Molecular Detection of *Brucella* in Milk using Polymerase Chain Reaction

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Abstract

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Brucellosis is a highly contagious disease affecting a wide variety of farm animals. It is also an important zoonosis, and man is often infected following contact with infected animals or the consumption of contaminated milk and milk products. At present, mainly bacteriological and serological detection methods are used. A bacteriological method takes days to weeks to grow the organism besides its health hazard. Serological tests are faster but antigen–antibody interactions can be faulted by non-specific interactions. A method for direct detection of *Brucella melitensis* in 1 ml of milk was developed on the basis of enzymatic treatment of milk components and subsequent PCR and line probe assay (LPA). After PCR, 3×10^4 CFU/ml sensitivity was obtained by agarose gel electrophoresis and LPA. The safety and sensitivity of LPA combined with its speed suggests the potential of this technique for diagnosis of brucellosis in milk rather than the time consuming classical methods.

Key words: *Brucella melitensis*; milk; polymerase chain reaction

Brucellosis is a very widespread and economically important zoonosis, which mainly infects cattle, sheep and goats. Sanitary control of animals to be used for consumption can be ineffective, as also is the control for milk and its transportation. This accounts for an increase in the number of affected people who are either exposed to infection because they work in the milk industry or who consume unpasteurized milk and milk products. Also, the lack of sanitary education particularly in developed countries causes people to maintain potentially dangerous feeding habits. At present, mainly bacteriological and immunological detection techniques are used (ALTON *et al.* 1988). Although the bacteriological method has advantage of detecting the organism directly, it needs days to weeks. It is cumbersome and represents a risk of infection for technicians performing it as well (MAZUELOS *et al.* 1994). For rapid testing to detect brucella antibodies persisting in milk, immunological methods as milk ring test or ELISA are used. However, these tests have limited reliability as they depend on the antibody titre in the milk, size of the dairy and frequency of testing to avoid nonspecific interactions (TOM 1997; BATRA 1998). Moreover, immunized animals give false positive results when tested (THOEN *et al.* 1983; FEKETE *et al.* 1992).

Recently, safe molecular genetic techniques such as polymerase chain reaction (PCR) have been incorporated in studies regarding taxonomy and diagnosis of brucella (KLEVEZAS *et al.* 1995; MATAR *et al.* 1996). However, none of PCR assays has been applied for detection of *Brucella* spp. in food products. All *Brucella* spp. are pathogenic for human beings but *Br. melitensis* leads to the most severe and disabling symptomatic infection (WALLACH *et al.* 1994). Hence, the goal of the present study was to use PCR and LPA methods for detection of brucella in milk.

MATERIAL AND METHODS

Brucella melitensis serotype 3 (the predominant strain in Egypt) was grown on tryptic soy agar (Oxoid Ltd., London, England) supplemented with yeast for 72 h and subsequently on brain heart infusion agar (Oxoid) supplemented with glucose (15 g/l) for 24 h. *Br. melitensis* pure culture was diluted in 1 ml of raw milk to the concentration 3×10^2 CFU/ml. Then the milk was heated in water-bath for 1 h at 80°C. Tris pH 7.7 (200 µl) was added with 20 mg lipase and 20 mg phospholipase (freshly prepared). After incubation for 1 h at 37°C, 500 µl trypsin 6.3%, 150 µl EDTA and 16 µl Triton X-100 were added. After further incubation for 1 h at 37°C, the sample was centrifuged for 30 min at 12 500 rpm. Then, the pellet was washed 3 times with 1 ml H_2O and centrifuged each time for 15 min. It was resuspended in 30 µl H_2O, 2.4 µl NaOH (2M) and 2.4 µl SDS (7%) and subjected to microwave treatment (700 waH) of 4 min. DNA was extracted with a mixture of phenol ($CHCl_3$)

isoamyl alcohol in a relative concentration of 24/24/2 in the presence of 450 µl guanidiniumthiocynate. After centrifugation for 5 min at 12 500 rpm, 0.5 ml $CHCl_3$ was added to supernatant and centrifuged once more. Ethanol 100% (1 ml) was added and the pellet was washed twice with 1.6 ml 80% ethanol. The final pellet was dissolved in 40 µl H_2O. For PCR reaction 5 µl was used as the template. PCR was performed as described by HERMAN and RIDDER (1992). BRU-P_5 Bio and BRU-P_8-Bio were used as primers (Table 1). Agar gel electrophoresis and LPA analyzed the PCR products. LPA was performed onto nitrocellulose strips as described by STUYVER *et al.* (1993) and RIJPENS *et al.* (1995). All reagents were provided in the Inno-LPA Kit (Innogenetic, Belgium).

