Assiut Vet. Med. J. Vol. 60 No. 140 January 2014

USING POLYMERASE CHAIN REACTION TECHNIQUE (PCR) FOR DETECTION *BRUCELLA MELITENSI*S IN ABORTED EWES' MILK IN NINEVEH, IRAQ

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ABSTRACT

Received at: 26/12/2013

Accepted: 13/2/2013

In this study, polymerase chain reaction technique (PCR) used to detect *Brucella melitensis* in aborted ewes' milk in Nineveh Province, Iraq using species specific primers of *Br. melitensis.* Fifty aborted ewes were involved. Blood and milk samples were taken from each ewe. Blood samples used to detect Brucella antibodies in serum using Rose-Bengal plate test (RBPT), whereas PCR were applied on the milk samples to detect *Br. melitensis* using one pairs of specific primers for the strain of *Br. melitensis.* Results indicate 35 out of 50 samples (70%) positive reaction by RBPT. From these 35 samples: 26 samples (74.3%) were positive by PCR, while 9 samples (25.7%) were negative. Samples that showed negative reaction by RBPT (15 out of 50 "30%") revealed 8 samples (53.3%) positive by PCR and 7 samples (46.7%) were negative. The total number of milk samples that gave positive reaction by PCR (*Br. melitensis*) were 34 out of 50 (68%) distributed on Nineveh's regions between 50–100%. The study concluded that using an accurate tests, such as species-specific PCR technique, is an important for reflecting an accurate incidence; since PCR revealed that *Br. melitensis* existed in aborted ewe's milk in a high percentage in Nineveh, Iraq, which has a public health importance.

Key word: *Brucella melitensis, sheep, milk, PCR*

INTRODUCTION

Brucellosis is a contagious bacterial infection of livestock, with important effects on both public health and animal health and production. It is continues to be of great health concern and economic importance worldwide (Anonymous, 2001; Ibrahim *et al*., 2012). Infection in animals frequently results in abortion and diminished milk production (Culter *et al*., 2005). Brucella organisms localize in the supra-mammary lymph nodes and mammary glands in 80% of infected animals, which continue to excrete these pathogens in their milk throughout their lives and have a significant role in the epidemiology of the diseases (Abd El Razik *et al*., 2007). Therefore, man is infected by animal's brucellosis through direct or indirect by ingestion of animal products as after drinking raw milk or eating unpasteurized cheese (Celebi *et al*., 2007).

The disease presents a great variety of clinical manifestations, making it difficult to diagnose clinically. Therefore, the diagnosis must be confirmed directly by isolation, or indirectly by the detection of immune response against its antigens (Anonymous, 2001). Bacteriological methods depends on the viability and numbers of *Brucella* in the sample. Thus, culture methods are not always successful as they are time-consuming and the handling of microorganism is hazardous (Refai, 2003; Kazemi *et al*., 2008) and serological methods are not conclusive; because not all infected animals produce significant levels of antibodies, and because cross-reaction with other bacteria can give false negative results (Abd El Razik *et al*., 2007; Ilhan *et al*., 2008). However, these methods are not wholly satisfactory, PCR assay has been used as an accurate and sensitive assay for detection of *Brucella spp* in a wide variety of clinical samples; such as, blood, milk, semen, and aborted fetuses even if samples contain low number of brucella (Anonymous, 2001; Dehkordi *et al*., 2012).

Bucellosis in Mosul, Iraq is a widespread, and many researches have been performed using various diagnostic methods in different samples and different animal species (Al-Farwachi *et al*., 2009; Al-Obaidi *et al*., 2009; Rhaymah *et al*., 2010 and Al-Farwachi *et al*., 2010). The diagnosis of brucellosis in sheep using serum and bacterial culture by PCR has been done in Mosul (Mohammad, 2006), while *Brucella spp* antibodies in raw milk has been detected using ELISA in sheep and goat (Al-Obaidi *et al*., 2009). The purpose of this study was to investigate the viability of the species specific PCR assay as a diagnostic tool for the detection of *B. melitensis* DNA in the milk of sheep after abortion using specific primers and comparing its results with the Brucella antibodies in serum using Rose-Bengal test in the same animal in Nineveh Province, Iraq.

