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Review of Detection of *Brucella* spp. by Polymerase Chain Reaction

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Here we present a review of most of the currently used polymerase chain reaction (PCR)-based methods for identification of Brucella bacteria in biological samples. We focused in particular on methods using single-pair primers, multiplex primers, real-time PCRs, PCRs for marine Brucella, and PCRs for molecular biotyping. These methods are becoming very important tools for the identification of *Brucella*, at the species level and recently also at the biovar level. These techniques require minimum biological containment and can provide results in a very short time. In addition, genetic fingerprinting of isolates aid in epidemiological studies of the disease and its control. PCR-based methods are more useful and practical than conventional methods used to identify *Brucella* spp., and new methods for *Brucella* spp. identification and typing are still being developed. However, the sensitivity, specificity, and issues of quality control and quality assurance using these methods must be fully validated on clinical samples before PCR can be used in routine laboratory testing for brucellosis.

Brucellosis is caused by *Brucella* spp. which is composed of eight terrestrial species and at least two marine species. Terrestrial *Brucella* spp. include *B. abortus, B. melitensis, B. suis. B. ovis, B. canis, B. neotomae,* and two new species, *B. microti* and *B. inopinata. Brucella* isolated from marine mammals are *B. ceti* and *B. pinnipedialis* (1). The first 3 terrestrial species include several biovars. The terrestrial *Brucella* species display a high degree of DNA homology based on DNA-DNA hybridization studies. Nevertheless, DNA polymorphism sufficient to differentiate the first 6 *Brucella* species and some of their biovars has been shown to exist (2). *Brucella* isolated from marine mammalian species is still under investigation. According to the classical criteria of host preference and DNA polymorphism at their outer membrane protein 2 (*omp2)* locus, at least 2 species that infect marine mammals exist (3).

The gold standard for the diagnosis of brucellosis is isolation of *Brucella* bacteria. However, to isolate *Brucella* bac-

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teria is time- and resource-intensive; it requires level 3 biocontainment facilities and highly skilled technical personnel to handle samples and live bacteria for eventual identification and biotyping. Handling all live *Brucella* involves risk of laboratory infection and very strict biosafety rules must be observed. In order to avoid these disadvantages, methods based on the polymerase chain reaction (PCR) are becoming very useful and considerable progress has been made recently to improve their sensitivity, specificity, and technical ease and to lower costs. To date, at least 400 reports have been published dealing with various PCR-based methods for *Brucellosis* detection. In this review, we discuss extraction of DNA and various PCR methods using different primers and reaction conditions.

PCR-BASED METHODS AND MOLECULAR DIAGNOSIS OF BRUCELLOSIS

1. Extraction of DNA from Brucella

Extraction of DNA is the first step in performing any PCR. Standardized procotols for DNA extraction exist (4,5) or commercial kits may be used, such as the FlexiGen DNA kit form QIAGEN (Hilden, Germany) and the DNA isolation kit for mammalian blood from Roche Applied Science (Laval, Quebec, Canada). Primary cultures of *Brucella* can be tested directly. The test samples from which DNA can be extracted most commonly for brucellosis diagnosis include tissues from neonates or aborted fetuses, milk, whole blood, serum, semen, body fluids, and foods such as cheese. Some samples are easily obtained from animals for DNA extraction, including milk and blood. For instance, an improved method for purification of bacterial DNA from bovine milk for detection of *Brucella* spp. by PCR has been reported (6). This method uses a lysis buffer with high concentrations of trishydroxymethylaminomethane, ethylene diamine tetraacetic acid disodium salt dehydrate (EDTA), and sodium chloride, high concentrations of sodium dodecyl sulfate, and proteinase K, and a high incubation temperature for the efficient extraction of *Brucella* DNA. The sensitivity of the PCR was 5 to 50 *Brucella* colony forming units (CFU)/mL of milk (6).

Blood samples are often used in PCR-based diagnosis of human brucellosis (7,8). However, inhibitors frequently affect PCR results (9). Washing the blood a few times with water or lysis buffer until all the hemoglobin disappears before extracting the DNA increases the PCR sensitivity substantially (10). A PCR method that incorporates this washing procedure, a higher number of PCR cycles (40 cycles instead of 35), and primers for the gene encoding the *Brucella* cell surface salt-extractable (BCSP) 31-kDa protein can detect 700 CFU/mL of peripheral blood (11).

