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Brucella spp. detection in dairy products using Nested and Hemi Nested PCR techniques

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ABSTRACT

Brucella spp. is a gram negative coco bacillus which has different hosts including human, cow, goat, sheep and horse. While infection with Brucella in endemic region still remains, rapid and simple detection of Brucella seems necessary to prevent and treatment of the disease. The aim of this study is Detection of Brucella spp. in dairy products using Nested and Hemi Nested PCR techniques and comparing the techniques' sensitivity and specificity. In this study 57 raw cow milk samples, 34 pasteurized cow milk samples, 28 pasteurized cheese samples, 23 traditional cheese samples, 33 raw sheep milk samples, 33 raw goat milk samples, were collected from Tehran province. DNA was extracted by using DNP kit from samples. After optimization of PCR for the two techniques of Hemi Nested and Nested PCR sensitivity and specificity of the two techniques were evaluated. In Nested PCR, among 57 samples of raw cow milk, 19 cases were PCR positive. In 34 pasteurized milk samples 10 cases were PCR positive, from 28 pasteurized cheese samples only 8 cases, from 23 traditional cheese DNA samples 14 cases, from 33 samples of raw goat milk 21 cases and finally from 33 samples of raw sheep milk 19 cases were PCR positive. With respect to these results, Nested PCR technique investigated in this study has significant sensitivity and accuracy compared to Hemi Nested PCR technique; therefore, the molecular technique as a confirmatory method in detection of Brucella spp.

Key words: *Brucella spp.*, milk, cheese, Hemi Nested PCR, Nested PCR

INTRODUCTION

Brucella is a mandatory parasite among humans and animals and its specification is the intracellular living. *Brucella* is gram-negative and aerobic. This organism is without spore and a capsule is seen in smooth or mucous colonies [9]. Although for each type of *Brucella* there is a host of choice, all of them are able to infect a variety of animals and humans [26]. The most common way to become infected is the digestive system (contaminated milk and dairy products), mucosa (droplets) and skin (contact with infected animal tissue). There are several Brucella detection methods such as culture, serological test, and molecular techniques. Each of them has their advantage and disadvantages. Culture has high laboratory risk and various sensitivity which led to the inadequate diagnosis [20]. In addition early in the disease, serological methods can be negative [24]. Therefore, laboratory tests should be repeated after 1-2 weeks in suspected cases. On the one hand, the detection of *Brucella* antibodies alone is not an indicator for the presence of pathogens. On the other hand, high titers of antibody after treatment is often the initial

phase of the disease. Chronic or acute disease is not always a sign of failure in the initial treatment [23]. Since the O polysaccharides of *Brucella* epitope are similar to many kinds of other bacteria, such as: *Yersinia Enterocolita O9*, *Salmonella Urbana*, *Vibro Cholera*, *Francisella Tularensis*, *Escherichia coli O157*, there is a possibility of cross-reactions; therefore, methods based on the detection of LPA[1] are very low in specificity [15]. Therefore although serological methods are well documented but they are not appropriate indicators for detection, as a result using specific molecular methods are evadible [4].

Many molecular technique including PCR and its derivatives, techniques based on hybridization, multiplex PCR, Real time PCR, SNP[2], NASBA[3] and Nucleic Acid-based diagnostics have been developed [21]. PCR in laboratory diagnosis of human Brucellosis demonstrated that acute and chronic blood is more sensitive than culture and more specific than serological methods. Furthermore, work on DNA reduces the risk of laboratory infection of the culture [6].

Nested PCR technique, a sub-division of PCR, is a solution to increase the sensitivity and accuracy of the PCR and also helps to the isolation of specific product from the mass of non-specific products. In this technique two pairs of primers are used so that the second pair is located in the first pair. Since infection with *Brucella* in endemic region remains a major health problem, time and accuracy in detection of *Brucella* seems vital to prevention and treatment of the disease. The aim of this study is *Brucella spp.* detection in dairy products using Nested and Hemi Nested PCR techniques and comparing the techniques' sensitivity and specificity.

