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Nucleotide Polymorphism-Based Single-Tube Test for Robust Molecular Identification of All Currently Described *Brucella* Species▽†

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Among the numerous molecular methods described during the last 20 years to identify *Brucella*, multiplexed amplification methods offer the cheapest and simplest technical solution for molecular identification. However, one disadvantage of such methods is their need to undergo technical revalidation each time a new marker is added to the system. Moreover, polymorphic markers cannot be assessed at the single-nucleotide level in these assays. Since new *Brucella* species are continuously being described, open methodologies able to accommodate new markers while preserving all other system parameters have an obvious advantage. We present a ligase chain reaction (LCR)-based method that simultaneously assesses multiple genetic markers at the single-nucleotide level. Most of the selected markers originate from a multilocus sequence typing (MLST) database that has been extensively validated on hundreds of different *Brucella* strains. When assayed on both reference and field strains, the method yields characteristic capillary electrophoresis profiles for each of the 10 *Brucella* species described to date and displays discriminatory potential below the species level for some. Since the LCR methodology is insensitive to interference resulting from the use of multiple oligonucleotides in a single mixture, the way is open for smooth future updates of the proposed system. Such updates are inevitable, given the pending description of new *Brucella* species.

Bacteria of the genus *Brucella* are causative agents of brucellosis, a widespread disease of various animal species and a zooanthroponosis characterized by chronic inflammatory lesions in the reproductive organs that may extend to joints and other organs (6). The disease remains endemic in many areas of the world and is associated with substantial economic losses (24). From a strict genotaxonomic point of view, all *Brucella* isolates should be considered a single species (7). However, nomenspecies designations have been assigned historically and are retained for practical reasons. There are 10 such recognized *Brucella* species, each having a preferential primary host or range of hosts, namely, *Brucella abortus* (bovine), *B. melitensis* (caprine and ovine), *B. ovis* (ovine), *B. canis* (canine), *B. suis* (porcine, rangiferine, and leporine), *B. neotomae* (rodent), *B. microti* (rodent), *B. pinnipedialis* (marine mammalian, preferentially pinnipedian), *B.ceti* (cetacean), and *B. inopinata* (isolated from a human breast implant). Three species are divided into biovars (*B. abortus*, *B.melitensis*, and *B. suis*), although some, particularly those of *B. abortus*, remain unresolved (7).

Biochemical typing methods are the reference methods for characterizing brucellae at both the species and the biovar levels. They involve metabolite detection, growth on dyes, sensitivity to bacteriophages, and agglutination with monospecific antisera (2). Biochemical typing is time-consuming and difficult to standardize and requires well-trained personnel operating in biocontainment level 3 facilities.

As an alternative to biotyping, the last 20 years have seen a rapid development of molecular techniques applicable to identifying brucellae and understanding their epidemiology (10, 32). The initial assays consisted of single PCRs targeting genes with genus level specificity. For species detection, both nucleotide sequence-based and amplification profile-based methods became available later. Combinations of individual PCRs reached excellent discriminatory potential at the species level but required multiple independent reactions for each analyzed strain. Multiplex PCR methods undoubtedly provided the easiest way to identify the species of an unknown *Brucella* strain, given the few technical steps and the lack of sophisticated instruments required. The most popular multiplex PCR assay developed initially for differentiating the five classical species of *Brucella* was the so-called AMOS PCR (5), which is based on the insertion sites of the IS*711* element in the genomes of *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis* (hence the name AMOS). Several extended versions of the assay that used either the historical primer pairs or variants of these combined with new ones were later published. These updated assays could differentiate vaccine strains from field strains and identify new species, including the marine mammal brucellae (18, 21). The most up-to-date version of this assay displays the expected specificity at species level, except for

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TABLE 1. Properties of primers and probes

