



Contamination of Bovine Milk With *Brucella* spp.: A Current Public Health Menace in Kurdistan Province of Iran

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Abstract

Background: Brucellosis, a deteriorating zoonotic disease, is very common in most parts of Iran. Consumption of contaminated milk and dairy products is one of the most significant ways for transmission of the infections to human. Since the close rearing of cattle and sheep is practiced in Kurdistan province of Iran, the infection of cow with non-specific species is not out of mind. The present study aimed to determine the frequency of bovine milk contamination with zoonotic *Brucella* spp.

Methods: A total of 240 milk samples, equally from traditional and industrialized dairy farms, were collected aseptically. Conventional microbiological method was used for isolation of the bacterium, followed by the genotypic identification of the isolates. Moreover, direct molecular processing of the samples was carried out for detection of the bacterial genome. The positive samples were further genotypically assessed to identify the contamination as *Brucella abortus* or *Brucella melitensis*.

Results: In general, 16 (6.66%) and 15 (6.25%) of the samples were contaminated with *Brucella* spp. in phenotypic and genotypic methods, respectively. The proportion of contamination with *B. abortus* and *B. melitensis* in phenotypic and genotypic methods were 5% and 1.66%, and 5% and 1.25%, respectively. The overall rate of contamination in traditional milk samples was more than industrialized samples.

Conclusions: Contamination of bovine milk with *Brucella* spp. is a serious threat to public health in the studied region. Continuous vaccination, application of test and slaughter policy, and presumption of pasteurized milk and dairy products are highly recommended.

Keywords: Bovine milk, *Brucella abortus*, *Brucella melitensis*, Western Iran



Background

Brucella spp., the etiological agent of brucellosis in domestic animals and Malta fever in human, is a Gram negative, facultative intracellular aerobic microorganism (1). The World Health Organization (WHO) has categorized the disease as one of the seven neglected zoonotic infections in human despite its worsening implications (2). The genus *Brucella* comprises of 12 species based on the main host and pathogen (3). Among the various species, *Brucella abortus*, *Brucella suis*, and *Brucella melitensis* cause infections in human, where the latter species is considered the most infectious one (4). Following the infection of a female animal, the bacterium affects the reproductive system, and finally localizes in mammary gland and retromammary lymph nodes everlastingly. Continues shedding of the bacteria in milk imposes a public health hazard (5). The main routes for bacterial transmission to human is through not only consumption of unpasteurized milk and dairy products, but also close contact with infected animals, as well as inhalation (6).

The Middle East and Central Asia are the hotspots with the highest incidence of the infection among

livestock and human (7). In Iran, vaccination with S19 or RB51 strains in cattle and Rev1 strain in sheep/goat, as well as test/eradication policy in domestic animals have been launched to control the infection in dairy industry (8,9). Although serological assays including milk ring test (MRT), Rose Bengal slide agglutination, and standard tube agglutination (STA) tests are frequently used in brucellosis surveillance and control programs, they may produce false-negative (10,11) or false-positive (12) results. Moreover, the lack of knowledge regarding the *Brucella* species involved in a brucellosis case in the serological tests is another negative point of this method. As a consequence, the probability of ascertaining the infection source and, therefore, applying the proper control procedures may have been precluded (13). In comparison, culture and microbiological isolation, as the gold standard technique, as well as molecular methods for detection of *Brucella* spp. may be more informative and trustable (10).

Although *Brucella* spp. attack preferred hosts, some species may infect the non-preferred hosts in close rearing system of different animal species. Due to the scant of recent information regarding the prevalence of *Brucella*

in bovine milk and keeping cows in close proximity of small ruminants in the same barn, the present study was conducted to evaluate the frequency of *B. abortus* and *B. melitensis* in cow milk samples collected from Kurdistan province of Iran.

Methods

Sample Collection

In a cross-sectional study, from December 2018 to May 2019, a total of 240 milk samples, equally from industrialized and agrarian farms in all over Kurdistan province of Iran, were collected aseptically in sterile bottle glasses after disinfecting four teats. The samples were chilled until their delivery to the laboratory within maximum five hours and divided into two parts. One part was scrutinized for bacteriological isolation and the other one was inactivated at 65°C for 30 min, and stored at -20°C until molecular analysis (14).