MATERIALS and METHODS

Fifty aborted ewes were involved in this study. Blood and milk samples were taken from each ewe. Blood samples used to detect Brucella antibodies in serum using

Rose-Bengal plate test (RBPT), whereas polymerase chain reaction technique (PCR) were applied on the milk samples to detect *Brucella melitensis* using one pairs of specific primers for the strain of *Br. melitensis.*

Preparation of milk samples for PCR:
Milk samples were prepared to improve extraction and purification of bacterial DNA from milk according to Romero and Lopez-Goni (1999): 10 mL of milk centrifuged at 3000 g/min for 15 min. The supernatant was removed and discarded. The pellet was resuspended in 500 µl sterile distil water, mixed with 100 µl buffer (1 mM EDTA, 10 mM Tris-HCl, 50 mM NaCl, 10% sodium dodecyl sulfate and 5 µl of proteinase K), Vortex and incubated in water bath at 65° C for 5 min, then centrifuged for 5 min at 3500 g/min**.**

DNA extraction:
Br. melitensis DNA was extracted using commercial DNA extraction kit (Sacace Biotechnologies Srl, Italy). According to manufacture instructions, 1.5 ml polypropylene tubes were used, 300 µl of lysis solution and 100 µl of the samples added to each tube, Vortex and incubated at 65° C for 5 min. All tubes centrifuged at 14000g for 5 min. The supernatant carefully transferred by a micropipette into new clean and sterile tubes, then 20 µl of Sorbent added to each tube and incubated at room temperature for 3 min, then centrifuged at 5000g for 30 sec. The supernatant carefully removed and discarded. Thereafter, 300 µl washing Solution 1 added to each tube, vortex vigorously and centrifuged at 8000g for 30 sec., then the supernatant removed and discarded. Then 500 µl washing solution 2 added, vortex vigorously and centrifuged at 8000g for 30 sec, then the supernatant removed. The pellet was resuspended in 50 µl of DNA-eluent, incubated at 65° C for 5 min and vortex periodically. The tubes centrifuged at maximum speed 12000g for 1 min to recover the DNA, which existed in the supernatant and became ready for amplification.

DNA Amplification:
For amplifying *Br. melitensis* DNA, the primers were used as an essential PCR elements which give bands (731 bp), and the primers sequences were 5'-AAA, TCG, CGT, CCT, TGC, TGG, TCT, GA-3' and the reverses were 5'-TGC, CGA, TCA, CTT, AAG, GGC, CTT, CAT-3' (Mohammad, 2006). The amplification reaction mixture (final volume: 25 µl) includes: 2.5 µl PCR buffer [10 mM Tris-HCl (pH 8), 50 mM KCl , 1.5 mM $MgCl_2$], 2.5 µl deoxynucleoside triphosphate (200 µM) (Germany, Eppendrof), 2.8 µl foreword primer, 2.6 µl reverse primer, 1 U of *Taq* DNA polymerase (Germany, Eppendrof), and 100 ng genomic DNA. The volume was completed to 25 µl with deionized distil water.

The amplification was carried out by the thermalcycler (Germany, Eppendrof) according to the commercial kit (Sacace Biotechnologies Srl, Italy). The following program was started when the temperature reaches 95° C: one cycle at 93° C for 5 min, 40 cycles of 90° C for 60 sec, 60 °C for 60 sec, 72° C for 60 sec. the final incubation was one cycle at 72° C for 10 min.

Analysis of results was based on the presence or absence of specific bands of amplified DNA in agarose gel 2% (U.S.A., Ammersham Bioscience) stained with ethidum bromide. The length of specific amplified DNA fragments for *Br. melitensis* (731 bp) was visualized using Gel documenting system.

Statistical Analysis
The data was analyzed statistically using SPSS 16.0 (SPSS, 2007). The Goodness-of-fit between RBPT and PCR was measured by McNemar's Chi-square test (Ott and Longnecker, 2004), whereas the agreement between the two was identified using Kappa Statistic (Viera and Garrett, 2005).

