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Rapid Multiplex PCR Assay for the Simultaneous Detection of the *Brucella* Genus, *B. abortus*, *B. melitensis*, and *B. suis*

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The routine identification and differentiation of *Brucella* species is a time-consuming and labor-intensive process, which frequently places personnel at risk of laboratory-acquired infection. Here, we describe the development of a rapid multiplex PCR assay for the confirmation of presumptive *Brucella* isolates. The assay was able to identify and differentiate major human pathogens, namely *B. abortus*, *B. melitensis*, and *B. suis*, in a single test of less than an hour and a half.

Keywords: Multiplex PCR, internal amplification control, brucellosis

Brucellosis is a worldwide zoonotic disease caused by facultative intracellular bacteria of the genus *Brucella*. The disease is a major cause of direct economic loss and an impediment to trade and transportation. The genus *Brucella* consists of six recognized species (*viz*., *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae*) according to host preference and pathogenicity. Despite this, the various *Brucella* species exhibit high degrees of genetic similarity [6, 8, 10, 19, 21]. Human brucellosis has been attributed to *B. abortus*, *B. melitensis*, and *B. suis*, and more recently to strains resembling *Brucella* isolated from marine mammals [26]. The routine identification and differentiation of *Brucella* species is based largely on culture isolation and phenotypic characterization. This process is lengthy, requiring experienced technicians, and carries with it a high risk of laboratory-acquired infections [4, 17]. In order to overcome these difficulties, PCR-based assays have been explored for the rapid detection and confirmation of *Brucella*, which almost completely obviate the need for direct handling of the pathogen.

Several conventional and real-time PCR assays have been developed. These assays usually target the nucleic acid sequences that are *Brucella* genus specific, including the 16S rRNA , the 16–23S intergenic spacer region, *omp2*, and *bcsp31* [3, 7, 11, 16, 23, 25]. Real-time PCR makes use of costly reagents and hence is poorly suited for use in general diagnostic laboratories. PCR identification of *Brucella* strains at the species level has also been reported. These target the specific integration of the IS711 element in the genome of the respective *Brucella* species [2, 9, 12, 18, 20, 22, 24, 27]. Most of these assays are monoplex PCRs, which rely on the amplification of a single target, and take an average of three hours to run to completion. Moreover, they lack internal amplification control (IAC), which has now become almost mandatory in diagnostic PCRs. Here, we present the development of a multiplex PCR (mPCR) assay that enables the rapid detection of the *Brucella* genus with simultaneous differentiation between *B. abortus*, *B. melitensis*, and *B. suis* isolates, in a single test of less than an hour and a half. IAC was incorporated to monitor for the presence of inhibitors in the PCR mixture. To the best of our knowledge, this is the first study in India that makes use of multiplex PCR for the identification of the major *Brucella* species implicated in human brucellosis.

The sequences of primers and IAC used in this study are listed in Table 1. Primers were designed using the Gene Runner software (Hastings Software, NY, USA) and synthesized at Operon Biotechnologies, Cologne, Germany. For *Brucella* spp. identification, the primers target the *bcsp31* gene (GenBank Accession No. M20404). The nucleic acid targets and the primers for *B. abortus, B. melitensis*, and *B. suis* identification are similar to those

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Table 1. Sequences of oligonucleotides used in the rapid multiplex PCR assay.

PCR identification	Primer sequence (5'–3')	Reference or source
Brucella genus (BG)	F: CAATCTCGGAACTGGCCATCTCGAACGGTAT R: ATGTTATAGATGAGGTCGTCCGGCTGCTTGG	This study
B. abortus (BA)	GACGAACGGAATTTTTCCAATCCC	[5]
B. melitensis (BM)	AAATCGCGTCCTTGCTGGTCTGA	[5]
B. suis (BS)	GCGCGGTTTTCTGAAGGTTCAGG	[5]
IS711 (IS)	TGCCGATCACTTAAGGGCCTTCA	[5]
IAC	F: CAATCTCGGAACTGGCCATCTCGAACGGTAT AACTACGATACGGGAGGGCTTACCATC	This study
	R: ATGTTATAGATGAGGTCGTCCGGCTGCTTGG TCTGCTATGTGGCGCGGTATTATCC	

IAC primers are flanked by *Brucella* genus (BG) primers on the 5' ends.

