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Validated 5′ Nuclease PCR Assay for Rapid Identification of the Genus *Brucella*

T. Bogdanovich,[1] M. Skurnik,[2] P. S. Lübeck,[3] P. Ahrens,[3] and J. Hoorfar[3]*

University of Turku, Turku,[1] and Haartman Institute, University of Helsinki, Helsinki,[2] Finland, and Danish Institute for Food and Veterinary Research, Copenhagen, Denmark[3]

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A real-time, genus-specific 5′ nuclease PCR assay for amplification of a 322-bp fragment of the *per* gene was developed for rapid (<2 h) identification of *Brucella* spp. from agar plates. The assay, including an internal amplification control (116 bp), identified *Brucella* strains ($n = 23$) and did not detect non-*Brucella* strains ($n = 174$), indicating its usefulness, particularly for laboratories with stringent quality assurance programs.

Owing to continuous efforts to control and eradicate brucellosis in domestic animals, the levels of brucellosis have been reduced in many countries (6). However, a natural reservoir of *Brucella* bacteria in wildlife, e.g., *Brucella suis* biovar 2 in hares in Denmark, can still pose a threat. Thus, the task of detection and identification remains challenging and requires reliable and sensitive diagnostics tools.

The diagnosis of brucellosis is based mainly on serological responses, which can be unspecific owing to cross-reaction or subsensitive reactions in samples from areas with a low or subclinical prevalence of brucellosis (5, 7, 9, 16). Culture-based verification of suspected cases, or pathological findings in clinical cases, can be time-consuming and also can impose a hazard to laboratory personal. Thus, numerous alternative verification methods, based mostly on amplification of universal genes in a conventional PCR, have been reported, although some have produced false-positive results (reviewed in reference 3).

The *Brucella*-specific perosamine synthetase (*per*) gene is highly conserved and present even in the naturally rough *Brucella* species *B. ovis* and *B. canis* and spontaneously rough strains of *B. abortus* and *B. melitensis* (4). The *per* gene is, with various degrees of similarity, present in a few other bacteria, including *Yersinia enterocolitica* serotype O:9, *Vibrio cholerae* O1, *Escherichia coli* O:157, some serovars of *E. hermanni* and *Stenotrophomonas maltophilia*, and *Salmonella* group N (O:30) (14). To the best of our knowledge, the only real-time PCR work reported is based on hybridization probes used in three different assays for identification of three *Brucella* species (15). This report describes for the first time the development of a ready-to-go, nonproprietary, open-formula (thus possessing the potential for standardization), 5′ hydrolysis probe-using real-time PCR assay including an internal amplification control (IAC) for direct verification of suspected *Brucella* colonies on agar plates.

The *Brucella*-specific primers were designed as previously described (11). The primer combination bruc1-bruc5 was found to be most selective (11). In the present study, three different TaqMan probes (6-carboxyfluorescein [FAM] labeled) were designed and compared in the assay: Bruc1, in close proximity to the 3′ end of the forward primer; Bruc2, in the middle of the amplified fragment; and Bruc3, within a few nucleotides of the 3′ end of the reverse primer (Table 1).

An artificially created chimerical DNA (12), a second set of primers, and a second TaqMan probe (VIC labeled) (12) were used for an IAC in every reaction mixture, except for the nontemplate control (Table 1). In addition, a negative control (nontarget DNA) and a nontemplate control containing water instead of any DNA were included in each run.

A typical 25-μl reaction mixture contained, in addition to the primers and probes (Table 1), each deoxynucleoside triphosphate at 0.3 mM, 2.5 μl of 10× reaction buffer (F-511, DyNAzyme II; Finnzymes, Espoo, Finland), 1.25 U of DyNAzyme II recombinant enzyme (Finnzymes), 5 mM $MgCl_2$, 1% glycerol, and 0.2 mg of bovine serum albumin per ml. Since it has been shown that polymerase choice is crucial in order to overcome PCR inhibitors (1), the DyNAzyme polymerase was chosen from among several tested (data not shown). All amplifications were performed with a RotorGene 3000 (Corbett Research, Mortlake, Australia) with the following thermocycler profile: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Fluorescence was measured once every cycle after the annealing step: FAM on channel 1 (excitation at 470 nm, detection at 510 nm) and JOE (6-carboxy-dichloro-dimethoxyfluorescein) on channel 2 (excitation at 530 nm, detection at 555 nm). The normalized fluorescence data were converted to a log scale, and the threshold was determined to calculate the threshold cycle value (*Ct*; the cycle at which the threshold line crosses the amplification curve). In every run, the threshold was set above the background (0.01) normalized fluorescence value.

