

Real-Time Multiplex PCR Assay for Detection of *Brucella* spp., *B. abortus*, and *B. melitensis*

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The identification of *Brucella* can be a time-consuming and labor-intensive process that places personnel at risk for laboratory-acquired infection. Here, we describe a real-time PCR assay for confirmation of presumptive *Brucella* isolates. The assay was designed in a multiplex format that will allow the rapid identification of *Brucella* spp., *B. abortus*, and *B. melitensis* in a single test.

Brucellosis is a zoonotic disease that causes abortion, fetal death, and genital infections in animals. In humans, this highly diverse illness initially presents as fever, malaise, and myalgia and may later develop into a chronic illness affecting various organs and tissues. The causative agents of brucellosis are facultative intracellular gram-negative bacteria belonging to the genus *Brucella*. This genus has been further classified into six species according to host preference and pathogenicity. Genetic studies, however, indicate that the six classical species of *Brucella* represent a single genomospecies based upon DNA-DNA reassociation (14). In spite of this evidence, the conventional classification scheme has been maintained. Human brucellosis has been attributed to *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis* and more recently to strains resembling *Brucella* isolated from marine mammals (13). Laboratory detection of *Brucella* and species identification is based largely on culture isolation and phenotypic characterization. This process is lengthy and labor-intensive and has been associated with a heightened risk of laboratory-acquired infections (7). To surmount these problems, nucleic acid amplification has been explored for the rapid detection and confirmation of *Brucella*.

A number of nucleic acid sequences have been targeted for the development of *Brucella* genus-specific PCR assays, including 16S rRNA, the 16S-23S intergenic spacer region, *omp2*, and *bcs31* (1, 9, 11, 12). The most frequently described PCR target for the diagnosis of human brucellosis is the *bcs31* gene encoding a 31-kDa antigen conserved among *Brucella* spp. (8, 9, 15). PCR identification of *Brucella* strains at the species or biovar level has been more challenging. Recently, Redkar et al. (10) described real-time PCR assays for the detection of *B. abortus*, *B. melitensis*, and *B. suis* biovar 1. These PCR assays target the specific integration of IS711 elements within the genome of the respective *Brucella* species or biovar. The assays, however, were designed to be tested in separate PCRs. Using similar PCR targets, but in a multiplex format, we have developed a real-time triplex assay that permits rapid confir-

mation of *Brucella* spp., *B. abortus*, and *B. melitensis* isolates in a single test.

The primers and TaqMan probes (Qiagen, Alameda, Calif.) utilized for the multiplex assay are shown in Table 1. All primers and TaqMan probes were designed using the multiplex TaqMan design feature of Beacon Designer software (Premier BioSoft International, Palo Alto, Calif.). For *Brucella* spp. identification, the primers and probe target the *bcs31* gene (GenBank accession number M20404). The nucleic acid targets for *B. abortus* and *B. melitensis* identification are similar to those described by Redkar et al. (10). However, the primers and probes to these targets were redesigned for the multiplex TaqMan format. The *B. abortus* primers and probe set targets the specific insertion of an IS711 element downstream of the *alkB* gene (GenBank accession number AF148682), whereas the *B. melitensis* primers and probe set targets the insertion of an IS711 element downstream of BMEI1162 (GenBank accession number NC_003317). Both targets share the same IS711 reverse primer, while the forward primers target either *alkB* (*B. abortus*) or BMEI1162 (*B. melitensis*). The *B. abortus* and *B. melitensis* TaqMan probes target the *alkB* and BMEI1162 gene, respectively. The 50- μ l multiplex PCR mixture consisted of 1 \times AmpliTaq Gold buffer (Applied Biosystems, Foster City, Calif.), 6 mM MgCl₂, 2 mM of deoxynucleoside triphosphate blend (Applied Biosystems), a 200 nM concentration of each primer, a 100 nM concentration of each probe, 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 5 μ l of a DNA extract. Amplification and real-time fluorescence detection was performed on the iCycler real-time PCR detection system (Bio-Rad Laboratories, Hercules, Calif.) using the following parameters: 10-min denaturation and polymerase activation step at 95°C followed by 45 cycles of 95°C for 15 s and 57°C for 1 min. A sample with a fluorescence signal 30 times greater than the mean standard deviation in all wells over cycles 2 through 10 was considered a positive result, whereas a sample yielding a fluorescence signal less than this threshold value was considered a negative result.

