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Polymerase Chain Reaction for the Direct Detection of *Brucella* spp. in Milk and Cheese

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ABSTRACT

A polymerase chain reaction test was developed to detect *Brucella* spp. directly in milk and cheese and optimized using primers for the BSCP-31 gene. A total of 46 cheese samples produced with sheep and goats milk were assayed, and *Brucella* spp. was detected in 46% of them, especially in cheese made from sheep milk. This method is of remarkable epidemiologic interest because it is an indirect test indicating the sanitary quality of milk used in dairy industries. The method showed good sensitivity and specificity. It is faster and less expensive than the conventional bacteriological assays.

The brucelleae are gram-negative, obligate parasites of animals and humans and are located intracellularly. In pregnant animals (cattle, sheep, goats, and swine) brucelleae produces placentitis and abortion. The disease in humans, brucellosis, is a consequence of accidental contact with infected animal feces, urine, milk, and tissues. The common sources of infection for humans are unpasteurized milk, milk products, cheese, and professional contact (farmers, veterinarians, slaughterhouse workers) with infected animals. In Italy, during 1997, 1,681 cases of human brucellosis were notified by the Minister of Health *(8)*.

Traditionally, identification of most microbial pathogens in food involves enrichment cultures, cultivation on selective media, and ultimately a series of biochemical tests to identify the organisms *(4)*. Such standard microbiological techniques are slow, laborious, and often require several days, even weeks, to be performed. Enrichment protocols also may fail to detect strains of bacteria present in food at low levels.

Recently, faster, more specific, and less expensive methods to detect and identify microbial pathogens in food that often cause disease outbreaks in humans have been made possible. Several reports indicate polymerase chain reaction (PCR) as a promising new diagnostic method to detect foodborne pathogens and so to monitor food safety *(5, 6)*. However, detection of microorganisms using PCR in complex biological matrices, such as food samples, is limited by the presence of substances that inhibit or reduce the amplification, especially the termostable DNA polymerase activity *(2, 9, 10)*.

In fact, the majority of methods described involve an enrichment culture from which the DNA is extracted for PCR assay *(14)*. Few reports describe the direct detection *(1)* of microbial pathogens in food samples using PCR.

The aim of this paper is to describe a PCR test for the detection of *Brucella* spp. in milk and milk products that does not include the enrichment cultures and dilutions *(3)* normally necessary to reduce the content of endogenous inhibitors of *Taq* polymerase.

MATERIALS AND METHODS

Bacterial strain, media, and growth conditions. A lyophilized *Brucella melitensis* Rev 1 strain, supplied by the Istituto Zooprofilattico Sperimentale of Lazio and Toscana, was used as the positive control. The Rev 1 strain was reconstituted in nutrient broth (Oxoid, Basingstoke, England) containing inactivated horse serum (Oxoid), processed according to the producer's instructions, and incubated at 37°C. After 3 days of incubation, serial 10-fold dilutions were performed in sterile saline (0.9% NaCl). Then, 0.1 ml of each dilution from 10^{-1} to 10^{-12} was plated in duplicate on Brucella medium base (Oxoid), containing 2% (vol/vol) Brucella selective supplement (Oxoid), reconstituted with methanol and sterile distilled water (1:1, vol/vol), and 5% (vol/vol) horse serum.

After 48 to 72 h of incubation at 37°C, the microbial concentration was determined and expressed as colony forming units (CFU/ml).

Laboratory samples. A PCR test was performed with 10 ml of sheep and goat milk samples and with 10 g of cheese sample homogenized with saline (1:2 wt/vol) for 3 min, using a stomacher (PBI International, Milan, Italy). Then, 1 ml of each sample was artificially contaminated with 0.1 ml of each serial dilution (10^{-1} to 10^{-12}) of the Rev 1 strain previously inactivated with 2 volumes of acetone.

Total DNA was extracted directly using DNA affinity columns (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany). First, 1 ml of contaminated milk and 1 ml of cheese homogenate were centrifuged for 10 min at 8,000 × *g* at room temperature. The supernatant was discarded and the pellet was resuspended in 180 μl of lysozyme (20 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) and incubated for 30 min at 37°C. Then, 200 μl of lysis buffer and 20 μl of proteinase K (20 mg/ml) were added, and the suspension was incubated at 56°C for 30 min and for 15 min at 95°C. The lysate was mixed with 200 μl of ethanol and then the mixture was applied to the QIAamp spin column (Qiagen). DNA was adsorbed onto the QIAamp silica-gel membrane during a brief cen-