MATERIALS AND METHODS

Preparation of samples: In this study, 57 samples of raw cow milk and 34 samples of pasteurized milk, 28 samples of pasteurized cheese, 23 samples of traditional cheese, 33 samples of raw goat milk and 33 samples of raw sheep milk were collected from the Tehran province.

DNA extraction from strains of *Brucella spp.:* To optimize PCR technique for detection of *Brucella*, from standard strain of the bacteria, DNA was extracted by boiling and DNG (CinnaGen, Iran).

DNA extraction by DNP kit: 100µl of milk sample mixed with 400µl of Lysis Solution and vortex for 10 sec. 300µl of Precipitation Solution were added, gently mix by inversion 10 times, then centrifuged at 12,000 g for 10min. By gently inverting the tube, supernatant were decanted. 1 ml Wash Buffer was added to pellet and mix gently by 10 times inversions, after that centrifuged at 12,000 g for 5min and followed by decanting the supernatant. Wash Buffer was poured off completely and pellet was dried by holding at 65^{o}C for 5 min. At least pellet was suspended in 50µl of Solvent Buffer by gentle shaking and placing at 65^{o}C for 5 min.

DNA extraction by Boiling: 100µl of milk sample was added in 1.5 cc micro tube. The tube was holding in water bath at 95 ^{o}C for 15 min then centrifuged at 12,000 g for 10min. The supernatant which contained DNA was isolated and transferred to new tube.

Hemi Nested PCR technique: Hemi Nested PCR was performed as described by Tantillo et al 2003[22].

Nested PCR Primers: PCR primers were designed for BCSP-31 gene encoding for an antigen localized at or near bacterial cell surface of brucella species (Gen bank accession no. HE603359.1) by employing the NCBI primer designing support software program. Primers specificity was evaluated by using alignment and nucleotide blast. The outer amplified region has 523 bp lengths and the inner amplified product has 275bp.

Nested PCR sensitivity and specificity: For determination PCR sensitivity, different dilutions of DNA virus from 152 ng to 10 pg were provided. The DNA's of Mice, human, *Toxoplasma gondii*, *Saccharomyces cerevisiae*, *Escherichia coli* ,*Shigella spp* and *salmonella spp.* were used to verify PCR specificity.

[1] Lipoply sacharide A
[2] single-nucleotide polymorphism
[3] Nucleic acid sequence based amplification

Nested PCR reaction: Each reaction involves 5µl of DNA, 2.5 µl 10X PCR Buffer, 1 µl of each outer primer F[1]/R[2] 10mM, 0.75 µl MgCl2 50mM, 0.5 µl dNTP (dATP, dCTP, dGTP, dTTP) 10mM, 0.4 µl 5u/µl Taq DNA Polymerase in a total volume of 25 µl. The second round of the reaction was optimized with the same values. In the second round, the reaction product from the first round was used instead of DNA template. Primers used in the second round are Inner F/R. Primer sequences are summarized in Table 1.

Table 1. Primers sequencing of Nested PCR

Primers name	sequences
Outer Primer F	5`AAGGG CAAGG TGGAA GATTT 3`
Outer Primer R	5`CCTCG TTCCA GAGAA CCTTG 3`
Inner primer F	5`GCGTA AGGAT GCAAA CATCA 3`
Inner primer R	5`AGATC GGAAC GAGCG AAATA 3`

Thermal cycle reactions Nested PCR : The mixture was subjected in the first round with initial denaturation temperature at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56/5°C for 1 min and extra 30s at 72°C. In addition, the mixture was subjected to 30 cycles in the second round with a denaturation at 94°C for 30 s, annealing at 58°C for 20s and extra 20s at 72°C were optimized.

Evaluation of PCR product: PCR products were analyzed by electrophoresis on 2% agarose gel in 0.5 X TBE buffer (CinnaGen, Iran) and visualized by 0/0001 SYBR green was stained (SYBR safe CinnaGen, Iran) and a U.V transilluminator.

