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Direct Detection of *Brucella* spp. in Raw Milk by PCR and Reverse Hybridization with 16S-23S rRNA Spacer Probes

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The 16S-23S rRNA spacer regions of *Brucella abortus*, *B. melitensis*, and *B. suis* were cloned and subcloned after PCR amplification. Sequence analysis of the inserts revealed a spacer of about 800 bp with very high (>99%) homology among the three species examined. Two genus-specific primer pairs, BRU-P5–BRU-P8 and BRU-P6–BRU-P7, that could be used in a nested PCR format and three genus-specific DNA probes, BRU-ICG2, BRU-ICG3, and BRU-ICG4, were deduced from this spacer. The specificity and sensitivity of both primer sets and probes were examined by testing them against a collection of 18 *Brucella* strains and 56 strains from other relevant taxa by using PCR and the Line Probe Assay (LiPA), respectively. A method for direct detection of *Brucella* spp. in 1 ml of raw milk was developed on the basis of enzymatic treatment of the milk components and subsequent PCR and LiPA hybridization. After a single PCR, sensitivities of 2.8×10^5 and 2.8×10^4 CFU/ml were obtained for detection by agarose gel electrophoresis and LiPA, respectively. Nested PCR yielded a sensitivity of 2.8×10^2 CFU/ml for both methods.

Brucellosis is a widespread zoonosis which infects mainly cattle, sheep, goats, and swine, resulting in a decrease in reproductive efficiency and abortion. All six *Brucella* spp., *Brucella abortus*, *B. suis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. canis*, are considered to be potentially pathogenic to humans (6). Transmission to humans occurs by exposure to infected animals or by ingestion of contaminated milk or milk products (25, 27). While eradication programs are being applied in several countries, brucellosis remains a worldwide public health problem with severe economic consequences.

As the different *Brucella* species are genetically very similar, a single species, *B. melitensis*, has been proposed by Verger et al. (24). With classical bacteriological detection methods, several days to weeks are needed to grow *Brucella* organisms. Identification is based on morphological, biochemical, and serological properties. These tests have limited reliability. Often, serological cross-reactions with *Yersinia enterocolitica* O9 occur. Because of the high-level pathogenicity of the organisms, *Brucella* cultures must be handled with great caution.

Nucleic acid-based detection methods, such as the PCR, are very promising tools for diagnostics. PCR assays that have been described for *Brucella* spp. use primers derived from the 43-kDa outer membrane protein gene of *B. abortus* (8–10), the 16S rRNA gene (12, 18), insertion sequence IS*711* (5), and the BCSP31 protein gene (2, 16). With the primers derived from the BCSP31 protein gene (16), discrimination between *B. ovis* and the other *Brucella* spp. is possible, while with those derived from insertion sequence IS*711* (5), discrimination between certain serovars of four *Brucella* species can be achieved. However, none of these PCR assays have been applied for detection of *Brucella* spp. in food products. Herein, we describe the use of a PCR and a reverse hybridization method, the Line Probe Assay (LiPA) (17, 23), for specific identification and direct detection of *Brucella* spp. in raw milk.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. *Brucella* strains were grown on tryptic soy agar (Oxoid Ltd., London, England) supplemented with yeast (Oxoid) for 72 h and subsequently on brain heart infusion agar (Oxoid) supplemented with glucose (15 g/liter) for 24 h. All other strains were grown as recommended by the LMG Culture Collection (Laboratory of Microbiology, University of Ghent, Ghent, Belgium).

DNA extraction from cultured cells. Whole-cell DNA was extracted as described by Flamm et al. (11). Crude cell lysates were prepared by addition of 100 μl of 0.1 M NaOH–0.25% sodium dodecyl sulfate (SDS) to the pellet obtained from a 2-ml volume of a pure bacterial culture and subsequent heating at 90°C.

Sample preparation for raw milk. To break the milk fat down enzymatically, 200 μl of a lipase-phospholipase solution (17,600 U of lipase [Type VII, isolated from *Candida cylindracea*; Sigma Chemical Co., St. Louis, Mo.], 73 U of phospholipase A_2 [isolated from porcine pancreas; Sigma], 10 mM Tris, pH 7.7) was added to 1 ml of raw milk. After incubation for 1 h at 37°C, 700 μl of a trypsin solution (0.016 Anson units of trypsin [isolated from beef pancreas; BDH Chemicals Ltd., Poole, England], 0.02 M EDTA, 2.5% Triton X-100) was added to break the milk proteins down. After incubation for 1 h at 37°C, the sample was centrifuged at 14,000 × *g* for 30 min. The pellet was washed three times with 1 ml of H_2O, resuspended in 35 μl of 0.15 M NaOH–0.5% SDS, and then subjected to a 4-min microwave treatment (700 W). DNA was extracted with phenol-chloroform-isoamyl alcohol (24:24:2) in the presence of 0.5 M guanidinium thiocyanate. A second extraction was done with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 100% ethanol in the presence of 0.4% Etachinmate (Eurogentec S.A., Seraing, Belgium) and 0.03 M Na acetate. The final pellet was dissolved in 40 μl of H_2O.

