

Isolation and biotyping of *Brucella* spp. from sheep and goats raw milk in southeastern Iran

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Abstract. Brucellosis is known as an important public health problem for humans around the world and is one of the most prevalent zoonotic disease as well. *Brucella* spp. is a Gram negative rod, facultative intracellular pathogenic and non motile bacteria. The aim of this study were to determine the frequency and biotyping of *Brucella* spp. isolated from sheep and goats from south eastern part of Iran. 700 samples (raw milk) from sheep and goats (90) flocks were collected randomly, from January to July 2015, samples were transferred packed in ice to microbiology lab in Kerman University, immediately. Samples were inoculated on *Brucella* agar which contains antibiotic and inactivated horse serum and incubate for 5 days. Gram smears were prepared from suspected colonies followed by oxidize and biochemical tests. Polymerase Chain Reaction (PCR) were used for confirmation. All positive samples were biotyped by phage typing. Nine milk samples out of 700 (1.28%) collected were positive by bacteriological method and all of them were *Brucella melitensis* Biotype 1 and one out of 700 samples was *Brucella ovis*. In relation to the National vaccination program in these areas the most contaminated region was Rabor, Rayen, Bardsir and Baft, respectively.

INTRODUCTION

Brucellosis are known to cause problems for animals and human beings worldwide and is the most common zoonotic disease, which is reported daily with over 500000 new cases (Kaya, 2012). Genus *Brucella* is an intracellular pathogenic, gram negative rod, non-motile and six species are known (*Brucella melitensis*, *B. abortus*, *B. ovis*, *B. suis*, *B. canis* and *B. neotomae*), which are named based on the kind of host (Gardoso *et al.*, 2006; Ihan *et al.*, 2008; Zowghi *et al.*, 2008). This bacterium causes Malta fever in human and is one of the important

factor in blood culture etiologic which can infected laboratory personals (Nielsen, 2002). This bacterium is transmitted by direct contact with animal tissues or via consuming unpasteurized dairies (Behroozikah *et al.*, 2012; Zambriski *et al.*, 2012). These micro-organisms mostly exist in lymphatic glands in animals breast and delivered through milk (AL-Garadi *et al.*, 2011; Zambriski *et al.*, 2012).

The majority of cases are reported from Mexico, Argentina and Peru in Latin America, where goats are important in local agriculture (Zambriski *et al.*, 2012). Human brucellosis is seldom life-threatening, but it does lead

to serious morbidity and as such as is an economic and public-health concern in developing countries (Pappas *et al.*, 2005; Franco *et al.*, 2007).

The aim of this study were to determinate of *Brucella* spp. frequency in sheep and goats of tribes which settled in suburban township of Kerman (Bardsir, Rayen, Rabor, Baft).

Dairy flock products are the main products in Iran, preventing flocks from this bacterial infection is important to prevent them from abortion. This study would also be helpful to eradicate Malta fever in humans.

MATERIAL AND METHODS

Sample collection

In this survey, 700 samples of milk were collected randomly from flocks from January to July 2015 in the region of Bardsir, Rayen, Rabor and Baft (Kerman province) in sterile container were gathered and transported to microbiology lab of Afzalipour Faculty of Medicine in 4°C (Zambriski *et al.*, 2012).

Isolation of bacteria

Milk sample were centrifuged in 3500 RPM for 20 min then culture a full swab of creamy upper layer and the sediments in Eugon broth contains tween 80 (in order to liquefy cream and make bacteria free) for 4 days under 10% CO₂ (Zambriski *et al.*, 2012; Baliley, Scotts 2007). Then 100 ml of broth were cultured in Brucella agar containing polymyxin B sulfate, Bacitracin, Nystatin, Cyclohexamide, Nalidixic acid and Vancomycin (Himedia) and inactivated horse serum and incubated for 5 days in 37°C with 10% CO₂. After 5 days the specified colonies (which have a blue shining under white light) were examined by preparing gram stain, oxidase, urease, sensitivity to thionin and fuchsin (Zambriski *et al.*, 2012; Baliley, Scotts 2007).

