Prevalence Study of *Brucella melitensis* and *Brucella abortus* in Cow's Milk using Dot Enzyme Linked Immuno Sorbent Assay and Duplex Polymerase Chain Reaction

Farhad Safarpoor Dehkordi*, Yousef Valizadeh, Tannaz Alipour Birgani and Khashayar Ghasemi Dehkordi

Young Researchers and Elites Club, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

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Ingestion of raw infected milk and dairy products is the most important way for human brucellosis. This study was carried out in order to investigate the prevalence of *Brucella* spp., *Brucella abortus* and *Brucella melitensis* in cow's milk using culture, dot-ELISA and duplex PCR. All of the 150 milk samples were cultured for *Brucella* spp. and the positive results have been evaluated for presences of *Brucella abortus* and *Brucella melitensis* using dot-ELISA and duplex PCR assays. Totally, 12% of milk samples were positive for *Brucella* spp. using culture. The dot-Elisa assay indicated that 12% and 2% of milk samples were positive for *Brucella abortus* and *Brucella melitensis*, respectively. Novel duplex PCR showed that 14%, 4% and 2% of milk samples were positive for *Brucella abortus*, *Brucella melitensis* and both bacteria. Isfahan had the highest incidence of *Brucella* spp. (17.5%), while Chaharmahal Va Bakhtiari had the lowest incidence of *Brucella* spp. (8%). There were significant differences about $P < 0.05$ for incidence of Brucella spp. between Isfahan with Chaharmahal Va Bakhtiari provinces. Our study showed that milk inspection should be performed in order to diminish the distribution of brucellosis in human populations.

Key words: Brucellosis, Milk samples, Culture, Dot-ELISA, Duplex PCR.

Brucellosis is a highly contagious, zoonotic, and economically important bacterial disease of human and animals caused by Gram-negative, aerobic and facultative intracellular bacteria of the genus *Brucella*. Disease is an important public health problem in many parts of the world, such as the Mediterranean littoral, the Middle East and parts of Latin America[1] where it causes loss in milk production and low fertility rates in animals. Fever, malaise and myalgia may later develop into a chronic illness affecting various organs and tissues in human. Human are commonly infected through ingestion of raw milk, cheese and meat or through direct contact with infected animals. Some serological tests, like dot-enzyme linked immune sorbent assay (dot-ELISA) can reduce the exposure of cross-reactions of *Brucella* spp. with other pathogens. More recently, molecular techniques have been developed for *Brucella* spp. detection, such as various types of Polymerase Chain Reaction (PCR) that are simpler, faster and less hazardous[2].

The two-fold purpose of the current study were to determine the prevalence rate of *B. abortus* and *B. melitensis* in cow's milk samples in Iran and evaluate the accuracy of dot-ELISA and duplex PCR assays for detection of *B. abortus* and *B. melitensis*.

MATERIALS AND METHODS

Samples and Brucella identification

A total 150 milk samples were randomly collected from cows of 3 major provinces located in south-west part of Iran (Table 2). Samples were

* To whom all correspondence should be addressed.
Tel.: +98 9365819491; Fax: 98 3813381892;
E-mail: Dr.Farhads@yahoo.com

collected from 21 randomly selected dairy herds at spring of 2011. The animals selected for this study were clinically healthy and the milk samples showed normal physical characteristics. Samples were collected under sterile hygienic conditions and were immediately transported at 4°C to laboratory in a cooler with ice packs. Samples were made on blood agar base (Oxoid) supplemented with 5% defibrinated sheep erythrocytes and antibiotics (Vancomycin, Nalidixic acid, bacitracin, nystatin and cyclohexamide at the doose recommended in OIE manual)[3]. Cultures were incubated for 10 days with and without 5% CO_2 at 37 °C. Bacterial isolated were identified according to the conventional procedures[4].

Dot-ELISA

The dot-ELISA was performed based on the method that was previously reported[5]. 20 µl of each milk samples and control were applied to nitrocellulose membrane (Amersham). The membrane was blocked in 5% skim milk in PBS for 45 min and was further washed 3 times, 5 min each, with PBS-T (PBS containing 0.05% Tween 20) was placed in 1:100 dilution of anti-*brucella* serum in PBS-T and incubated at room temperature for 1 hr. the membrane were then incubated in conjugate rabbit anti-sheep peroxidase (sigma) diluted 1:1000 in PBS-T for 1 hr. the same washing procedure was repeated after incubation with the anti-sheep peroxidase conjugate, Followed by reaction with a chromogen substance solution consisting of 30 µl of 30% H_2O_2 in 50 ml of PBS mixed with 30mg of 4-chloro-1-naphtol (sigma) in 10 ml of cold methanol. The results were determined by observation of violet-stained spots on the membrane. The sensitivity and specificity of 80% and 85% were determined by manufacturer for ELISA kit, respectively.