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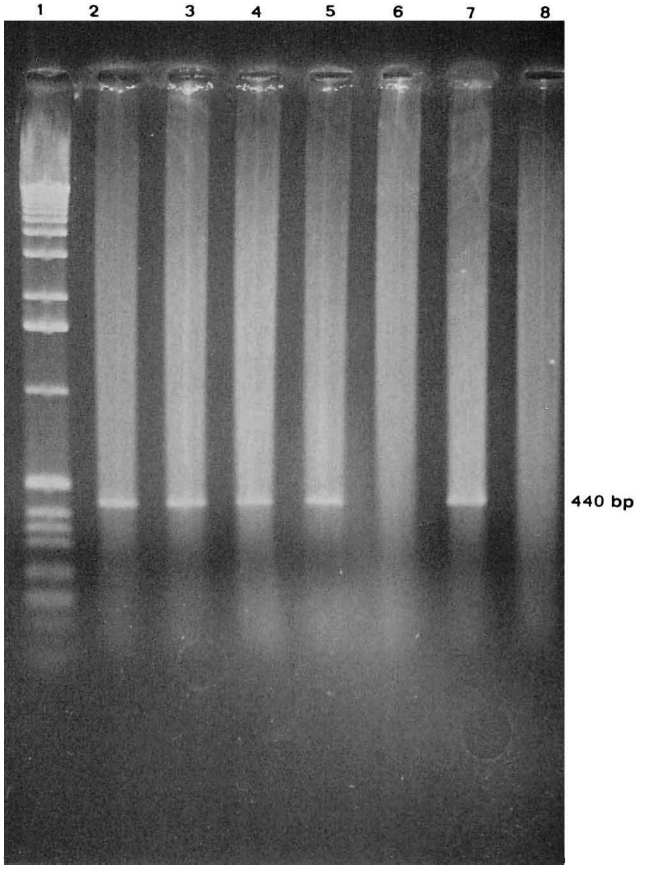

FIGURE 1. *Electrophoretic profile of amplification products of artificially contaminated milk samples with serial dilutions of* B. melitensis *Rev 1 strain. Lane 1, 1-kb DNA ladder (Gibco); lane 2, 10^3 CFU/ml; lane 3, 10^2 CFU/ml; lane 4, 10 CFU/ml; lane 5, <10 CFU/ml; lane 6, negative; lane 7, positive (purified* B. melitensis *DNA) control; and lane 8, negative (no DNA) control.*

trifugation step. Protein and other contaminants that can inhibit PCR were not retained on the QIAamp membrane. Then, DNA bound to the QIAamp membrane was washed with two different wash buffers, provided in the kit, in two centrifugation steps to improve the purity of the eluted DNA. Finally, DNA was extracted with 200 μl of elution buffer, and 10 μl of DNA were used as the PCR template.

Primers. The oligonucleotide primers used were BRU UP (5′ GGG CAA GGT GGA AGA TTT 3′) and BRU LOW (5′ CGG CAA GGG TCG GTG TTT 3′) *(7),* (Gibco BRL Life Technologies, Paisley, Scotland) that amplify a 440-bp fragment of the BSCP-31 gene coding for a 31-kDa membrane protein of the *Brucella* genus.

PCR assay. Amplification of DNA was performed in a total volume of 50 μl using 25 μl of 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), 2× PCR buffer (20 mM of Tris-HCl, pH 8.3, 100 mM KCl, 3 mM $MgCl_2$), and 1μM of each primer. The sample volume used was 10 μl.

The mixture was processed in a Mastercycler 5330 plus (Eppendorf) with an initial denaturation step of 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension of 1 min at 72°C; the reaction was completed by a final extension step of 7 min at 72°C.

Agarose gel electrophoresis. After amplification, 8 μl of the reaction mixture was analyzed by electrophoresis on 1% (wt/vol) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1× TBE buffer (100 mM Tris-HCl, 90 mM boric acid, 1 mM EDTA, pH 8.4) (Gibco BRL) *(11)* and visualized by ethidium bromide staining and UV transilluminator. A 1-kb DNA ladder (Gibco BRL) was used as the molecular weight marker (500 bp, 12 kb).

Cheese samples. Tests were done on 46 cheese samples, in particular 15 samples made from milk of sheep, 18 samples from milk of goats, and 13 samples from a mixture of milk from these species. Ripening times ranged from 30 to 45 days. Samples were