PCRIS711

PCR assays of raw milk, with primers derived from the IS711 element of the *Brucella* genome, were used. The primers were used to amplify a 317-bp target sequence that included the gene-coded IS711 region of the *Brucella* genome (Tab. 1)(Ning *et al.*, 2012). The 25-µL PCR mixtures contained 2.5 U of Easy-A Taq DNA polymerase, 1 × Easy-A Taq buffer (Kiagen, Iran), 2 mM MgCl₂, 2.5 mM concentration for each deoxynucleosidetriphosphate, 40 pmol of each primer, and 100 ng of purified genomic DNA. PCR was performed in a Mjthermalcycler (Bio-Rad, USA) as follows: 95°C for 3 min for denaturation, 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and finally 72°C for 10 min. The PCR products were fractionated in a 1.5% agarose gel containing and then stained with an ethidium bromide solution (0.5 mg/mL), visualized under an UV transilluminator (Bio-Rad, USA). Visible bands of sizes appropriate to be representative of the 317-bp target sequence of *Brucella* were considered IS711-positive products (Ning *et al.*, 2012).

Determination of Biotype

Brucella spp. which isolated from Brucellosis department of Razi vaccine serum Research Institute, Karaj, Iran, have been specified by phage typing method (Alton *et al.*, 1988).

For Data analysis, *Brucella*'s prevalence in raw milk was reported by Confidence Interval 95%.

RESULTS

From 700 samples (raw milk) which were collected from flocks (300 sheep, 400 goats) of suburban township of Kerman (Baft, Bardsir, Rayen, Rabor), 10 isolates were identified, including nine [1.28% (95% CI: 0.58,

Tab. 1. Sequences primer for detection *brucella* spp.

Primer	Sequence	Fragment size
IS711	GAGAATAAAAGCCAACACCCGGATGGACGAAACCCACGAAT	317 bp

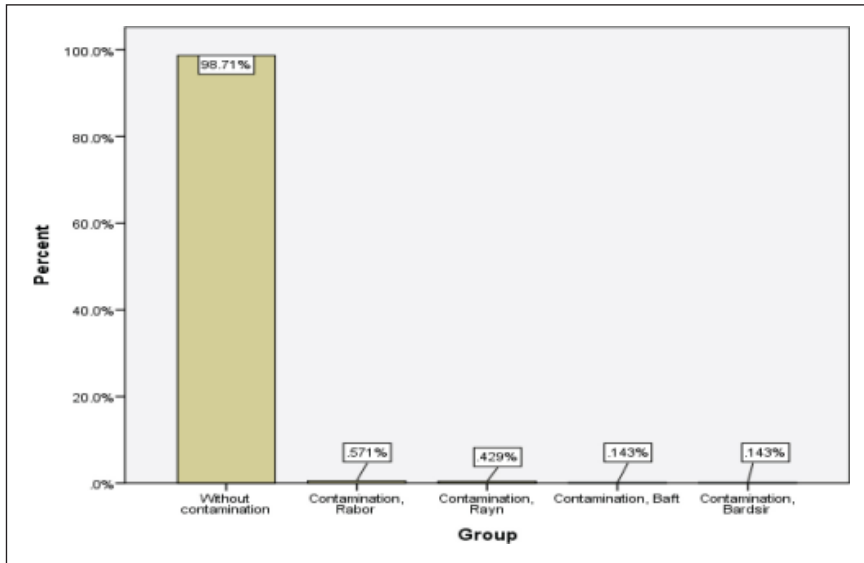


Figure 1. Prevalence of *B. melitensis* in Rabor, Rayen, Bardsir and Baft in Kerman.

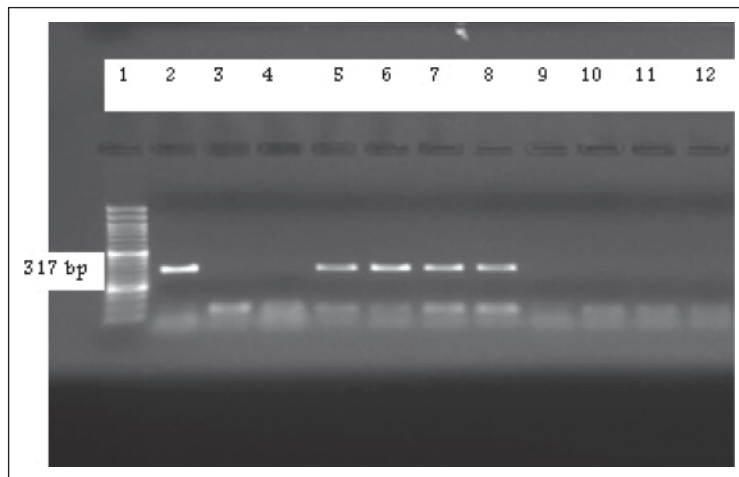


Figure 2. Detection of *Brucella* DNA in milk collected from southern Iran by IS711 PCR, Lane 1, molecular size markers. (50-bp DNA ladder), Lanes 2, positive control, Lanes 3, NTC (Negative Template Control), Lane 4 Extraction Control, Lanes 5 to 8, positive samples, 9-12 Negative samples.