A total of 174 non-*Brucella* (8, 11) and 23 *Brucella* isolates and strains were used to evaluate the selectivity (a combination of inclusivity and exclusivity) and detection limit of the assay (Table 2). The *Brucella* isolates had the following strain no.: *B. melitensis* biovar 1 (16 M, $n = 2$), *B. melitensis* biovar 2 (86/8/59; $n = 2$), *B. melitensis* biovar 3 (Ether; 73862 Tgb. Nr. 126087), *B. abortus* biovar 1 (544, $n = 2$), *B. abortus* biovar 2 (86/8/59), *B. abortus* biovar 3 (Tulya; Tgb. No. 1766/98), *B. abortus* biovar 4 (292; Tgb. No. 292/85), *B. abortus* biovar 5

* Corresponding author. Mailing address: Danish Institute for Food and Veterinary Research (DFVF), Bülowsvej 27, DK-1790 Copenhagen V, Denmark. Phone: 45-72346251. Fax: 45-72346001. E-mail: jho@dfvf.dk.dk.

TABLE 1. Primers and probes used for amplification and identification of *Brucella* spp. in a 5′ nuclease real-time PCR

Primer or probe	Nucleotide sequence (5′ → 3′)	Amplicon size (bp)	Final concn (nM)
Brucella-specific PCR			
Primers			
bruc1	CGG TTT ATG TGG ACT CTC TCG	322	200
bruc5	CAG TAT TCT CGT GTA GGC GAA GTA		200
Probes tested			
Bruc1[a]	(FAM) ATTGCAGATAGATCCAGAGGGGGT (TAMRA)[b]		50
Bruc2	(FAM) TCGATCCGAGAGATTTGTGACGAA (TAMRA)		50
Bruc3	(FAM) AGATGTCGGCATTTTTCCAGTACT (TAMRA)		50
IAC			
Primers			
OT1559	CTGCTTAACACAAGTTGAGTAG	116	50
18-1	TTCCTTAGGTACCGTCAGAA		50
Probe			
Int. Camp.	(VIC)TTCATGAGGACACCTGAGTTGA (TAMRA)		50

[a] Selected for use in the final PCR assay.
[b] TAMRA, 6-carboxytetramethylrhodamine.

(B3196), *B. abortus* biovar 6 (870; Kuh 781), *B. abortus* biovar 7 (63/75), *B. abortus* biovar 9 (C68), *B. suis* biovar 1 (1330; $n = 2$), *B. suis* biovar 2 (Thomsen; 589 Tgb. No. 103707), *B. suis* biovar 3 (686), *B. suis* biovar 4 (40/67), *B. suis* biovar 5 (513), *B. ovis* (2604 f.PCR), *B. canis* (RM6/66), and *B. neotomae* (NCTC 10084). In addition, DNA samples of all known biovars of *B. abortus*, *B. melitensis*, and *B. suis* ($n = 13$) were obtained from the Veterinary Laboratories Agency, Weybridge, Surrey, United Kingdom. For the non-*Brucella* bacteria, 4 μl of template DNA was used from material extracted by a resin-based method (Chelex) as previously described (13). The final assay was evaluated directly on colonies from blood agar plates by placing one loopful of bacteria (= one small colony) into an Eppendorf tube containing 100 μl of double-distilled water and keeping it at 95°C for 10 min before centrifuging it for 5 min at 13,000 × *g*. A 3-μl supernatant was used as the template in the final PCR assay.

The detection limit was evaluated in triplicate with purified DNA samples serially diluted in Tris-EDTA buffer (with 0.1 mM EDTA). In addition, the amplicons from the real-time PCR were verified by gel electrophoresis (12).

Among the three FAM probes tested, Bruc1 showed *Ct* values as low as 11 (best result), in contrast to the Bruc2 probe, which was homologous to the middle part of the amplicon and showed *Ct* values higher than 40. A three-step profile was found to be more efficient than a two-step profile with combined annealing and extension steps, perhaps owing to the size of the amplicon (322 bp).

