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Detection of *Brucella* spp. in soft cheese by semi-nested polymerase chain reaction

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Brucellae are Gram-negative bacteria with a predilection for the reticuloendothelial system and the reproductive tract of cattle, sheep, goats and pigs. In these species brucellosis is of great economic significance because of the resulting abortion and infertility (Samartino et al. 1993; Corbel, 1997; Altecruse et al. 1998). Human brucellosis, caused by contamination from infected animals (percutaneous, conjunctival or nasal infection), is very common and remains a serious health hazard in Mediterranean Europe, western Asia, and parts of Africa and South America (Boschiroli et al. 2001). Raw milk, butter, ice-cream and unpasteurized cheese are also potential vehicles for the transmission of *Brucella*.

The control of dairy products for the absence of *Brucella* spp. is important for public health, and rapid, sensitive, specific diagnostic tests are needed to identify infected animals and contaminated foods. Several PCR assays have been developed recently (Allmann et al. 1995) for the detection of genus-specific or species-specific sequences of *Brucella*. The tests were used on blood samples (Ouahrani-Bttache et al. 1996; Tcherneva et al. 1996; Morata et al. 1999) and on milk and cheese samples (Serpe et al. 1999; Tantillo et al. 2001), but are not yet in routine use. The objective of this study was to improve the sensitivity of the PCR method for *Brucella* spp. described previously (Tantillo et al. 2001) for screening dairy products, which are complex food matrices with a high content of endogenous inhibitors of Taq polymerase (Rossen et al. 1992; Wilson, 1997; Al-Soud et al. 1998).

Materials and Methods

Bacterial strain, media and growth conditions

Lyophilized *Brucella melitensis* Rev 1 strain, supplied by the Istituto Zooprofilattico Sperimentale of Lazio and Toscana and *Br. abortus* and *Br. ovis* from our institute, used as positive controls, were reconstituted in Nutrient Broth (Oxoid, Basingstoke, Hampshire, UK) containing heat-inactivated horse serum (Oxoid) processed according to the producer's instructions and incubated at 37 °C for 3 d. Then, serial ten-fold dilutions were prepared in sterile physiological saline (9 g NaCl/l) and plated in duplicate on Brucella Medium Base (Oxoid), supplemented with 2% (v/v) Brucella Selective Supplement (Oxoid) reconstituted with methanol and sterile distilled water (1:1; v/v) and 5% (v/v) horse serum. Duplicate plates were incubated at 37 °C for 10 d in aerobic conditions. The number of colony-forming units (cfu/ml) was determined.

Sample preparation

Semi-nested PCR was performed with 25 g soft cheese, homogenized with 50 ml physiological saline using a stomacher (PBI International, Milan, Italy) for 3 min. Samples of cheese homogenate were artificially contaminated with each dilution of bacterial suspension. Then, 0·1 ml of artificially contaminated homogenate was plated on selective medium and incubated in aerobic conditions at 37 °C for 10 d. For PCR assay, 0·1 ml of each serial dilution was treated with 2 volumes of acetone for bacterial inactivation and was added to the cheese homogenate. Each sample was then centrifuged at 8000 ***g*** at room temperature for 10 min. The pellet was suspended in 180 µl of a solution containing 20 mg lysozyme/ml (Sigma Chemical Co, St. Louis, MO, USA) and incubated for 30 min at 37 °C. Then, 200 µl lysis buffer (QIAGEN, Hilden, Germany) and 20 µl Proteinase K (20 mg/ml) were added and the suspension was incubated at 56 °C for 30 min, and for a further 15 min at 95 °C. After adding 200 µl ethanol, the mixture was applied to the QIAamp spin column (QIAGEN). DNA was adsorbed onto the QIAamp silica-gel membrane during one centrifugation step, after which the DNA bound to the QIAamp membrane was washed by centrifugation using two different washing buffers. Purified DNA was eluted from the QIAamp spin column in 200 µl elution buffer. DNA concentration and the purity of the eluate were then measured by absorbance at 260 nm and by calculating the ratio of absorbance at 260 nm to absorbance

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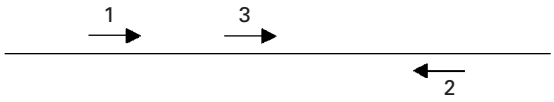

Fig. 1. Schematic representation of position of the primers of BCSP-31 gene. BRU UP=1; BRU LOW=2; BRU IN=3.