Bacterial Culture

Following the centrifuging of the milk samples in 3000 g for 15 minutes, the pellet and cream were streaked on Brucella selective agar (Quelab, Canada) complemented with Brucella selective supplement (Oxoid, UK). The plates were incubated at 37°C for 14 days under 5%-10% CO₂ tension and examined for *Brucella* spp. on daily base after the fourth day (15). Resultant colonies representing the morphology of *Brucella* spp. were further subcultured on Blood agar (BA, Quelab, Canada) containing 7% defibrinated sheep blood sample. Conventional identification of the isolates as *Brucella* spp. was based on Gram and modified Ziehl-Neelsen (MZN) stainings, catalase, oxidase, H₂S production, and nitrate and urea reactions (16).

Genomic DNA Extraction

For initiation, the samples were thawed, 1.5 mL of each one was poured into sterile two µL tube, and centrifuged for 10 min at 6000 g. Three layers including the cream (the upper fat), milk whey, and the protein deposition (the lower layer) were separated. The milk whey was

collected and discarded, then 200 µL Tris-EDTA (TE) solution was added to each microtube and homogenized thoroughly (8). In addition, the overnight culture of the bacteria isolated in phenotypic method was used for DNA extraction.

DNA was extracted from both pure culture of the isolates and directly from each milk sample using Gram Negative Bacterial DNA Extraction Kit (CinnaGen, Iran, Cat no. EX6011), in accordance with the manufacturer's guidelines.

Molecular Assessment

Molecular detection of *Brucella* spp. was carried out with the partial amplification of *bcsp31* gene using primer pair and thermal condition introduced elsewhere (17) (Table 1). In the next round of polymerase chain reaction (PCR) reaction, the identified bacteria were categorized as *B. abortus* (all biovars) or *B. melitensis* (all biovars) based on the method represented by Whatmore et al (18) and Bricker and Halling (19) (Table 1). All molecular assays were repeated twice.

RB51 and Rev1 strains (kindly provided by Veterinary Administration Office of Kurdistan province) were used as positive controls and sterile distilled water was exercised as negative control in the survey. The amplicons were electrophoresed into 1.2% agarose gel in 80 V for 70 minutes and visualized under UV light.

Statistical Analysis

The statistical association of the frequency of *Brucella* spp. and the two studied species in phenotypic and genotypic methods with the rearing system (traditional and industrialize) was analyzed in SPSS software (version 21.0, Chicago, IL) using chi-square test. A *P* value ≤ 0.05 was considered statistically significant.

Results

Cultivation of the milk samples demonstrated the growth of pinpoint, dew-drop typical round, glistening, convex, and translucent colonies in 16 (6.66%) cases. An individual colony from each plate was subcultured

Table 1. Details of Primer Sequences and Thermal Conditions Used in the Present Study

Gene	Primer Sequence (5'→3')	PCR Thermal Condition				Product Size (bp)	Reference
		Initial Denaturation	Denaturation	Annealing	Extension		
<i>Bcsp31</i> (<i>Brucella</i> spp.)	TGGCTCGGTTGCCAATATCAA CGCGCTTGCCCTTCAGGTCTG	95°C for 4 min	94°C for 1 min	60°C for 1 min 40 cycles	72°C for 45 sec	72°C for 5 min	223 (17)
<i>Omp25</i> (<i>B. abortus</i>)	ATGCGCACTCTTAAGTCTC GCCSAGGATGTTGTCCGT	95 °C for 4 min	94 °C for 1 min	59 °C for 1 min 35 cycles	72 °C for 1 min	72 °C for 7 min	490 (18)
IS711 (<i>B. melitensis</i>)	AAATCGCGTCCTTGCTGGTCTGA TGCCGATCACTTAAGGCGCCTTCAT	95 °C for 4 min	95 °C for 1:15 min	55/5 °C for 2 min 30 cycles	72 °C for 1 min	72 °C for 7 min	731 (19)