RESULTS

The total number of animals that gave positive reaction by RBPT was 35 out of 50 serum samples (70%), while by PCR was 34 out of 50 milk samples (68%). Statistically, this result indicates no significant difference between RBPT and PCR ($P<0.01$).

The agreement between RBPT and PCR was measured by comparing the results of the two tests in each animal of this study. The observed agreement was 0.66 (66%), while Kappa was 0.206 indicating slight agreement between the two tests (Table 1).

Table 1: Comparison between the number and percentage of the RBPT and PCR results in 50 sheep.

RBPT / PCR	Positive	Negative	PCR Total Result
Positive	26 (74.3%)*	8 (53.3%)	34 (68%)
Negative	9 (25.7%)	7 (46.7%) **	16 (32%)
RBPT Total Result	35 (70%)	15 (30%)	50

* indicates the number of animals that gave positive result in both RBPT and PCR in samples from the same animal.

** indicates the number of animals that gave negative result in both RBPT and PCR in samples from the same animal.

DNA amplification of *Br. melitensis* using Species-specific Primers electro-phorized on (1.2 %) agarose gel indicates positive sample that showed single band of 731 bp (Fig. 1).

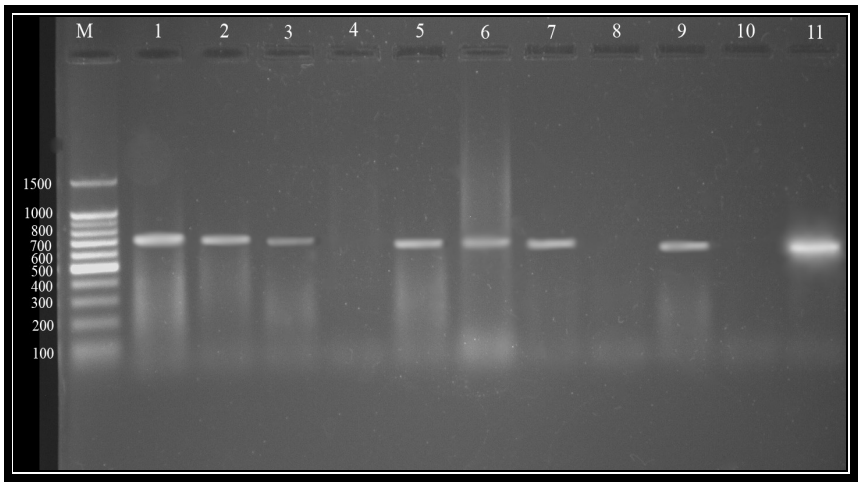

Fig. 1: Result of DNA amplification of *Br. melitensis* using Species-specific Primers electrophorized on (1.2 %) agarose gel. A single band of 731 bp in lanes (1, 2, 3, 5, 6, 7, 9, 11) indicates positive sample, while lanes (4, 8, 10) representing negative samples. Lane (M) indicates the DNA Ladder 100bp as DNA marker.

DISCUSSION

Brucellosis is a major zoonotic disease endemic in many parts of the world. It is characterized by chronic infection in animals leading to abortion and infertility. The disease is caused by *Brucella* which is gram negative, non spore forming, facultative intracellular organism (Cardoso *et al.,* 2006).

In the present study, a PCR has been compared with one of serological methods for detection of brucellosis, RBPT is the most sensitive and rapid screening test used in detection of Brucella infection in animals, but it may produce false-positive and false-negative results (Ilhan *et al*., 2008). PCR has been used as an accurate and sensitive assay for detection of *Brucella spp* in a wide variety of clinical samples even if samples contain low number of brucella (Anonymous, 2001; Dehkordi *et al*., 2012).

In this research, there were differences between results of RBPT and PCR (Table 1). Studying milk samples using PCR to same animals their serums gave positive reactions by RBPT indicates that 74.3% positive with both tests (Table 1), which confirm that the infection of that animals caused by *Br. melitensis*, since we used one pairs of specific primers for the strain of *Br. melitensis.* Furthermore, there was 25.7% of milk samples presented negative reaction by PCR, while their samples gave positive reaction by RBPT (Table 1), which may assign to antibodies of other bacteria, not *Br. Melitensis*, or may produced due to *B. abortus* S19 vaccination or exposure to gram-negative bacteria, including include *Vibrio cholerae* O1, *E. coli* O:157, *Salmonella* group N (O:30) and *Yersinia enterocolitica* O:9, which has lipopolysaccharide (LPS) O-chains similar to those of brucellae (Charisis, 1998, OIE, 2004, Munoz *et al*., 2005).