To test the specificity of the multiplex assay, an extensive panel of well-characterized *Brucella* and non-*Brucella* strains was assembled and tested (Table 2). Identification of *Brucella* strains was performed using standard classification tests, in-

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TABLE 2. Performance of a real-time multiplex PCR assay for the detection of *Brucella* spp., *B. abortus*, and *B. melitensis*

Strain	PCR identification		
	<i>Brucella</i> spp.	<i>B. abortus</i>	<i>B. melitensis</i>
<i>B. abortus</i>	25/25 ^a	23/25	2/25
<i>B. melitensis</i>	25/25	0/25	25/25
<i>B. suis</i>	10/10	0/10	0/10
<i>B. canis</i>	1/1	0/1	0/1
<i>Brucella</i> spp. ^b	2/2	0/2	0/2
Non- <i>Brucella</i> spp. ^c	0/59	0/59	0/59

^a Number of PCR-positive isolates per number of isolates tested.

^b Human isolates genetically related to marine mammal strains of *Brucella*.

^c *Acinetobacter baumannii*, *Actinobacillus actinomycetemcomitans*, *Afiopia broomeae*, *Afiopia* genomospecies 4, *Agrobacterium radiobacter*, *Agrobacterium* spp., *Bartonella bacilliformis*, *Bartonella henselae*, *Bartonella quintana*, *Bordetella bronchiseptica*, *Burkholderia cepacia*, *Burkholderia pseudomallei*, *Capnocytophaga canimorsus*, *Cardiobacterium hominis*, *Eikenella corrodens*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli* O157:H7, *Francisella tularensis*, *Haemophilus aphrophilus*, *Haemophilus haemolyticus*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Haemophilus paraphrophilus*, *Kingella kingae*, *Moraxella catarrhalis*, *Neisseria meningitidis*, *Oligella ureolytica*, *Ochrobactrum anthropi* (*n* = 9), *Ochrobactrum* spp. (*n* = 5), *Pasteurella aerogenes*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Psychrobacter phenylpyruvicus*, *Ralstonia pickettii*, *Roseomonas* genomospecies 5, *Roseomonas gilardii*, *Roseomonas* spp., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus bovis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Stenotrophomonas maltophilia*, *Vibrio cholerae*, and *Yersinia enterocolitica* O:9.

cluding growth characteristics, oxidase activity, urease activity, CO₂ requirement, H₂S production, dye tolerance, sero-agglutination, and susceptibility to the Tbilisi phage. Non-*Brucella* strains were identified using standard biochemical and immunological procedures and, in some instances, cellular fatty acid analysis and partial 16S rRNA sequencing. Crude nucleic acid extracts were prepared by resuspending a 1-μl loop of bacteria into 100 μl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8), boiling the suspension for 10 min, and pelleting the cellular debris by centrifugation. The supernatant was collected as the crude DNA extract. In some cases, nucleic acids were purified using DNeasy spin columns (Qiagen Inc., Valencia, Calif.) according to the manufacturer's recommendations. The ability to amplify DNA from non-*Brucella* strains was demonstrated using a real-time PCR assay targeting the 16S rRNA gene (4) (data not shown).

Amplification results for *Brucella* and non-*Brucella* strains using the multiplex assay are shown in Table 2. The sensitivities and specificities of the assay for *Brucella* spp., *B. abortus*, and *B. melitensis* identification were 100 and 100%, 92.0 and 100%, and 100 and 97.9%, respectively. For *Brucella* spp. identification, perfect agreement was found between the phenotypic and genotypic determinations. The inclusion of a genus-specific primer-probe set facilitates the identification of rare or unusual strains of *Brucella*. The applicability of this approach was recently (13) and presently (Table 2) demonstrated by the identification of two patient isolates that were ultimately found to be closely related to marine mammal strains of *Brucella*. Further resolution of *Brucella* strains was provided by the species-specific primer-probe sets of the multiplex assay. Primers and probes for the detection of *B. abortus* and *B. melitensis* were selected for inclusion in the multiplex assay because these strains represent greater than 90% of the *Brucella* spp. isolated in California (6). With regards to the identification of *B. abortus*

TABLE 1. Oligonucleotide primers and probes used in the real-time multiplex PCR assay for the detection of *Brucella* spp., *B. abortus*, and *B. melitensis*

PCR identification	Forward primer ^a	Reverse primer ^a	Probe ^a	5' Fluorophore/3' quencher ^b
<i>Brucella</i> spp.	GCTCGGTTGCCAATATCAATGC	GGGTAAGCGCTCGCCAGAAG	AAATCTTCACCTTGCCCTTGCCATCA	6-FAM/BHQ1
<i>B. abortus</i>	GCGGCTTTTCTATCAGCGTATTC	CATGGCGTATGATCTGTGTTACG	CGCTCATGTGCTGGCAGACTTCAATG	HEX/BHQ1
<i>B. melitensis</i>	AACAAGCGGCGACCCCTAATAA	CATGCGCTATGATCTGTGTTACG	CAGGAGTGTTCGGCTCAGAAATTAATCCACA	Texas Red/BHQ2

^a Oligonucleotide sequence provided in 5' to 3' orientation.