on BA. The pure colonies with an appearance of pink small cocobacilli in Gram and MZN stainings, catalase and oxidase positive, representing positive reactions in nitrate reduction and urease production tests, were biochemically identified as *Brucella* spp. In addition, a trace of H₂S production in four (1.66%) samples and no evidence of H₂S production in 12 (5%) samples primarily categorized the isolates as *B. melitensis* and *B. abortus*, respectively. Thirteen (5.41%) and three (1.25%) isolates were obtained from traditional and industrialized milk samples. The frequency of the isolates in traditional and industrialized milk samples are illustrated in Table 2. All of the isolates were molecularly confirmed as *Brucella* spp. in the *bcs31* (genus)-specific PCR reaction (Figure 1). In the next step, the isolates generated the expected amplicons in species-specific reactions, confirming as *B. abortus* and *B. melitensis* (Figures 2 and 3)

Moreover, the contamination of 15 (6.25%) out of 240 milk samples with *Brucella* spp. was detected in molecular approach (Figure 1), among which 12 (5%) and three (1.25%) were recognized as *B. abortus* and *B. melitensis* (Figures 2 and 3), respectively. The details of *Brucella* spp. contamination in traditional and industrialized milk samples are depicted in Table 2.

Besides, the statistical relationships were observed between the frequencies of *Brucella* spp. ($P \leq 0.05$) and *B. abortus* ($P \leq 0.05$) with the rearing system, which was the opposite to the result obtained for *B. melitensis* ($P > 0.05$).

Discussion

Milk and dairy products are incriminated as potential vectors for transmission of some notorious zoonotic diseases to human. In some previous studies undertaken by the author and colleagues in the studied region, the contamination of milk with *Coxiella burnetii* and aflatoxin M1 (20), Shige-toxicogenic *Escherichia coli* (21), and methicillin resistant *Staphylococcus aureus* has also been proved (22). Based on the mistaken beliefs regarding the benefits of unpasteurized milk and dairy products compared to pasteurized form, the consumption of these products are very popular in the district. The results represented the average 6% frequency of milk contamination with *Brucella* spp., which is a matter of concern due to the worsening implications of the disease in both veterinary and public sector. On the other hand, empirical evidence has manifested the habit of using

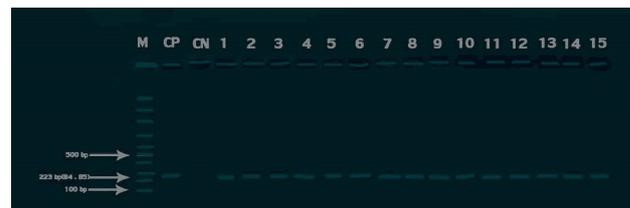


Figure 1. Agarose Gel Electrophoresis of PCR Products Generated From *bcs31* gene Amplification in *Brucella* spp. M: 100 bp DNA Ladder (Sinaclon, Iran), CP: Positive Control (RB51 Strain), CN: Negative Control, Lanes 1-15: Field Samples.

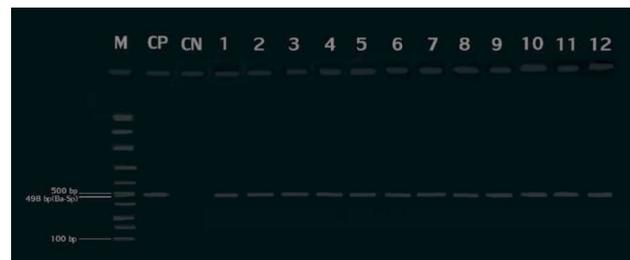


Figure 2. Agarose Gel Electrophoresis of PCR Products Generated From *omp25* gene Amplification in *B. abortus*. M: 100 bp DNA Ladder (Sinaclon, Iran), CP: Positive Control (RB51 Strain), CN: Negative Control, Lanes 1-12: Field Samples.

communal grazing or keeping different species of animals (cattle, sheep, and goat) tethered or at limited pastures. This provides favorable conditions for propagation of non-preferred bacterial species among domestic animals (23). The spillover of *B. melitensis* from sheep and goat to cattle has previously been demonstrated in other studies (8,14). Generally, the animal breeding capacity of Kurdistan, an interest in consumption of unpasteurized milk and dairy products, and deregulation of trade and decreased animal traffic control in borders with neighboring countries are the plausible reasons for the burden of the infection in the given province (8,24-26). Notably, the results implied that the infection in small ruminants' population is currently prevalent in the region which should be considered in the future epizootiological surveillance programs.