PCR techniques to detect *Brucella* in samples: In this study, 208 DNA samples of dairy products (57 samples of raw cow milk and 34 samples of pasteurized milk, 28 samples of pasteurized cheese, 23 samples of traditional cheese, 33 samples of raw goat milk and 33 samples of raw sheep milk) were collected, and DNA samples for each population as template in the PCR technique were evaluated.

RESULTS

Optimization of Hemi Nested PCR and Nested PCR techniques (first and second rounds): Results shown in Table 2, Figure 1 and 2.

Table 2.First and second rounds of PCR results

Sample (n)	Nested PCR Result		Hemi Nested PCR Result	
	First round n (%)	Second round n (%)	First round n (%)	Second round n (%)
Raw cow milk (57)	12 (21%)	19 (33%)	10 (17%)	14 (24%)
Pasteurized milk (34)	3 (9%)	10 (29%)	3 (9%)	6 (17%)
Pasteurized cheese (28)	5 (18%)	8 (28%)	3 (10%)	5 (18%)
Traditional cheese (23)	6 (26%)	14 (60%)	5 (21%)	8 (34%)
Raw goat milk (33)	11 (33%)	21 (63%)	6 (18%)	15 (45%)
Raw sheep milk (33)	9 (27%)	19 (57%)	6 (18%)	11 (33%)

