



ISOLATION OF *BRUCELLA MELITENSIS* STRAINS FROM SYRIAN BOVINE MILK SAMPLES

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Summary

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Brucellosis is a worldwide zoonotic disease that still constitutes a major public health problem in Syria. In the current study, *Brucella* has been isolated from cow milk samples and typed by both molecular and conventional techniques. Over a 6-year period (2002–2007) 2372 milk samples were collected from Syrian cow herds. The milk ring test (MRT) was performed where 57% of samples were found to be positive. Milk samples were cultured on the *Brucella* selective medium and incubated for 24–48 h. One-quarter of milk samples were contaminated with *Brucella*, which is considered as a relatively high rate of contamination. In addition, biochemical properties of isolates have been identified. All isolated *Brucella* strains were positive for catalase, oxidase and urea reaction, did not need CO₂ to grow and rarely produced H₂S, could grow in the presence of fuchsin and thionin. The agglutination test was negative with anti-R and positive with anti-M and anti-A sera; so it was determined as *B. melitensis* strain, biovar 3. A specific PCR assay targeting the *bcsp31* gene and the specific integration of IS711 elements within the genome of *Brucella* species has been used. Molecular typing results were compatible with conventional methods.

Key words: agglutination test, biochemical test, cow herds, milk ring test

INTRODUCTION

The term brucellosis is applied to a group of closely related infectious diseases, all caused by Gram-negative bacterial pathogens from the genus *Brucella*. Phenotypic characteristics, antigenic variation, and prevalence of infection in different animal hosts have resulted in the initial recognition of six species: *B. melitensis*, *B. suis*, *B. abortus*, *B. canis*, *B. ovis* and *B. neotomae* (Vizcaino *et al.*, 2004). In addition,

in the 1990s, new brucellae have been isolated from marine mammals and a new species, *Brucella marina* was proposed (Nymo *et al.*, 2011). Manifestations of the disease may range from abortion in the cow to orchitis or epididymitis in the bull (Dougherty *et al.*, 2013). This disease is transmitted by direct or indirect contact with infected excreta. The most important

routes of transmission are the oral and venereal ones.

B. melitensis infections are widespread worldwide, particularly in the Mediterranean countries, including Syria (Safi *et al.*, 2013). *B. melitensis* is a small Gram-negative coccobacillus that occurs invariably in the smooth phase, does not require CO₂, is catalase and oxidase positive and does not produce H₂S or no more than a trace (Ali *et al.*, 2013). It hydrolyses urea and grows in the presence of basic fuchsin or thionin, at the standard concentrations. It has three recognised biovars that are typed on the basis of biochemical tests and serological reactivity (Ali *et al.*, 2014). The economic importance of brucellosis requires the use of sensitive and rapid diagnostic methods. At present, the diagnosis of brucellosis in live dairy cattle involves either the isolation of *Brucella* from milk samples or the detection of anti-*Brucella* antibodies in milk (Hamdy & Amin, 2002). Recently, polymerase chain reaction (PCR)-based detection of organisms has been found to be more convenient as compared to cultural isolation. PCR is an option for brucellosis diagnosis; however, a few studies have been carried out with field samples in a way to use the reaction as a diagnostic tool (O'Leary *et al.*, 2006). AMOS (from the initial letters of *abortus*, *melitensis*, *ovis* and *suis*) PCR assay can identify *B. abortus*, *B. suis*, *B. melitensis*, *B. ovis* (Bricker *et al.*, 2003).

The aim of the present study was to evaluate the species and biovar of *Brucella* strains isolated from bovine milk samples collected from Syrian provinces over a period of 6 years using conventional biotyping and specific PCR for the differentiation of *Brucella* species.

MATERIALS AND METHODS

Milk samples

A total of 2372 milking cows were sampled between 2002 and 2007 from 140 cattle herds with abortion history in all Syrian provinces. To get the most reliable results, the milk samples (15 mL) obtained from the animals (in the evening) were kept at 4 °C overnight. Each milk sample was centrifuged at 2000 ×g for 15 min and the cream and deposit were spread on solid selective medium (Anonymous, 2012).

