receptor is included as a control gene for the *Brucella* genus. A multiplex PCR was established in this study to differentiate *B. melitensis* reference strain 63/9 from 39 *B. melitensis* strains, 13 *B. abortus* strains, 5 *B. suis* strains, 6 *B. canis* strains, 3 *E. coli*, and 4 *Salmonella* strains by targeting the BMEA_B0162 and the BMEA_A1238 in the genome. This method allows at least 100 pg of *B. melitensis* 63/9 genomic DNA to be detected. We established a fast, and a cost-effective method to distinguish *B. melitensis* 63/9 from other *Brucella* strains and some non-*Brucella* bacteria strains with high sensitivity and specificity, making the first report about the identification of *Brucella* reference strain recognized by World Organization for Animal Health at the strain-level.

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INTRODUCTION

Brucellosis is one of the world's most infectious and contagious zoonoses caused by the genus *Brucella*, leading to breeding difficulty and the mankind of febrile diseases (Glowacka *et al.*, 2018; Dadar *et al.*, 2021). According to the corresponding preferred hosts, *Brucella* has been divided into twelve species, such as *Brucella melitensis* (goats and sheep), *Brucella abortus* (cattle), *Brucella suis* (pigs), *Brucella ovis* (sheep), *Brucella canis* (dogs), *Brucella neotomae* (desert woodrats), *Brucella microti* (Microtus voles), *Brucella papionis* (Baboons), *Brucella pinnipedialis* (pinnipeds), *Brucella ceti* (whales), *Brucella inopinata* (Unknown) and *Brucella vulpis* (Red foxes) (El-Sayed and Awad, 2018). All species belonging to the genus *Brucella* are closely related and *Brucella melitensis* is the most pathogenic *Brucella* in humans and also certain animals (Bayu, 2018; Parks *et al.*, 2020).

Brucella melitensis reference strain 63/9, corresponding to ATCC 23457, was originally isolated from human blood and bone marrow samples in India (Mathur, 1963). For many years, it has been widely used as a reference strain in research studies all over the world (Elfaki *et al.*, 2005; Bounaadja *et al.*, 2009; Pisarenko *et al.*, 2018). Up to the present, a quick and reliable assay for distinguishing *B. melitensis* 63/9 from field strains is not available. The objective of this study was to explore a genetic marker and a universal strategy for quick and specific identification of *B. melitensis* 63/9.

MATERIALS AND METHODS

Bacteria strains and genomic DNA preparation: A total of 63 *Brucella* strains representing *Brucella* common species and 7 non-*Brucella* strains referred in the study were summarized in Table 1. All *Brucella* reference strains were provided from China Veterinary Culture Collection Center (CVCC, Beijing, China). Other bacterial strains were isolated previously after routine clinical monitoring and all the strains were identified using the Bruce-ladder multiplex PCR assay in The National Reference Laboratory for Animal Brucellosis (Beijing, China) according to OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Brucellosis, 2022). All *Brucella* strains were routinely cultivated in Tryptic Soy Broth (TSB, BD Difco) at 37°C or on Tryptic Soy Agar (TSA, BD Difco) medium incubated at 37°C with 5% CO_2. Bacteria were then resuspended with sterile normal saline and inactivated at 80°C for 2 h. The 7 non-*Brucella* species were grown, harvested and inactivated as previously mentioned (Capobianco *et al.*, 2020; Zhou *et al.*, 2020). Bacterial genomic DNA was extracted with Bacterial DNA Extraction Kit (Omega Bio-Tek). The concentration and purity of bacterial DNA were measured by NanoDrop ND-1000 spectrometer (Thermo Scientific, USA).

Primer design: To differentiate and characterize *B. melitensis* 63/9 by a multiplex PCR method, we utilized the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1990) from the National Center for Biotechnology Information (NCBI). The common *Brucella* genome sequences of *Brucella* strains *B. melitensis* 63/9, *B. melitensis* 16M, *B. melitensis* Ether, *B. melitensis* M28, *B. melitensis* Rev.1, *B. abortus* 2308, *B. abortus* A19, *B. suis* 1330, *B. suis* S2 and *B. canis* 6/66 were respectively used for the non-redundant nucleotide collection database searching. To make certain that aligned subject sequences exist in the database totally, the expect value (E-value) of nucleotide sequences was set at 20,000. The BLAST Ring Image Generator (BRIG) analysis (Alikhan *et al.*, 2011) was performed to visualize the comparative genomic analysis among the different *Brucella* strains mentioned above. All primers utilized in the study were designed using the online software Primer3 (Untergasser *et al.*, 2012) and produced by Shanghai Sangon (Shanghai, China).

