Diagnosis of Brucellosis in Dairy Animals Using Nested Polymerase Chain Reaction

K.A. Abd El-Razik, E.M. Ismail, H.M. Youssef and M.A. Hashad
1National Research Center, Dokki, Giza, Egypt
2Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

Abstract: In this study, a total number of 92 milk samples collected from obligatory slaughtered 32 cattle, 30 buffaloes and 30 sheep suspected for brucellosis in different governorates of Egypt were tested using the nested Polymerase Chain Reaction (nPCR). Thirty eight out of the 92 milk samples (41.3%) gave the expected product. The sensitivity of nested PCR using vaccinal strains S19 and Rev 1 vaccine, ranged between as high as 10-10^6 cfu mL^-1. The nesting of PCR resulted in rise of PCR sensitivity; by about 10 folds. Bacteriological studies yielded 16 (17.3%) Brucella isolates recovered from milk (6 from cattle, 4 from buffaloes and 6 from sheep), they were typed as Brucella melitensis biovar 3. It could be concluded that using nested PCR as confirmatory test for detection of susceptible dairy animals, is of great value to avoid condemnation of false positive reactors, which reflects economically on animal resources.

Keywords: Brucella, diagnosis, PCR, milk, isolation, dairy animals

INTRODUCTION

Brucellosis is economically considered as one of the most important diseases affecting cattle leading to great losses in calves through abortion, reduction in milk yield and decreased fertility.

Unfortunately, colonization of the udder is frequent and the excretion of the organism in the milk may be frequently led to epidemics of brucellosis in people working with cattle or drinking their milk leading to undulant fever (Alton, 1990; Amin et al., 1995).

At present, diagnosis of brucellosis in dairy cattle involves the isolation of Brucella from milk samples or the detection of Brucella antibodies in serum or milk. However, these methods are not actually satisfactory. Bacteriological isolation is a time-consuming procedure and handling the microorganisms is hazardous. Serological methods are not conclusive, because not all infected animals produce significant levels of antibodies and cross-reactions with other bacteria can give false-positive results (Alton et al., 1988).

Molecular techniques offer many advantages over phenotypic methods, because they are more sensitive, reproducible and applicable on a wide range of microorganisms with only slight modifications (Mercier et al., 1996).

Polymerase Chain Reaction (PCR) is a new tool for molecular biology, it is so sensitive that a single DNA molecule has been amplified and single copy-genes are routinely extracted out of complex mixtures of genomic sequences then visualized as distinct bands on agarose gels (Perring, 1991).

PCR provides a promising option for the diagnosis of brucellosis. A potentially useful method has been used alone or in combination with labelled probes for the detection of Brucella sp. from isolated bacteria or highly contaminated aborted tissues (Fekete et al., 1990a; Fekete et al., 1992; Herman and Herman, 1992; Kulakov et al., 1992; Ghazi et al., 2006; Abd-El-Razik et al., 2006, 2007 a, b).

Corresponding Author: K.A. Abd El-Razik, National Research Center, Dokki, Giza, Egypt
Therefore, the aim of the present study was to investigate the application of nested Polymerase Chain Reaction (nPCR) in comparison to bacteriological examination for detection of *Brucella* in milk samples of obligatory slaughtered cattle, buffaloes and sheep suspected for Brucellosis. Moreover, detection of the sensitivity of this nested PCR technique using reference and vaccinal *Brucella* strains.

**MATERIALS AND METHODS**

**Milk Samples**

Ninety two milk samples (each sample is a mixed sample from all quarters) were collected from different obligatory slaughtered, *Brucella* sero-active lactating cattle, buffaloes and sheep species in different governorates (serologically positive in both Rose Bengal Plate and Tube Agglutination Tests by the veterinary authority).

**Brucella Strains**

Six reference *Brucella* strains were used in this study for the DNA experiments:

- *Brucella abortus* virulent strain 544 (biovar 1) and vaccinal strains (S19 and RB51).
- *Brucella melitensis* virulent strains (biovar 3 and M16) and vaccinal strain Rev 1.

**Bacteriological Examination**

It was performed according to the recommendation of the FAO/WHO Expert committee on brucellosis (Alton *et al.*, 1988).

Media used for Isolation of *Brucella* microorganisms from milk (Alton *et al.*, 1988) were Bacto-*Brucella* agar (Difco Laboratories, Detroit, USA), Selective medium for isolation of *Brucella* and a freeze-dried vial of selective antibiotic supplement of Farrel (1974) for the isolation of *Brucella* sp. (Oxoid Ltd., Basingstoke, Hampshire RG 24 OPW, UK).

**Chemicals and Reagents Used for Extracting Genomic DNA of Brucella**

Four primers were used. These primers were called BRU-P5, BRU-P6, BRU-P7 and BRU-P8 as shown in Table 1. These primers were selected from the 16S-23S rRNA spacer regions of *Br. abortus*, *Br. melitensis* and *Br. suis* according to Ripens *et al.* (1996).

