Single-step method for rapid detection of *Brucella* spp. in soft cheese by gene-specific polymerase chain reaction

By LUIGI SERPE*, PASQUALE GALLO, NICOLETTA FIDANZA, ALFREDO SCARAMUZZO and DOMENICO FENIZIA

Istituto Zooprofilattico Sperimentale del Mezzogiorno, via Salute 2, I-80055 Portici (NA), Italia

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Brucellosis can be transmitted to man by direct contact with infected animals or through contaminated meat, milk and dairy products (Nicoletti, 1989). The analysis of *Brucella* spp. is carried out in the laboratory by microbiological or serological assays (Alton *et al*. 1988). The first are more specific but are also time-consuming and expose the analyst to the risk of infection (López-Merino, 1991). However, the latter can result in false positives owing to cross reactivity with other Gram-negative bacteria (Diaz-Aparicio *et al*. 1994). Because of these limitations, the amplification *in vitro* of specific DNA regions by the polymerase chain reaction (PCR) could represent a powerful tool for rapid and specific diagnostic analysis. In recent years, several PCR methods have been developed to amplify specific DNA sequences of *Brucella* strains (Herman & de Ridder, 1992; Romero *et al*. 1995; Valentino *et al*. 1997). In addition, direct analysis of *Brucella* in contaminated abortive tissues (Fekete *et al*. 1992), milk and blood (Leal-Klevezas *et al*. 1995; Rijpens *et al*. 1996) has been reported.

In this paper we describe a method for gene-specific PCR amplification of a 443 base pair (bp) fragment of *Brucella* DNA that belongs to a gene encoding for a 31 kDa outer membrane protein. This protein (BCSP-31) is a membrane antigen characteristic of the *Brucella* genus (Mayfield *et al*. 1988). The PCR method was developed for the analysis of soft cheeses. We focused our attention on Mozzarella, Pecorino and ricotta samples, because such products are not subjected to the natural microbial autopurification process of maturing. They are widely consumed in Italy and a relationship between infected foods and the areas where brucellosis is a human zoonosis is a possibility.

The analysis was performed without purification of DNA from bacteria. Indeed, after homogenization, the sample was subjected to thermal shock by freeze–thaw cycles that lysed bacteria and solubilized nucleic acids for subsequent PCR amplification. Amplified DNA fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Several brands of soft cheeses and ricotta contaminated at different levels with *Brucella* cells were analysed by our procedure to evaluate the detection sensitivity and the repeatability of the method.

* For correspondence.

materials and methods

Brucella strains

Br. abortus 544, *Br. abortus* S19, *Br. melitensis* list 2, *Br. suis* 1330 biovar 1, *Br. canis*, *Br. melitensis* Rev 1 (vaccine strain), *Br. melitensis* biovar 2 and *Br. ovis* were isolated from infected materials in our Institute. The isolates were characterized at the Institute of Infectious Diseases, University of Pisa, I-56100 Pisa, Italy, by biochemical tests (CO_2 requirement, H_2S production), growth in the presence of dyes (fuchsin, thionin), agglutination with monospecific sera (anti-A, anti-M), sensitivity to phages and oxidative–metabolic activities (Farina & Scatozza, 1995). Bacteria were grown in brucella broth (Biolife, I-20153 Milano, Italy) or plated on to brucella agar (Biolife) and incubated at 37 °C for 48 h.

Primers and gene-specific polymerase chain reaction amplification

Theoretical analysis of the two PCR primers used to set up the method was carried out using the OLIGO™ 5·0 software package (Valentino *et al.* 1997). The primers were synthesized by Genenco (I-50132 Firenze, Italy). The designations and sequences of PCR primers were as follows: BRU-UP (GGG CAA GGT GGA AGA TTT) and BRU-LOW (CGG CAA GGG TCG GTG TTT), targeting the outer membrane protein BCSP-31 (Mayfield *et al.* 1988). PCR amplification was performed using a *Taq* DNA Polymerase kit (Promega Corp., Madison, WI 53711–5399, USA) in a final volume of 25 μl. The basic amplification reaction mixture contained 50 mM-KCl, 10 mM-Tris-HCl, pH 8·3, Triton X-100 (1 g/l), 1·5 mM-$MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP (Promega), 1 μM of each primer, *Taq* DNA polymerase (0·08 units/μl) and 10 μl sample solution. The reaction mixtures were placed into a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer, I-00157 Roma, Italy) and amplified by denaturation at 94 °C for 30 s, followed by 29 denaturation cycles at 94 °C for 1 min, annealing at 48 °C for 1 min and extension at 72 °C for 2 min. After a final extension time of 7 min at 72 °C, the reaction was stopped by 10 min incubation at 4 °C.