Oligonucleotide synthesis, purification, and labelling. The oligonucleotides to be used as primers or probes were synthesized by the phosphite-triester method on an ABI 392 DNA synthesizer (Applied Biosystems, Foster City, Calif.). After deprotection and precipitation with ethanol, they were redissolved in distilled H_2O and used without further purification. Oligonucleotides were biotinylated at the 5′ end by adding biotin-phosphoramidite during synthesis. Efficiency of biotinylation was checked by reversed-phase high-pressure liquid chromatography.

Preparation of LiPA strips and hybridization. Oligonucleotide probes BRU-ICG1, BRU-ICG2, BRU-ICG3, and BRU-ICG4 were dT tailed and fixed onto nitrocellulose strips as described by Stuyver et al. (23). The strips were incubated with an alkali-denatured biotinylated PCR product (10 μl) in 1 ml of hybridization buffer (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 20% deionized formamide, 0.5% blocking reagent [Boehringer GMbH, Mannheim, Germany], 0.1% *N*-lauroylsarcosinate) for 1 h at 50°C in a shaking water bath. The strips were subjected to two washes at room temperature for 1 min each with 1 ml of wash buffer (3× SSC, 20% deionized formamide), followed by a stringent wash at 50°C for 15 min. After a brief rinse in 1 ml of rinse solution at room temperature, the strips were incubated with 1 ml of conjugate solution (streptavidin coupled to alkaline phosphatase) for 30 min at room temperature. The strips were then washed three times with 1 ml of rinse solution and once with 1 ml of substrate diluent. Color development was achieved by addition of the substrate 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) plus nitroblue

TABLE 1. Bacterial strains used and hybridization and PCR results obtained with probes BRU-ICG1, BRU-ICG2, BRU-ICG3, and BRU-ICG4 and primer pairs BRU-P5–BRU-P8 and BRU-P6–BRU-P7, respectively

Species	Strain	Source[a]	BRU-ICG1	BRU-ICG2	BRU-ICG3	BRU-ICG4	BRU-P5–BRU-P8[b]	BRU-P6–BRU-P7[b]
B. abortus serotype 1	L308	NIDO	+	+	+	+	+	+
B. abortus serotype 2	86/8/89	NIDO	+	+	+	+	+	+
B. abortus serotype 4	292	NIDO	+	+	+	+	+	+
B. abortus serotype 6	870	NIDO	+	+	+	+	+	+
B. abortus serotype 9		NIDO	+	+	+	+	+	+
B. abortus serotype 3	Tulya	NIDO	+	+	+	+	+	+
B. canis	RM 6/66	NIDO	+	+	+	+	+	+
B. canis	10854^T	NCTC	+	+	+	+	+	+
B. melitensis serotype 1	RZSP4	NIDO	+	+	+	+	+	+
B. melitensis serotype 1	16M	NIDO	+	+	+	+	+	+
B. melitensis serotype 2	63/9	NIDO	+	+	+	+	+	+
B. melitensis serotype 3	Ether	NIDO	+	+	+	+	+	+
B. neotomae	10084^T	NCTC	+	+	+	+	+	+
B. ovis	1051^T	NCTC	+	+	+	+	+	+
B. ovis	63/290	NIDO	+	+	+	+	+	+
B. suis serotype 1	RZSP6	NIDO	+	+	+	+	+	+
B. suis serotype 1	1401	NIDO	+	+	+	+	+	+
B. suis	40	NIDO	+	+	+	+	+	+
Agrobacterium rhizogenes	150^T	LMG	−	−	−	−	0	0
Agrobacterium tumefaciens	196	LMG	−	−	−	−	0	0
Agrobacterium tumefaciens	C58C1RifR	RZS	−	−	−	−	−	−
Bacillus brevis	S12	RZS	−	−	−	−	0	0
Bacillus cereus	S1	RZS	−	−	−	−	0	0
Bacillus coagulans	S13	RZS	−	−	−	−	0	0
Bacillus firmus	7125	LMG	−	−	−	−	0	0
Bacillus lentus	MB7	RZS	−	−	−	−	0	0
Bacillus macerans	S21	RZS	−	−	−	−	0	0
Bacillus megantum	S11	RZS	−	−	−	−	0	0
Bacillus pumilis	S19	RZS	−	−	−	−	0	0
Bacillus subtilis	S20	RZS	−	−	−	−	0	0
Bradyrhizobium japonicum	4252	LMG	−	−	−	−	−	−
Brochothrix campestris	4712	DSM	−	−	−	−	0	0
Brochothrix thermosphacta	20171	DSM	−	−	−	−	0	0
Clostridium acetobutyricum	5711	LMG	−	−	−	−	0	0
Clostridium perfringens	12224	LMG	−	−	−	−	0	0
Clostridium sporogenes	126B	INRA	−	−	−	−	0	0
Clostridium tyrobutyricum	620B	INRA	−	−	−	−	0	0
Enterococcus durans	EDS 1	INIA	−	−	−	−	0	0
Enterococcus faecalis	EFS 1	INRA	−	−	−	−	0	0
Enterococcus faecium	EFM 1	INRA	−	−	−	−	0	0
Escherichia coli	MB60	RZS	−	−	−	−	0	0
Hafnia halvei	711	INRA	−	−	−	−	0	0
Lactobacillus bulgaricus	RR1	RZS	−	−	−	−	0	0
Lactobacillus casei	MB57	RZS	−	−	−	−	0	0
Lactococcus lactis	1363	LMG	−	−	−	−	0	0
Leuconostoc lactis	774	INIA	−	−	−	−	0	0
Listeria innocua	19	IFM	−	−	−	−	0	0
Listeria ivanovii	MB4	RZS	−	−	−	−	0	0
Listeria grayi	68.18	CIP	−	−	−	−	0	0
Listeria monocytogenes	1003	RZS	−	−	−	−	0	0
Listeria murrayi	76.124	CIP	−	−	−	−	0	0
Listeria seeligeri	265/65/90	IHE	−	−	−	−	0	0
Listeria welshimeri	382	IFM	−	−	−	−	0	0
Mycoplana dimorpha	4961^T	LMG	−	−	−	−	−	−
Ochrobactrum anthropi	33	LMG	+	−	−	−	−	−
Ochrobactrum anthropi	401	LMG	+	−	−	−	−	−
Ochrobactrum anthropi	2134	LMG	+	−	−	−	−	−
Ochrobactrum anthropi	3301	LMG	+	−	−	−	−	−
Ochrobactrum anthropi	3309	LMG	+	−	−	−	−	−
Ochrobactrum anthropi	3331^T	LMG	+	−	−	−	−	−
Ochrobactrum anthropi	3402	LMG	+	−	−	−	−	−
Ochrobactrum anthropi	5436	LMG	+	−	−	−	−	−
Ochrobactrum anthropi	5446	LMG	+	−	−	−	−	−
Phyllobacterium rubiacearium	$1\ (t1)^T$	LMG	−	−	−	−	−	−
Rhizobium leguminosarum	8820	LMG	−	−	−	−	−	−
Rhizobium loti	4284	LMG	+	−	−	−	−	−