Milk ring test (MRT)

This test was performed by adding 30 µL of antigen (Institute Proquier, Montpellier, France) to 1 mL volume of whole milk that has been stored for at least 24 h at 4 °C. The height of milk column in the tube must be at least 25 mm. A positive reaction was indicated by the formation of a blue ring above a white milk column or at the milk-cream interface. The test was considered to be negative if the ring does not appear and the colour of milk becomes blue.

Bacterial culture

For the isolation, identification and typing of *Brucella* spp., 2YTA (20 g agar, 10 g peptone, 5 g sodium chloride, 5 g meat extract, 1 L distilled water) plates with 5% sterile horse serum, polymyxin B (5 U/mL), bacitracin (25 U/mL) and cycloheximide (100 µg/mL) were used (Parlak *et al.*, 2013). To prepare solid selective media, the basal medium was melted and then cooled to 56 °C in a water bath and stock solutions of the antibiotics were added. Plates were placed in an incubator for 48 h at 37 °C with 10% CO₂ tension adjusted automatically. Bacterial strains were stored in *Brucella* broth (BD, Spark,

USA) at $-20\text{ }^{\circ}\text{C}$ until susceptibility testing.

Identification of *Brucella* isolates

Typing of *Brucella* isolates was made according to the CO_2 requirement, H_2S production, growth in the presence of dyes (thionin and basic fuchsin), and reaction with monospecific sera anti-A, anti-M and anti-R (Parlak *et al.*, 2013). The laboratory personnel working with these isolates wore impermeable protective clothing, gloves, and a face mask during contact with the organism.

Agglutination reaction

An isolated colony have been mixed with a monospecific sera anti-A and anti-M or anti-R (ARCOMEX, Amman, Jordan) on a clean glass slide, agitated gently for 30 s and examined for agglutination.

Bacterial DNA extraction

Brucella DNA was extracted using proteinase K and sodium dodecyl sulfate method. The DNA was purified twice with phenol-chloroform using Phase Lock Gel Heavy tubes (Eppendorf AG, Hamburg, Germany). The DNA was also precipitated and washed, and the pellet was re-suspended in $50\text{ }\mu\text{L}$ of nuclease-free water. Two hundred nanograms of DNA template were used afterwards for PCR amplifications.

Brucella genus-specific DNA amplification

To diagnose the *Brucella* positive samples, the first PCR amplification was carried out using primers (Table 1) designed to target the *bcsP31* gene (B4/B5) which encodes an immunogenic membrane protein of a 31 kDa antigen of *B. abortus* and is conserved in all *Brucella* biovars (Baily *et al.*, 1992). All the primers were obtained from AECS, Damascus, Syria. PCR was performed in a $25\text{ }\mu\text{L}$ mixture containing template DNA; PCR buffer (10 mM Tris HCl with pH 8.4, 50 mM KCl, 1.5 mM MgCl_2); 10 pmoL of each primer; 200 μM (each) of dATP, dCTP, dTTP and dGTP (Bioline, Inc.), and 1.25 U of Taq polymerase (Qiagen, Chatsworth, NJ, USA). The cycle consisted of a preheating step at $95\text{ }^{\circ}\text{C}$ for 5 min followed by 35 cycles of $90\text{ }^{\circ}\text{C}$ for 1 min, $60\text{ }^{\circ}\text{C}$ for 30 s, and $72\text{ }^{\circ}\text{C}$ for 1 min with a final incubation at $72\text{ }^{\circ}\text{C}$ for 10 min. Twenty percent of each PCR product was visualised on a 1% agarose gel stained with $2\text{ }\mu\text{g}/\text{mL}$ of ethidium bromide.