Sample preparation

Different brands of Mozzarella, Pecorino and ricotta made from bovine and buffalo milk produced in the Campania and Calabria regions were used for the experiments; the age of these products ranged from 2 to 15 d. Cheese or ricotta samples (10 g) were weighed into 20 ml 150 mM-Tris-HCl buffer, pH 8·0 and homogenized in a Stomacher 400 digester (PBI, I-20153 Milano, Italy) for 3 min at medium power. Homogenate (1·2 ml) was transferred into an Eppendorf tube and Tween 20 added to a final concentration of 20 ml/l. The contents were mixed and centrifuged at 10000 ***g*** and 20 °C for 30 min in a model 5402 Eppendorf centrifuge (I-20138 Milano, Italy). The supernatant was discarded and the pellet resuspended in 150 μl sterile water and sonicated at 20 °C for 5 min. The bacteria were lysed by the following freeze–thaw steps: 20 min at −60 °C, 10 min at 100 °C, 20 min at −60 °C, 10 min at 100 °C. Finally, the sample was centrifuged at 10 000 ***g*** and 4 °C for 10 min, and 10 μl of the supernatant withdrawn for PCR amplification.

Spiking suspensions

Br. abortus S19 and *Br. melitensis* list 2 cells were suspended in 10 mM-Na_2HPO_4–1·7 mM-KH_2PO_4–137 mM-NaCl–2·7 mM-KCl buffer, pH 7·4 at 3×10^7, 3×10^6, 3×10^5, 3×10^4 and 3×10^3 cfu/ml and used to spike samples and homogenates prior to the analyses in order to evaluate DNA recovery and method sensitivity. The

concentration was calculated both by nephelometry (measurement on the McFarland scale by comparison with a standard suspension of $BaSO_4$ in water) and by plating bacteria on to agar brucella plates.

Electrophoretic analysis

PCR products were analysed by standard electrophoresis on agarose gels (10 g/l) in 89 mM-Tris–89 mM-sodium borate–2 mM-EDTA buffer, pH 8·0 and stained by ethidium bromide (0·5 μg/ml). A 100 bp DNA ladder and λ DNA–*Eco*RI + *Hind*III markers (Promega) were included. Electrophoresis was carried out with a model GPS 200/400 power supply and a model GNA 100 horizontal electrophoresis system (Pharmacia Biotech, I-20093 Milano, Italy).

RESULTS

During the first part of this study, we purified the DNA from each *Brucella* strain according to the procedure of Anderson *et al.* (1984), and carried out PCR amplification with the selected primers. All DNA gave the same PCR product of $\sim$ 440 bp.

Subsequently, we developed the best experimental procedure to extract DNA from *Brucella* cells in the presence of the matrix. The procedure for sample preparation was optimized to obtain effective gene-specific PCR amplification of target DNA. To buffer the matrix acidity and carry out PCR amplification at pH 8 (recommended for *Taq* polymerase activity) we tested 10 mM-Na_2HPO_4–1·7 mM-KH_2PO_4–137 mM-NaCl–2·7 mM-KCl buffer, pH 7·4, 150 mM-Tris-HCl buffer, pH 8·0 and 150 mM-Tris-HCl buffer pH 9·0. The best results were obtained using the pH 8·0 Tris-HCl, as we found by analysing a Mozzarella sample spiked with *Br. melitensis* list 20 at 6×10^6 cfu. We also optimized centrifugation time to ensure complete sedimentation of the bacteria in the sample. In addition, as lipids adsorb bacteria, we used the non-ionic detergent Tween 20 to emulsify the fat fraction of the sample. Different amounts of Tween 20 were tested and we found that a concentration of 20 ml/l gave the best recovery of bacteria from spiked samples. When the concentration of the detergent was decreased to 0·2 ml/l amplification products were detected in the supernatant. Similar results were observed with Pecorino and ricotta samples spiked with *Br. melitensis* list 2.

We also evaluated matrix effects on PCR amplification by analysing several samples of Mozzarella, Pecorino and ricotta spiked with 200 μl *Br. abortus* S19 or *Br. melitensis* list 2 suspensions at different concentrations. All experiments were conducted in triplicate. The results obtained for Mozzarella are shown in Fig. 1. PCR products of $\sim$ 440 bp were observed only in the samples spiked with 6×10^6, 6×10^5, 6×10^4 and 6×10^3 cfu. Similar results were obtained for Pecorino and ricotta samples.

The specificity of the BRU-UP and BRU-LOW primers was tested by PCR amplification of the DNA purified by the procedure of Anderson *et al.* (1984) from *Rhizobium meliloti*, *Yersinia enterocolitica*, *Escherichia coli*, *Pasteurella multocida*, *Bacillus subtilis*, *Streptococcus thermophilus*, *Listeria monocytogenes* and *Salmonella haard*. We chose a microorganism genetically related to *Brucella* spp. (*Rhizobium meliloti*) and bacteria that are often present in dairy products and found that their DNA was not amplified by the primers selected (Fig. 1).