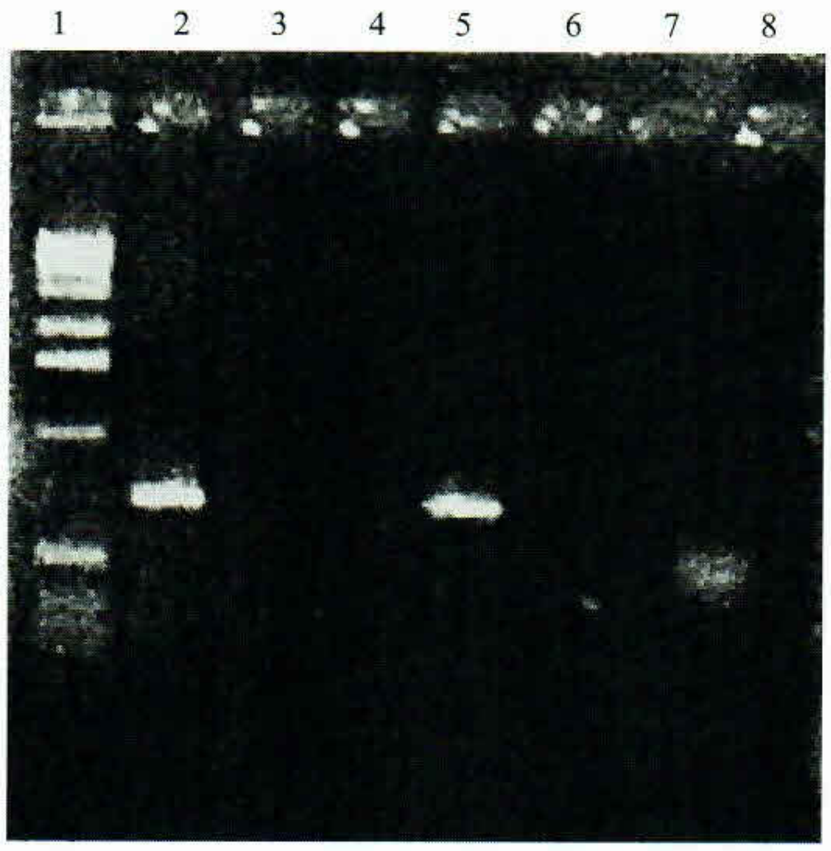
Table 1. Nucleotide sequences of primer set

Primer	Sequence (5′ to 3′)
BRU-P_5-Bio	TCGAGAATTGGAAAGAGGTC
BRU-P_8-Bio	GCATAATGCGGCTTTAAGA