Multiplex PCR procedure: Premix Taq (Ex Taq Version 2.0 plus dye) (Takara, Dalian, China) was utilized for the Multiplex PCR reactions in a total volume of 50 µL comprising 10 ng bacterial DNA, 25 µL 2× Premix Taq, and 10 pmol of each primer. The multiplex PCR amplification was conducted utilizing a Veriti 96-Well Fast Thermal Cycler (Applied Biosystems, Waltham, Massachusetts, United States) with the following protocol: an initial denaturation (98°C for 5 min), continued with 35 cycles of denaturation (98°C for 30 sec), primer annealing (58°C for 30 sec) and product extension (72°C for 30 sec), and final product extension at 72°C for 5 min. The amplification products were run in 1% gel and stained with GoldView (Solarbio, Bejing, China). The fragments of PCR products were visualized by exposing the gel to trans-UV and images were captured by Gel Doc (Bio-Rad, United States) apparatus.

Discrimination and sensitivity of the multiplex PCR system: Bacterial genome template prepared from 63 various *Brucella* strains and 7 distinct non-*Brucella* bacteria were utilized to evaluate the discrimination and consistency of the primers in this PCR system. The sensitivity of this PCR-based method indicating the minimum amount of DNA that can be detected by photography of GoldView stained gels was decided by analyzing diluted DNA templates from reference strain *B. melitensis* 63/9. The genomic DNA of *B. melitensis* 63/9 was serially 10-fold diluted with distilled water from 1 ng/µL to 1 pg/µL. These diluted genomic DNA were respectively worked as template in this developed method.

RESULTS

Analysis of bioinformatics and primer design: The Bruce-ladder multiplex PCR assay validated in this study identified the different species of *Brucella* (Fig. 1). *B. melitensis* 63/9 can grow on dye thionin and basic fuchsin, showing positive with *Brucella* anti-A monospecific serum, and negative to Tb phage, confirming belonged to *B. melitensis* biotype 2 (Fig. 2). *B. melitensis* 63/9 has two circular chromosomes, a larger chromosome of 2,125,701 bp and a smaller chromosome of 1,185,518 bp. The comparative analysis shows that BMEA_B0162 is a promising gene for differentiating *B. melitensis* 63/9 from other *Brucella* strains, as a 116 bp fragment is inserted in all the other *Brucella* strains. Fig. 3 indicates the location of BMEA_B0162 encoding hypothetical protein unique to strain *B. melitensis* 63/9, and the gene conferring TRAP transporter solute receptor (BMEA_A1238) is proved well to be conserved in all *Brucella* species (Imaoka *et al.*, 2007).

The further analysis of BMEA_B0162 was done using Jalview tool (Waterhouse *et al.*, 2009) that performed the MUSCLE algorithm and gave a detailed output with colorful DNA bases (Fig. 4). B0162 F/R primers were chosen for the specific detection of *B. melitensis* 63/9 based on a 116 base pair gap in the BMEA_B0162. In addition, A1238 F/R acting as control primers were selected from BMEA_A1238 (Table 2). The primer set in the PCR reaction produced two fragments of 452 bp and 224 bp specific to BMEA_B0162 and BMEA_A1238 respectively for *B. melitensis* 63/9. Non-*B. melitensis* 63/9 *Brucella* strains showed 2 fragments, 568 bp and 224 bp, and no amplicon was observed for non-*Brucella* bacteria.

Analytical discrimination of the multiplex PCR system: The multiplex PCR discrimination was conducted by amplifying the bacterial genomic DNA prepared from 63 strains of different *Brucella* biovars and 7 strains of distinct non-*Brucella* bacteria. The PCR reaction results exhibited that two specific fragments of 452 bp and 224 bp corresponding to the amplification of part regions of BMEA_B0162 and BMEA_A1238 from *B. melitensis* 63/9 were generated. In contrast, two amplifications of 568 bp and 224 bp were produced in the other 62 strains of *Brucella*. No amplified fragment was observed in the strains not belonging to *Brucella*, implicating that the established multiplex PCR assay had a perfect performance with 100% inclusivity of *Brucella* species and exclusivity of non-*Brucella* bacteria (Fig. 5).

