

DETECTION OF *BRUCELLA* SPECIES IN APPARENTLY HEALTHY COWS AND GOATS RAW MILK BY PCR

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ABSTRACT

Brucellosis an intracellular pathogen capable of infecting animals and humans. The aim of this study was to identify *Brucella spp* in apparently healthy cow and goat raw milk samples by a polymerase chain reaction (PCR)-based method. A total of 75 cow milk samples(55 direct and 20 indirect) and 50 goat milk samples were examined by PCR. To establish a PCR protocol for diagnosis of brucellosis, DNA was extracted from the milk samples by using a commercial kit. PCR amplification was done for detection of *Brucella* DNA using *BCSP31* target gene and IS711 locus. The PCR assay showed that an amplicon of 223 bp was obtained in 28% (21/75) and 8% (4/50) samples of cow and goat tested milk using primers (B4/B5) derived from a *BCSP31* gene encoding the 31-kDa *Brucella abortus* antigen. In another PCR, an amplicon of 498 bp was obtained in 100% (21/21) of the brucella genus *BCSP31*-PCR positive cow milk samples using *Brucella abortus*-specific primers derived from a locus adjacent to the 3'-end of IS711, and also an amplicon of 731 bp was produced in 100% (8/8) of the *BCSP31*-PCR positive goat milk samples using *Brucella melitensis*-specific primers. PCR positive results for brucella genus and brucella abortus species were observed in all (100%) cow's milk samples (n=11) with clinical history of abortion. As a single PCR product, no *B. abortus* was detected in goat milk samples also *B. Melitensis* was not detected in cow milk samples.

INTRODUCTION

Brucellosis is an important infectious re-emerging bacterial zoonosis of public health and economic significance. It affects the health and productivity of livestock as well as that of their owners and can have a deep economic impact (1). Brucellosis is

classified among the top seven. world neglected zoonotic diseases (2).The highest annual incidence rates are reported from the Middle Eastern countries, such as Syria, Iraq, Iran, and Saudi Arabia (3; 4).The disease affects cattle, swine, sheep, goats, camels and dogs. It may also infect other ruminants and marine mammals It is an important zoonotic disease and causes significant reproductive losses in sexually mature animals with excretion of the organisms in uterine discharges and milk (5; 6). Brucellae are facultative intracellular coccobacilli belonging to the order Rhizobiales of the α -2 subgroup of Proteobacteria. (7).The genus *Brucella* encompasses 11 accepted nomo-species. Each species was named based on antigenic and biochemical characteristics and primary its host species specificity. The ‘classical’ six species are *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae* which are primarily isolated from small ruminants, bovines, pigs, dogs, sheep and desert wood rats, respectively(8). *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis* are pathogenic to humans. Brucellosis can be transmitted either by direct contact with infected animals and animal excreta or indirect contact through ingestion of contaminated food and water containing large quantities of bacteria (9).

The clinical picture of brucellosis is so strange and protean that it can be easily bewildered with other infectious and noninfectious diseases, leading to diagnostic delays and late onset of therapy (10). Therefore, laboratory confirmation is needed for de- tection of *Brucella*. There are currently three major approaches for the diagnosis of brucellosis including microbiological, serological, and molecular techniques (11). The diagnostic standard remains the isolation of *Brucella* from blood cultures or host tissues (12). However, in the absence of adequate culture facilitates the diagnosis of brucellosis depends on serological tests (11), but the specificity of these techniques are low in endemic areas, in persons exposed professionally to *Brucella* or in patients with relapse or a recent history of brucellosis (13). Alternatively, molecular techniques could be used for diagnosis of brucellosis, especially that these kinds of procedures are useful for diagnosis of several infectious diseases caused by fastidious or slowly growing bacteria and also have detected the small amounts of DNA in different samples (10;14;15).For these reasons, the aim of this study was to develop a one-stage diagnostic PCR test to detect brucellosis in previously aborted and apparently healthy cow milk samples.

MATERIALS AND METHODS

Sample collection

The tested goats(n=50)and 75 cows raw milk samples composed of 55 direct samples(including 11 samples from cows having a history of abortion)and 20 indirect samples. These samples were collected in a sterile cup with a lid from different local markets and household animals in some regions of Basrah governorate during the period from October 2016 to May , 2017.The samples were brought to the laboratory as soon as possible, divided into 0.5 mL of sterile 2-mL Eppendorf tubes, and kept frozen until use (16;17).