Despite the application of routine vaccination and eradication programs for *Brucella* spp. in Iran, the serology screening is not trustable due to the positive and negative false results (10-12). Sampling at an early stage of the infection (e.g., within the first 14 days (27), reduced antibody titers over time (28), generation of a shorter duration of humoral antibody response in cattle due to

Table 2. Details Regarding the Frequency of *Brucella* Spp. in Milk Samples

Samples	No. (%) of Samples	No. (%) of <i>Brucella</i> spp. in Phenotypic Analysis	No. (%) of <i>B. abortus</i> in Phenotypic Analysis	No. (%) of <i>B. melitensis</i> in Phenotypic Analysis	No. (%) of <i>Brucella</i> spp. in Molecular Analysis	No. (%) of <i>B. abortus</i> in Molecular Analysis	No. (%) of <i>B. melitensis</i> in Molecular Analysis
Traditional milk samples	120 (50%)	13 (5.41%)	10 (4.16%)	3 (1.25%)	12 (5%)	10 (4.16%)	2 (0.83%)
Industrialized milk samples	120 (50%)	3 (1.25%)	2 (0.83%)	1 (0.41%)	3 (1.25%)	2 (0.83%)	1 (0.41%)
Total	240 (100%)	16 (6.66%)	12 (5%)	4 (1.66%)	15 (6.25%)	12 (5%)	3 (1.25%)



Figure 3. Agarose Gel Electrophoresis of PCR Products Generated from IS711 gene Amplification in *B. Melitensis*. M: 100 Bp DNA Ladder (Sinaclon, Iran), CP: Positive Control (Rev1 Strain), CN: Negative Control, Lanes 1-3: Field Samples.

infection with *B. suis* (29), previous vaccination against brucellosis (30), and latent infectious status in the cases of inter-uterus infection or in the early postnatal period (5) are possible reasons producing false negative results. In addition, false positive serological results might be generated because of cross-reaction with *E. coli*, *Vibrio cholera*, *Yersinia enterocolitica*, and *Francisella tularensis* (5).

Although the bacterial isolation is the gold standard diagnostic method for *Brucella* spp. (31), there are some obstacles. A plausible reason for avoiding the application of phenotypic method in a routine manner may be related to the highly hazardous and contagious zoonotic nature of the bacterium, which requires appropriate biosecurity facilities and personnel having expertise in diagnostic laboratory (32). Besides, the bacterium is classified as a class-B bio-weapon and the procedure is time-consuming (32). In our study, a total number of 16 *Brucella* spp. was isolated using microbiological procedure. All of the isolates were molecularly confirmed as the genus and further characterized as *B. abortus* and *B. melitensis*. Some other internal (33,34) and external (35,36) researches have also been documented the identification of zoonotic *Brucella* spp. in ruminants' milk samples using conventional bacteriological method and molecular confirmation of the isolates using PCR. The reported contamination rate was from 1.28% to 25% (33-36).

On the other hand, not only does the replacement of a fast, simple, and accurate complementary diagnostic method for direct molecular detection of the bacterium in milk provide precise detection of the infection, but it also protects laboratory staff against contamination. Herein the subtle higher identification rate of the bacterium in the phenotypic method compared to the direct molecular approach may be explained by the fact

that the different compounds of the milk such as proteins and fats may exert inhibitory effect on direct DNA extraction protocols. Also, a drawback of the used kit is that it was not specific for bacterial DNA extraction from milk samples. Consequently, this may influence the final outcome. Remarkably, some factors, namely the amount of the bacteria shedding through the milk, disease phase, and fastidiously-growing nature of the genus may affect the bacterial detection ratio (37). Similar to our study, another survey compared the culture and PCR methods for detection of *Brucella* spp. in milk samples (38). The results represented a significant higher proportion of bacterial detection in PCR in comparison with the phenotypic method, which is in contrast with the data obtained herein. A possible reason for this incompatibility may be related to the DNA extraction method applied in the both studies.