Code	Target gene/ SNP[a]	Nucleotide sequence	Size (nt)
UR[b]		GACGATGAGTCCTGAGTAA	19
UF[c]		CCGAGATGTACCGCTATCGT	20
cUR		TTACTCAGGACTCATCCTC	19
A	*glk*-1403G	GCCGACAAGATCACGCCCA-cUR-AA-UF-CTGGGCATCTGCGCG	75
B	*glk*-1344G	GACACGCCCTTCGATGCGT-cUR-AA-UF-AGAATTTGCTCGCCGGC	77
C	*trpE*-2858A	CCAGACGGGCGCCAAG-cUR-AA-UF-CATACGCTTGCCAATTATTTCCA	80
D	*omp25*-3627A	TAGCCAAGGTAAAGACCGGTATAGCC-cUR-AA-UF-GGCCTTGTTCCAGCCA	83
E	*cobQ*-3445A	AAGCCTCGCTGGATATTGATGGC-cUR-TT-UF-ATTATCTGGCTGAAGGGCTGA	85
F	*glk*-1557A	GCGGCGTTTATCTTTCGGGTAGCTA-cUR-AA-UF-GCTCATTTTCATGGCGCATA	86
G	*aroA*-677A	ACCCGCACCGGCCTG-cUR-AA-UF-TGATACTACTATGCAATGTGCTGATGAACCCA	88
H	*dnaK*-1654A	CATCGACCTGAAGAACGACAAGC-cUR-TT-UF-TATCCGAGTTCAAGAAGGAAAGTGA	89
I	*ptsP*-1677G[d]	GCCTTCAATAGCGCGCGC-cUR-AA-UF-CGTCGCGTTAGACAGCTCATGGCCACCCGCC	90
J	*cobQ*-3224A	CACCAGCGGGCCGGA-cUR-AA-UF-TAGTCACATATCATGCTATGAAATCCACATCGGGCA	92
K	*pyrH*-817G[d]	TTCTCGATCGCGGGC-cUR-AA-UF-GGTTCGCTTACGTTGCATAGTGCTCACCCACAAGGAAG	94
L	*dnaK*-1928T	ACCAGAACCACTTCGTCAATTTCG-cUR-AA-UF-ATCCGGTCTCATCGCTGAATGGTCATGCCGCCA	98
M	*trpE*-2796A	GTTTCGATCCTGCTGGTCGATCA-cUR-T-UF-ATGGTCGCCTATACTTATATCAAAGGTGGCTGA GGGA	100
N	*omp25*-3715A	CTGGAAGTTCCAGCCAGCAAACG-cUR-AA-UF-CGATCCGATTACAGGCCGATCCGTATACGATCT GGTCCTT	104
O	IS*711*	ACTGTCCGCAAGCTTCAAGC-cUR-TT-UF-AAAATTTAACGTTCCTAAAGCTGAGTCTGCCCGGCCA TTATGGTG	106
P	*rpoB*-265A[e]	ATGAATGCCGTCAGCGCG-cUR-TT-UF-ATTTGACGAACGTATGCCGCTTAACTCAAATCATCCACC GAAGTTGGATGTTA	112

[a] Unless otherwise indicated, marker position refers to the polymorphic nucleotide assessed with each PLP according to the nucleotide numbering used in reference 29.
[b] UR, universal reverse primer.
[c] UF, universal forward primer.
[d] Marker position refers to the nucleotide numbering used in reference 9.
[e] Marker position refers to the nucleotide numbering used in reference 20.

some strains belonging to the *B. canis* and *B. suis* species (16, 21).

From the start of the new millennium, whole-genome sequences became available and were used to develop new typing tools characterizing genetic diversity. Multilocus sequence typing (MLST) confirmed the genetic individuality of *Brucella* species defined by biochemical typing (29). Canonical single-nucleotide polymorphisms (SNPs) retrieved from compiled MLST sequences or from related markers that could be exploited as the basis of diagnostic tests were identified. Such assays could rapidly identify *Brucella* isolates to the species level (8, 11, 31), identify vaccine strains (12), or even identify to the biovar level where biovars reflect true genetic entities (9). VNTR (variable-number tandem repeat)-based typing schemes that reached the highest resolution ever reported in the context of *Brucella* molecular characterization assays have been described (15, 17, 30). However, both MLST- and VNTR-based analyses questioned the validity of some of the biovars established by classical biotyping, particularly those of *B. melitensis* (1, 29).

In the present work, we describe the setup of a multiplex assay able to identify 16 genetic signatures at once. Fifteen of the investigated markers were selected within genes previously characterized for their ability to identify *Brucella* at the species level according to the present list of recognized names (23), and one was used for genus assignment. Moreover, the assay was designed to display discriminatory potential to the biovar level for *B. suis* and to an intermediate level for *B. abortus*, *B. melitensis*, and *B. ceti*.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. All bacterial strains used in this study are listed in Table S1 in the supplemental material. Purified genomic DNA was obtained from agar-grown material with the DNeasy blood and tissue kit according to the manufacturer's instructions for Gram-negative bacteria (Qiagen, Valencia, CA).