Serum samples are often used for extraction of DNA for PCRs. One study compared the relative recovery of bacterial DNA extracted from human serum spiked with known concentrations of *B. melitensis* Rev 1. Seven commercial kits were examined: UltraClean DNA BloodSpin Kit (MO BIO, Carlsbad, CA, USA), Puregene DNA Purification System (Gentra Minneapolis, MN, USA), Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), High Pure PCR Template Preparation Kit (Roche), GFX Genomic Blood DNA Purification Kit (GE Healthcare, Little Chalfont, UK), NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany), and QIAamp DNA Blood Mini Kit (Qiagen) (12). These commercial kits were compared with a genus-specific real-time PCR method. The study revealed that some kits were more sensitive than others and that the most efficient kits could isolate sufficient DNA for detection of as little as 100 fg of *Brucella* DNA, in some cases without any contamination. The other procedures yielded DNA isolation results that were less sensitive and the negative samples were always contaminated with *Brucella* DNA. The results show that commercial extraction kits are capable of extracting low amounts of relatively pure *Brucella* DNA from animal serum (12).

Another commercial product for DNA extraction is the FTA paper card (Whatman; Maidstone, UK). Quantification of DNA from EDTA-animal blood deposited on an FTA card was shown to be accurate and reproducible (13). The results also showed that fractions of recipient cell DNA detected by real-time reverse transcription-PCR were similar between the FTA and a salting-out method, the standard DNA extraction method. Furthermore, the two methods showed similar sensitivity of detection of recipient cells (13). FTA cards have more recently been used for DNA extraction from body fluids (unpublished data, cited in 13). Tests showed that *Brucella* DNA was isolated with good yield, but the sensitivity of the method was not determined.

2. Single pairs of PCR primers

Primer pairs used to identify *Brucella* spp. at the genus-specific level include the primers for sequences encoding BCSP 31(B4/B5) (14), 16SrRNA(F4/R2) (15), 16s-23S 16S–23S intergenic transcribed spacers (ITS) (Bru ITS-S/Bru ITS-A) (16,17), 16S-23S rDNA interspace (ITS66/ITS279) (18),

IS711 (IS313/IS639) (19), *per* (*bruc1/bruc5*) (20), *omp2* (*JPF/JPR*) (21), outer membrane proteins (*omp 2b*, *omp2a* and *omp31*) (22), proteins of the *omp25/omp31* family of *Brucella* spp. (23), and arbitrary primers (24). The sensitivity and specificity vary substantially for the different pairs of primers. Primers *B4* and *B5* targeting *BCSP 31* are often used for human brucellosis diagnosis. If combined with an increase in cycle numbers, this pair of primers can provide the greatest sensitivity when testing human blood samples (11).

A study (25) analyzed the sensitivity and specificity of the 3 established genus-specific PCR methods targeting *bcsp31*, *omp2* and 16S rRNA gene sequences, and it also compared their efficiencies for the simple detection of the *Brucella* genus directly from blood samples in a large-scale screening of individual animals from seropositive Indian field buffalo and cattle herds. The results showed that the 16S rRNA gene used for detection of bovine blood samples was insensitive. The *bcsp31* PCR was the most sensitive: it had a sensitivity similar to that obtained using the combination of *omp2* blood PCR and ELISA. The use of more than one marker-based PCR gave increased sensitivity and higher specificity providing a better molecular diagnostic approach for screening of field animals.

Recently, a combined PCR has been applied to detect *Brucella* spp. at the genus level. Four pairs of primers derived from *bcsp*31 and outer membrane proteins (*omp2b, omp2a, omp31*) were used in 4 individual PCRs in different combinations to identify *B. abortus, B. melitensis, B. canis,* and *B. suis* (22). These PCRs were shown to be ideal methods for detection of human brucellosis.

A novel PCR assay for the rapid detection of members of the *Brucella* genus that could differentiate among the 6 recognized *Brucella* species (excluding *B. microti*) in 7 single PCR reactions has been described (26). The assay was shown to be highly specific, with the additional advantage of being suitable for both conventional and real-time PCR formats. The only disadvantage was the similarity of the PCR patterns for *B. suis* biotype 4 and *B. canis* (2).

In some geographic areas, one species of *Brucella* may be more prevalent than others. Some PCRs have been developed to differentiate and identify *Brucella* biotypes. One such PCR can separate *B. abortus* biovars 1, 2, and 4 from other *Brucella* species (27).