described by Bricker and Halling [5]. The IAC was constructed as per the method of Kumar *et al.* [14, 15]. The IAC primers used in this reaction had 5' overhanging ends, which were identical to the primers (BG-F and -R) used in mPCR specific for the *bcsp31* gene, whereas the 3' ends were complementary to a DNA sequence of pUC 18 (Table 1). The PCR reaction mixture for generation of the IAC DNA contained 400 nM of each primer, 0.2 mM of each dNTP (MBI Fermentas, Vilnius, Lithuania), 0.5 U of *Taq* polymerase (MBI Fermentas), and 1.5 mM $MgCl_2$ in 1× PCR buffer (MBI Fermentas) with 400 pg of template DNA. Reaction parameters included initial denaturation at 95°C for 5 min, 30 cycles of amplification with denaturation at 95°C for 1 min, annealing at 64°C for 1 min, extension at 72°C for 1 min, and final extension of the incompletely synthesized DNA at 72°C for 5 min, in a Bio-Rad myCycler thermal cycler (Bio-Rad Laboratories, CA, USA). The PCR product was purified using a commercially available kit (Qiagen, Hilden, Germany). The concentration of IAC DNA was determined spectrophotometrically at 260 nm and was stored at –20°C. Multiplex PCR was performed in a 20-µl reaction volume containing 1× PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, primers (800 nM BG-F and -R; 600 nM IS; 350 nM BA; 450 nM BS; 200 nM BM), 2.0 µl (~500 pg) of template DNA, and 1 U of *Taq* polymerase (MBI Fermentas). Various concentrations of IAC DNA were tried before choosing 10^4 copies per reaction. Amplification consisted of initial denaturation at 95°C for 1 min, 30 cycles of amplification with denaturation at 95°C for 5 s, annealing at 64°C for 10 s, extension at 72°C for 30 s, and final extension of the incompletely synthesized DNA at 72°C for 1 min in the myCycler thermal cycler. The PCR products were analyzed in 2.0% agarose gels containing 0.5 µg/ml of ethidium bromide and subjected to electrophoresis in a 1× TAE buffer.

In order to evaluate the specificity of the multiplex assay, an extensive panel of well-characterized *Brucella* and non-*Brucella* strains was employed in the test (Table 2). Identification of *Brucella* strains was performed using standard classification tests, including observing growth characteristics, oxidase activity, urease activity, CO_2 requirements, H_2S production, dye tolerance, and seroagglutination [1]. Non-*Brucella* strains were identified using standard biochemical and immunological procedures. Crude nucleic acid extracts were prepared by resuspending a loop of bacteria in 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 [28]. The supernatant was collected as the crude DNA extract and employed in PCR. Boiling of the organisms greatly reduces the risk of laboratory-acquired infection with *Brucella*. In some cases, nucleic acids were purified using a DNeasy tissue kit (Qiagen, Germany) in accordance with the manufacturer's recommendations. Amplification results of *Brucella* and non-*Brucella* strains using the multiplex assay are shown in Table 2. The primers BA, BM, and BS with IS produced amplicons of 498 bp, 731 bp, and 285 bp for *B. abortus*, *B. melitensis*, and *B. suis*, respectively, along with amplicons of 208 bp being produced by primers BG-F and -R with all *Brucella* spp. simultaneously. The primers did not amplify any product from non-*Brucella* species (Table 2). The IAC was coamplified with target DNA and revealed an amplification of 621 bp (Fig. 1) in all the DNA samples isolated from bacterial cultures, as shown in Table 2. IAC was included in the mPCR to increase the confidence of the assay by pinpointing false negatives that may be the result of assay failure, and/or reaction inhibition [13]. A major advantage of this assay is the speed with which the assay is performed, whereas conventional methods of *Brucella* identification can take from days to weeks.

The detection limit of each primer set in the multiplex format was also tested (data not shown). For this, serial 10-fold dilutions of a known amount of *B. abortus, B. melitensis*, and *B. suis* purified DNA were tested in triplicate using the multiplex format along with 10^4 copies of IAC. The detection limit for the primer set of *B. melitensis* was

Table 2. Bacterial strains used for the evaluation of specificity of PCR primers.