Continued on following page

TABLE 1—*Continued.*

Species	Strain	Source[a]	BRU-ICG1	BRU-ICG2	BRU-ICG3	BRU-ICG4	BRU-P5–BRU-P8[b]	BRU-P6–BRU-P7[b]
Rhizobium meliloti	4289	LMG	–	–	–	–	–	–
Salmonella enteritidis	P35	RZS	–	–	–	–	0	0
Salmonella typhimurium	P34	RZS	–	–	–	–	0	0
Staphylococcus aureus	P30	RZS	–	–	–	–	0	0
Streptococcus thermophilus	RR2	RZS	–	–	–	–	0	0
Yersinia enterocolitica	P95	RZS	–	–	–	–	0	0
Yersinia enterocolitica O9	4567	NIDO	–	–	–	–	0	0
Yersinia enterocolitica O9	MB 537	NIDO	–	–	–	–	0	0

[a] Abbreviations: CIP, Collection de Bactéries de l'Institut Pasteur (France); DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany); IFM, Institute für Milchhygiene (Austria); IHE, Institute for Hygiene and Epidemiology (Belgium); INIA, Instituto Nacional de Investigaciones Agrarias (Spain); INRA, Institut Nationale de Recherches Laitières (France); LMG, Culture Collection of the Laboratory of Microbiology Ghent (Belgium); NCTC, National Collection of Type Cultures (Public Health Laboratory Service, United Kingdom); NIDO, National Institute for Veterinary Research (Belgium); RZS, Government Dairy Research Station (Melle, Belgium).

[b] The primer annealing temperature was 55°C. 0, not tested.

tetrazolium and incubation of the strips at room temperature for 30 min on an orbital shaker. The color reaction was stopped by replacing the substrate with TE buffer (0.05 M Tris, 0.02 M EDTA, pH 8). All reagents except TE buffer were provided in the Inno-LiPA kit (Innogenetics, Antwerp, Belgium).