Extraction of genomic DNA from milk samples

Total genomic DNA was extracted from milk using a modified method(18)of Favor Prep Genomic DNA Mini Kit(BioNeer, Korea) according to the manufacturer's instructions for blood samples. Concentration and purity of the DNA samples were determined spectrophotometrically (Quawell, USA) by readingA260 and A280.

Brucella genus-specific DNA amplification

To diagnose the Brucella positive samples, the first PCR amplification was carried out using primers designed to target fragment of the bcp31 gene. A pair of primers described previously (19) specific for *Brucella* genus including, (B4: 5' - TGGCTCGGTTGCCAATATCAA-3')as forward primers andB5 reverse primers(B5: 5' -CGCGCTTGCCCTTCAGGTCTG-3) were obtained From(BioNeer, Korea).PCR reaction consisted of 12.5 µL 2x PCR master mixes (promega USA), 1 µg DNA template, 100 nM of each primers, and nuclease free water up to 25 µL. PCR profile was performed on a thermocycler (Techne, UK) using the following parameters: Denaturation primal for 5 minutes 95 °C, 35 cycles of template denaturation for 1 minutes 94 °C, 30 s of primer annealing at 64 °C, and 60 s of primer extension at 72 °C with a final extension cycle for 7 minutes 72 °C. PCR product was visualized on a 1% agarose gel stained with safety dye (Green-DNA DYE; Biotech,USA).

species-specific Brucella DNA amplification

All samples positive using the B4/B5 primers were subjected other primers described previously (20) specific for *Brucella* spp designed to target a 498 and 731bp

fragments including, forward primers(F: 5' -TGCCGATCACTTAAGGGCCTTCAT-3') which were derived from insertion sequence 711 (IS711) unique to identification of *Brucella* species but the reverse primers are different and were derived from *B. abortus*(F: 5' -GAC GAACGGAATTTTTCCAATCCC-3')and *B. melitensis* (R: 5' -AAA TCGCGTCCTTGCTGGTCTGA-3')specific locus on chromosomal DNA. IS711-PCR assay were done in a total volume of 25 μ L containing the same mixture were used for BCSP31-PCR. The amplification programs for *B. abortus* and *B. melitensis* consisted of initial DNA denaturation for 3 min at 95 °C and then cycled 35 times for 90 sec at 95 °C, for 1 min at 65 °C, and for 1 min at 72 °C. Final extension step of 5 min at 72 °C was performed. The reaction products (5 μ L) were detected by electrophoresis on 1.5% agarose gel and visualized with UV transilluminator after staining with Safety dye (Green-DNA DYE; Biotech, USA) to determine the size of amplified products.

Statistical analysis

To demonstrate any association between results, the exact Fisher test and Pearson's chi-squared test with 1 Yates correction were used with the limit of significance being set at 5%. Statistical analysis is done by using SPSS software version 11.

RESULTS

PCR detection of *Brucella* spp in animals milk samples

Cow milk

Total of 55 and 20 direct and indirect cow milk samples respectively were collected from the different regions of Basrah province city to use in this study. The PCR assays resulted in the amplification of 223- bp bands from the targeted *bcsp31* gene of the *Brucella* are shown in Figure 1. Nineteen direct (34.5%) and Two indirect (10%) milk samples were found to be positive for brucellosis. The association between type of milk sample and brucella genus positive results is considered to be statistically significant ($P < 0.05$). (Table.1).

DNA from these *Brucella* positive milk samples were subjected to the IS711-PCR *Brucella* species-specific PCR. PCR electrophoresis results were shown in Figure (2,3), which illustrates the presence of 498 bp bands specific

The number of single product *B. abortus* detected by IS711-PCR was 19(100%) and 2(100%) from respectively direct and indirect milk samples were found to be positive for *B. abortus* only. Among the 19 and 2 direct and indirect milk samples respectively, *B. abortus* alone was evident in all of these tested milk samples (100%) and *B. melitensis* alone was not detected in any of these samples (Table.1). There was no significant difference ($P > 0.05$) in *Brucella* species distribution