n: number

[1] Forward
[2] Reverse

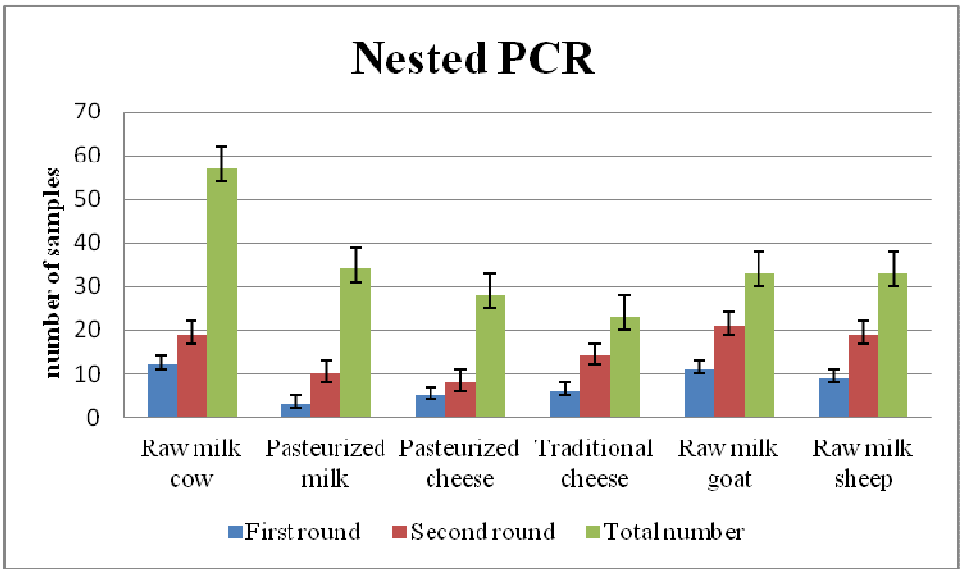

Figure 1. First and second rounds of Nested PCR

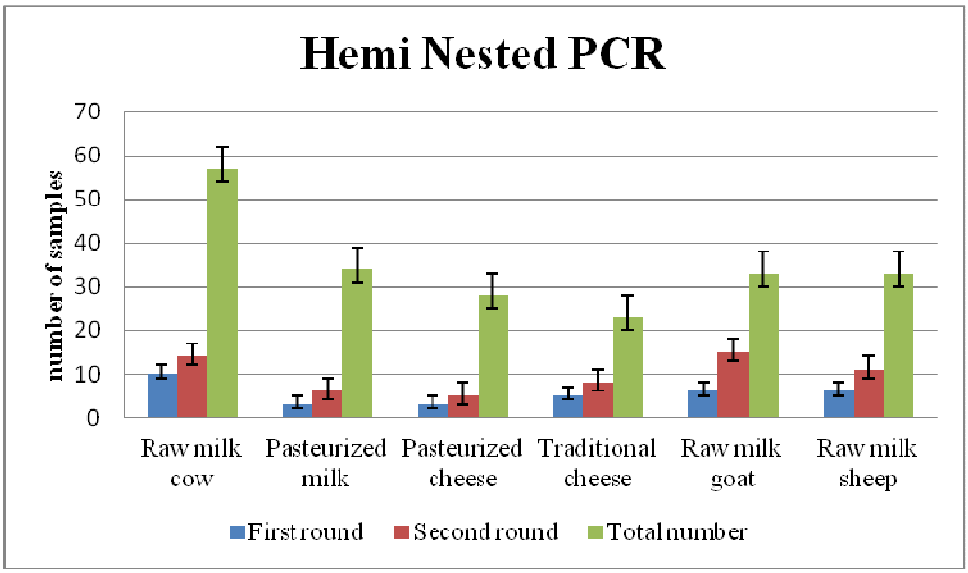

Figure 2. First and second rounds of Hemi Nested PCR

Optimization of the first and second rounds of Hemi Nested PCR technique electrophoresis gel: PCR products were loaded on 2% agarose gel. Fragment size obtained with external primers (first round) was 443 bp, and with internal primers (second round) it was 225bp, just as shown in (Figure3).

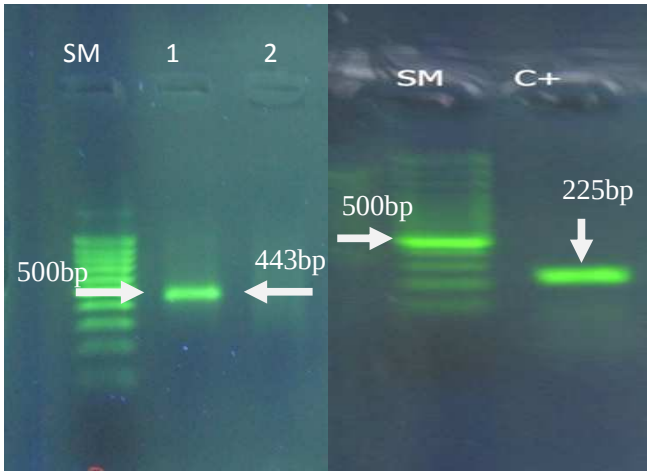

Figure 3. Primers optimized for gel electrophoresis in the first and second rounds of Hemi Nested PCR. SM.100bp DNA Ladder (Fermentas, USA), 1. Positive control in first round, 2.Negative control, C+. Positive control in second round

Optimization of the first and second rounds of Nested PCR technique electrophoresis gel: PCR products were loaded on 2% agarose gel. Fragment size obtained with external primers (first round) was 523 bp and in internal primers (second round) was 275bp (figure 4).

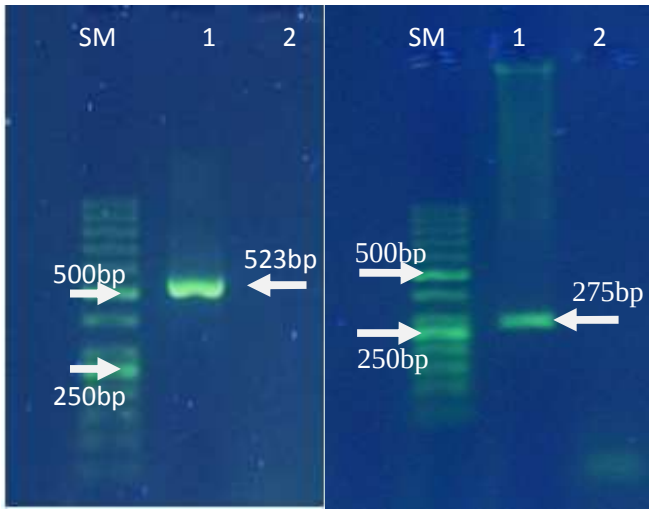

Figure 4. Primers optimized for gel electrophoresis in the first and second rounds Nested PCR SM.50bp DNA Ladder (Fermentas, USA), 1.Positive control, 2.Negative control