**Extraction of Genomic DNA from Cultivated Brucella Strains**

Two different methods were applied for the extraction of the DNA from different *Brucella* cultures as:

Method 1: (Cetinkaya *et al.*, 1990) and Method 2: (Husseinin, 1993).

**Extraction of DNA from Milk**

Two different methods were applied for extraction of DNA from field milk samples collected from cattle, buffaloes and sheep.

Method 1: (Modification of the method described by Felste *et al.* (1992) and Method 2: (Romero and Lopez-Goni, 1999). Nested Polymerase Chain Reaction according to

**Table 1: Nucleotide sequence of the Brucella specific primers annealing sites**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5</td>
<td>TGAGAAATTGAGAAGGATGC</td>
<td><em>Br. abortus</em> 16S-23S rRNA spacer, bp 1-22</td>
</tr>
<tr>
<td>P6</td>
<td>AAGAGCTGAGTTATCCCG</td>
<td><em>Br. abortus</em> 16S-23S rRNA spacer, bp 20-40</td>
</tr>
<tr>
<td>P7</td>
<td>CGAGCATTTGGACTGGAA</td>
<td><em>Br. abortus</em> 16S-23S rRNA spacer, bp 682-699</td>
</tr>
<tr>
<td>P8</td>
<td>GGATAATGGCTTTAACA</td>
<td><em>Br. abortus</em> 16S-23S rRNA spacer, bp 717-735</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Bacteriological examination of 92 milk samples resulted in recovery of 16 Brucella isolates (6 from cattle, 4 from buffaloes and 6 from sheep).

Nested Polymerase Chain Reaction (nPCR) revealed specific products in all these Brucella reference and vaccinal genomic DNA.

Sensitivity of the nested PCR technique to detect Brucella microorganisms was performed in terms of the number of Brucella cfu mL⁻¹ using vaccinal strains (Br. abortus strain S19 and Br. melitensis Rev. 1 vaccines) according to Alton et al. (1988).

Figure 3 shows the sensitivity of nested PCR on Br. abortus S19 experimentally infected milk where as low as 10⁴ cfu mL⁻¹ in the first PCR and 10⁵ cfu mL⁻¹ in the second PCR were successfully amplified using the PCR carried out in the study.

Figure 4 shows the sensitivity of nested PCR on Br. melitensis Rev. 1 experimentally infected milk (10⁵ cfu mL⁻¹ in the 1st PCR and 10 cfu mL⁻¹ in the second PCR).

Figure 5 Shows the PCR amplification products of DNA extracted from positive (38 out of 92)

![Fig. 1: Agarose gel electrophoresis of the first PCR products following amplification of DNA extracted from different Brucella reference strains. Lane (1): Br. abortus S44, Lane (2): Br. abortus S19, Lane (3): Br. abortus RB51, Lane (4): Br. melitensis 3, Lane (5): Br. melitensis 16 M, Lane (6): DNA ladder and Lane (7) Br. melitensis Rev. 1.](image1)

![Fig. 2: Agarose gel electrophoresis of the second PCR products following amplification of DNA extracted from different Brucella reference strain cultures. Lane (1): Br. abortus S44, Lane (2): Br. abortus S19, Lane (3): Br. abortus RB51, Lane (4): Br. melitensis 3, Lane (5): Br. melitensis 16 M and Lane (6): Br. melitensis Rev. 1. (7): DNA ladder.](image2)
field milk samples (1st PCR), while Fig. 6 shows the second PCR products of the same positive milk samples (38 out of 92).

The development of the PCR was directed towards the detection of those pathogens which conventional diagnostic techniques are either too insensitive, too slow, or can not discriminate prognostic or therapeutically important subgroups, e.g., Mycobacteria, Brucella, Mycoplasma pneumoniae (Collins et al., 1993; Fafi et al., 1995; Cetinkaya et al., 1996).

Testing milk samples have some advantages over testing serum samples as sampling is not invasive, therefore, the accidental transmission of diseases by needle and reduction of production due to stress can be reduced. Moreover, sample condition is not critical and it can be stored frozen or refrigerated without preservative (Vancini et al., 1998).

Fig. 3: Sensitivity of nested PCR on Br. abortus S19 experimentally-infected milk

Fig. 4: Sensitivity of nested PCR on Br. melitensis Rev-1 experimentally-infected milk, D-Application of nested PCR assay to detect Brucella in milk samples from obligatory slaughtered cattle, buffaloes and sheep suspected for brucellosis

Fig. 5: Agarose gel electrophoresis of PCR amplification products of DNA extracted from field milk samples (1st PCR) using Brucella specific primers

Fig. 6: Agarose gel electrophoresis of PCR amplification products of the 2nd PCR products from 1st ten positive samples using Brucella specific primers
Although both PCR and bacterial examination depends on the presence of the microorganisms in the milk, yet our results differ where PCR was higher (41.3%) than the bacterial isolation (17.3%). The negative culture results may be attributed to the presence of microbial contaminants in the sample, loss of viability of the organism before culturing as a result of freezing and thawing of the sample or the inhibition of some Br. melitensis strains in the selective medium (Blasco, 1992). The presence of these factors does not affect PCR detection of Brucella DNA.

