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Diagnosis of Brucellosis in Dairy Animals Using Nested Polymerase Chain Reaction

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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 Riv I vaccine, ranged between as high as $10\text{-}10^2$ 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.

Key words: *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 (Persing, 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).

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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., Basingstokes, 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 Rijpens *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 Fekete *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

Primer	Sequence (5′ to 3′)	Location
P5	TCGAGAATTGGAAAGAGGTC	*Br. abortus* 16S-23S rRNA spacer, bp 1-029
P6	AAGAGCTCGATTTATCCG	*Br. abortus* 16S-23S rRNA spacer, bp 22-40
P7	CGAGCATTTGCACTCGAA	*Br. abortus* 16S-23S rRNA spacer, bp 682-699
P8	GGATAATGCGGCTTAACA	*Br. abortus* 16S-23S rRNA spacer, bp 717-735

Rijpens *et al*. (1996). Identification of the sensitivity of the nested PCR technique according to Alton *et al*. (1988).

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 10^3 cfu mL^{-1} in the first PCR and 10^2 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 (10^2 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)

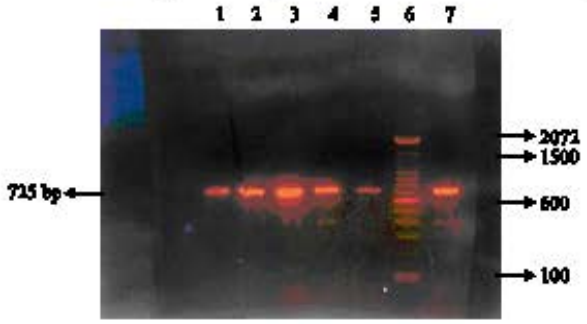

Fig. 1: Agarose gel electrophoresis of the first PCR products following amplification of DNA extracted from different *Brucella* reference strains, Lane (1): *Br. abortus* 544, Lane (2): *Br. abortus* S19, Lane (3): *Br. abortus* RB51, Lane (4): *Br. melitensis* 3, Lane (5): *Br. melitensis* 16 M, Lane (6): DNA ladder and Lane (7) *Br. melitensis* Rev. 1

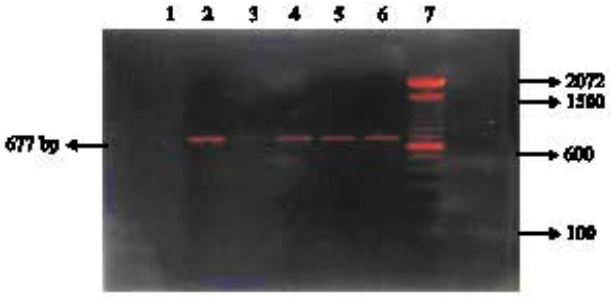

Fig. 2: Agarose gel electrophoresis of the second PCR products following amplification of DNA extracted from different *Brucella* reference strain cultures, Lane (1): *Br. abortus* 544, Lane (2): *Br. abortus* S19, Lane (3): *Br. abortus* RB51, Lane (4): *Br. melitensis* 3, Lane (5): *Br. melitensis* 16 M and Lane (6): *Br. melitensis* Rev. 1. (7): DNA ladder

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 pneumonis* (Collins *et al*., 1993; Rafi *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).

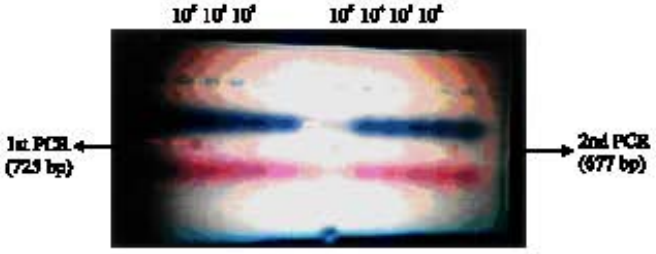

Fig. 3: Sensitivity of nested PCR on *Br. abortus* S19 experimentally-infected milk

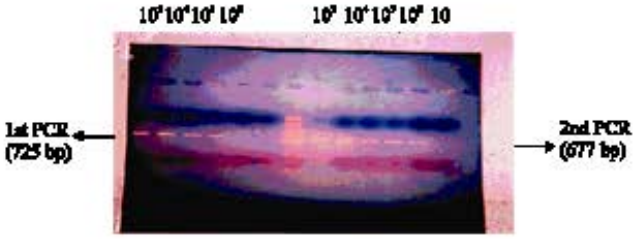

Fig. 4: Sensitivity of nested PCR on *Br. melitensis* Rev-1 experimentally infected milk, D-Application of nested PCR assay to detect *Brucella* in milk samples from obligatory slaughtered cattle, buffaloes and sheep suspected for brucellosis

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

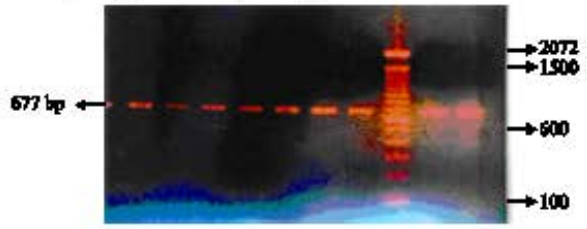

Fig. 6: Agarose gel electrophoresis of PCR amplification products of the 2nd PCR products from 1st ten positive samples using *Brucella* specific primers

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 Husseinin (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 10^3 cfu mL^{-1} in the 1st PCR and 10^2 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 10^2 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 2.8×10^5 cfu mL^{-1} after a single PCR and 2.8×10^2 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 plating 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).

Table 2: Results of PCR on milk samples of obligatory slaughtered cattle, buffaloes and sheep suspected for brucellosis. N.B: DNA extraction was according to the second method (Romero and Lopez-Goni, 1999)

Species	Test	Milk PCR
Cattle (32)	No.	13.0
	(%)	40.6
Buffaloes (30)	No.	9.0
	(%)	30.0
Sheep (30)	No.	16.0
	(%)	53.3
Total (92)	No.	38.0
	(%)	41.3

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 electropheretic 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.

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