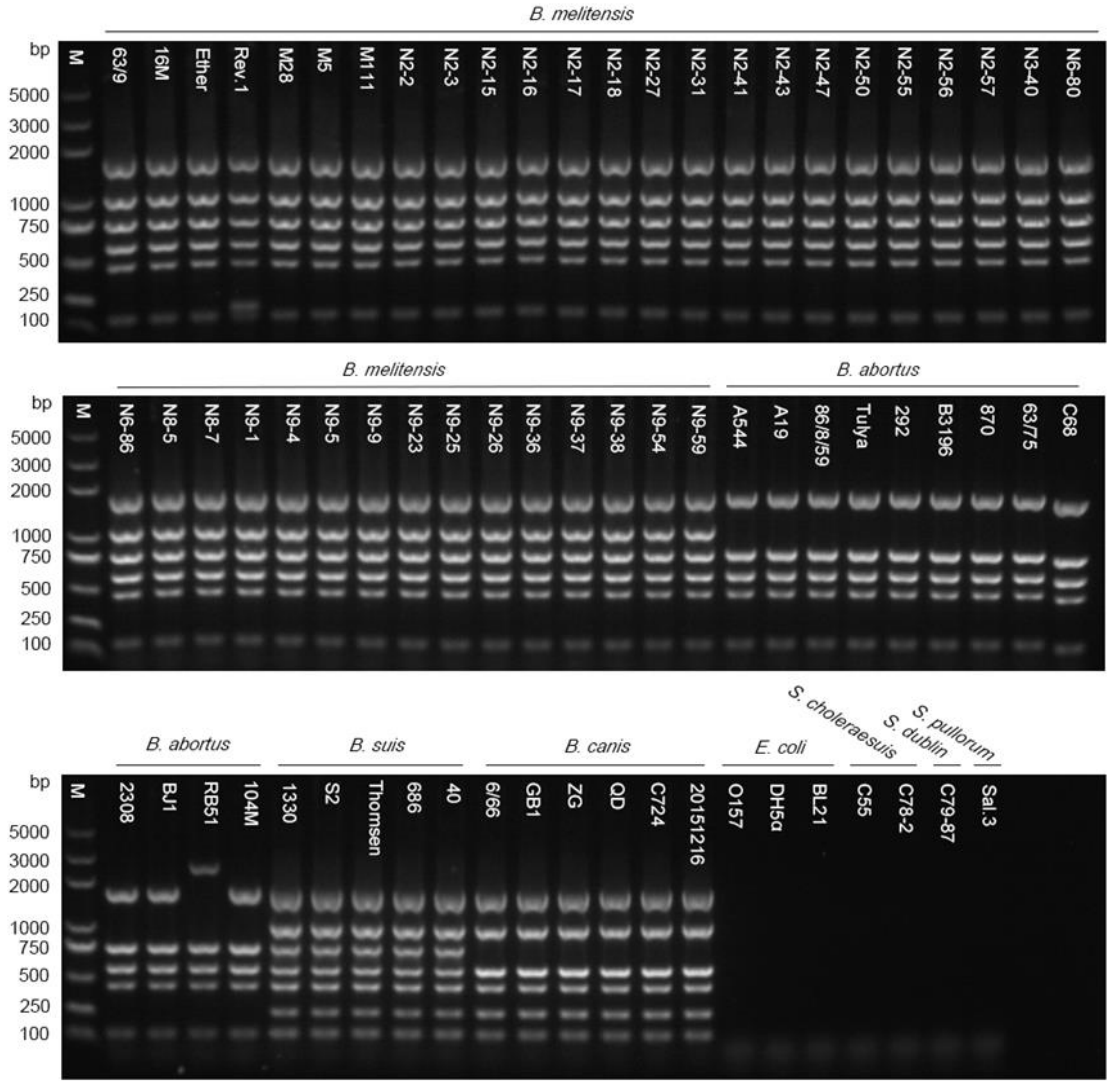

Fig. 1: Species level identification using the Bruce-ladder multiplex PCR assay.