^b 6-FAM = 6-carboxyfluorescein; HEX = 6-hexachlorofluorescein; BHQ1 = Black Hole Quencher 1; BHQ2 = Black Hole Quencher 2.

tus and *B. melitensis*, two discrepant results were observed (Table 2). Two strains identified phenotypically as *B. abortus* were identified as *B. melitensis* in the multiplex PCR assay. Upon review of the phenotypic results, one of the two strains demonstrated an aberrant test reaction: no lysis by phage Tbilisi.

To assist in the resolution of the discrepant PCR results, partial DNA sequencing of the *omp2a* and *omp25* genes from these two isolates was performed. Polymorphisms in the sequences of these genes can be used to classify strains at the species and sometimes biovar level (3, 5). Sequencing of the *omp2a* gene was performed as previously described (13). A 523-bp fragment of the *omp25* gene was similarly amplified and sequenced. The primers used for amplification and sequencing of *omp25* were 5'-GTTCCGGCTCCGGTTGAAGTA and 5'-CGCGGATATCCTGCGTGTC. The *omp2a* sequence for both isolates demonstrated 100% sequence identity with the *omp2a* sequence (GenBank accession number U26438.1) of *B. abortus* and an *omp2a* sequence (GenBank accession number AE009569.1) of *B. melitensis* strain 16 M. Polymorphisms in the 3' end of *omp2a* have been reported to discriminate between *B. abortus* and *B. melitensis* (5). Apparently, this observation was based on an earlier GenBank submission of the *omp2a* sequence (GenBank accession number U26440.1) for *B. melitensis* strain 16 M. The 3' ends of these two sequences for strain 16 M differ at three nucleotide positions. The reason for this sequence discrepancy is unclear, but discriminating between *B. abortus* and *B. melitensis* using this region of *omp2a* is difficult given these confounding sequence entries. Sequencing of *omp25* also revealed that both isolates possessed identical sequence. A search of GenBank indicated that this *omp25* sequence differed from that of *B. melitensis*, *B. suis*, and *B. abortus* at 1, 3, and 5 nucleotide positions, respectively. Thus, our data suggest that these two isolates represent atypical *Brucella* strains that share phenotypic and genotypic characteristics of both *B. abortus* and *B. melitensis*. Interestingly, both isolates were derived from patients who likely acquired their infections in the Middle East. In contrast, most *Brucella* spp. isolated from patients in California originate from infections acquired in Mexico or from unpasteurized dairy products derived from Mexico (6).

No significant fluorescence was observed for the non-*Brucella* strains tested in the multiplex assay (Table 2). The specificity panel included strains phenotypically, genetically, and serologically related to *Brucella* and included 14 strains of *Ochrobactrum*. Amplification has been reported for some *Ochrobactrum* strains using a *bcs31* primer set originally described by Baily et al. (1, 2). One of these strains, *O. anthropi* LMG 3331 (ATCC 49188), tested negative using the *bcs31* primer-probe set in our assay. The increased specificity of our assay represents a significant improvement over previous PCR assays targeting *bcs31*.

The detection limit of each primer-probe set in the multiplex format was also tested (data not shown). Serial 10-fold dilutions of a known amount of *B. abortus* or *B. melitensis* purified DNA were tested in triplicate using the multiplex format. The detection limit for each set of primers and probe was 150 fg of purified DNA. No fluorescence was detected at the level of 15 fg of purified DNA. The analytical sensitivity of this assay

suggests its usefulness for the direct detection of *Brucella* in clinical specimens.

As described, the real-time multiplex PCR assay will permit the confirmation of bacterial isolates as *Brucella* spp., *B. abortus*, or *B. melitensis* within 2 to 3 h. The inclusion of a genus-specific primers-probe set assists in the identification of infrequently isolated *Brucella* species and the recognition of atypical *Brucella* strains. Conventional methods for *Brucella* isolation and identification may take days to weeks to perform and often require the preparation of heavy suspensions of these highly infectious pathogens. Our laboratory performs Gram stain, oxidase, and urease testing as a primary screening test for suspected *Brucella* isolates. If a slow-growing, oxidase- and urease-positive, gram-negative coccobacillus is observed, the isolate is then tested by PCR. The PCR method described here uses heat to inactivate the organisms and greatly reduces the risk of laboratory-acquired infection with *Brucella*. Finally, the multiplex format of the assay will reduce reagent cost and staff time required to perform testing for brucellosis.

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