Primers and probes. The primers and probes used are listed in Table 1. Sixteen "padlock-shaped" probes (PLPs) were designed that contained a number of common features, including (i) a 5′-terminal phosphate group, (ii) a 3′-terminal nucleotide corresponding to the polymorphic nucleotide in the template sequence, (iii) a hybridization site for the universal reverse primer, and (iv) a nucleotide sequence stretch matching the universal forward primer but in the opposite orientation. The 5′ and 3′ arms of the PLPs were designed asymmetrically to display melting temperature (T_m) values differing by about 5°C, as described previously (27). The PLPs were engineered in such a way as to return free energy (ΔG) scores of intramolecular folding not exceeding -10 kcal mol^{-1} upon assessment with the standard settings of the MFOLD algorithm (33). To allow size-based identification of the amplified probes by capillary electrophoresis, each PLP was designed in such a way as to display unique sizes chosen within a range of 75 to 112 nucleotides. All PLPs were purified by polyacrylamide gel electrophoresis.

LCR procedure. A ligase chain reaction (LCR) was conducted in three successive steps according to the procedure of Gaszczyk et al. (K. Gaszczyk, E. Verstappen, O. Mendes, C. Schoen, and P. Bonants, unpublished data) with minor modifications. Briefly, the first step (ligation) was conducted in a 10-μl mixture containing 10 ng purified genomic DNA, 4 U of *Pfu* DNA ligase (Agilent, Santa Clara, CA), 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% Igepal, 0.01 mM ATP, 1 mM dithiothreitol (DTT), and 100 pM of each PLP except the IS*711* probe (50 pM) and the *ptsP* probe (300 pM). Reaction mixtures were made up on ice and transferred rapidly onto a thermal cycler. After 3 min at 95°C, 25 cycles of 30 s at 95°C and 5 min at 65°C were performed, followed by a 2-min final denaturation at 98°C. The second step (exonuclease treatment) started with the addition of 15 μl of exonuclease mixture consisting of 67 mM glycine-KOH, pH 9.4, 2.5 mM $MgCl_2$, 50 μg/ml bovine serum albumin (BSA), and 1 U exonuclease λ (New England BioLabs, Ipswich, MA). The

TABLE 2. Multiplex LCR profiles of the different *Brucella* species

Species	Biovar	ST[c]	Presence of peak[a]: A[b] (72.3)	B (73.9)	C (76.9)	D[b] (80.3)	E (81.7)	F[b] (82.9)	G[b] (85)	H (85.6)	I (87.2)	J[b] (88.5)	K (91.7)	L (95.2)	M[b] (99.3)	N[b] (101.3)	O (103.3)	P (109)
B. suis	1, 3	14, 17	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−
B. suis	2	15, 16	−	−	−	−	−	−	−	−	+	−	+	+	−	−	+	−
B. suis	3 (ref)[f]	17	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+
B. suis	4	17, 18	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−
B. suis	5	19	−	−	−	−	−	−	−	−	+	−	+	−	−	−	+	−
B. canis		20, 21	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−
B. ovis		13	−	−	−	−	−	+	−	−	+	−	+	−	−	−	+	−
B. neotomae		22	−	+	−	−	−	−	−	−	+	−	+	−	−	−	+	−
B. ceti		23	−	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−
B. ceti		26	−	−	+	−	+	−	−	−	+	−	+	−	−	−	+	−
B. pinnipedialis		24, 25	−	−	+	−	−	−	−	−	+	−	+	−	−	−	+	−
Brucella sp.[d]		27	−	−	+	−	−	−	−	−	+	−	+	−	+	−	+	−
B. abortus	1, 2, 3, 4, 5, 6, 9	1 to 5	−	−	−	+	−	−	−	−	+	−	+	−	−	−	+	−
B. abortus	3 (ref)[f]	6	−	+	−	+	−	−	−	−	+	−	+	−	−	−	+	−
B. melitensis	1, 2, 3	7[e], 8[e], 10, 11[e], 12	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−
B. melitensis	2, 3 (ref)[f]	7[e], 8[e], 9, 11[e]	+	−	−	−	−	−	−	−	+	−	+	−	−	−	+	−
B. microti		NA[g]	−	−	−	−	−	−	+	−	+	−	+	−	−	−	+	−
B. inopinata		NA	−	−	−	−	−	−	−	−	−	+	+	+	−	−	+	−
O. intermedium			−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] +, present; -, absent. The experimental electrophoretic mobility (mean of 10 values obtained from independent runs) (nt) is shown in parentheses.
[b] Species-specific marker.
[c] ST as defined in the MLST scheme described in reference 29.
[d] Nonascribed *Brucella* species of probable marine origin; includes strains F5/02 and F5/99 (28).
[e] Marker K (*pyrH*-817G) is variable in ST7, ST8, and ST11 (*B. melitensis*).
[f] Profile observed only for the reference strain (ref) of biovar 3 (*B. abortus* and *B. melitensis*).
[g] NA, not applicable.