PCR amplification with whole-cell DNA or cell lysates. For cloning, the 16S-23S rRNA spacer was amplified by PCR with quasiuniversal primers P1 and P2 (Table 2 and Fig. 1), located about 50 bp upstream from the 3′ end of the 16S rRNA gene and about 500 bp downstream from the 5′ end of the 23S rRNA gene, respectively. A recognition site for the *Not*I restriction enzyme was added to primer P2 to facilitate cloning of the PCR products obtained. For sensitivity and specificity testing of the probes by reverse hybridization on LiPA strips, the spacers were amplified with the same primers except that these were biotinylated at their 5′ ends.

PCR was performed in a total volume of 50 μl containing 1.5 U of AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, Conn.), 50 mM KCl, 2.0 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 0.5% Tween 20, each deoxynucleoside triphosphate at 200 μM, 50 pmol of each primer, and 10 to 50 ng of DNA or 1 μl of crude cell lysate. The mixture was subjected to 30 cycles of amplification in a thermal cycler (Cetus 9600; Perkin Elmer). The first cycle was preceded by initial denaturation for 1 min at 95°C. Each cycle consisted of denaturation for 15 s at 95°C, annealing for 15 s at 50°C, and extension for 30 s at 72°C. The last cycle was followed by a final extension step for 8 min at 72°C.

PCR amplification with raw milk samples. PCR was performed in a total volume of 50 μl containing 1.5 U of AmpliTaq DNA polymerase (Perkin Elmer), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 0.5% Tween 20, each deoxynucleoside triphosphate at 200 μM, and 50 pmol of each primer. For the first PCR, BRU-P5-BIO and BRU-P8-BIO (Table 2 and Fig. 1) were used as primers and 5 μl of the sample DNA was used as the template. For nested PCR, BRU-P6-BIO and BRU-P7-BIO (Table 2 and Fig. 1) were used as primers and 5 μl of the first PCR product was used as the template. The thermal cycling program used was the same as that described above, except that the annealing temperature was 55°C. The PCR products were analyzed by agarose gel electrophoresis and/or LiPA hybridization.

Cloning. For cloning, the PCR product was loaded onto a 1% (wt/vol) GTG agarose gel (FMC Bioproducts, Rockland, Maine) and electrophoresed for about 90 min at 8 V/cm. The gel portion containing the amplified DNA fragment was removed, and the DNA was extracted with the Geneclean Kit (Bio 101, Inc., La Jolla, Calif.).

The gel-purified PCR fragment was digested with *Not*I to enable directional cloning of the amplification product in the *Eco*RV-*Not*I-digested pBluescript SK^+ vector (Stratagene, La Jolla, Calif.). After overnight ligation at 15°C, the recombinant plasmid was transformed in competent *Escherichia coli* DH5α (Life Technologies Inc., Gaithersburg, Md.) and the cell suspension was plated on Luria broth (Life Technologies) agar containing carbenicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactopyranoside (IPTG) by a standard protocol (21). White colonies were subjected to clone analysis as described by Birnboim and Doly (4).

Nucleotide sequence analysis. Plasmid DNA was prepared by the Qiagen method (Qiagen Inc., Chatsworth, Calif.). The inserts of the recombinant plasmids were sequenced by the dideoxy-chain termination method of Sanger et al. (22). Sequencing reactions were performed with the reagents from the Deaza G/A^{T7} Sequencing Mixes Kit (Pharmacia, Uppsala, Sweden) and [α-^{35}S]dATP (Amersham, Buckinghamshire, United Kingdom) and were analyzed with the Bethesda Research Laboratories sequencing system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

The sequencing primers used were T7 and T3, two promoters-primers adjacent to the multiple cloning site of pBluescript SK+, and P4 (Table 2 and Fig. 1), a quasiuniversal primer located 40 bp downstream from the 5′ end of the 23S rRNA gene of *E. coli*. Nucleotide sequence information was also obtained directly from PCR products by using P1 and P4 (Table 2 and Fig. 1) as PCR primers as well as sequencing primers. In the latter case, sequencing reactions were performed with the Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.) and the results were analyzed on a 373A automated DNA sequencer (Applied Biosystems). Sequence alignment was performed with the PC/GENE software provided by Intelligenetics Inc. and Genofit SA (Geneva, Switzerland).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the EMBL database under accession numbers X95889 to X95892.

RESULTS

PCR amplification and cloning. PCR amplification of chromosomal DNAs from all six *Brucella* species with primers P1 and P2 yielded one amplification product of about 1,500 bp. Amplification products of *B. abortus* Tulya biovar 3, *B. suis*