Brucella abortus strain S19 and *B. abortus* strain RB51 are used as vaccines for cattle. Therefore, it is very important to be able to identify and differentiate these vaccine strains from pathogenic *B. abortus* strains. One pair of primers based on sequences of the *ery* locus was used to identify S19 (28), while an RB51-specific PCR was used to identify vaccine RB51 from other *Brucella* species using differentiation based on *wboA* gene mutations (29).

3. Multiplex PCRs

Several multiplex PCRs have been described for identification of *Brucella* at the species level and partly at the biovar level using different primer combinations. The first multiplex PCR, called AMOS PCR for *Brucella abortus, B. melitensis, B. ovis,* and *B. suis,* was published in 1994. It used five primers to identify *Brucella* at the species level (30). This method could detect selected biovars of 4 species of *Brucella*; biovars 1, 2, and 4 of *B. abortus*; all 3 biovars of *B. melitensis*; biovar 1 of *B. suis*; and biovar 1 of *B. ovis*. This assay could not differentiate individual biovars within a species. The PCR was used to evaluate animal field samples and was found to be in 100% agreement with the conventional biotyping methods. In order to distinguish *B. abortus* vaccine strains S19 and RB51 from field strain isolates, 3 additional primers were added to the original AMOS PCR (31). The eight primer mixtures could differentiate most of *Brucella* strains expected to occur in the US. Based on this AMOS PCR format, another primer was designed and added. This refined AMOS PCR produced an extra band found only in *B. abortus* biovars 3b, 5, 6 and 9 (32).

An improved PCR, the *B. abortus* species-specific PCR, was subsequently used to specifically recognize field strains of *B. abortus* biovars 1, 2, and 4, which were the only biovars occurring in the US. This method was also used with bovine tissue samples to distinguish the aforementioned strains from vaccine strains, other *Brucella* species, and *Brucella*-related or -unrelated bacteria that might give cross reactions (33). *Brucella suis* biovars 1, 2, and 3 were identified by a multiplex PCR, which included primers based on sequence variation of the *omp2b* gene. However, the use of this PCR was limited because the *B. suis* biovar 1 pattern produced from animal field isolates of *B. suis* was similar to that of *B. suis* biovars 2 and 3, based on identification by bacteriological methods (34). A multiplex PCR using 8 multi-locus variable number tandem repeat analysis (MLVA) primers was able to distinguish *B. melitensis* from other *Brucella* species and allowed strain typing (35). This method was used to identify 7 epidemiologically-linked clusters of *B. melitensis* and the source of a laboratory-acquired infection. The assay was found to be practical for technical and economical reasons.

More recently, a multiplex PCR assay (Bruce-ladder) has been used to identify all *Brucella* sp. at genus level, including 6 terrestrial species, the marine species of *Brucella,* and the vaccine strains S19, RB51, and Rev. 1 (36). Based on the Bruce-ladder PCR, an improved multiplex PCR was developed that differentiates all 9 currently recognized *Brucella* species, including the recently described species *B. microti, B. inopinata, B. ceti* and *B. pinnipedialis*. The method was used to identify all known *Brucella* strains and their biotypes in one test (37).

A new PCR-based test method, random amplified polymorphic DNA PCR, was performed to identify primers for differentiating *Brucella* at the species and biovar levels (38). Based on the results, 19 primers were used to develop a multiplex PCR. This multiplex PCR method specifically identified *B. neotomae, B. pinnipedialis, B. ceti,* and *B. microti*. The assay also differentiated *B. abortus* biovars 1, 2, 4 from biovars 3, 5, 6, 9; futhermore, it differentiated among *B. suis* biovar 1, biovars 3 and 4, and biovars 2 and 5. This method gave identical results to previous typing for all *Brucella* types and reference strains and for the majority of 118 field strains. One group of *B. canis* strains exhibited a unique pattern, while a second group produced the same pattern as *B. suis* biovars 3 and 4. No cross reactions were detected.