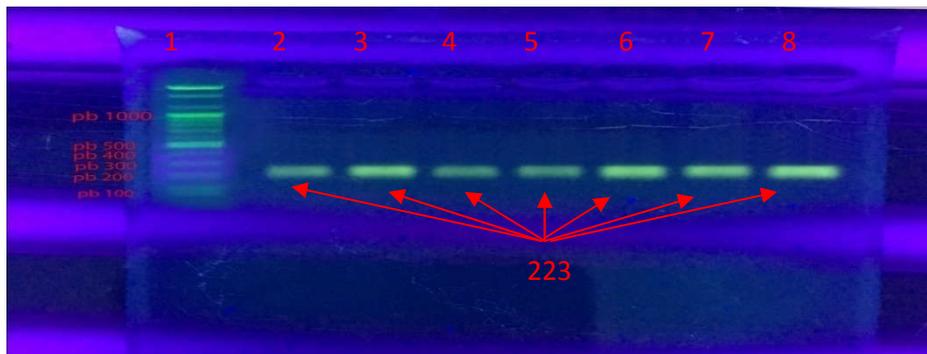


Figure 1 - Identification of DNA amplified fragments by agarose gel electrophoresis and Green-DNA DYE. An amplicon size of 223 bp was obtained by PCR using Baily's primers (B4, B5) and template DNA cow and goat milk. Lanes: 1, 1 kb Ladder; 2, 3, 4, 5, cow milk; 6, 7, 8, goat milk.

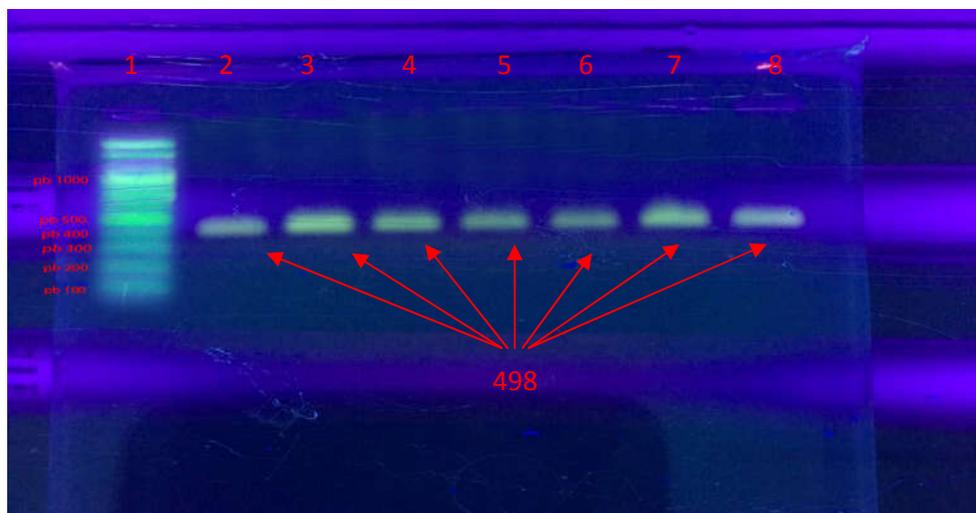


Figure 2 - Identification of DNA amplified fragments by agarose gel electrophoresis and Green-DNA DYE staining. An amplicon of 498 bp was obtained by PCR using *B. abortus*- specific primers and cow milk DNA extract as template. Lanes: 1, 1 kb Ladder; 2-8, positive *B. abortus*.