TABLE 2. Nucleotide sequences and locations of primers and probes used

Primer or probe	Sequence (5′ to 3′)	Location
P1	TGGGGTGAAGTCGTAACAAGGTA	*E. coli* 16S rRNA, bp 1484–1506
P2	GCATGCGGCCGCCCTTTCCCTCACGGTACTGGT[a]	*E. coli* 23S rRNA, bp 454–474
P4	CACGTCCTTCGTCGCCT	*E. coli* 23S rRNA, bp 44–60
BRU-ICG1	CGTGCCGCCTTCGTTTCTCTTT	*B. abortus* 16S-23S rRNA spacer, bp 107–128
BRU-ICG2	TTCGCTTCGGGGTGGATCTGTG	*B. abortus* 16S-23S rRNA spacer, bp 229–250
BRU-ICG3	GCGTAGTAGCGTTTGCGTCGG	*B. abortus* 16S-23S rRNA spacer, bp 255–275
BRU-ICG4	CGCAAGAAGCTTGCTCAAGCC	*B. abortus* 16S-23S rRNA spacer, bp 582–602
BRU-P5	TCGAGAATTGGAAAGAGGTC	*B. abortus* 16S-23S rRNA spacer, bp 10–29
BRU-P6	AAGAGGTCGGATTTATCCG	*B. abortus* 16S-23S rRNA spacer, bp 22–40
BRU-P7	CGAGCATTTGCAGTCGAA	*B. abortus* 16S-23S rRNA spacer, bp 682–699
BRU-P8	GCATAATGCGGCTTTAAGA	*B. abortus* 16S-23S rRNA spacer, bp 717–735

[a] The *Not*I site is underlined.

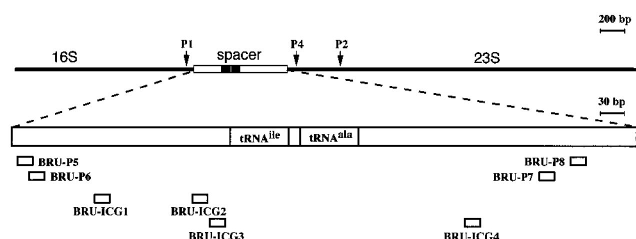

FIG. 1. Locations of the primers and probes used in this study.

RZSP6 biovar 1, and *B. melitensis* RZSP4 biovar 1 were cloned in pBluescript SK+.

For each of the three species, one recombinant plasmid was digested with *Hin*dIII, producing three fragments of about 3,800, 360, and 300 bp, respectively. The largest fragment, containing the vector and 900 bp of the original amplification product, was self-ligated. The two smaller fragments were subcloned in *Hin*dIII-digested, calf intestinal phosphatase-treated pBluescript SK+.

Nucleotide sequence analysis. For each *Brucella* strain, the three subclones with inserts of 900, 360, and 300 bp were sequenced. Results revealed that the 16S-23S rRNA spacer of *Brucella* comprises 765 bp (Fig. 2). The 16S-23S rRNA spacer sequences of *B. abortus* Tulya biovar 3 and *B. melitensis* RZSP4 biovar 1 were found to be identical. The *B. suis* RZSP6 biovar 1 sequence differed from those of both other species at three positions only (see alignments in Fig. 2). The spacer sequence of *Ochrobactrum anthropi* LMG 3331^T was completely determined by direct sequencing of the PCR product. The alignment with the spacer sequence of *B. abortus* is shown in Fig. 2. The *O. anthropi* spacer encompassed 706 bp. The homology value obtained with the *Brucella* spacers was about 80%. In the three *Brucella* spacers, as well as in the *O. anthropi* spacer, coding sequences for tRNA(Ile) and tRNA(Ala) were found.

Sensitivity and specificity of probes and primers. Four oligonucleotide probes were deduced from the *Brucella* spacer (BRU-ICG1, BRU-ICG2, BRU-ICG3, and BRU-ICG4 [Table 2 and Fig. 1]). Specificity and sensitivity testing of these probes was performed by using a reverse hybridization format on LiPA strips. The four oligonucleotide probes were immobilized on these strips as parallel lines, after which the strips were hybridized with the biotinylated amplification products from a large collection of bacterial strains. Purified DNA or cell lysates were amplified with biotinylated quasiuniversal primers P1 and P2; these primers were able to amplify the target from all of the organisms examined in this study. The hybridization and amplification results are presented in Table 1. All four probes hybridized strongly with amplified DNAs from all 18 strains of the six *Brucella* species tested. False-positive reactions with 56 non-*Brucella* strains were not observed, except for probe BRU-ICG1, which cross-reacted with all of the *O. anthropi* strains tested and one *Rhizobium* strain. Some representative hybridization results on LiPA strips are shown in Fig. 3. The presence of the expected amplicons was confirmed by electrophoresis of 8 μl of the amplification mixture on a 1.5% agarose gel and visualization after ethidium bromide staining.