RESULTS AND DISCUSSION

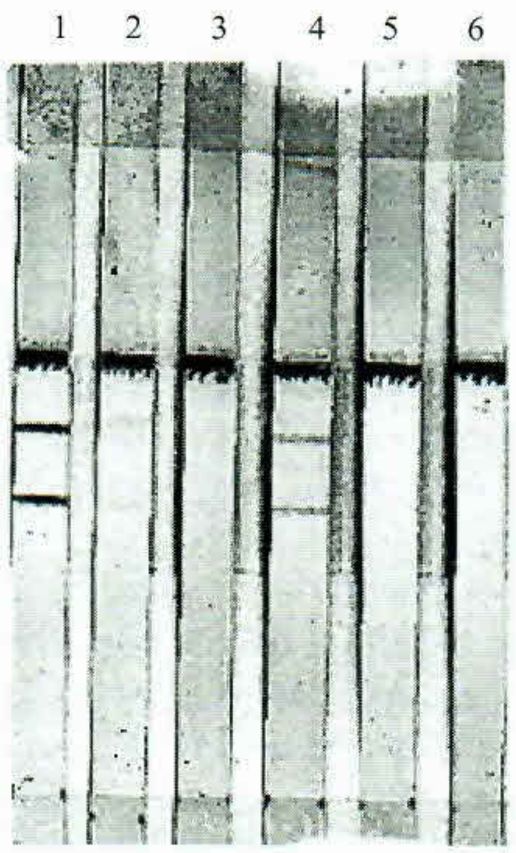
Brucella melitensis is a zoonosis which occurs in rural areas where sheep and goats are raised. The organism is highly pathogenic and readily affects cattle and other animals (RIBEIRO *et al.* 1990). Human infection is usually associated with the consumption of raw milk and milk derivatives (YOUNG 1983). Therefore, fast and accurate diagnosis is very important for a positive outcome of control programs. PCR is a promising alternative for the problematic culture and identification of brucella by conventional techniques (HERMAN & RIDDER 1992). As an alternative to chemical extraction of milk components used by HERMAN *et al.* (1995) for direct detection of *Listeria* spp. in 25 ml of milk, an enzymatic approach was followed. Unlike *Listeria* spp., brucella has very high affinity for the fat phase of milk so that after chemical extraction brucella cells adhere to interphase. The use of lipase, phospholipase and trypsin ensured efficient pelleting of brucella cells during centrifugation. Addition of EDTA with trypsin reduced the content of milk proteins in the pellet. An extra phenol extraction was necessary to remove the inhibitory effect of the last remaining proteins with this sample preparation procedure, the sensitivity of LPA for detection of brucella in milk was investigated. When PCR was carried out with primer set, the sensitivity of 3×10^4 CFU/ml was achieved for both agarose gel electrophoresis (Fig. 1) and LPA (Fig. 2). Consequently, it could be concluded that the sample preparation protocol described here, followed by PCR and subsequent LPA is a very useful tool for detection of brucella in milk. Moreover, LPA could be a good alternative for agarose gel electrophoresis because it is not necessary to use ethidium bromide or UV transilluminator which must be handled carefully for their health hazard.

At present, the reaction is fairly expensive to perform but it is ultimately faster than bacteriological tests and is more accurate than immunologic methods because it directly detects the presence of the organism. Comparing to the high cost of travel and handling the animals for the collection of blood samples, as well as the inconvenience and disruption of normal management practices, LPA is considered a desirable method for detecting brucella in milk.



Lanes: (1) molecular weight markers; (2) 3×10^4 CFU; (3) 3×10^3 CFU; (4) 3×10^2 CFU; (5) positive control PCR; (6) negative control PCR; (7) uncontaminated milk

Fig. 1. Detection of *Brucella melitensis* in 1 ml of milk by PCR on agrose gel



Lanes: (1) 3×10^4 CFU; (2) 3×10^3 CFU; (3) 3×10^2 CFU; (4) positive control PCR; (5) negative control PCR; (6) uncontaminated milk

Fig. 2. Detection of *Brucella melitensis* in 1 ml of milk by PCR on LPA stripes

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Souhrn

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Brucelosa je závažná, vysoce nakažlivá choroba postihující různé druhy zvířat chovaných na farmách, která je kontaktem s infikovanými zvířaty nebo konzumací kontaminovaného mléka a mléčných výrobků přenosná na člověka. K její detekci jsou využívány především bakteriologické a sérologické metody. Při bakteriologickém stanovení trvá kultivace organismů několik dní až týdnů a metoda může být nebezpečná pro zdraví člověka. Sérologické testy jsou rychlejší, ale vzájemné působení antigen–protilátka může být negativně ovlivněno nespecifickými interakcemi. Na bázi enzymatického ošetření mléčných komponentů s následující polymerasovou řetězovou reakcí a LPA byla vyvinuta metoda přímé detekce organismu *Brucella melitensis* v 1 ml mléka. Po provedení polymerasové řetězové reakce elektoforesou s agarosovým gelem a LPA jsme získali citlivost 3×10^4 CFU na 1 ml. Bezpečnost a citlivost LPA a rychlost stanovení zvýhodňují tuto techniku při diagnostice brucelosy v mléce před klasickými, časově náročnými metodami.

Klíčová slova: *Brucella melitensis*; mléko; polymerasová řetězová reakce

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