resulting samples (25 μl) were incubated at 37°C for 45 min, followed by inactivation at 95°C for 10 min. Upon completion of the second step, a 3.5-μl sample was pipetted into a new microtube and supplemented with universal reverse primer and 5′ Cy5-labeled universal forward primer (8 pmol each), 12.5 μl of 2-fold real-time PCR mix (ABGene, Epsom, United Kingdom), and MiliQ water up to 25 μl. After 10 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C were performed, followed by a 15-min final elongation at 72°C and denaturation at 98°C for 2 min.

Sample analysis. LCR products were analyzed by capillary electrophoresis on a CEQ8000 instrument (Beckman Coulter, Fullerton, CA). One microliter of a 20-fold dilution of the LCR product made in MiliQ water was added to 39 μl of sample loading solution (Beckman Coulter, Fullerton, CA) containing 0.3 μl of internal size standard 400, which consists of a proprietary cocktail of 22 DNA molecular size standards with sizes ranging from 60 to 420 bases (Beckman Coulter, Fullerton, CA).

RESULTS

SNPs were selected within (i) DNA fragments included in a 9-locus MLST scheme reported previously (29), (ii) the *ptsP* and *pyrH* genes (9), (iii) the *rpoB* gene (20), and (iv) the IS*711* insertion element (14). Some of the selected markers allow *B. suis* characterization up to the biovar level (*ptsP*, *pyrH*, and *rpoB*) or genus confirmation (IS*711*). The developed assay thus involves the interrogation of the bases present at 15 different SNP sites. Six of these are specific for a particular species. They are a T at position 151 of *omp25*, i.e., *omp25*-151T, for *B. abortus* (equivalent to *omp25*-3627T in reference 29); *glk*-427A for *B. ovis* (equivalent to *glk*-1557A); *omp25*-239A for *B. canis* (*omp25*-3715A); *glk*-255G for *B. melitensis* (equivalent to *glk*-1403G); *aroA*-377A for *B. microti* (equivalent to *aroA*-617A); and *cobQ*-3224A for *B. inopinata*. A seventh SNP, *trpE*-290A (equivalent to *trpE*-2858A), is group specific, as it identifies *Brucella* strains of marine mammalian origin. This group comprises *B. ceti* and *B. pinnipedialis.* These two species, together with *B. suis* and *B. neotomae*, are further identified on the basis that they possess an overall multiplex profile that is unique for the *Brucella* species considered. An eighth SNP, *glk*-196G (equivalent to *glk*-1344G), is found only in *B. neotomae* and in the reference strain of *B. abortus* biovar 3. The remaining seven SNPs assayed in our multiplex LCR are not specific for a particular species or group but rather are intended for *B. suis* biovar differentiation (*ptsP*-1677G, *pyrH*-817G, *dnaK*-1928T, and *rpoB*-265A), for *B. ceti* differentiation below the species level (*cobQ*-3445A and *dnaK*-1654A), or for the identification of *Brucella* strains of marine origin (strains F5/02 and F5/99) that cluster poorly with other marine mammal-derived *Brucella* isolates and to which a species name has not yet been formally assigned (*trpE*-2796A) (13, 19, 28).