Organism	Strains	PCR detection of				
		BG	*BA*	*BM*	*BS*	*IAC*
Reference strains						
Brucella abortus S99	IVRI[a]	+	+	-	-	+
B. abortus S19	IVRI	+	+	-	-	+
B. abortus 544	IVRI	+	+	-	-	+
B. melitensis 16M	IVRI	+	-	+	-	+
B. melitensis Rev1	IVRI	+	-	+	-	+
B. suis 1330	IVRI	+	-	-	+	+
Field strains						
B. melitensis	59/98	+	-	+	-	+
	184/01	+	-	+	-	+
	11/03	+	-	+	-	+
	12/03	+	-	+	-	+
	11/04	+	-	+	-	+
	184/04	+	-	+	-	+
	59/05	+	-	+	-	+
	95/05	+	-	+	-	+
	150/6	+	-	+	-	+
	22/09	+	-	+	-	+
	B101	+	-	+	-	+
	B065	+	-	+	-	+
	B225	+	-	+	-	+
	B606	+	-	+	-	+
	B607	+	-	+	-	+
	B608	+	-	+	-	+
B. abortus						
	Br 19	+	+	-	-	+
	Br 32	+	+	-	-	+
	Br 62	+	+	-	-	+
	Br 71	+	+	-	-	+
	Br 82	+	+	-	-	+
	Br 101	+	+	-	-	+
	Br 129	+	+	-	-	+
	Br 130	+	+	-	-	+
	Br 173	+	+	-	-	+
	Br 200	+	+	-	-	+
Non-Brucella spp.[b]		-	-	-	-	+

+: Positive result, -: negative result;
BG: *Brucella* genus.
BA: *B. abortus.*
BM: *B. melitensis.*
BS: *B. suis.*
IAC: Internal amplification control.
[a]IVRI: Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.
[b]*Bacillus cereus, Bacillus circulans*, *Bacillus licheniformis, Bacillus megaterium, Bacillus subtilis, B. sphaericus, Bacillus thuringiensis, Burkholderia cepacia, Burkholderia pseudomallei, Escherichia coli, Listeria monocytogenes, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella* Typhi, *Shigella flexneri, Shigella boydii, Shigella sonnei, Staphylococcus aureus, Streptococcus pyogenes, Vibrio cholerae, Yersinia enterocolitica* O:9, *Yersinia pseudotuberculosis.*

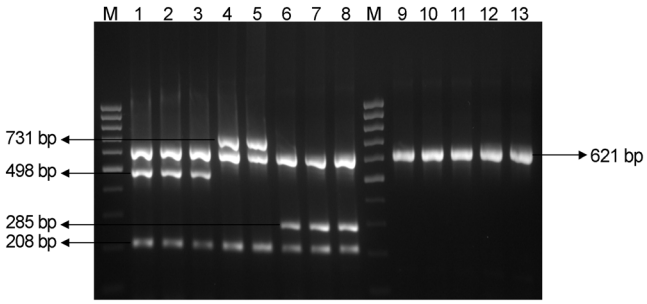

Fig. 1. Multiplex PCR for simultaneous detection of *Brucella* genus, *Brucella abortus, B. melitensis*, and *B. suis*, along with coamplification of IAC.
Lane 1, *B. abortus* 544; lane 2, *B. abortus* S19; lane 3, *B. abortus* S99; lane 4, *B melitensis* 16M; lane 5, *B melitensis* Rev1; lanes 6–8, *B. suis* 1330 (in triplicates); lane 9: *Vibrio cholerae*; lane 10, *Yersinia enterocolitica* O:9; lane 11, *Escherichia coli*; lane 12, *Salmonella* Typhi; and lane 13, no template control.

found to be 10 pg, and for *B. abortus* and *B. suis* was 1 pg of purified DNA. The analytical sensitivity of this assay suggests its usefulness for the direct detection of *Brucella* isolates from agar plates. Furthermore, to increase the confidence of this assay, a panel of 8 blood samples that were culture positive for *B. melitensis* was also used. The results were seen to be in excellent agreement. This shows that the assay along with the incorporated IAC may have the potential to diagnose brucellosis where a blood sample is used as the clinical specimen.

This work demonstrates that the rapid mPCR assay is simple and easy to perform and represents a highly specific approach for the simultaneous identification of the *Brucella* genus as well as the differentiation between the *Brucella* species known to cause debilitating infection in human beings. After picking up an isolated colony from an agar plate, the result delivery takes place within an hour and a half. This should allow for prompt and appropriate public health measures to be immediately implemented. A preliminary evaluation of this assay, with blood samples from acute brucellosis patients, suggests that it can be effectively used directly with any clinical specimen. As this method reduces the risk of laboratory-acquired infection, and the multiplex format of the assay reduces reagent costs and staff time, this technique appears to be a very useful tool for the diagnosis of brucellosis.

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