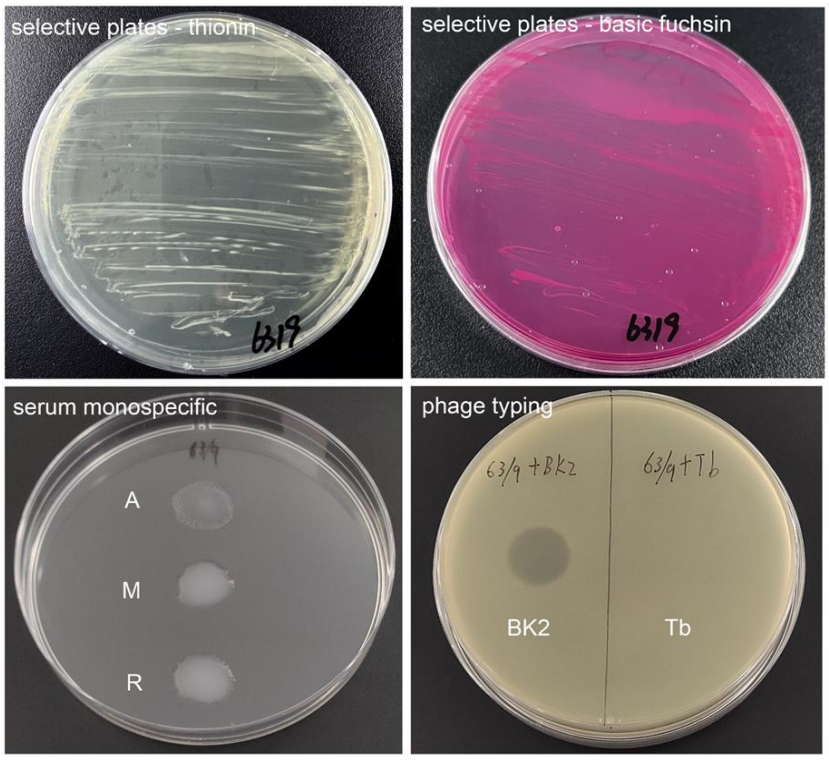

Fig. 2: Classical bacteriology test for *Brucella* typing.