Species-specific *Brucella* DNA amplification

All samples that tested positive using the B4/B5 primers were subjected to second PCR to determine which *Brucella* species might have caused the infection. The sequences of primers used in the study are listed in Table 1. Species-specific DNA

Table 1. Oligonucleotide primers used in this study

Target gene	Sequence 5'-3'	Product size (bp)
<i>bcsP31</i> - B4	TGG CTC GGT TGC CAA TAT CAA	223
<i>bcsP31</i> - B5	CGC GCT TGC CTT TCA GGT CTG	
<i>IS711</i> - <i>B. abortus</i> -F	CAT GCG CTA TGT CTG GTT AC	113
<i>IS711</i> - <i>B. abortus</i> -R	GGC TTTTCT ATC ACG GTA TTC	
<i>IS711</i> - <i>B. melitensis</i> -F	AGT GTT TCG GCT CAG AAT AAT C	252
<i>IS711</i> - <i>B. melitensis</i> -R	ACC GGA ACA TGC AAA TGA C	

segments of *B. abortus* and *B. melitensis* were targeted for amplification using specific primers derived from the IS711 element (Baily *et al.*, 1992; Bricker *et al.*, 1994). Amplification conditions were the same as for the first PCR, except for the use of an annealing temperature of 58 °C. A positive control based on DNA from a *B. abortus* 2308 and *B. melitensis* 16M references strains was included in all tests, as well as a negative control containing DNA from *Yersinia enterocolitica* O9 and *E. coli* O157. PCR products were visualised on a 1% agarose gel as previously described.

RESULTS

Table 2 shows that MRT was positive in 57% of cases (1352 out of 2372 bovine milk samples). The number of milk samples contaminated with *Brucella* after culturing was 596 (25%), and this percentage is considered relatively high.

Table 2. Conventional methods (milk ring test and culture on *Brucella* selective medium) and PCR results of testing bovine milk samples (n=2372)

Method		Number (%)
MRT	negative	1020 (43%)
	positive	1352 (57%)
Culture	negative	1776 (75%)
	positive	596 (25%)
PCR	negative	1776 (75%)
	positive	596 (25%)

Our results demonstrate that the isolates belonged to *B. melitensis* biovar 3 since they are catalase and oxidase positive, produced a trace of H₂S when grown on recommended media; could hydrolyse urea. They could grow in the presence of basic fuchsin or thionin at standard con-

centrations and without needing supplementary CO₂. All bacterial isolates contained smooth surface antigens and reacted in agglutination reactions with the anti-A and anti-M antibodies only (no reaction with anti-R antibodies).

To confirm our results, all bacteria grown on solid selective media were subjected to *Brucella* genus amplification using specific primers that amplify a conserved region in *Brucella*. Agarose gel electrophoresis of the *bcs31* conventional PCR amplification gave a product with size of 223 bp, indicating the presence of *Brucella* genus in milk sample (Fig. 1). The PCR control of the *Brucella* reference strain amplified a product of a similar size. No amplification was detected in the negative control subjects (*Y. enterocolitica* O:9; and *E. coli* O157). No cow brucellosis was detected in the samples which were negative in the MRT (results are not presented).

The DNA from *Brucella* positive milk samples was subjected to the species-specific PCR. The second PCR electrophoresis results are shown in Fig. 1, which illustrates the 113 bp and 252 bp bands specific for *B. abortus* and *B. melitensis*, respectively. Among the milk samples, we detected only *B. melitensis* (100%). *B. abortus*, *Y. enterocolitica* and *E. coli* O157 were not detected in any of the samples. These results confirmed the specificity and sensitivity of these primers for the targeted region in *Brucella* DNA.

DISCUSSION

The epidemiological surveillance of human and animal brucellosis is among the methods considered as a high priority and of essential strategic importance for endemic countries. The Food and Agricultural Organization, the World Health Or-