It is assumed that the frequency of brucellosis is underestimated in the present research as no ovine and caprine milk samples were evaluated. Likewise, the predominant biovar of *B. abortus* identified in different internal studies in Iran is biovars 3 (8,39-41). RB51 or S19 strains of the bacterium, used as a vaccine, are biovar 1 strains. The latter may be also disposed in milk like a wild-type strain and inaccurately recognized as a severe strain (8). Therefore, further research is mandatory to draw firm conclusions about the exact distribution of wild-type *Brucella* spp. in milk. Some PCR-based techniques can simply discriminate between the wild-type and vaccine strains based on the amplicon size (32,42).

Considering the endemic status of brucellosis in Iran, epidemiological investigation of the disease is of great significance. Some literature has documented the frequency of Malta fever or brucellosis in recent years in Kurdistan province of Iran. Demographic information relating to the distribution of Malta fever among 1997 to 2003 documented the statistical association of gender and place of living with the frequency of the disease, as males in villages and bricklayer in cities were the most affected groups. Moreover, the highest level of incidence was in 2003 and the lowest in 2000 (43). Norouzinezhad et al have assessed the epidemiological characteristics and trend of the incidence of human brucellosis in Kurdistan province from 2009 to 2016. The results depicted the highest incidence as 103.54 in 100 000 in 2014 and the lowest as 23.86 in 100 000 in 2010. According to an 8-year analysis, the highest incidence rate was seen in Bijar county among farmers, housewives, ranchers, male sex, rural dwellers, and those aged 24-25 years. Majority of the patients reported contact with livestock (26). In another study analysing the seroepidemiology of brucellosis in 2014, the rate was 6.4% with the highest burden among butchers (12%) (24). Besides, the distribution of *Brucella* spp. in bovine and caprine milk in 2012 in Kurdistan was stated to have been 33.33% and 44% with the frequency

of *B. abortus* and *B. melitensis* as 15% and 3.33%, and 2% and 30%, respectively (8). The frequency of the infection in cow milk and its traditional products in Isfahan and Chaharmahal and Bakhtiari provinces of Iran in 2012 was reported to have been 1% *B. abortus* in milk, 2.5% *B. abortus* and *B. melitensis* in cheese, and 1% *B. abortus* in cream (44). In contrast, the all 12 *Brucella* spp. detected in raw and unpasteurized bulk cow milk tanks of traditional domestic dairy sale centers in Khorramabad was *B. abortus* (9). The plausible explanations for the discrepancy in various studies may be related to the type and number of sampling, methodology, geographical and socioeconomical conditions, vaccination, and disease controlling measures (9,45). Moreover, the frequency of *Brucella* spp. DNA in bovine milk samples in small-scale urban and peri-urban farming in Tajikistan was reported to have been 10.3% in seropositive and seronegative cows. Two individual strains, one as *B. abortus* and one as *B. melitensis* were recognized (14). This proportion was 7.1% in Samsun, and 95% in Erzurum, Turkey, with all the strains identified as *B. abortus*, respectively (44,46). It is noteworthy to state that since vaccination against brucellosis is infrequently applied in Mediterranean and Central Asian countries, this may clarify the high rate of the infection in some cases (14,47).

Conclusions

Holistically, given the evidence regarding the contamination of milk with zoonotic species of *Brucella* in Kurdistan province of Iran, continuous vaccination of both industrialized and traditional rearing ruminants, test and slaughter policy, accurate evaluation of the contamination status of milk, and encouraging the consumption of pasteurized milk and dairy products may warrant the protection against brucellosis or Malta fever in the region. Moreover, it is highly recommended to use Rev1 as well as RB51 for vaccinating cows.

Conflict of interests

The authors declare that there is no conflict of interests.

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Ethical Approval

The samples were taken from the animals with official permission and under the supervision of the Institutional Animal Ethics and Research Committee of Islamic Azad University, Sanandaj Branch (Certificate No. 98/101/96500).

Authors' contribution

This study was conceived by EA and the Sampling was performed by MHA. EA and MHA carried out the laboratory works and EA wrote this article. All the authors have read and approved the manuscript.

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