Nested PCR sensitivity and specificity result: Nested PCR had high specificity showed no reaction to the other infectious agents except DNA of *Brucella spp*. The PCR sensitivity up to 100 pg was observed (Fig 5).

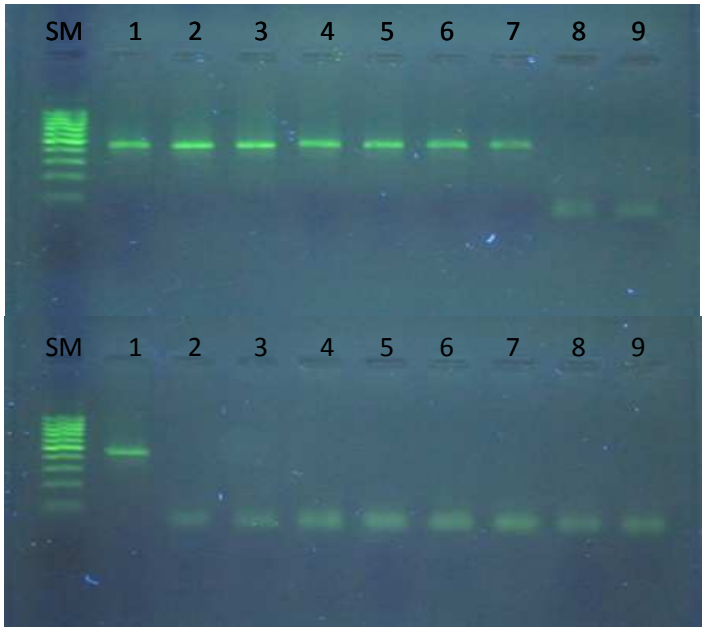

Figure 5a. Senseitivity of Nested PCR first round product. SM.100bp DNA Ladder (Fermentas, USA).1. Positive control, 2;152 ng DNA, 3; 76 ng DNA, 4; 38 ng DNA, 5;19 ng DNA, 6;1900pg DNA, 7;100 pg DNA, 8;10 pg DNA,9; negative control
Figure 5b. Specificity of Nested PCR first round product. SM.100bp DNA Ladder (Fermentas, USA), 1. Positive control, 2; PCR product of Mice DNA., 3 Human DNA, 4; *E.Coli* DNA., 5; *Saccharomyces cerevisiae* DNA., 6; *Toxoplasma gondii* DNA., 7; *salmonella spp*. DNA., 9; *Shigella* DNA, 9;negative control.

DISCUSSION

Milk and dairy products play a significant role in human nutrition. *Brucella* is an intracellular parasite for humans and animals which cause infections especially in the reticuloendothelial system and reproductive organs. Brucellosis is a fever disease passing at regular intervals [25]. Despite many improvements, Brucellosis still remains a major public health threat in some endemic areas. Probability of infection with Brucellosis is still remaining because of misdiagnosing or late diagnosing. Brucellosis is an endemic disease in Middle East [10, 11]. Consumption of contaminated raw milk and unpasteurized dairy products like cream, fresh cheese, ice cream and colostrums, are the main ways of disease transmission. In addition, consumption of contaminated animal products can cause illness in human. Therefore, traditional foods play an important role in disease catching. According to researchers, it is shown that from 7% of local fresh cheese sold in various food stores in Iran, goat bacteria were isolated. It was also possible to separate the bacteria for up to 11 weeks after the cheese production [1]. There are several methods for *Brucella* detection including agglutination methods, blood culture, PCR and ELISA[1]. Blood culture has variable sensitivity between 53 to 90 percent, which causes detection inadequacy [2]. In addition, when there is not essential condition for culture, serological methods like agglutination methods are used. False positive results due to cross-reactivity with other bacteria antibodies are the main problem of using serologic methods. Thus, for exact detection of Brucellosis, complementary techniques for serological methods should be used [3, 16].