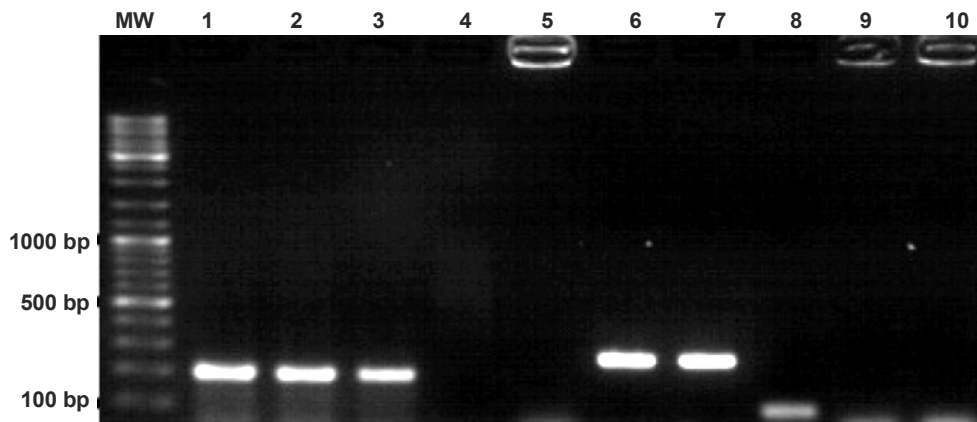


Fig. 1. Agarose gel electrophoresis of PCR assay products. Lane MW: 100 bp ladder (HyperLadder, Bioline, Inc., MA, USA); lanes 1 and 6: *B. melitensis* 16M reference strain (223 bp); lane 2: *B. abortus* 2038 reference strain (223 bp); lane 3: *B. melitensis* from bovine milk sample (223 bp); lanes 4 and 9: *Y. enterocolitica* O:9; lanes 5 and 10: *E. coli* O157; lane 7: *B. melitensis* from bovine milk sample (252 bp), lane 8: *B. abortus* 2038 reference strain (113).

ganization and the World Organisation for Animal Health consider brucellosis as the most widespread zoonosis in the world because it has long been established among livestock in all countries of North Africa and the Middle East including Syria (Anonymous, 2009). The importance of this highly contagious disease is due to: (1) appreciable economic losses caused to the livestock industry in infected areas resulting from abortions, sterility, decreased milk production, veterinary care costs and the cost of culling and replacing animals; and (2) the severe hazard to human health, through either direct contact with infected animals or, more frequently, the consumption of contaminated milk and dairy products (Adone & Pasquali, 2013).

Currently, the diagnosis of brucellosis in bovine milk sample is based almost entirely on milk ring test, which indirectly detects the brucellosis in the host (Abdalla & Hamid, 2012). Numerous studies have shown that the MRT can be used to scan a herd of animals, and further demonstrated

that such reaction possesses sufficient sensitivity and specificity to diagnose *Brucella* in a single animal (Trangadia *et al.*, 2010). In other words, bacteria isolation is necessary to determine whether the animal itself is infected with *B. melitensis*, or it contains only antibodies against such bacteria. The most reliable and the only unequivocal method for the diagnostic of animal brucellosis is based on the isolation of *Brucella* spp. Therefore, the isolation of *B. melitensis* on appropriate culture media is recommended for an accurate diagnosis (Traxler *et al.*, 2013).

PCR is an option for brucellosis diagnosis; however a few studies have been carried out with field samples in a way to use the reaction as a diagnostic tool (Marianelli *et al.*, 2007). In Syria, in spite of the importance of bovine brucellosis, it is important to determine the different *Brucella* biovars among bovines. Therefore, this experiment analysed the presence of *B. melitensis* in milk from the Syrian provinces using microbiological culture, biochemical test and PCR.

We describe a PCR assay that can distinguish *B. melitensis* from *B. abortus*. The assay exploits the polymorphism arising from species-specific localisation of the genetic element *IS711* in the *Brucella* chromosome. Identity is determined by the size(s) of the product(s) amplified from primers hybridising at various distances from the element. Two closely related bacteria (*Y. enterocolitica* O:9 and *E. coli* O157) have been tested negative by the assay. Primer pairs used to identify *Brucella* spp. at the genus-specific level include the primers for sequences encoding BCSP 31(B4/B5) (Baily *et al.*, 1992), 16SrRNA(F4/R2) (Romero *et al.*, 1995), 16s-23S 16S–23S intergenic transcribed spacers (ITS) (Bru ITS-S/Bru ITS-A) (Bricker *et al.*, 2000), 16S-23S rDNA interspace (ITS66/ITS279) (Keid *et al.*, 2007), IS711 (IS313/IS639) (Hénault *et al.*, 2000), per (bruc1/bruc5) (Bogdanovich *et al.*, 2004), omp2 (JPF/JPR) (Leal-Klevezas *et al.*, 1995), outer membrane proteins (omp 2b, omp2a and omp31) (Imaoka *et al.*, 2007), proteins of the omp25/omp31 family of *Brucella* spp. (Vizcaino *et al.*, 2004), and arbitrary primers. In this study, the infection was diagnosed using multiple PCR assay, in addition to the conventional methods (biochemical and cultural tests). The assay described in the present study has several advantages over the conventional methods to identify *B. melitensis* infection. A major advantage is the speed with which the assay can be performed, i.e. within a day. The cultural methods require at least several days. Another major advantage of the multiple PCR assay is that no live *Brucella* organisms are necessarily applied in this assay. This is important because *B. melitensis* is a human pathogen.