DNA extraction, Primer design and duplex PCR assay

For *Brucella* DNA detection, the assay that was introduced by Consuelo Vanegas *et al*., (2009)[6] was used. Purification of DNA was achieved using a genomic DNA purification kit (Fermentas GmbH, St. Leon-Rot, Germany), and the total DNA was measured at an optical density of 260 nm according to the method described by Sambrook and Russell (2001)[7]. In the present study, the duplex PCR assay has been designed by the authors. This duplex PCR to screen the *B. abortus* and *B. melitensis* detected the DNA sequence of the gene coding the *B. abortus* UV endonuclease (uvrA) gene target (GenBank: L10843.1) and the DNA sequence of the gene coding the *B. melitensis* 16S ribosomal RNA (16S rRNA) gene (GenBank: L26166.1) in the GenBank database of the National Center For Biotechnology Information (NCBI).In order to design primers, recorded sequences of the uvrA and 16S rRNA targets have been gotten from the NCBI. The CLS sequence viewer software (Version 6/4) has been used for alignments of the uvrA and 16S rRNA targets. Forward and reverse primers have been designed based on the protected are in these sequences. Thermodynamic properties of designed primers were studied using the Gene Runner software (Version 3.05). In order to ensure the specificity of designed primers, the Basic Logical Alignment Search Tool (BLAST) service, has been used. The forward and reverse primers to screen the *B. abortus* and *B. melitensis* have been shown in Table 1.

The PCR reaction was performed in a total volume of 50 µl containing 5 µL PCR buffer 10X, 200 µM dNTP (Fermentas), 2 mM $Mgcl_2$, 0.5 µM of each primers F & R, 1.5 U Taq DNA polymerase (Fermentas) and 2 µL DNA template. Reactions were initiated at 94°C for 5 min, followed by 32 cycles of 94°C for 50 sec, 57°C for 1 min, 72°C for 1 min and a final elongation step at 72°C for 5 min, with a final hold at 4°C in a DNA thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). A negative control (sterile water), and a positive control DNA from *B. abortus* strain S19 (S19 vaccine strain) (Razi Institute, Karaj, Iran), were included in each amplification run.

Gel electrophoresis

The PCR-amplified products (UvrA: 892 bp and 16S rRNA: 687 bp) were examined by electrophoresis (120 V/208 mA) in a 1.5% agarose gel, stained with a solution of ethidium bromide (0.004 µg/ml) and examined under UV illumination. *Brucella* DNA extracted from the S19 vaccine strain was used as positive control.

Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (SPSS Inc., Chicago, IL, USA) and analyzed using SPSS 18.0 statistical software (Microsoft Corp., Redmond, WA, USA) chi-square test analysis was performed and differences were considered significant at values of $P < 0.05$.

RESULTS

Culture showed that 18 out of 150 milk samples (12%) were positive for *Brucella* spp. (Table 1). The positive culture results have been studied for presences of *B. melitensis* and *B. abortus* using dot-ELISA and duplex PCR assays. Totally, 13 out of 150 (12%) and 3 out of 150 (2%) milk samples were positive for *B. abortus* and *B. melitensis* using the dot-ELISA technique, respectively (Table 2). The results of the duplex PCR has been confirmed the results of dot-ELISA. As far as the duplex PCR assay had been done, 21 out of 150 (14%) and 6 out of 150 (4%) milk samples were positive for *B. abortus* and *B. melitensis*. The results of the duplex PCR showed that 3 out of 150 (2%) milk samples were positive for presences of both *B. abortus* and *B. melitensis*.