4. Real-time PCR

Real-time PCR has recently been developed (39-41). The major advantages of real-time PCR are that it can be performed in a very short time, does not require electrophoretic analysis, and avoids contamination. The samples that can be tested by real-time PCR include cultured *Brucella* cells (39), serum (40), blood, and paraffin-embedded tissues (41). Real-time PCRs can be used for the diagnosis of human brucellosis and discriminated among inactive, seropositive, and active states when it was used to test serum samples for which clinical findings were known (41). In an extension of the AMOS PCR, 3 separate real-time PCRs were developed to specifically identify *B. abortus* biovars, *B. melitensis,* and *B. suis* at the species level using fluorescence resonance energy transfer (39). The upstream primers used in these real-time PCRs were from insertion sequence 711; the downstream primers and adjacent hybridization probes were species-specific. The real-time PCR was complete in about 30 minutes and it was specific and sensitive based on evaluation of reference and field strains.

A real-time PCR using primers *B4* and *B5* primers (targeting *bspc*31) and SYBR Green I to diagnose brucellosis at genus level was compared with PCR-enzyme-linked immunosorbent assay (40). This method was applied to blood cultures of serum samples and to whole blood samples in human brucellosis cases. The results showed that real-time PCR applied to serum samples was more sensitive than other methods. A quantitative real-time PCR with primers and a *Taqman* probe of *bcsp*31 was developed to differentiate inactive, seropositive, and active human brucellosis in serum samples. The results showed that the sensitivity was 10 fg of *Brucella* DNA and was useful for both initial diagnosis and differentiation between chronic and active brucellosis (42).

Three real-time PCRs for diagnosis of human brucellosis at genus level were developed and evaluated with whole blood and paraffin-embedded tissues (41). The primers and adjacent hybridization probes were from 16S-23S ITS, *omp25* and *omp31*. According to the results, real-time PCR with 16S-23S ITS primers and its probes was the most sensitive and could be used for the diagnosis of human brucellosis in the clinical laboratory.

One study compared real-time PCRs to identify *Brucella* spp. at genus level using primers and *TaqMan* probes targeting the IS711, *bcsp*31 and *per* genes. The results showed that the IS711-based assay was the most sensitive, specific, efficient, and reproducible method to detect *Brucella* spp. (43).

Novel primers and *TaqMan* probes specific for the 6 classic *Brucella* spp. were designed and the primers used for 6 single real-time and conventional PCRs to identify and differentiate *Brucella* spp. at the species level. These PCRs were verified to be highly specific and suitable for use with real-time and conventional PCRs (27).

Four single real-time PCRs used to identify and differentiate *B. suis* at biovar level were developed based on primers and *TaqMan* probes capable of discriminating 4 single nucleotide polymorphisms. The results showed that allelic profiles were unique for each *B. suis* biovar and the most relevant signatures of 137 field strains of worldwide origin characterized previously were collected and verified with real-time PCRs. One disadvantage of the real-time PCRs was that some *B. suis* biovar 3 field strains matched the allelic profile of *B. suis* biovar 1 (44).

5. PCRs for marine species of Brucella

In recent years, marine mammal *Brucella* species have been found in diverse sea mammals (45). Phylogenetic trees constructed based on the *omp2b* sequence showed divergence between *omp2b* and between *omp2a*

nucleotide sequences, indicating that marine mammal *Brucella* isolates form a more heterogeneous group than do isolates from terrestrial mammals. Analyses combining infrequent restriction site (IRS)-derivative PCR, restriction enzyme digestion (RFLP), -PCR of outer membrane protein genes, and IS*711* fingerprint profiles to analyze *Brucella* originating from 11 species of marine mammals showed that isolates originating from cetaceans, corresponding to *B. ceti*, fell into two clusters that corresponded to isolates from either dolphins (cluster 3 or ISR-PCR IV) or porpoises (cluster 2 or ISR-PCR II, III). Their preferred host and isolates from seals fell into another major group (cluster 1 or ISR-PCR I), corresponding to *B. pinnipedialis.* This major group was further subdivided (cluster 4 or ISR-PCR I), with isolates from hooded seals making up a distinct group (46). Based on analysis of 45 marine mammal isolates with the multilocus sequencing method, 5 sequence types (ST) were found and labeled ST23 to ST27. ST 23 was predominantly associated with porpoises; ST26 was isolated from dolphin species only; ST24 and ST25 were largely associated with seals. ST27 was isolated only once from a bottlenose dolphin and was more closely related to the seal STs than to those associated with porpoises and dolphins (47).