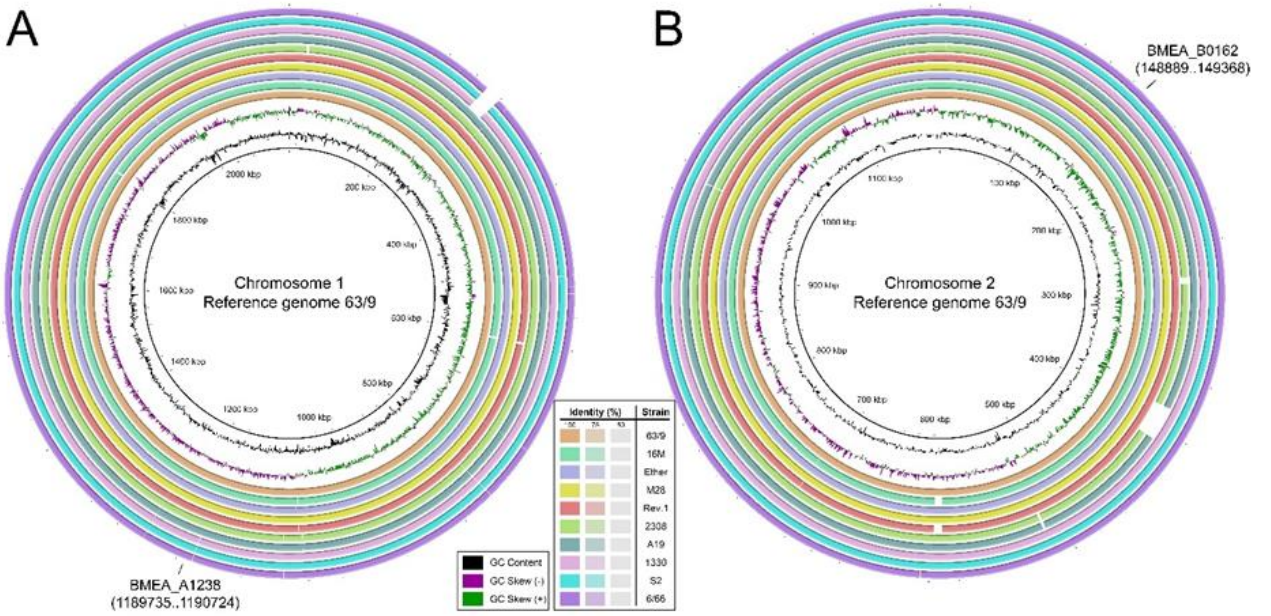

Fig. 3: Comparative genomic analysis of chromosome 1 (A) and chromosome 2 (B) in strain 63/9, 16M, Ether, M28, Rev.1, 2308, A19, 1330, S2 and 6/66. The innermost ring represents the scale for *B. melitensis* 63/9 in kilobase pairs. The next two rings represent the GC content and GC skew, respectively. The remaining 10 colored rings represent regions of sequence identity detected by BRIG (version 0.95) conducted between 63/9 and the other strains in this study. Regions of the target genes are labelled on the outermost ring.