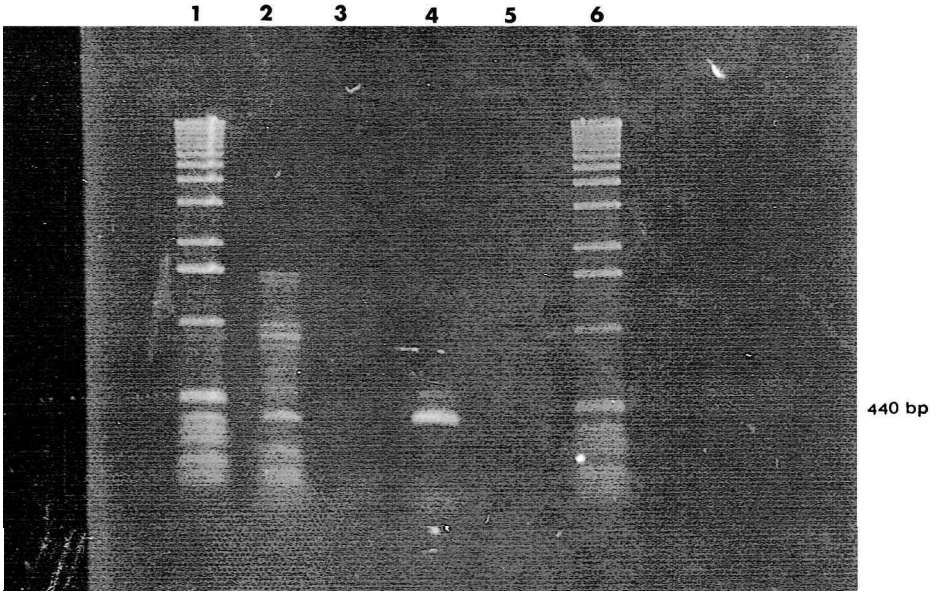

FIGURE 2. *Electrophoretic profile of amplification products of artificially contaminated cheese samples with serial dilutions of* B. melitensis *Rev 1 strain. Lane 1, 1-kb DNA ladder (Gibco); lane 2, 10^2 CFU/ml; lane 3, 10 CFU/ml; lane 4, positive (purified* B. melitensis *DNA) control; lane 5, negative (no DNA) control; and lane 6, 1-kb DNA ladder (Gibco).*

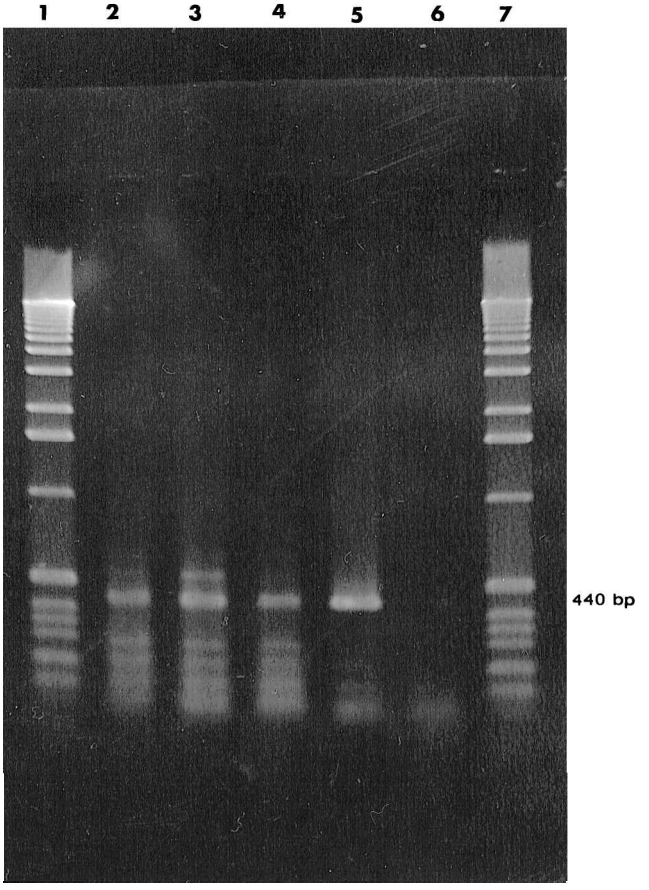

FIGURE 3. *Electrophoresis of PCR products obtained from naturally contaminated cheese samples. Lane 1, 1-kb DNA ladder (Gibco); lane 2, positive sample; lane 3, positive sample; lane 4, positive sample; lane 5, positive (purified* B. melitensis *DNA) control; lane 6, negative (no DNA) control; and lane 7, 1-kb DNA ladder (Gibco).*

collected from farms and cheese markers of southern Italy (Calabria and Puglia). They were tested for the presence of *Brucella* spp. by conventional culture methods and the direct PCR method. Also, *Brucella*-positive and -negative cheeses, made from milk of sheep, from Calabria were included as controls.

Ten grams of each cheese samples were homogenized as previously described in Laboratory samples. One milliliter of each homogenate was treated for PCR assay while the remaining part of the same homogenate was centrifuged at 8,000 × *g* for 10 min at room temperature and 10 g of pellet were resuspended in 90 ml of nutrient broth (Oxoid) containing 5% (vol/vol) inactivated (at 56°C for 30 min) horse serum (Oxoid) and 2% (vol/vol) *Brucella* selective supplement (Oxoid) and incubated for 24 h at 40°C. Then, 0.1 ml was plated in duplicate on *Brucella* medium base (Oxoid) containing 2% *Brucella* selective supplement (Oxoid) and 5% horse serum (Oxoid) at 37°C for 10 days.