A PCR was designed to discriminate all terrestrial *Brucella,* including *B. ovis,* from *Brucella* sp. isolated from marine mammals (48). The method uses primers A26 and B26, which amplify the entire *bp26* gene and its flanking sequences using the IS711 element downstream of the *bp26* gene as a specific marker. Subsequently, specific PCRs were developed based on infrequent restriction site-PCR. Using specific PCRs I, II, III, IV, *Brucella* sp. from marine mammals was divided into 4 groups. Samples positive for specific PCR I were *B. pinnipediae* strain B2/94 (common seal); those positive for specific PCR II and III were *B. cetaceae* (now identified as *B. ceti*) strain B1/94 (porpoise); and those positive for specific PCR IV were *B. ceti* strains B14/94 (49). All these PCR results match the results obtained using IRS-derivative PCR, PCR-RFLP of outer membrane protein genes (*omp*), and IS711 fingerprint profiles (46). Specific V and VI PCRs were developed according to specific IRS-PCR fragments of the marine mammal *Brucella* isolates. These PCRs were specific for *B. pinnipedialis* strains. The strains from marine mammals which were positive for specific PCRs I and VI were also positive for specific PCR V (50).

6. Molecular biotyping

There are a number of methods for genotyping *Brucella* strains. However, most were limited to use with livestock isolates until hypervariable octameric oligonucleotide fingerprints ("HOOF-Prints") technology was developed (51). The HOOF-Print method used variable number tandem repeats of the DNA sequence "AGGGCAGT" at 8 loci in the genome of *Brucella* to identify the specific alleles at each of the repeat loci. The PCR products are analyzed by Metaphor agarose gel and fluorescence tagged capillary electrophoresis. After verifying the classical *Brucella* species and biovars, field strains from infected cattle and wildlife herds were examined. The method was able to discriminate all species and biovars and field strains. This method was as rapid as other methods and the results were reproducible. One disadvantage was that no species- or biovar-specific alleles were found and it could therefore be used only after identification by other methods. More recently, a *Brucella* MLVA-15 typing assay was developed (52). The 15 markers were divided into two panels. Panel 1 included 8 user-friendly minisatellite markers with good species identification capabilities. Panel 2 had 7 microsatellite markers with higher discriminatory power.

Twenty one variable number tandem repeat markers were used for typing *Brucella* spp. including 8 pairs of HOOF-PCR primers (53). The results showed that 6 loci were sufficient to determine the species designation. When more variable loci were analyzed, the assay was able to discriminate isolates originating from some different geographical sources. This method has great potential for further development and application to both epidemiological tracing of *Brucella* spp. transmissions and in determining the relationship among isolates worldwide (53).

The methods for characterization of the genetic diversity of *Brucella* spp. include multilocus sequencing (54). Methods for identification of *Brucella* spp. include multiplex assays based on single-nucleotide polymorphisms identifying the major *Brucella* spp. isolates at the species level (55), real-time PCR of single-nucleotide polymorphisms defining the major *Brucella* spp. clades for distinguishing bacteria with clonally derived population structures (56), and rapid identification of *Brucella* spp. isolates at the species level by real-time PCR-based single-nucleotide polymorphism analysis (57). All these methods are somewhat more complicated than other PCR methods and require special equipment, making them of limited use in the diagnostic laboratory. Additional time is needed to streamline and adapt these methods for use with diagnostic samples.

CONCLUSION

The gold standard for diagnosis of Brucellosis remains isolation of *Brucella* spp. bacteria from samples. However, PCR-based methods that identify nucleic acid fragments from the bacteria are more useful and practical. Most of the new methods for *Brucella* spp. identification and typing are still being developed and still await validation for use with clinical samples. This is especially true for PCR tests targeting new species of *Brucella* spp. from marine mammals.

Most of these PCR-based methods were developed using *Brucella* spp. DNA prepared directly from cultured bacteria or extracted from the culture. The quality and purity of *Brucella* spp. DNA is very important in performing these methods, especially for multiplex PCR methods. Any inhibitor in DNA samples from any sources may limit the use of these methods. False-negative reactions can occur through a number of mechanisms such as specimens that contain EDTA, RNase or DNase, heme, heparin, phenol, polyamines, plant polysaccharides, urine, calcium alginate, and probably a host of other reagents. False-positive reactions resulting from specimen contamination or amplicon carryover also require attention (58).

The sensitivity and specificity of most PCR-based methods are not well established and their real value for use with clinical samples and hence diagnosis has not been validated. There is still a great deal of work required for verification, validation, establishment of standard positive and negative controls, internal and inhibition control, reagents, quality assurance, and contamination before any of these methods may be used in routine laboratory testing for brucellosis (59).

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