To determine the sensitivity of the method, we added $1{\cdot}2 \times 10^6$, $1{\cdot}2 \times 10^5$ and $1{\cdot}2 \times 10^4$ cfu to 10 g Mozzarella samples; the samples were spiked by injecting

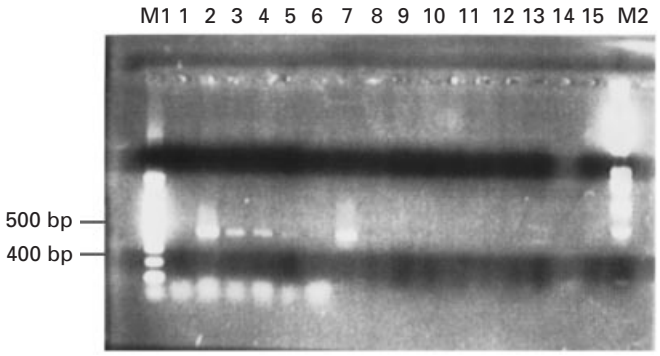

Fig. 1. Determination of matrix effects on polymerase chain reaction (PCR) amplification and specificity of selected primers. Lane M1, 100 bp ladder DNA; lane 1, blank sample; lanes 2–6, sample (10 g) of Mozzarella cheese spiked with *Brucella melitensis* list 2 at 6×10^6, 6×10^5, 6×10^4, 6×10^3 and 6×10^2 cfu respectively; lane 7, positive control: PCR of 10 ng DNA from *Brucella melitensis* list 2; lane 8, PCR of 10 ng DNA from *Rhizobium meliloti*; lane 9, PCR of 10 ng DNA from *Escherichia coli*; lane 10, PCR of 10 ng DNA from *Pasteurella multocida*; lane 11, PCR of 10 ng DNA from *Bacillus subtilis*; lane 12, PCR of 10 ng DNA from *Streptococcus thermophilus*; lane 13, PCR of 10 ng DNA from *Listeria monocytogenes*; lane 14, PCR of 10 ng DNA from *Salmonella haard*; lane 15, PCR of 10 ng DNA from *Yersinia enterocolitica*; lane M2, λ DNA marker.

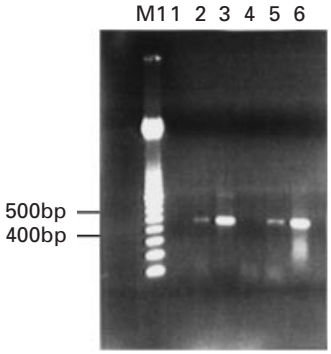

Fig. 2. Determination of sensitivity of method for detecting *Brucella* spp. in 10 g Mozzarella samples using a gene-specific polymerase chain reaction. Samples were spiked prior to homogenization with *Brucella melitensis* list 2 at lanes 1 and 4, $1{\cdot}2 \times 10^4$; lanes 2 and 5, $1{\cdot}2 \times 10^5$; lanes 3 and 6, $1{\cdot}2 \times 10^6$ cfu. The experiments were performed in duplicate. Lane M1, 100 bp DNA ladder.

bacteria (4 ml of 3×10^5, 3×10^4 and 3×10^3 cfu/ml suspensions respectively) into four different points of the matrix. The samples were thoroughly mixed and then homogenized in 20 ml 150 mM-Tris-HCl buffer, pH 8·0; DNA extraction and PCR amplification were carried out as described previously. Experiments were carried out in duplicate and the results are shown in Fig. 2. The 443 bp PCR products were detected only in samples spiked with $1{\cdot}2 \times 10^6$ and $1{\cdot}2 \times 10^5$ cfu. Similar results were obtained for Pecorino and ricotta samples

The repeatability of the method was evaluated by analysing on three different days three samples (10 g Mozzarella, Pecorino and ricotta) spiked with $1{\cdot}2 \times 10^5$ cfu *Br. melitensis* list 2. All samples were positive.

DISCUSSION

The PCR method we have developed for detecting *Brucella* spp. in soft cheeses and ricotta was simple, specific and rapid. Targeted amplification was not affected by the presence of other microorganisms in the sample. The lysis of bacteria was

single-step and did not require DNA extraction and purification prior to amplification. Only 150 min were necessary to prepare the samples for the PCR experiment.

PCR amplification of microbial DNA directly in the food sample is relatively difficult, because of the possible presence of *Taq* polymerase inhibitors. However, the extraction of microorganisms and purification of their nucleic acids is a multi-step, time-consuming and expensive procedure, involving greater risk to the analyst. We calculated the detection sensitivity of the method to be $1{\cdot}2 \times 10^4$ cfu/g sample. Although this detection limit is relatively high, it should be noted that classical microbiological methods for detection of *Brucella* spp. in foods are difficult to standardize, owing to the growth conditions of these bacteria. Thus, no sensitivity limit is reported for these procedures.

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