Four oligonucleotides, BRU-P5, BRU-P6, BRU-P7, and BRU-P8 (Table 2 and Fig. 1), were selected for use as primers in a nested PCR format. The specificity of the two primer pairs BRU-P5–BRU-P8 and BRU-P6–BRU-P7, producing amplification products of 726 and 678 bp, respectively, was evaluated against very close relatives of *Brucella* spp. When amplification was performed at an annealing temperature of 50°C, neither of

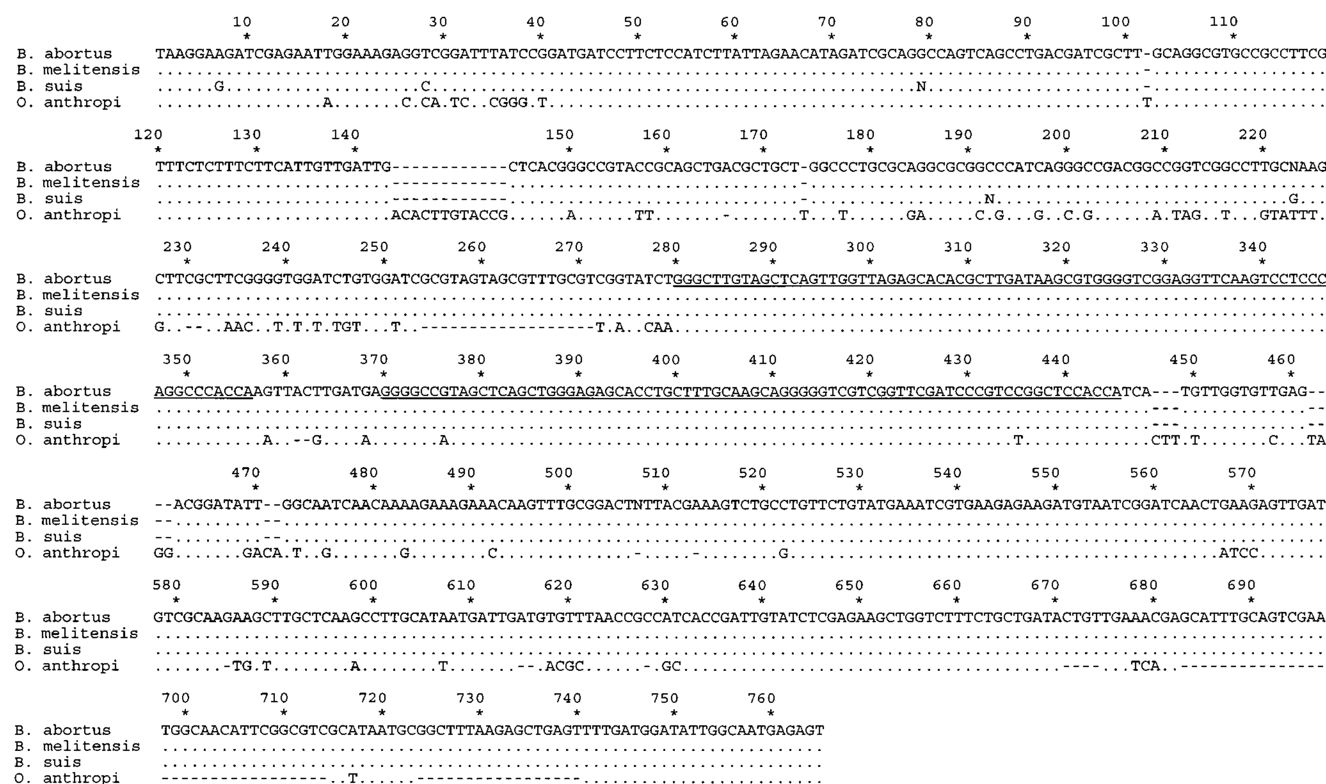

FIG. 2. Alignment of the 16S-23S rRNA spacer sequences of *B. abortus* Tulya, *B. melitensis* RZSP4, *B. suis* RZSP6, and *O. anthropi* LMG 3331^T. Sequences coding for the tRNA^{Ile} and tRNA^{Ala} genes are underlined.

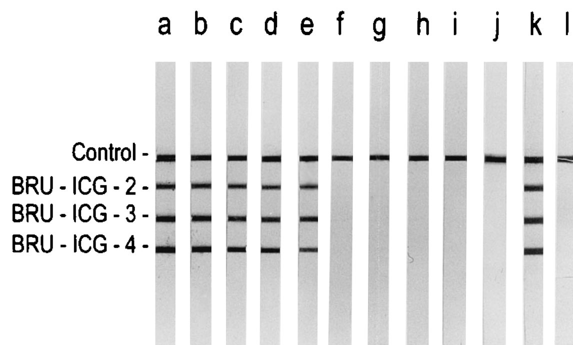

FIG. 3. Examples of hybridization results obtained with LiPA strips. Strips: a, *B. abortus* Tulya; b, *B. melitensis* RZSP4; c, *B. suis* RZSP6; d, *B. neotomae* NCTC 10084^T; e, *B. ovis* NCTC 1051^T; f, *O. anthropi* LMG 3331^T; g, *P. rubiacearium* LMG 1 (t1)^T; h, *Rhizobium leguminosarum* LMG 8820; i, *R. loti* LMG 4284; j, *Mycoplana dimorpha* LMG 4961^T; k, *B. canis* RM 6/66; l, PCR blank.