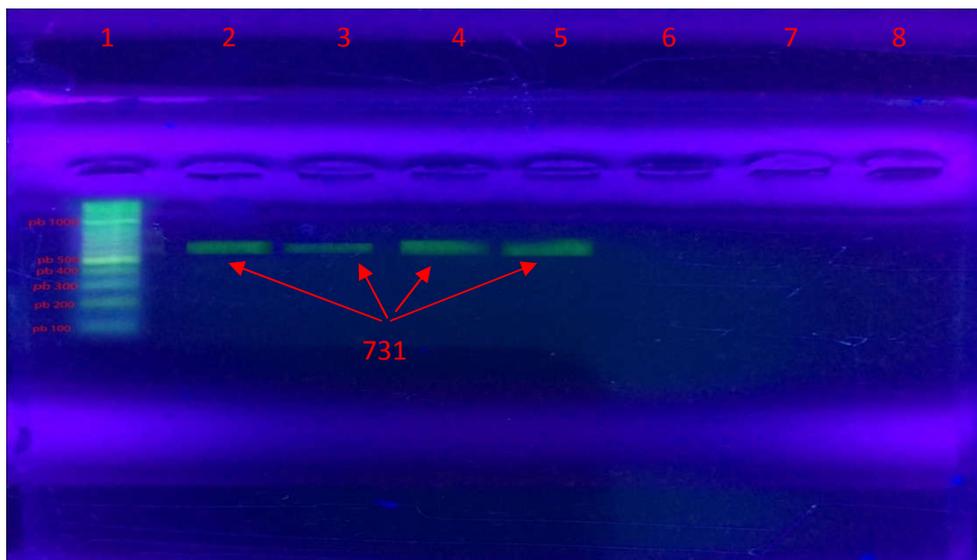


Figure 3 - Identification of DNA amplified fragments by agarose gel electrophoresis and Green-DNA DYE staining. An amplicon size of 731 bp was obtained by PCR using *B. melitensis*- specific primers and goat milk DNA as template. Lanes: 1, 1 kb Ladder; 2, 3, 4, 5, positive *B. melitensis*.

Table-1 PCR amplification for Brucella of the BCSP31 and IS711 target gene with respect to source of cow milk sample

Source of milk samples	PCR for <i>Brucellagenus</i>			PCR for <i>Brucellaspecies</i>		
	-ve amplification n. (%)	+ev amplification n. (%)	P value	Single product <i>B. abortus</i> n. (%)	Single product <i>B. melitensis</i> n. (%)	P value
Direct n.=55	36(65.5)	19(34.5)	0.0438	19(100)	0	1.0000
Indirect n.= 20	18(90)	2(10)		2(100)	0	
Total n.= 75	54(72)	21(28)		21(100)	0	

Effect of clinical status on PCR results

The relationship between positive PCR results and abortion in tested cow was displayed in Table (2). All milk sample 11(100%) obtained from aborted cow showed higher detectable PCR positive result for each of brucella genus and *B. abortus* compared with non aborted animals 8(18.2%). There was highly significant ($P < 0.01$) in the association between PCR positive results and abortion.

Table-2 The relationship between positive PCR results and abortion

PCR results	Source of milk samples			
	Aborted cow tested n.	Positive n.(%)	Non aborted cow tested n.	Positive n.(%)
Brucella genus	11	11(100%)	44	8(18.2)
<i>B. abortus</i>	11	11(100%)	8	8(100%)
Test of significance	$X^2=40.95; DF : 3; P=0$			

Goat milk

Conventional PCR confirmed that 4(8%) out of 50 goat milk samples were diagnosed as Brucella positive. No *B. abortus* was detected in these samples. DNA from the 4 Brucella positive milk samples were subjected to the species-specific IS711 PCR. PCR electrophoresis results are shown in Figure (3) which illustrates the presence of 223 bp and 731 bp bands specific for Brucella genus and *B. melitensis*, respectively. *B. melitensis* amplification product was detected in all four (8%) Brucella genus positive milk sample. There was no significant difference ($P > 0.05$) in Brucella species distribution.

Table-3 PCR amplification for Brucella of the BCSP31 and IS711 target gene in goat milk sample

Milk samples lok	PCR		Total
	-ve amplification	+ev amplification	
PCR for <i>Brucellagenus</i>	46(92)	4(8)	50(100)
Single product <i>B.</i> <i>abortus</i>	0	0	0
Single product <i>B.melitensis</i>	46(92)	4(8)	50(100)
Test of significance	$X^2=2.41$:DF : 2; P=0.299		

DISCUSSION

Molecular diagnosis of brucellosis by PCR techniques has increasingly been used as a supplementary method (21). Genus-specific PCR assays are inexpensive tests for screening and have the capability to detect low concentrations of DNA (14). The current study is the first Iraqi study using the *Bcsp31* and *IS711* target genes for the detection of *Brucella genus and spp* respectively in the milk samples compare to other one Iraqi study using these primers set particularly on blood and serum (22) and other studies previously conducted in different countries used these primers for the detection of *Brucella genus and spp* on the reference strains and clinical samples such as blood and serum (23; 24; 25). On the other hand many studies supporting the usage of these primers in the detection of *Brucella genus and spp* in raw milk (14;26-29).