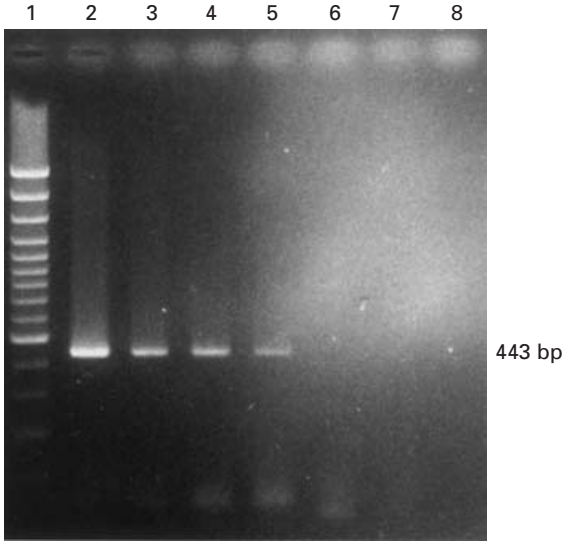

Fig. 2. Electrophoretic profile of standard amplification products of artificially contaminated soft cheese samples with serial dilutions of *Br. melitensis* Rev 1 strain. Lane 1, 100 bp DNA ladder (Fermentas); lane 2, 10^5 cfu/ml; lane 3, 10^4 cfu/ml; lane 4, 10^3 cfu/ml; lane 5, 10^2 cfu/ml; lane 6, negative; lane 7, negative; lane 8, negative control.

at 280 nm using a spectrophotometer (DU-600, Beckman Instruments, Fullerton, CA, USA).

Oligonucleotide primers

The primer pair, BRU UP (5′ GGG CAA GGT GGA AGA TTT 3′) and BRU LOW (5′ CGG CAA GGG TCG GTG TTT 3′) targeted a 443-bp fragment of *BCSP-31* gene encoding for an antigen localized at or near the bacterial cell surface of *Brucella* species (Mayefield et al. 1988). The primer used for semi-nested PCR, BRU IN (5′ GGG ACC GGC AGG CGA GAG 3′) selected from the published sequences, amplifies a 225-bp internal region (Fig. 1). The primer specificity was also tested on *Br. abortus* and *Br. ovis* strains from the Institute collection. Cross-reactivity was evaluated by testing the following reference strains *Listeria monocytogenes* ATCC 7646, *Salmonella* spp. ATCC 35664, *Escherichia coli* ATCC 35421, *Pasteurella aerogenes* ATCC 27883 (DID, Milan, Italy). These reference strains were grown according to the suppliers' instructions. Genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN).

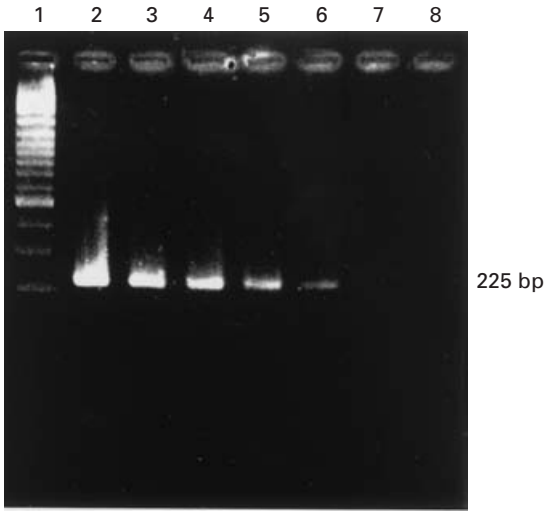

Fig. 3. Electrophoretic profile of semi-nested amplification products of artificially contaminated soft cheese samples with serial dilutions of *Br. melitensis* Rev 1 strain. Lane 1, 100 bp DNA ladder (Fermentas); lane 2, positive control; lane 3, 10^4 cfu/ml; lane 4, 10^3 cfu/ml; lane 5, 10^2 cfu/ml; lane 6, 10 cfu/ml; lane 7, negative <10 cfu/ml; lane 8, negative control.

PCR assay

Amplification of DNA was performed in a total volume of 50 µl using 25 µl Premix Taq (TaKaRa Taq™ Version; Takara Shuzo Co., LTD, Otsu, Japan) containing 1·25 units of DNA Polymerase, 0·4 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 2X PCR buffer (20 mM-Tris-HCl pH 8·3, 100 mM-KCl, 3 mM-$MgCl_2$), and 1 µM of each primer (BRU UP and BRU LOW). Sample volume was 2 µl. The mixture was processed in a Mastercycler personal (Eppendorf) with an initial denaturation step of 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and an extra 30 s at 72 °C. Then 0·5 µl of the first amplification reaction were further amplified in a volume of 50 µl with 25 µl Premix Taq (TaKaRa Taq™ Version; Takara Shuzo Co., LTD, Otsu, Japan) containing 1·25 units DNA Polymerase, 0·4 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 2X PCR buffer (20 mM-Tris-HCl pH 8·3, 100 mM-KCl, 3 mM-$MgCl_2$) and 1µM of each primer (BRU IN and BRU LOW). The mixture was subjected to 35 cycles with a denaturation at 94 °C for 30 s, annealing at 70 °C for 30 s and an extra 30 s at 72 °C.

