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 *bcsp31* (1, 9, 11, 12). The most frequently described PCR target for the diagnosis of human brucellosis is the *bcsp31* 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 I. 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 confirmation 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 *bcsp31* 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-μl multiplex PCR mixture consisted of 1× 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 μl 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-
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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Brucella spp.</th>
<th>B. abortus</th>
<th>B. melitensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. abortus</td>
<td>25/25</td>
<td>23/25</td>
<td>2/25</td>
</tr>
<tr>
<td>B. melitensis</td>
<td>25/25</td>
<td>25/25</td>
<td>0/10</td>
</tr>
<tr>
<td>B. suis</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>B. canis</td>
<td>1/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Brucella spp.</td>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Non-Brucella spp.</td>
<td>0/59</td>
<td>0/59</td>
<td>0/59</td>
</tr>
</tbody>
</table>

* Number of PCR-positive isolates per number of isolates tested.
* Human isolates genetically related to marine mammal strains of Brucella.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>PCR identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. abortus</td>
<td>23/25</td>
</tr>
<tr>
<td>B. melitensis</td>
<td>25/25</td>
</tr>
<tr>
<td>B. suis</td>
<td>0/10</td>
</tr>
<tr>
<td>B. canis</td>
<td>0/1</td>
</tr>
<tr>
<td>Brucella spp.</td>
<td>0/2</td>
</tr>
<tr>
<td>Non-Brucella spp.</td>
<td>0/59</td>
</tr>
</tbody>
</table>

Excluding growth characteristics, oxidative 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-l 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. abor-
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'-GTTCCGCGCCTCGTTGAAGTA and 5'-CGCAGATATTCGCTGTC. 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 bcsP3 primer set originally described by Baily et al. (1, 2). One of these strains, O. anthropi LMG 3331 (ATCC 49188), tested negative using the bcsP3 primer-probe set in our assay. The increased specificity of our assay represents a significant improvement over previous PCR assays targeting bcsP3.

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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REFERENCES