Total of 55 and 20 direct and indirect cow milk samples respectively were collected from the different regions of Basrah province city used in this study. The PCR assays resulted in the amplification of 223- bp bands from the targeted *bcp31* gene of the *Brucella*. Nineteen direct (34.5%) and Two indirect (10%) milk samples were found to be positive for brucellosis. The present data showed that these assays can be used for risk analysis investigation during routine control of milk, especially as they were able to detect *Brucella* DNA in clinical samples.

The current results demonstrate higher percentage for Genus-specific *bcp31* PCR positivity compare with previous study(30) in which that the Genus-specific *bcp31* PCR amplified *Brucella*-specific DNA from 9/72(12.5%) and 1/15(6.7%) milk samples obtained from cows, and a milk tank, respectively. Other studies also recorded lower PCR positive results compare to the present study .In Pakistan (31) identified *Brucella spp.* at a rate of 9.5% in bovine raw milk, in (32) identified it in cow milk at a rate of 5.31%, and in Turkey (33) identified it in bovine raw milk at a rate of 2%. (34) have been found that only 4 /215 (1.86%) samples was *Brucella* positive. On the other hand the present results were lower than previous study(14) in which the number of brucellosis positive samples detected by *bcp31*-PCR was 317 (94.9%) out of 334 milk samples. Also Romero *et al.* (35) determined that 87.5% of the milk samples were positive for *Brucella* DNA . This conflict in the results may be explained by different PCR programs, sample type and preparation, storage conditions, and DNA extraction procedures.

Beside that the current study found the prevalence of *Brucella* in indirect milk samples was determined to be low (10%) compare with 34.5% of direct milk samples. This may not mean that all of these animals are healthy or not infected with *Brucella spp.* The pathogen *Brucella* can be located in the lymph nodes of animals and may not be transferred or reach the milk during the time is sample taken, or there may be a low number of bacteria present in these milk samples (36). Shedding in milk can be prolonged or lifelong or may be intermittent (37).

Among the 19 and 2 direct and indirect milk samples respectively, *B. abortus* alone was evident in all of these tested milk samples (100%) and *B. melitensis* alone was not detected in any of these samples. Based on previous publication about brucellosis in Iraq, this study is the first to record direct detection for *B.abortus* DNA in milk samples of cows. This finding dis agreement with previous (38,30,15)in which that the *B. Melitensis* DNA can be PCR amplified from bovine milk samples, the present study confirmed negative *B. melitensis* DNA amplification.

Brucella organisms were not isolated in this study. *Brucella* culturing is hazardous,. Isolation rate is very low even in experienced laboratories(30) . The probability of successful isolation of *B. abortus* markedly reduced when a few organisms are present in the samples or the material is heavily contaminated. Negative culture results cannot exclude infection with *Brucella* (37). Nevertheless, clinical

presentation, abortion and strong seropositive results finally led to the diagnosis of brucellosis. Serological diagnosis from freshly aborted animals may fail because antibody titers against *B. abortus* rise only 1–2 weeks after infection (39), however circulating *Brucella* DNA may be detected with molecular techniques.

Cows infected with *B. abortus* usually abort only once, and following that give birth to healthy or weak calves. Some cows may not exhibit any clinical signs of the disease and give birth to healthy calves (40). Those animals can be the source of continual infection (41). In infected herds, PCR may be a very valuable tool in reducing the time to eradicate the disease by identifying anergic shedders or newly infected animals that should be removed from the herds immediately. As such in the current study although all tested milk samples obtained from apparently healthy cows, PCR positive results for brucella genus and brucella species were observed in all (100%) previously aborted 11 cows. The current result is in agreement with the previous study (15) in which that all serum samples collected from aborted cows (n=10), buffaloes (n=5), ewe (n=1) and goats (n=9) were positive with the genus specific bcp31 real-time PCR assays. But other results of (15) oppose the present results concerning the identification of *B. abortus* DNA in all serum samples collected from cows, buffaloes, ewe and goats. The present results also is in agreement with previous study (29) in which that out of 273 cow milk samples 18 (6.6%) samples were positive with the genus and spp (*B. abortus*) specific bcp31 and IS711 PCR assays and no *B. melitensis* was detected in cow milk samples.