Table 1. Prevalence and Percentage of Positive Milk for *Brucella melitensis*

Species	Number	Total	Culture positive	Culture negative
Sheep	300	300	3 (1%)	297
Goat	400	400	6 (1.5%)	394
Total	700	700	9 (1.28%)	691

2.42)] *B. melitensis* Biotype I and one *B. ovis* [0.14% (95% CI: 0.004, 0.8)]. From 9 isolated which *B. melitensis*, 4 cases were isolated from Rabor, 3 cases from Rayen, one isolate from Bardsir and one case from Baft (Fig. 1).

PCR amplification targeting the IS711 element was performed to determine the presence of *Brucella* DNA in milk samples. Among 700 samples of raw milk, 10 cases were PCR positive (Fig. 2).

DISCUSSION

Brucellosis like tuberculosis can cause an infectious chronic granulomatous reaction which are caused by intracellular organisms. This disease needs to be treated by synthetic antibiotics for a long time. Acute clinical symptoms due to this disease in animals cause, decreasing dairy production in developing countries (Pappas *et al.*, 2005).

Brucellosis transmission to the human is via consuming unpasteurized dairies or direct contact to infectious parts of animal (placenta) or inhaling aerosols (Abbas & Talei, 2010; Behroozikah *et al.*, 2012; Pappas *et al.*, 2005).

Since 1950, when *B. melitensis* from goat milk was reported in Iran, this organism has been isolated sporadically from different regions in a study by Zowghi from 1971 to 1984, 0.24% *B. melitensis* were isolated from flocks milk which were biotype I, II (Zowghi & Ebadi, 1985).

In present study which was carried out in Kerman, 1.28% of sheep and goats milk were contaminated by *B. melitensis* biotype 1 and 0.14% by *B. ovis*.

In a study in Malaysia, 4 out of 300 samples from goats vaginal swabs, isolated *B. melitensis* biovar in 1 (1.3%) (AL-Garadi *et al.*, 2011).

In a study by Abbas & Talei in Iraq (2010) 5 out of 100 samples from cheese were *B. melitensis* biotype II (5%) (Abbas & Talei, 2010).

In another study, carried out by Behroozikah in Iran (2012) 92.8% of biovar I, were responsible for miscarriages in sheep and goats. In Saudi Arabia in 1992, *B. melitensis* biovar I, II were the most frequent infection reported (Radwan *et al.*, 1992). In Turkey (2008) 7.8% of samples (milk) were contaminated by *B. melitensis* biovar III (Najum, 2014).

Results related to present study were the same as results reported from Iraq, Turkey and Malaysia. Reported contaminations were mainly from suburban township of Kerman.

B. melitensis biotype I isolated in our study is an endemic biotype in Iran, it was also was reported in a study by Behrouzika

in Malaysia and Saudi Arabia (AL-Garadi *et al.*, 2011; Behroozikah *et al.*, 2012; Radwan *et al.*, 1992).

In a study was carried out by Najum to diagnose *B. melitensis* infection in goat's milk, two methods were done, Milk Ring Test and PCR. In MRT test non specific antibodies from *Yersinia*, *E. coli*, *Salmonella* and *Vibrio* spp. were detected as well. PCR which is a high sensitivity and precise method could be benefit and helpful as an objective complement and confirmatory test besides other common tests for diagnosis of Malta fever in human (Najum, 2014).

In our study isolation was carried out by cultivation and confirmatory test was done by PCR for detecting species and biotype, biochemistry tests and phage typing were used.

CONCLUSION

In recent years vaccination has been a effective method to prevent flocks from brucellosis. Goat and sheep flocks vaccine which used to inculcate flocks prepared from Razi research center but as regards some flocks which are originally from south Kerman and neighbor province.

Migrate to highlands of Kerman and not yet vaccinated can transmit to other flocks and subsequently to human.

Sheep and goats play an important roles for tribes and this study will help the economically in according to present study the most contaminated suburban townships of Kerman are Rabor, Rayen, Bardsir and Baft respectively. These regions needs to be vaccinated more precisely until the rate of miscarriage decrease and milk producing increases and in the other hand controlling and, eradicating Malta fever in human indeed. Analogous study and research is suggested on raw milk and other dairies products in other different regions a province such as Sirjan and Shahr e Babak. This study would be profitable and helpful to controls and prevents brucellosis in Kerman.

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Conflict of interest:

There is no conflict between the authors.

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