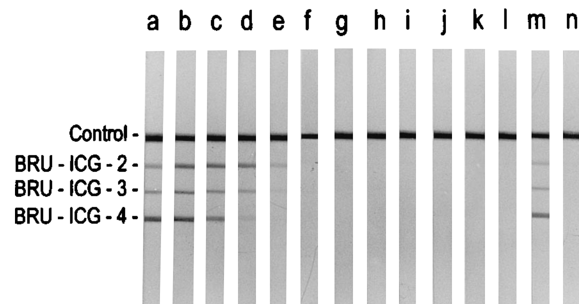

FIG. 5. Detection of *B. abortus* in 1 ml of raw milk by PCR with primers BRU-P5 and BRU-P8 on LiPA strips. One milliliter of raw milk was artificially contaminated with different numbers of *B. abortus* CFU. Strips: a, 2.8×10^8 CFU; b, 2.8×10^7 CFU; c, 2.8×10^6 CFU; d, 2.8×10^5 CFU; e, 2.8×10^4 CFU; f, 2.8×10^3 CFU; g, 2.8×10^2 CFU; h, 2.8×10^1 CFU; i, 2.8 CFU; j, 0.28 CFU; k and l, uncontaminated raw milk; m, positive control PCR; n, negative control PCR.

the primer pairs was specific for *Brucella* spp. Aspecific amplification occurred with some *O. anthropi* strains. With an annealing temperature of 55°C, both primer pairs proved to be 100% specific; amplification of all of the *Brucella* strains tested was maintained (Table 1).

Direct detection of *Brucella* spp. in raw milk. A 10-fold dilution series of a *B. abortus* Tulya pure culture was prepared in 1 ml of raw milk. The concentration of the undiluted *Brucella* culture estimated by plating on tryptic soy agar was 2.8×10^9 CFU/ml. Sample preparation was carried out, and a PCR was performed with primers BRU-P5 and BRU-P8. Sensitivities of 2.8×10^5 and 2.8×10^4 CFU/ml were achieved after agarose gel electrophoresis (Fig. 4) and LiPA hybridization (Fig. 5), respectively. A nested PCR was carried out with BRU-P6 and BRU-P7 as inner primers. The same sensitivity of 2.8×10^2 CFU/ml was achieved for both agarose gel electrophoresis and LiPA hybridization (results not shown). If the developed sample preparation is 100% efficient, the accomplished sensitivity of 2.8×10^2 CFU in 1 ml of raw milk corresponds to a maximum of 35 CFU in the first PCR.

DISCUSSION

Fast and accurate diagnosis of brucellosis is very important for a positive outcome of eradication programs. PCR is a promising alternative for the problematic culturing and identification of *Brucella* spp. by conventional techniques. The *Brucella*-specific PCR primers and DNA probes described here were derived from the 16S-23S rRNA spacer regions of one strain each of *B. abortus*, *B. melitensis*, and *B. suis*. This eubacterial spacer region has been successfully used before as a source of specific DNA probes (3, 17, 19, 20). Initially, four DNA probes were selected from the spacer region, three of which (BRU-ICG2, BRU-ICG3, and BRU-ICG4) proved to be 100% sensitive and specific for the genus *Brucella*. Only probe BRU-ICG1 cross-reacted with *O. anthropi* (formerly Centers for Disease Control and Prevention group Vd) and one *R. loti* strain. From the spacer region, we also derived two different primer sets, BRU-P5–BRU-P8 and BRU-P6–BRU-P7, which specifically amplified DNAs from *Brucella* strains at an annealing temperature of 55°C but cross-reacted weakly with *O. anthropi* at 50°C. This cross-reaction of probes and primers was not completely unexpected: *O. anthropi* is the closest relative of *Brucella* spp. at the DNA level (7, 26), and *Rhizobium* spp. are also genetically related to *Brucella* spp. (7, 26). We examined *O. anthropi* more extensively than in previous studies to prove the specificity of *Brucella* primers and probes. The primers described by Herman and De Ridder (12) and Romero et al. (18) cross-reacted with *O. anthropi* LMG 3331^T and LMG 3301, respectively, whereas Bricker and Halling (5) proved the specificity of their primers to only one *O. anthropi* strain. Fekete et al. (8–10), Baily et al. (2), and Ouahrani et al. (16) did not test any *O. anthropi* strains.