In this study, Brucella genomic DNA from different reference strains were extracted by two methods according to Cetinkaya et al. (1990) and Hussein (1993), respectively. The results of agarose gel electrophoresis showed the simplicity and efficiency of the 1st method in preparing purified and highly concentrated DNA to be used for PCR amplification.

PCR amplification results shown in Fig. 1 and 2 revealed no detectable differences between tested Br. abortus and Br. melitensis, in either sensitivity or size of amplification products. Similar observations were recorded by Fekete et al. (1990 a, b), Baily et al. (1992), Romero et al. (1995 a, b), Leal-Klevezas et al. (1995) and Rijpens et al. (1996).

The second PCR enhanced the intensities of the bands; this may be due to the reduced effect of inhibitory substances found in the first PCR (Matter et al., 1996).

Genetic analysis of Brucella species showed high DNA homology percent estimated as more than 95% between most strains (Verger et al., 1985). Moreover, the 16S-23S rRNA spacer region of Br. abortus (800 bp) shows a very high (79%) homology among the three species examined.

The second phase of this experiment was performed to investigate the sensitivity of nested PCR in form of cfu mL^-1 milk.

Figure 3 shows the sensitivity of nested PCR on Br. abortus S19 experimentally infected milk where as low as 10^5 cfu mL^-1 in the 1st PCR and 10^4 cfu mL^-1 in the second PCR were evident. While Fig. 4 shows the sensitivity of nested PCR on Br. melitensis Rev. 1 experimentally infected milk as 10^5 cfu mL^-1 in the 1st PCR and 10 cfu mL^-1 in the second PCR. This difference in sensitivity limit between S19 and Rev. 1 vaccines might be due to differences in the effectiveness of the DNA extraction protocol (Romero et al., 1995a). This proves that the sensitivity could be increased approximately 10 folds by using nested PCR. The method of milk DNA extraction improves the sensitivity of PCR instead of 2.8×10^6 cfu mL^-1 after a single PCR and 2.8×10^5 cfu mL^-1 after nested PCR as applied by Rijpens et al. (1996). This was agreed by Cetinkaya et al. (1990) 200 bacteria mL^-1, Baily et al. (1992) 60 fg of DNA, Leal-Klevezas et al. (1995) 10 cells mL^-1 milk, Romero et al. (1995a) 20 cells mL^-1, Romero et al. (1995b) 170 cfu mL^-1 for Brucella abortus 2308 and 1,700 cfu mL^-1 for Brucella melitensis 115 and Romero and Lopez-Goni (1999) 5 cfu mL^-1 milk.

In the present study, nested Polymerase Chain Reaction (PCR) applied on milk of 92 seropositive animals intended for obligatory slaughter due to brucellosis was more sensitive and revealed 41.3% as shown in Table 2 which was higher than Brucella isolation (17.3%). Similar observations were recorded by Ghazi et al. (2006) and Abd EL-Razik et al. (2006, 2007a, b).

The DNA was extracted by two different methods according to Fekete et al. (1992) and Romero and Lopez-Goni (1999), respectively. The two methods were applied on milk fat. Unlike Listeria sp., Brucella sp. has a very 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 pelting of the upper cream phase is a frequently applied classical bacteriological method for detection of Brucella sp. in milk (Rijpens et al., 1996).

These methods of DNA extraction depend upon that the Brucella cell wall is highly resistant to non-ionic detergents, EDTA and Tris, therefore the use of high concentrations of SDS, proteinase K and high temperatures of incubation was necessary for the efficient extraction of Brucella DNA (Oriyon and Berman, 1982).
The second method was proved simpler and efficient due to the appearance of sharp bands of highly concentrated DNA of low protein contamination and low RNA content.

Figure 5 demonstrates the electrophoretic picture of product of DNA amplification of Brucella DNA using P5 + P8 primers (725 bp) while, Fig. 6 shows, the product of the 2nd PCR amplification of the 1st PCR product using P6 + P7 primers (677 bp).

From these investigations, it could be concluded that using nested PCR as confirmatory test for detection of susceptible dairy animals, is of great value to avoid condemnation of false positive reactors, which reflects economically on animal resources. In addition, laboratory identification and typing of Brucella species based on biochemical and serological tests is a time consuming process. Selection of single set or mixture of primers from hyper variable regions of Brucella genome to allow rapid differentiation between Brucella species will be a good point for following research studies. Further investigations on the Restriction Fragment Length Polymorphism (RFLP) and southern blot hybridization technique to confirm Brucella specific identity of amplified products are needed.

REFERENCES