The PCR products derived from the amplification of the ligated PLPs range from 73 to 110 bp in size, although migration does not exactly correspond to the PCR fragment size (Table 2). For ease of identification, the assay was engineered as far as possible to display electrophoresis profiles made of peaks differing in size by at least 4 bp. This rule is not fulfilled for the profiles generated for *B. canis*, *B. ceti* ST23 (29), and *B. microti*, for which a 2-bp offset is displayed, and for *B. inopinata* (3-bp offset). Nevertheless, overall profiles are readily distinguished. Figure 1 shows typical reaction profiles obtained with the multiplex LCR procedure, demonstrating each of the 18 specific profiles that the assay generates. To validate the assay,

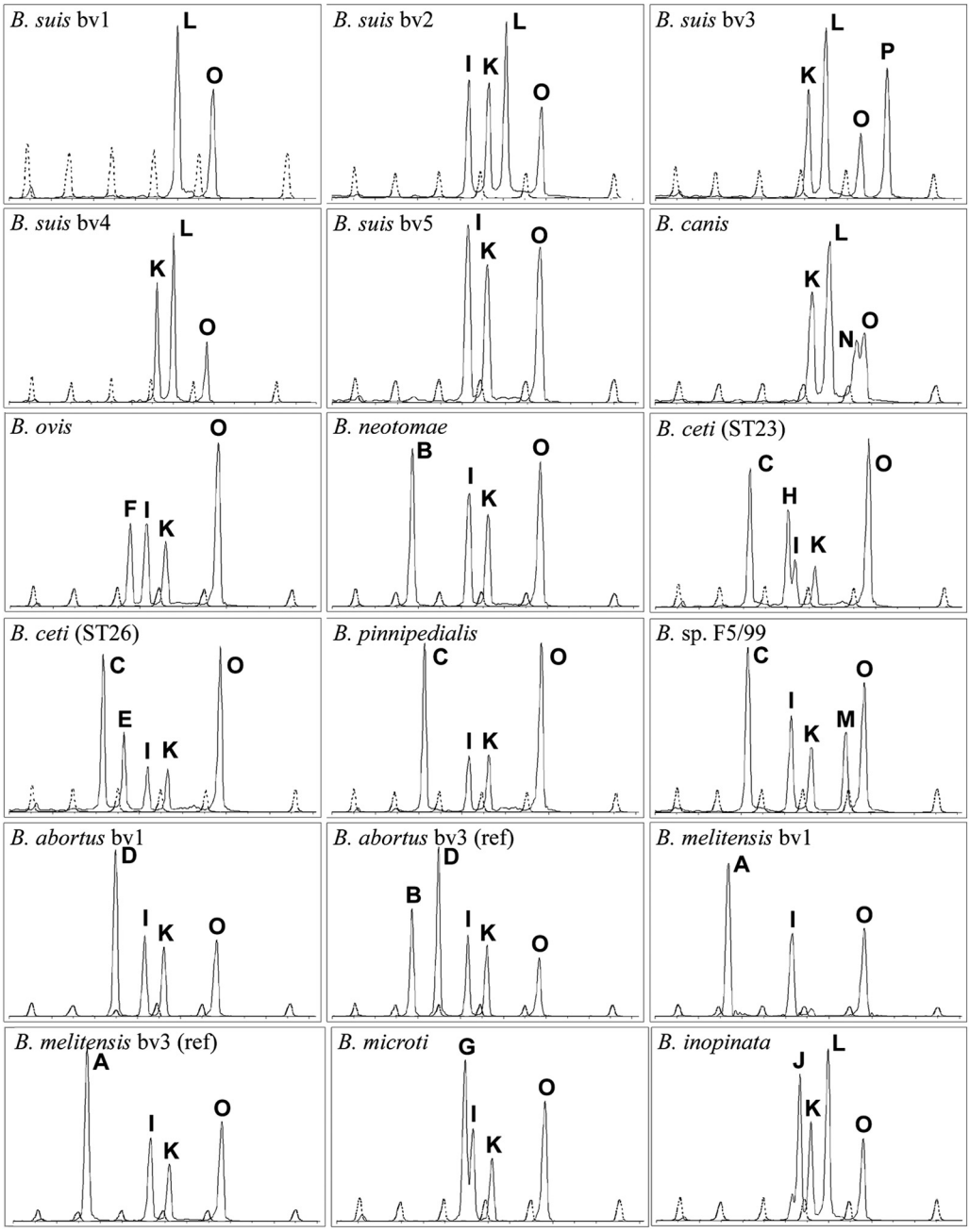

FIG. 1. Fragment analysis of representative LCR assays. The fragments were analyzed in two fluorescence channels. The dotted line is the signal of internal molecular size standards (channel 1). They have the following sizes (from left to right): 60, 70, 80, 90, 100, and 120 nucleotides. The solid line is the signal of the amplified LCR products (channel 2). A through P refer to the amplified probe codes listed in Table 1.