PCR products (8 µl) were analysed by electrophoresis on 2% (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X TBE buffer containing 0·89 M-Tris, 0·89 M boric acid, 0·02 M EDTA, pH 8·0 (USB, Cleveland, OH, USA) (Sambrook et al. 1989) and visualized by ethidium bromide staining and a u.v. transilluminator. A Gene Ruler™ 100-bp DNA Ladder Plus (MBI Fermentas, Vilnius,

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Lithuania) consisting of DNA fragments ranging in size from 3000 bp to 100 bp was used as marker.

Results

The concentrations of *Brucella* Rev 1, *Br. abortus* and *Br. ovis* reference strains, calculated by the plating method, were 2×10^9, 3×10^8 and 2×10^6 cfu/ml respectively. Similar concentrations were recorded in artificially contaminated cheese prior to inactivation. PCR tests on cheese samples artificially contaminated with all reference strains confirmed a detection limit of 10^2 cfu/ml (Fig. 2). The semi-nested PCR assay carried on the PCR products was able to detect 10 cfu/ml of *Brucella*. Agarose gel electrophoretic analysis of semi-nested PCR products (Fig. 3) showed the size of the amplified fragment to be, as expected, 225 bp. The method confirmed the specificity of 443 bp products and increased sensitivity in detecting target DNA of *Brucella* spp. 10-fold compared with the standard PCR assay (Fig. 2) described previously (Tantillo et al. 2001). The results presented in this study are from ten experiments.

Discussion

Semi-nested PCR amplification using primers BRU UP, BRU LOW, BRU IN allowed detection of *Brucella* spp. without cross-reactivity with the other microorganisms tested in the present study, which are often present in dairy products. The semi-nested PCR developed to detect *Brucella* spp. in soft cheese was specific, rapid, simple and was not affected by the presence in the samples of Taq polymerase inhibitors removed during the DNA extraction step.

In view of the increasing importance of brucellosis, the test described may be considered a genuine advance because it could offer rapid and specific screening for the presence of *Brucella* spp. in routine quality control. Furthermore, in also detecting inactivated microorganisms, the test may be used to monitor brucella in the entire cheese production chain production (animals, milk, cheese) (Allmann et al. 1995).

References

Allmann M, Hofelein C, Koppel E, Luthy J, Meyer R, Niederhauser C, Wegmuller B & Candrian U 1995 Polymerase chain reaction (PCR) for detection of pathogenic microrganisms in bacteriological monitoring of dairy products. *Research in Microbiology* **146** 85–97

Al-Soud WA & Radstrom P 1998 Capacity of nine Thermostable DNA Polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. *Applied and Environmental Microbiology* **64** 3748–3753

Altecruse SF, Timbo BB, Mowbray JC, Bean NH & Potter ME 1998 Cheese-associated outbreaks of human illness in the United States 1973 to 1992: sanitary manufacturing practices protect consumers. *Journal of Food Protection* **61** 1405–1407

Boschiroli ML, Foulongne V & O'Callaghan D 2001 Brucellosis: a worldwide zoonosis. *Current Opinion in Microbiology* **4** 58–64

Corbel MJ 1997 Brucellosis: an overview. *Emerging Infectious Diseases* **3** 213–221

Mayefield JE, Bricker BJ, Godfrey H, Crosby RM, Knight DJ, Halling SM, Balinski D & Tatabai LB 1988 The cloning expression and nucleotide sequence of a gene coding for an immunogenic *Brucella abortus* protein. *Gene* **63** 1–9

Morata P, Queipo-Ortuno MI, Reguera JM, Garcia-Ordonez MA, Pichardo C & Colmenero JD 1999 Post-treatment follow-up of brucellosis by PCR assay. *Journal of Clinical Microbiology* **37** 4163–4166

Ouahrani-Bettache S, Soubrier MP & Liautard JP 1996 IS6501-anchored PCR for detection and identification of *Brucella* species and strains. *Journal of Applied Bacteriology* **81** 154–160

Rossen L, Norskov P, Holmstromet K & Rasmussen OF 1992 Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA extraction solution. *International Journal of Food Microbiology* **17** 37–45

Samartino LE & Enright FM 1993 Pathogenesis of abortion of bovine brucellosis. *Comparative Immunology Microbiology and Infectious Diseases* **16** 95–101

Sambrook J, Fritsch EF & Maniatis T 1989 *Molecular Cloning: a laboratory manual*, 2nd Edn. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory

Serpe L, Gallo P, Fidanza N, Scaramuzzo A & Fenizia D 1999 Single-step method for rapid detection of *Brucella* spp. in soft cheese by gene-specific polymerase chain reaction. *Journal of Dairy Research* **66** 313–317

Tantillov G, Di Pinto A, Vergara A & Buonavoglia C 2001 Polymerase chain reaction for direct detection of *Brucella* spp in milk and cheese. *Journal of Food Protection* **64** 164–167

Tcherneva E, Rijpens N, Naydensky C & Herman L 1996 Repetitive element sequence based polymerase chain reaction for typing of *Brucella* strains. *Veterinary Microbiology* **51** 169–178

Wilson IG 1997 Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* **63** 3741–3751