Furthermore, Out of 15 animals their serum negative by EBPT there were 8 (53.3%) milk samples positive by PCR (Table 1) which may indicates early incubation of disease that results in false negative RBPT (Radostits *et al*., 2007). Furthermore, the false negative of RBPT in this research me referred to testing of animals immediately after abortion, since many of owners test their animals immediately after abortion. On the other hand, there were 7 milk samples (46.7%) present negative reaction by PCR out of 35 animals their serum also negative by EBPT (Table 1).

According to results of present research, the percentage of *Br. melitensis* infection in aborted ewes in Nineveh is 68%, which support by Anonymous (2001) who indicates that *Br. melitensis* is the main etiological agent of brucellosis in small ruminants. Furthermore, presence of positive results on milk sample by PCR means that the animals shed the bacteria in their milk, since PCR detect the organism itself (Ilhan *et al*., 2008) and the PCR was found highly sensitive and specific for identification of *Brucella* from milk even if as low as 40 cells/ml of milk (Romero *et al.,* 1995).

The study concluded that using an accurate tests, such as species-specific PCR technique, is an important for reflecting an accurate incidence; since PCR revealed that *Br. melitensis* existed in aborted ewe's milk in a high percentage in Nineveh, Iraq, which has a public health importance.

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Assiut Vet. Med. J. Vol. 60 No. 140 January 2014

استخدام تقنية تفاعل البلمرة المتسلسل (PCR) لتشخيص جرثومة البروسيلا المعزية في حليب النعاج المجهضة في محافظة نينوى – العراق

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في هذه الدراسة تم استخدام تقنية تفاعل البلمرة المتسلسل (PCR) للكشف عن جرثومة البروسيلا المعزية في حليب النعاج المجهضة " في محافظة نينوى وباستخدام بادئات نوعية متخصصة لجرثومة البروسيلا المعزية. تضمنت الدراسة فحص خمسون نعجة مجهضة ، أخذت عينات الدم والحليب من كل نعجة من هذه النعاج المجهضة ، استخدمت عينات الدم للكشف عن الأجسام المضادة بعد الحصول على مصل الدم بواسطة اختبار الروز بنغال (RBPT)، في حين تم تطبيق تقنية PCR على عينات الحليب بعد استخلاص دنا جرثومة البروسيلا وتضاعفه باستخدام زوج من البادئات المتخصصة. وتشير النتائج أن ٣٥ من مجموع ٥٠ عينة (٧٠٪) أعطت نتيجة إيجابية بواسطة اختبار RBPT. من هذه العينات ٣٥ الموجبة كان هناك ٢٦ عينة موجبة (٧٤.٣٪) باستخدام تقنية الـ PCR ، في حين كانت هناك تسعة عينات سالبة وشكلت نسبة (٢٥.٧٪). العينات التي أظهرت نتيجة سالبة في فحص RBPT وهي بحدود (١٥ من أصل ٥٠ "٣٠٪") طبقت عليها تقنية الـ PCR كان منها ثمانية عينات (٥٣.٣٪) موجبة في فحص الـ PCR و ٧ عينات (٤٦.٧٪) كانت سالبة. العدد الكلي للعينات الحليب التي أعطت نتيجة إيجابية بفحص الـ PCR كان ٣٤ من أصل ٥٠ (٦٨٪) موزعة على مناطق نينوى بين ٥٠-١٠٠٪. استنتجت الدراسة إلى أن استخدام اختبارات دقيقة ، تقنية تفاعل البلمرة المتسلسل للنوع المتخصص Species-specific PCR مهم جدا ليعكس نسبة حدوث الإصابة الدقيقة ، وأن كشف تواجد هذه الجراثيم في حليب النعاج المجهضة وبنسبة عالية في نينوى والعراق له أهمية كبيرة على الصحة العامة.