RESULTS

Microbial concentration of the Rev 1 reference strain, measured by plating method, was 3×10^{10} CFU/ml. The conventional microbiological method, applied on serial dilutions (10^{-1} to 10^{-12}) of broth culture, was able to detect 30 CFU; while the PCR method was able to detect < 10 CFU.

The same results were obtained in milk laboratory samples artificially contaminated with Rev 1 strain (Fig. 1). In artificially contaminated cheese samples (Fig. 2) the PCR test was positive at the 10^{-7} dilution of cheese homogenate (detection of 10^2 CFU of Rev 1 strain).

The control cheeses (*Brucella* positive and *Brucella* negative) gave the expected results in PCR and bacteriological assays. PCR detected the presence of *Brucella* spp. in 46% of the cheese samples from farms and industry (Figs. 3 and 4), whereas no test by the cultural method was positive. In particular, 10 cheese samples (67%) made from sheep milk, 7 samples (39%) from goat milk, and 4 samples (31%) from a mixture of sheep and goat milk were positive.

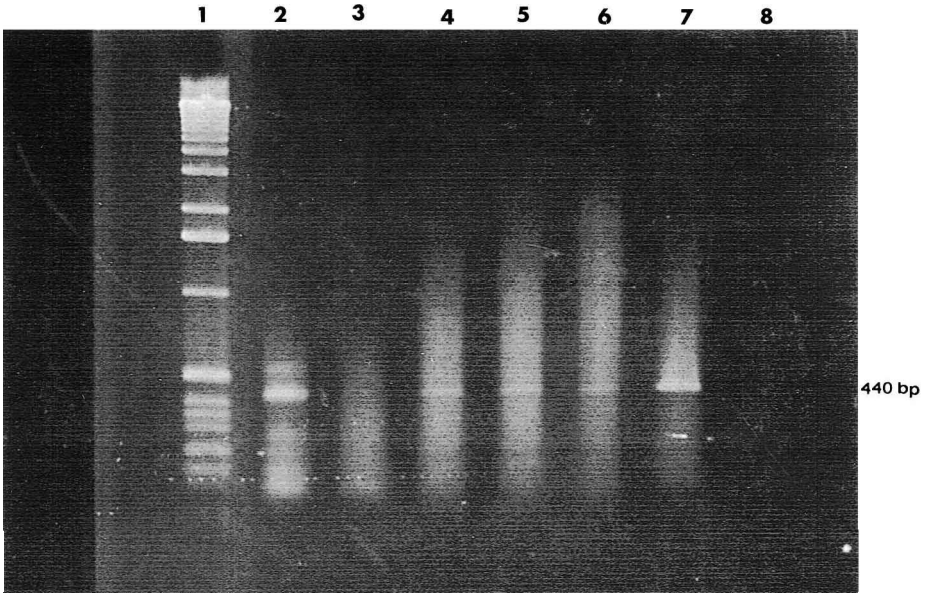

FIGURE 4. *Electrophoresis of PCR products obtained from some naturally contaminated cheese samples. Lane 1, 1-kb DNA ladder (Gibco); lane 2, positive sample; lane 3, negative sample; lane 4, positive sample; lane 5, positive sample; lane 6, positive sample; lane 7, positive (purified* B. melitensis *DNA) control; and lane 8, negative (no DNA) control.*

Failure to detect *Brucella* in culture tests may have resulted from the lack of viable cells because of low pH and/or water activity *(12)*.

DISCUSSION

The present study has confirmed that PCR is a rapid and sensitive test for direct detection and identification of *Brucella* spp. in a complex food matrix, such as milk and cheese, without employing the enrichment culture and dilution step as described by Serpe et al. *(13)*. Sensitivity and specificity of the direct method suggests it is an adequate and a reliable alternative procedure for routine microbial screening and monitoring, although the PCR test is not able to discriminate viable and nonviable cells.

The high percentage of positive samples pointed out the existence in southern Italy of a sanitary problem related to the incomplete eradication of sheep and goat brucellosis. The developed method is of great epidemiologic interest because it represents an additional test for the evaluation of the sanitary quality of milk.

The presence of *Brucella* spp. in commercial cheese is very important from the control agency's point of view because directive no. 92/46/EEC says that raw milk from goat and sheep farms with brucellosis may be used only to produce cheese with a ripening time of no less than 60 days, the time necessary to inactivate pathogenic microorganisms.

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