Purified DNAs from all of the *Brucella* strains tested, as well as crude DNA and lysate of *B. suis* biovar 2, produced *Ct* values (FAM) in the range of 11 to 20 (strongly positive), while the non-*Brucella* DNA produced *Ct* values (FAM) of >40 (negative). In addition, VIC *Ct* values (IAC) were all <30 (i.e., no PCR inhibition). Particularly bacteria containing the *per* gene, such as *Y. enterocolitica* O:9, *E. coli* O157 (strains 100, 120, 239, and 455), *E. hermannii* (strains CCUG 21202, CCUG 21152, CCUG 26042, and CCUG 26553), *V. cholerae* (strains 1083/30, 531D, C-230, VC-VN-182, and 1407), and *S. maltophilia* (strains CECT 112 and CECT 113), gave negative responses (FAM *Ct* of >40) in the *Brucella* PCR, which indicates the specificity of the target sequence selected. Interestingly, during gel electrophoresis of all of the real-time PCR products, two of the five *V. cholerae* O1 strains tested (531D and C-230) produced strong bands (but no positive-fluorescence *Ct* values) of similar sizes but approximately 50 bp longer than the positive control fragment of *B. suis* biovar 1 (strain 1330). The nucleotide sequences of the fragments revealed similarity to the mating pair stabilization protein gene of *V. cholerae* (accession no. AAL59755).

The detection limits of the assay varied from 200 fg (approximately 40 CFU) for *B. neotomae* (strain NCTC 10084) and *B. ovis* (strain 2604 f.PCR) to 2 pg (approximately 400 CFU) for *B. melitensis* biovar 1 (strain 16 M), *B. abortus* biovars 1 (strain 544) and 5 (strain B3196), and *B. suis* biovars 1 (strain 1330) and 2 (strain Thomsen). The observed differences in the detection limits could most likely be attributed to the quality of the DNA template used in the PCR since the DNA samples were obtained from different sources.

It was possible to use ready-to-go PCR mixtures stored for at least 2 months at −20°C (in the dark) containing all of the reagents except the sample DNA. In diagnostic laboratories with quality assurance programs, this timesaving exercise would reduce the risk of contamination and alleviate batch, pipetting, and personal variations.

The real-time PCR assay developed allows correct identification of all *Brucella* species and can simplify the procedure by testing presumptive *Brucella* colonies taken directly from agar plates. Use of the TaqMan probe offers a specificity higher than that of gel electrophoresis, as exemplified in this work by the two *V. cholerae* strains (531D and C-230) that produced slightly longer nonspecific amplicons in the PCR but gave no positive fluorescence signal. In addition, this real-time PCR can substantially decrease the risk of carryover contamination.

To the best of our knowledge, this is the first ready-to-go real-time *Brucella* PCR assay that includes an IAC (which is mandatory for quality assurance [2]), has no need for sample

TABLE 2. Bacterial strains used to test the selectivity of the *Brucella* real-time PCR assay

Organism(s)	No. of strains
Agrobacterium radiobacter	1
Campylobacter spp.	7
Citrobacter braakii	1
Citrobacter amalonaticus	1
Citrobacter freundii	17
Citrobacter koseri	1
Edwardsiella hoshinae	1
Escherichia coli	11
Escherichia coli O157	4
Erwinia herbicola	1
Enterobacter aerogenes	2
Enterobacter agglomerans	1
Enterobacter amnigenus	2
Enterobacter asbunae	1
Enterobacter asburiae	1
Enterobacter cloacae	1
Enterobacter gergoviae	2
Enterobacter sakazakii	2
Enterobacter tarda	1
Erwinia herbicola	1
Enterobacter taylorae	2
Escherichia fergusonii	1
Escherichia hermannii	4
Ewingella americana	1
Hafnia alvei	2
Klebsiella oxytoca	3
Klebsiella pneumoniae	2
Kluyvera ascorbata	1
Koserella trabulsii	1
Leminorella grimontii	1
Listeria monocytogenes	5
Micrococcus kristinae	1
Micrococcus luteus	1
Moellerella wisconsensis	1
Moraxella bovis	1
Morganella morganii	3
Obesumbacterium proteus	1
Ochrobactrum anthropi	1
Proteus agglomerans	1
Proteus mirabilis	3
Proteus vulgaris	2
Providencia heimbachae	1
Providencia stuartii	1
Pseudomonas aeruginosa	2
Pseudomonas alcaligenes	1
Rahnella aquatilis	1
Salmonella sp. group N (0:30)	5
Shigella sonnei	1
Shigella flexneri	1
Serratia marcescens	3
Serratia odorifera	2
Stenotrophomonas maltophilia	2
Vibrio cholerae OI	5
Yersinia enterocolitica	3
Yersinia enterocolitica 0:9	49
Brucella spp.	23

treatment, and is thus able to directly differentiate true-negative results from false-negative results, which are usually caused by the presence of PCR inhibitors (1, 10). The inclusion of an IAC with an irrelevant set of primers (for a *Campylobacter* PCR assay) has the advantage of minimal competition with the *Brucella* target with regard to the detection limit. The assay described is currently being adapted for testing of relevant clinical samples, and the robustness of the DNA polymerase to PCR inhibitors is being investigated.

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