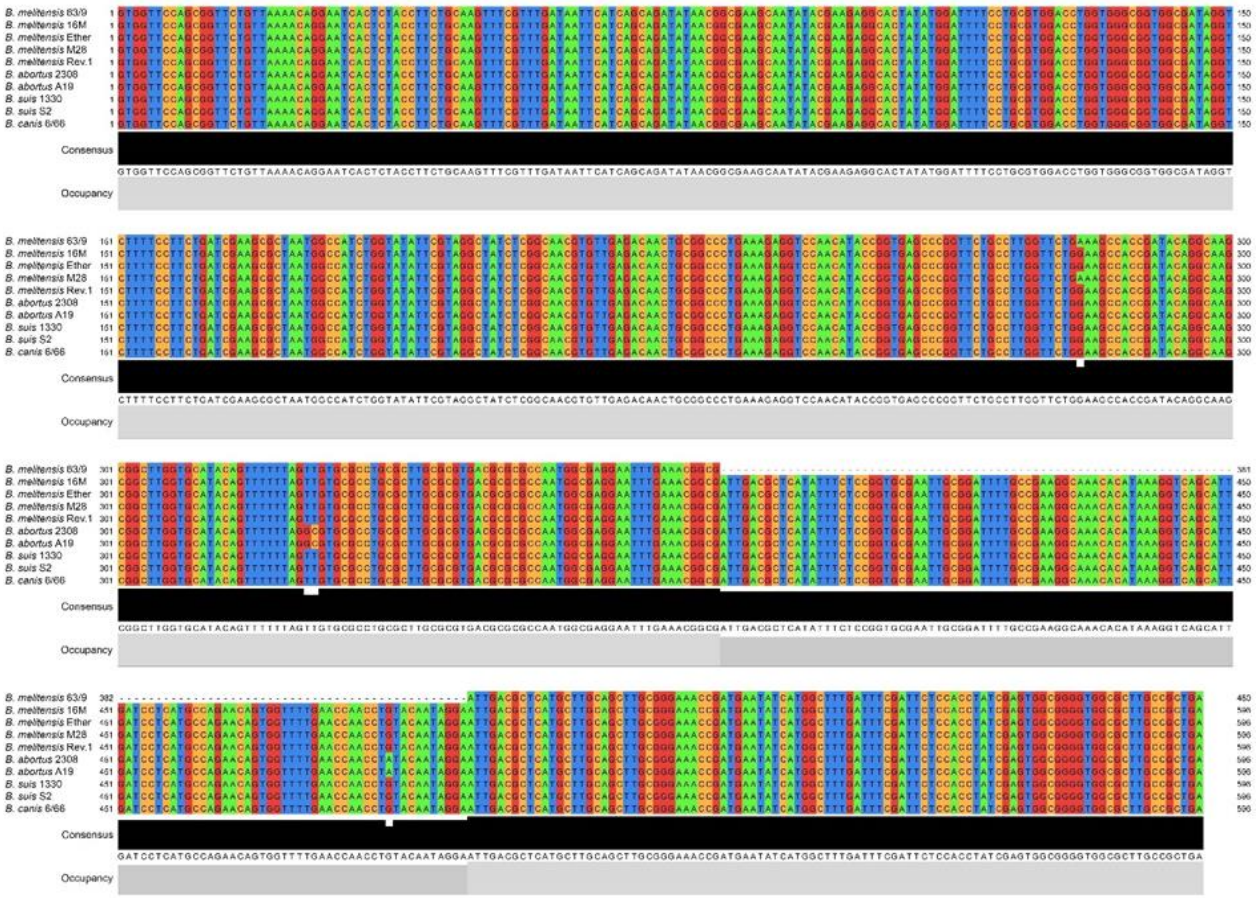

Fig. 4: The Syntenic region among the BMEA_B0162 gene of different *Brucella* strains generated using Jalview tool. The color variations represent the similarity and homology among the genes.

Sensitivity of the multiplex PCR system: The sensitivity of established multiplex PCR system was appraised by detecting serially diluted bacterial genomic template from *B. melitensis* 63/9. The results showed that BMEA_B0162 and BMEA_A1238 could be detected with the minimum concentration of 100 pg/µL, indicating that 100 pg/µL of bacterial DNA is necessary so as to identify and distinguish *B. melitensis* 63/9 using this multiplex PCR assay.

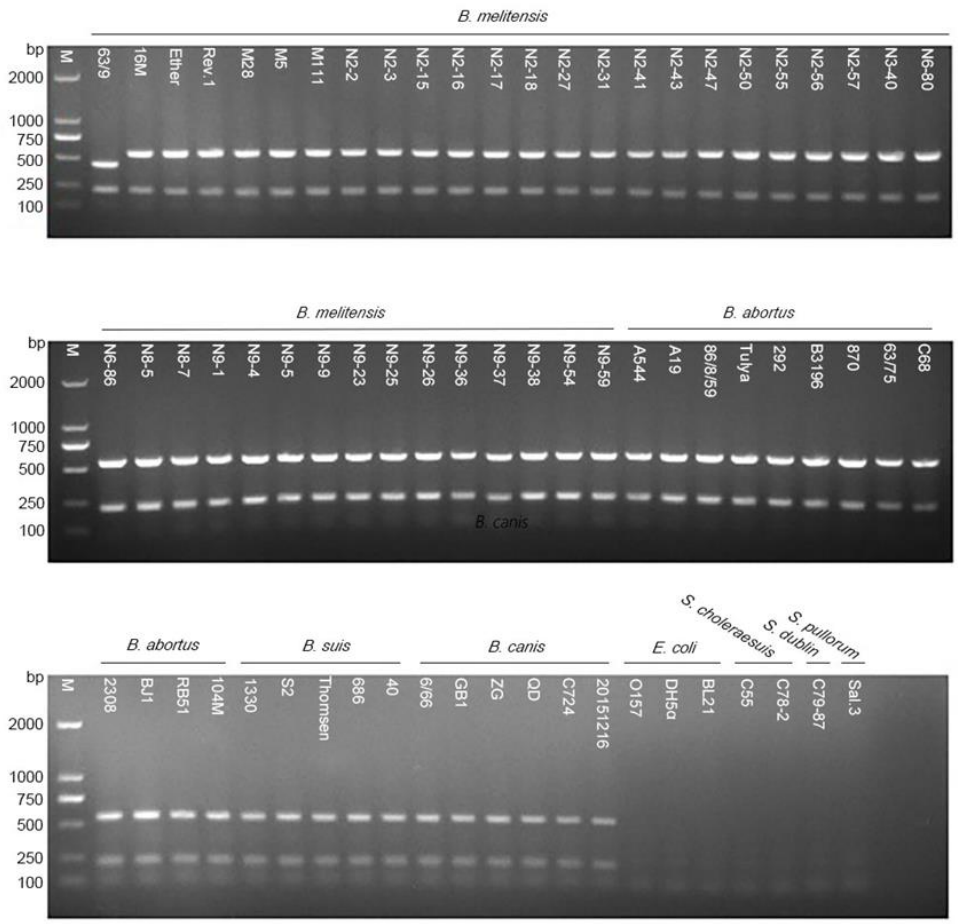

Fig. 5: Strain *B. melitensis* 63/9 was identified using designed primers targeting BMEA_B0162 (452/568 bp). The *Brucella*-specific PCR fragment was amplified with primers targeting BMEA_A1238 (224bp).