we examined the profiles of 103 cultured *Brucella* isolates, including all species and biovar reference strains, as well as a number of field isolates. In total, 18 *B. abortus*, 22 *B. melitensis*, 20 *B. suis*, 5 *B. canis*, 4 *B. ovis*, 14 *B. ceti*, 16 *B. pinnipedialis*, 1 *B. neotomae*, 1 *B. microti*, and 1 *B. inopinata* strains and 2 *Brucella* strains with no defined species name (*Brucella* sp.) were examined and found to give the predicted genotype. In order to assess the specificity of the assay, it was applied to 19 non-*Brucella* species, including *Ochrobactrum intermedium*, which belongs to a genus closely related to *Brucella*. The assay gave no signal on those species, confirming its specificity for the genus *Brucella* as currently defined.

DISCUSSION

The assay described here proposes a novel approach to identifying *Brucella* isolates to the species level. The molecular signatures addressed by the assay—polymorphic nucleotides located in genes with housekeeping functions scattered around the *Brucella* genome—were shown in earlier studies to display

appropriate discriminatory potential for genotaxonomic characterization (9, 20, 29). Moreover, most of these signatures have been validated on >500 strains, thereby guaranteeing the accuracy of the identifications. An MLST scheme (29) was the main source of molecular signatures for our assay. It was extended to *B. microti* and *B. inopinata* (25, 26), which were not yet described at the time the initial MLST scheme was published.

The rationale behind the assay was the selection from a number of previously sequenced housekeeping genes of a series of SNPs whose nucleotide identities, when combined, allow the identification of any *Brucella* species. The assay is basically a three-step, single-tube assay analyzed by capillary electrophoresis and applicable to purified DNA extracts. To minimize laboratory work, reagents required for the second and third steps are adapted for successive addition to the reaction tube, allowing smooth sample processing with no intermediate extraction or purification. The assay was developed using the Beckman 8000 genetic analysis system but should be easily transferable to other fragment analysis platforms. The principle of the assay relies on the conditional ligation of a series of single-stranded DNA probes displaying SNP-specific 3′-terminal nucleotides (3). The probes are padlock shaped (PLPs) so that ligation, which is conditioned by a perfect match between the template DNA and the 3′ terminus of the probe, generates a circular molecule (22). Each PLP has a unique length and includes an annealing site for a universal amplification primer and a sequence stretch identical to another universal primer in inverted-repeat orientation. The first stage involves multiplex PLP ligation using a thermostable DNA ligase. The second step involves nuclease removal of noncircular DNA molecules, i.e., template DNA and nonligated PLPs. The third step consists of PCR amplification of the ligated (circularized) PLPs. Thus, only PLPs matching the template sequence perfectly at the critical 3′-terminal nucleotide will be PCR amplified and detected.

The final assay conditions presented in this study and described in Materials and Methods were established after performance assessment of the individual PLPs and optimization of their nucleotide sequences and relative concentrations in the multiplex assay.

Compared to biotyping techniques, the assay has substantial advantages in terms of speed, robustness, and biosafety. It reaches the same sensitivity at species level. Due to the lack of genetic evidence to support the classification of *Brucella* into biovars as defined by biotyping, the assay presented here can assess ranking below the species level only partially. Compared to multiplex PCR (18), identification with the new assay is more amenable to automation but requires a capillary electrophoresis instrument. Moreover, in its actual design, it cannot differentiate field strains from vaccine strains. Compared to multiple-locus variable-number tandem-repeat analysis (MLVA) (4, 17, 30), it is less sensitive but also cheaper, requires less pipetting work, and is based on genetic markers with sound phylogenetic value. Technically, it is a single-tube test performed in three steps within a working day and requiring no subsequent extraction or purification. Assuming that carefully purified, phosphorylated PLPs are available in nonlimiting quantities, other reagents and consumables are reduced, resulting in a cost per analysis not exceeding $9.00 in our laboratory. Given the low sensitivity of the assay to interference due to the use of multiple PLPs in a single mixture, it lends itself to future expansion. Such expansion may target novel *Brucella* species, vaccine strains, or taxonomic ranks below the species level, when such ranks can be defined genetically.

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