Table 1. Oligonucleotid primers for *B. abortus* and *B. melitensis* amplification

Bacteria	Gene target	Sequence (5'-3')	Size of product (bp)	Genbank No
Brucella abortus	uvrA	F: CTTCCAGGATTGAGCGCGTAR: CGTTCCTTTCCGGTCAGACG	892	L10843.1
Brucella melitensis	16S rRNA	F: GTAACCGGAGAAGAAGCCCCR: TATCACCGGCAGTCCCCTTA	687	L26166.1

Table 2. Distribution of *Brucella* spp., *Brucella abortus* and *Brucella melitensis* in cows milk samples using Culture and dot-ELISA assays

Provinces	No. samples	*Brucella* spp. by Culture (%)	Dot-ELISA (%)	
			B. abortus	*B. melitensis*
Isfahan	40	7 (17.5)	6 (15)	2 (5)
Tehran	35	5 (14.28)	5 (14.28)	1 (2.85)
Chaharmahal Va Bakhtiari	75	6 (8)	7 (9.33)	-
Total	150	18 (12)	18 (12)	3 (2)

Table 3. Distribution of *Brucella abortus* and *Brucella melitensis* in cows milk samples using duplex PCR assays

Provinces	No. samples	*Brucella* spp. by Culture (%)	Duplex PCR		
			B. abortus	*B. melitensis*	Both bacteria
Isfahan	40	7 (17.5)	8 (20)	3 (7.5)	2 (5)
Tehran	35	5 (14.28)	6 (17.14)	2 (5.71)	1 (2.85)
Chaharmahal Va Bakhtiari	75	6 (8)	7 (9.33)	1 (1.33)	-
Total	150	18 (12)	21 (14)	6 (4)	3 (2)

Geographical distribution of *Brucella* spp. in various provinces showed that Isfahan had the highest incidence of *Brucella* spp. (17.5%), while Chaharmahal Va Bakhtiari had the lowest incidence of *Brucella* spp. (8%) (Table 2). Statistical analyses were significant for incidence of *Brucella* spp. which were isolated from the milk samples of Isfahan province with Chaharmahal Va Bakhtiari province ($P < 0.05$). There were no significant differences between the ability of dot-ELISA and duplex PCR for detection of *B. abortus* and *B. melitensis* in milk samples.

DISCUSSION

The moderate prevalence of *B. melitensis* in cow's milk samples in Isfahan (7.5%), Tehran (5.71%) and even Chaharmahal Va Bakhtiari (1.33%)

province maybe showed that the cows of these province, maintained in close association with infected sheep and goats. Our results showed that *B. abortus* had more prevalence rate than *B. melitensis* in cow's milk samples. In this present study we used from milk samples for detection of *Brucella* spp. because detection of bacteria in milk samples has some advantages since milk samples can be obtained cheaply and more frequently than blood samples and is often available at dairies. In addition to this, the milk and dairy products are the most frequent sources of infection of brucellosis to human. Previously report from Iran (East of Iran) indicate that the prevalence rate of brucellosis during 2002-2006 in Human was 37/100,000, in sheep and goat was 340/10,000 and in cattle was 56/10,000 (8). Following control programs for Brucellosis in Iran, the prevalence of *Brucella* spp. in milk decrease from 25.21% (1632 positive from 6472) in 1990 (9) to 12% in 2013 (this study). The prevalence of total *B. abortus* observed in cow milk samples in this study (14%) was higher than Egypt (5.44%) (10) but our results is lower than Punjab (India) (20.67%)[11], kenya (77.5%)[12] and Sokoto State (25.25%)[13]. These high differences in prevalence of *Brucella* spp. in milk maybe due the facts that type of samples (meat, milk, vegetable and clinical samples), number of samples, methods of sampling, method of experiment, geographical area and even climate of area which samples were collected are different in each investigation. As far as we know, bacteria have the higher growth and surveillance in warm condition. After analyzing the average temperatures of these 3 study area (12 °C for Chaharmahal Va Bakhtiari, 29 °C for Isfahan, 19 °C for Tehran), it was recognized that the prevalence rate of *Brucella* spp. in each area is related with their average temperatures. Therefore, the highest levels of milk inspection should be performed on the warm regions.

CONCLUSION

The dot-ELISA which was presented in this study can be adapted for detection of *B. abortus* and *B. melitensis* in milk samples. The results showed that dot-ELISA is rapid diagnostic test and the results are obtained within three hrs. The test is easy and can be performed in the least equipped laboratories. High rate of bacterial milk contamination showed that may be some food safety and quality standards (good agricultural practices (GAPs), good manufacturing practices (GMPs), and the hazard analysis and critical control point (HACCP) system need to be applied and performed in most of milk halls to control growth of *Brucella* during distribution and storage periods.

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