Table 1: *Brucella* spp. strains used in the present study.

Species(biovar)	Strain	Type	Host	Source
B. melitensis (2)	63/9	Reference strain	Caprine	India
B. melitensis (1)	16M	Reference strain	Caprine	United States
B. melitensis (3)	Ether	Reference strain	Caprine	Italy
B. melitensis (1)	Rev.1	Vaccine	-	-
B. melitensis (1)	M28	Field strain	Caprine	China
B. melitensis (1)	M5	Vaccine	-	-
B. melitensis (3)	M111	Vaccine	-	-
B. melitensis (3)	N2-2	Field strain	Bovine	China
B. melitensis (3)	N2-3	Field strain	Caprine	China
B. melitensis (3)	N2-15	Field strain	Caprine	China
B. melitensis (3)	N2-16	Field strain	Caprine	China
B. melitensis (3)	N2-17	Field strain	Caprine	China
B. melitensis (2)	N2-18	Field strain	Bovine	China
B. melitensis (3)	N2-27	Field strain	Caprine	China
B. melitensis (3)	N2-31	Field strain	Caprine	China
B. melitensis (3)	N2-41	Field strain	Caprine	China
B. melitensis (3)	N2-43	Field strain	Caprine	China
B. melitensis (3)	N2-47	Field strain	Caprine	China
B. melitensis (2)	N2-50	Field strain	Caprine	China
B. melitensis (3)	N2-55	Field strain	Caprine	China
B. melitensis (3)	N2-56	Field strain	Caprine	China
B. melitensis (3)	N2-57	Field strain	Bovine	China
B. melitensis (3)	N3-40	Field strain	Caprine	China
B. melitensis (3)	N6-80	Field strain	Caprine	China
B. melitensis (3)	N6-86	Field strain	Caprine	China
B. melitensis (3)	N8-5	Field strain	Bovine	China
B. melitensis (3)	N8-7	Field strain	Bovine	China
B. melitensis (3)	N9-1	Field strain	Caprine	China
B. melitensis (3)	N9-4	Field strain	Caprine	China
B. melitensis (3)	N9-5	Field strain	Caprine	China
B. melitensis (3)	N9-9	Field strain	Caprine	China
B. melitensis (3)	N9-23	Field strain	Caprine	China
B. melitensis (3)	N9-25	Field strain	Bovine	China
B. melitensis (3)	N9-26	Field strain	Caprine	China
B. melitensis (2)	N9-36	Field strain	Caprine	China
B. melitensis (3)	N9-37	Field strain	Caprine	China
B. melitensis (3)	N9-38	Field strain	Bovine	China
B. melitensis (3)	N9-54	Field strain	Bovine	China
B. melitensis (3)	N9-59	Field strain	Caprine	China
B. abortus (1)	A544	Reference strain	Bovine	United Kingdom
B. abortus (1)	A19	Vaccine	-	-
B. abortus (2)	86/8/59	Reference strain	Bovine	United Kingdom
B. abortus (3)	Tulya	Reference strain	Bovine	Uganda
B. abortus (4)	292	Reference strain	Bovine	United Kingdom
B. abortus (5)	B3196	Reference strain	Bovine	United Kingdom
B. abortus (6)	870	Reference strain	Bovine	Africa
B. abortus (7)	63/75	Reference strain	Bovine	Poland
B. abortus (9)	C68	Reference strain	Bovine	United Kingdom
B. abortus (1)	2308	Field strain	Bovine	United States
B. abortus (1)	BJ1	Field strain	Deer	China
B. abortus (-)	RB51	Vaccine	-	-
B. abortus (-)	104M	Vaccine	-	-
B. suis (1)	1330	Reference strain	Porcine	United States
B. suis (1)	S2	Vaccine	-	-
B. suis (2)	Thomsen	Reference strain	Porcine	Denmark
B. suis (3)	686	Reference strain	Porcine	United States
B. suis (4)	40	Reference strain	Porcine	Russia
B. canis	6/66	Reference strain	Canine	United States
B. canis	GB1	Field strain	Canine	China
B. canis	ZG	Field strain	Canine	China
B. canis	QD	Field strain	Canine	China
B. canis	C724	Field strain	Canine	China
B. canis	20151216	Field strain	Canine	China
Escherichia coli	O157	Field strain	-	China
Escherichia coli	DH5α	Competent strain	-	-
Escherichia coli	BL21	Competent strain	-	-
Salmonella choleraesuis	C55	Field strain	Porcine	China
Salmonella choleraesuis	C78-2	Field strain	Porcine	China
Salmonella dublin	C79-87	Field strain	Bovine	China
Salmonella pullorum	Sal.3	Field strain	Chicken	China

Table 2: Multiplex PCR primers used for identification and discrimination of *B. melitensis* 63/9.