PCR sensitivity is more than culture. In Some studies it has been reported that the sensitivity of the PCR technique for the detection is 98% [14, 17]. As it is mentioned the most common method for *Brucella* detection in milk and milk products is MRT[2] method having low sensitivity and accuracy. Furthermore, serological methods do not have enough accuracy and efficiency for diagnosis especially in dairy products. Hence, using the molecular methods as a confirmatory method is unavoidable [12].

Miyashiro et al in 2007 were detecting *Brucella Abortus* in cheese samples by PCR. They used B4, B5 primers; the PCR product amplified by the primers was 223 bp. From 192 samples which were examined in this study, all

[1] Enzyme-linked immunosorbent assay
[2] Milk Ring Test

samples were culture negative, but 19/27% of them were PCR positive [18]. The novel multiplex PCR in detecting BCSP31of *Brucella* confected with Leptospira and Bovine herpesvirus-1 was developed by Bhure in 2012. The technique used by them had high sensitivity and specificity for diagnosis even in infected [7].

Comparison between direct culture and PCR was done by Buyukcangaz et al in Turkey. They showed that PCR sensitivity and specificity was more significant than bacteriological methods [8]. Serological and molecular diagnosis of human Brucellosis was studied by Asaad AM et al. They compared ELISA and PCR as diagnostic tools. They stated that ELISA offers a significant advantage over conventional serological methods in the diagnosis of Brucellosis in endemic areas, while PCR technique results can be particularly important in patients with clinical signs and symptoms and negative serological results allowing the early and rapid confirmation of the Brucellosis [5]. In this study used Nested and Hemi Nested PCR techniques and the advantages of this approach can be identified as high sensitivity, high specificity and its needing less to further acceptance such as probing to confirm the presence of the amplified product [13, 19]. No such research has been done upon comparing Nested and Hemi Nested PCR for detection of *Brucella* in same samples to find the most accurate technique in diagnosis. The results show that Nested PCR is a reliable technique with high sensitivity and accuracy so that it is able to detect pathogen in the samples even in pasteurized cheese. This is a disturbing result because it shows that pasteurization methods are not accurate enough and after this process high percentage of samples remain contaminated. This increase was also observed in other samples. Thus, supervision processes must be considered in production.

Although it was seems that Hemi Nested primers which have been obtained from another study [22] has high sensitivity but unfortunately the result obtained in this study demonstrate that these primers react with *Salmonella*, *Escherichia coli* and *Pasteurella Aerogenes* causing a significant reduction in accuracy. Nevertheless, as result shown the Nested primers used in this study, have high sensitivity so that they only react with *Brucella spp.* The results also show that a significant percentage of the pasteurized milk samples in dairy factories are infected with *Brucella*. This is a warning for the effective methods of removing pathogens to be considered. Furthermore, methods results on traditional and pasteurized cheese in the city show that some of them also have a positive reaction; this is a concern factor for public health. Also, the presence of viable but non-cultivable forms must be examined. Finally, it might be very useful to consider factors such as milking devices' health, milk transportation and the management in dairy manufacturing as prevention policies.

CONCLUSION

With respect to these results it can be described that the studied Nested PCR has higher sensitivity and accuracy than Hemi Nested PCR, and common PCR (one pair primer) has much lower sensitivity and specificity comparing to the two techniques. It seems that molecular technique for detection of *Brucella spp.* must be used alongside with conventional detection methods as a complementary technique to improve dairy products and public health as well.

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