The identification of *B. melitensis* biovar level is currently performed by 4 main

tests; i.e., CO₂ dependence, production of hydrogen sulphide, dye (thionin and basic fuchsin) sensitivity, and agglutination with monospecific A and M anti-sera (Fiori *et al.*, 2000). Smooth *B. melitensis* strains are classified into three serotypes: biovar 1 (A–M+), biovar 2 (A+M–), and biovar 3 (A+M+), according to slide agglutination with A and M monospecific polyclonal anti-sera (Liu *et al.*, 2012). This study indicated that bacterial isolates from bovine milk belonged to *B. melitensis* biovar 3. Moreover, Darwish & Bemkerias (2001) reported that the Syrian *Brucella* isolates (between 1990–1996) belonged to *B. melitensis* biovars 2 and 3 in sheep and to *B. abortus* biovar 9 in cattle. In previous biotyping studies in the Middle East (Turkey, Egypt and Jordan), it has been shown that the most common variant was *B. melitensis* biovar 3 using both conventional and molecular methods, (Şimşek *et al.*, 2004; Samaha *et al.*, 2008; İça *et al.*, 2012).

Sheep and goats and their products remain the main source of infection, but *B. melitensis* in cattle has emerged as an important problem in Syria (FAO, 1998).

Most cattle and sheep farms in Syria do not have adequate hygiene precautions, and these animals live in a natural environment together with people (Al-Mariri *et al.*, 2011). In Syria, the sheep production system was based on seasonal movement between the rangelands in the east and southeast, and the dry and irrigated cropping areas in the west where the sheep are grazed on crop residues. This sheep movement allows them to contact cows in Syria farms (FAO, 1998). Therefore, we believe that the prevalence of *B. melitensis* among sheep is the reason behind the infection of cows with *B. melitensis*. In addition, there is another factor to such infection namely the conventional

grazing which is still the prevailing method in Syria, and which increases the percentage of contamination with *Brucella* after each abortion case. Bovine *B. melitensis* infection is emerging as an increasingly serious public health problem because *B. abortus* vaccines (S19) do not protect effectively against *B. melitensis* infection (Lucero *et al.*, 2006). Moreover, this strain does not lose its pathogenicity in cattle (Liu *et al.*, 2012). *B. melitensis* is highly virulent and causes higher abortion rates in cattle and camels and a much more severe human disease than *B. abortus*, the usual agent affecting cattle (Liu *et al.*, 2012). Thus we believe that cows must be vaccinated with Rev. 1 vaccine instead of S19 vaccine because it is more effective against *B. melitensis*. Until now, *B. melitensis* Rev.1 vaccine has not been perfectly evaluated for use in cattle, and therefore there are now difficulties in some countries to entirely eradicate the *Brucella* infection.

In conclusion, this study was designed to evaluate MRT, conventional technique and PCR to detect brucellosis in milk, collected from Syrian cow herds. *B. melitensis* was detected in 1352 (57%) out of 2372 milk samples by MRT, while only 596 (25%) samples were positive by both bacteriological isolation methods and PCR. The findings indicated the sensitivity and the specificity of the PCR assay as a useful tool for the rapid diagnosis of *B. melitensis* in bovine milk.

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