Primers	Sequence (5'→ 3')	Size (bp)	Target gene
B0162F	AAAGCCACCGATACAGGCAA	452/ 568	BMEA_B0162
B0162R	GCGTTTTGGTGTCGCTCTTT		
A1238F	TGGCTCGGTTGCCAATATCAA	224	BMEA_A1238
A1238R	CGCGCTTGCCTTTCAGGTCTG		

DISCUSSION

Brucella identification methods itself have actually gone through many successive stages of development. At the beginning, the classical assays (oxidase activity, urease activity, phage susceptibility, CO_2 requirement, H_2S production, dye tolerance, and agglutination pattern) were explored for *Brucella* identification, but they are solely responsible for species level taxonomic profiling (Kumar *et al.*, 2011). Afterwards, the most famous and conveniently used multiplex PCR techniques (AMOS PCR and the Bruce ladder) were developed for *Brucella* identification, though these methods are still limited to *Brucella* differentiation at species level (Bricker and Halling, 1994; Lopez-Goni *et al.*, 2008; Lopez-Goni *et al.*, 2011). However, identification at strain level offers the possibilities and opportunities to explore the functional capacity of reference strains. Recently, various genotypic identification methods for *Brucella* vaccines at the strain level have been developed. For example, a set of primers based on the sequences of the locus ery was utilized to identify *Brucella* S19, a *Brucella* RB51 specific PCR was exploited to identify *Brucella* vaccine RB51 from other *Brucella* strains using detection based on wboA gene mutations, and a SNP based MGB PCR assay targeted at the DnaK gene was used that could unambiguously and straightforwardly distinguish *Brucella* vaccine 104M (Yu and Nielsen, 2010; Nan *et al.*, 2018). However, few have been developed to identify *Brucella* reference strains up to the strain level (Garin-Bastuji *et al.*, 2014; Kurmanov *et al.*, 2022). Although the PCR instrument is a kind of equipment with high capital cost, PCR is still an inexpensive method with high efficiency and specificity. This kind of method offers practical assistance for identifying microorganisms in a single reaction when compared with other identification methods. Therefore, PCR has been extensively utilized for clinical diagnostic in animals and humans.

In our study, we report a natural mutation in the gene BMEA_B0162 and established a multiplex PCR approach to identify *B. melitensis* reference strain 63/9 at the strain level, based on genetic characterization, with a sensibility of 100pg. Two genes (BMEA_A1238, BMEA_B0162) were selected after genetic screening among 63 *Brucella* strains representing *Brucella* common species and 7 non-*Brucella* strains. BMEA_A1238 was proved to be genus specific in *Brucella*, and thus served as internal amplification control in order to avoid false negative results. BMEA_B0162 was utilized for specific detection of *Brucella melitensis* reference strain 63/9. The combination of BMEA_B0162 with BMEA_A1238 enables the identification of *Brucella melitensis* reference strain 63/9 at the strain level.

Conclusions: Strain-specific primers of BMEA_B0162 were incorporated for identification of the *Brucella melitensis* reference strain 63/9. To our knowledge, this is the first report about the identification of *Brucella* reference strain at strain-level using multiplex PCR method. This method can be utilized together with classical bacteriology assay and Bruce-ladder multiplex PCR for a reliable Brucella typing, and also will prompt infection model investigation, as well as for the pharmaceutical product development based on reference strains.

Author contribution: Jiabo Ding, Xingjia Shen concepted the experiment. Ge Zhang, Hui Jiang, Guangzhi Zhang conducted research and wrote the manuscript. Peng Li contributed to data analysis. Yu Feng participated in sample collection. All authors contributed to the article and have given their approval for the final submitted version.

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