Conventional PCR confirmed that 4 (8%) out of 50 milk samples collected from apparently healthy goat diagnosed as *Brucella* positive. The *B. melitensis* amplification product was detected in all four (8%) *Brucella* genus positive milk sample. The present results demonstrate agreement

with previous study (29) in which that out of 90 goat milk samples 3 (3.3%) samples were positive with the genus and spp (*B. melitensis*) specific bcp31 and IS711 PCR assays and no *B. abortus* was detected in goat milk samples also *B. melitensis* was not detected in cow milk samples. However Ali *et al.* (42) study who provides first evidence that *Brucella abortus* the causative agent of brucellosis in small ruminants in Pakistan. In addition 17/24 (71%) were positive in the *Brucella* genus-specific (bcp31) and *Brucella abortus*-specific (IS711) qRT-PCR.

Furthermore(43) found species-specific PCR assays have a lower analytical sensitivity than do genus-specific PCRs and. *B. abortus* was confirmed as the causative agent of ovine and caprine brucellosis in previous studies using PCR assays .This conflict may be explained by different PCR programs, sample type and preparation, storage conditions, and DNA extraction procedures.

B. melitensis one of the major causes of abortion in small ruminants; other ruminants may be infected occasionally (44). It is also the main agent responsible for brucellosis in humans, as it is highly virulent for humans. Circulation of this species in goats milk is of special concern to public health; control and eradication programs have to be adapted to this special situation accordingly. As such, species-specific PCRs are valuable tools in screening programs to identify the prevalent *Brucella* species. Transmission of *Brucella* through contaminated milk and milk products is an increasing threat not only for individuals, but also for whole families in urban and rural settings of endemic countries (45). In these areas, trade of non-pasteurized fresh milk and raw dairy products should be strictly controlled and limited to certified *Brucella*-free farms The present results showed that PCR is a sensitive tool for the control of brucellosis in raw milk. Basic health.

CONCLUSIONS

the present study adds to the data available regarding *B. abortus* and *Brucella melitensis* infections in cattle and goats and highlights the effectiveness and advantages of PCR in detection of brucellosis in raw milk.

الكشف عن نوع البروسيليا في الحليب الخام للأبقار والمعز السليمة ظاهريا بواسطة PCR

إيناس اسماعيل جابر ، فوزيه علي عبدالله

الخلاصة

البروسيليا كائن ممرض داخل خلوي يصيب الانسان والحيوان .ان الهدف من هذه الدراسة هو تعيين نوع البروسيليا في عينات حليب الابقار والمعز بواسطة طريقة تفاعل البلمرة المتسلسل. فحصت عينات حليب الحيوانات السليمة من المرض ظاهريا وبمجموع خمسة و سبعون عينة (55عينة مباشرة و 20 عينة غير مباشرة) من حليب الابقار و50عينة من حليب المعز بطريقة تفاعل البلمرة المتسلسل PCR ولتأسيس نظام PCR خاص بتشخيص مرض الحمى المتوجه تم استخلاص الحامض النووي DNA من عينات الحليب باستخدام عدة استخلاص تجاريه . للكشف عن البروسيليا بطريقة PCR تم تضخيم الحامض النووي DNA باستهداف

المورث BCSP31 والموقع IS711. اظهرت نتائج فحص PCR ان ناتج التضخيم ذو الحجم 223 bp قد لوحظ في (21/75) 28% و (4/50) 8% من عينات حليب الابقار والمعز على التوالي وباستعمال البادئات (B4/B5) المشتقة من المورث الذي يشفر لمستضد البروسيللا ذو الوزن الجزيئي 31-kDa. اظهر فحص PCR اخر اجري على عينات حليب الابقار التي اظهرت نتائج ايجابية في فحص BCSP31-PCR حيث استخدمت البادئات الخاصة بنوع *Brucella abortu* المشتقة من موقع IS711 بالقرب من النهاية 3'-end ان ناتج تضخيم بحجم 498 bp قد لوحظ في (21/21) 100% من عينات الحليب وكذلك ظهر ناتج تضخيم بحجم 731 bp في (8/8) 100% من عينات حليب المعز الموجه بفحص BCSP31-PCR وباستعمال البادئات الخاصة بنوع *Brucella melitensis*. النتائج الموجهة لجنس ونوع الروسيلا لوحظت في جميع عينات حليب الابقار (100%;n=11) التي لها تاريخ سريري للاجهاض. لم يتم الكشف عن *B. abortus* في حليب المعز وكذلك لم يكشف عن وجود *B. melitensis* في حليب الابقار

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