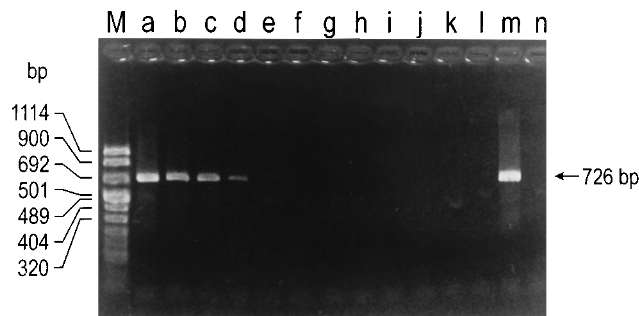

FIG. 4. Detection of *B. abortus* in 1 ml of raw milk by PCR with primers BRU-P5 and BRU-P8 on a 1.5% agarose gel. One milliliter of raw milk was artificially contaminated with different numbers of *B. abortus* CFU. Lanes: M, molecular weight markers (pUCBM21 digested with *Hpa*I plus pUCBM21 digested with *Dra*I-*Hin*dIII); a, 2.8×10^8 CFU; b, 2.8×10^7 CFU; c, 2.8×10^6 CFU; d, 2.8×10^5 CFU; e, 2.8×10^4 CFU; f, 2.8×10^3 CFU; g, 2.8×10^2 CFU; h, 2.8×10^1 CFU; i, 2.8 CFU; j, 0.28 CFU; k and l, uncontaminated raw milk; m, positive control PCR; n, negative control PCR.

Because of the extremely high (>99%) homology found among the 16S-23S rRNA spacers of the three different *Brucella* species sequenced, we did not attempt to deduce species-specific primers or probes. The fact that all four probes hybridized with all strains of the six *Brucella* species reflects high interspecies relatedness and indicates that *B. ovis*, *B. canis*, and *B. neotomae* also have highly similar spacer sequences. The very high genotypical similarities among the six *Brucella* species have been demonstrated earlier by DNA-DNA hybridizations (14, 15, 24); on the basis of these data, a single genus species, *B. melitensis*, was proposed (24). *Brucella* species also exhibit identical or nearly identical nucleotide sequences at the 16S rRNA level. In this respect, it is not surprising that until now, neither PCR primer sets nor DNA probes which are able to identify and differentiate all six *Brucella* species unambiguously have been described. However, for food safety analysis of *Brucella* spp., identification at the genus level is sufficient because all species are considered potentially pathogenic.

With one of the three primer sets (P1-P2, BRU-P5–BRU-P8, or BRU-P6–BRU-P7) and at least one of the genus-spe-

cific spacer probes, a LiPA which permits 100% specific identification of *Brucella* spp. can be designed. This simple test can be applied for culture confirmation of *Brucella* spp., thus eliminating time-consuming biochemical and serological tests. A LiPA for *Brucella* spp. should be of even greater value if this test could be applied directly to samples of interest because conventional culturing is very slow and inefficient. However, samples often contain components which may inhibit the amplification reaction. For some samples, efficient recovery of the organisms is also problematic. However, both obstacles can be overcome by appropriate sample preparation.

A method for direct detection of *Listeria monocytogenes* in 25 ml of raw milk was described recently by Herman et al. (13). However, this method, based on chemical extraction of the milk components, is not applicable for sensitive detection of *Brucella* spp. in (raw) milk (unpublished results). Unlike *Listeria* spp., *Brucella* spp. have a very high affinity for the fat phase of milk, so that after chemical extraction, *Brucella* cells adhere to the interphase. In fact, centrifugation of the milk and subsequent plating of the upper cream phase is a frequently applied classical bacteriological method for detection of *Brucella* spp. in milk (1). As an alternative to chemical extraction of the milk components, an enzymatic approach was followed. The use of lipase, phospholipase, and trypsin ensured efficient pelleting of the *Brucella* cells during centrifugation. This was proven by microscopic examination of the different phases after centrifugation. However, the remaining milk proteins lowered the solubility of the pellet. Addition of EDTA with trypsin reduced the content of milk proteins in the pellet by 90%, as was demonstrated by SDS-polyacrylamide gel electrophoresis analysis (data not shown). Addition of Triton X-100 made it much easier to quantitatively remove the supernatant. An extra phenol extraction was necessary to remove the inhibitory effect of the last remaining milk proteins on the PCR. With this sample preparation protocol, the sensitivity of the LiPA for detection of *Brucella* spp. in raw milk was investigated. To avoid interference from bacterial background flora in the milk, only the specific primer sets were used. When PCR was carried out with primer set BRU-P5-BIO–BRU-P8-BIO and followed by a LiPA, 2.8×10^4 *Brucella* CFU/ml of raw milk could be directly detected. The sensitivity could be increased approximately 100-fold by using a nested PCR. However, when a nested PCR is applied in practice, one has to consider stringent measures to avoid contamination.

The sample preparation described here, followed by PCR and subsequent LiPA hybridization, is a very useful alternative to the time-consuming classical method of *Brucella* detection in raw milk. It is possible that this sample preparation can also be used for detection of other bacterial pathogens in raw milk.

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