Iraqi Journal of Veterinary Sciences

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Serological and molecular investigations of brucellosis in dairy cows at certain areas of Al-Sulaymaniyah governorate, Iraq

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Article information

Article history:
Received July 18, 2020
Accepted April 16, 2021
Available online October 1, 2021

Keywords:
Brucellosis
iELISA
PCR
RBPT

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Abstract

This study aimed to detect *Brucella* antibodies in the sera of dairy cows and to identify *Brucella* species in the milk of seropositive cows. A total of 100 sera and 100 milk samples were collected from two 50-cows groups (group 1 with and group 2 without a history of reproductive problems and/or decreased milk production). Rose Bengal plate test and indirect ELISA were used to explore *Brucella* antibodies in the serum samples and thereafter milk samples of seropositive cows were undergone PCR analysis using *Brucella* genus specific primers and 3 pairs of species specific primers for identification of *B. abortus*, *B. melitensis* and *B. suis*. The RBPT showed 22 cows were carriers for the *Brucella* antibodies, 18 in group 1 and 4 in group 2 whereas the iELISA showed only 10 cows out of these 22 cows were positive, 9 in group 1 and only 1 cow in group 2. The PCR assay, which was performed on milk samples of the RBPT positive cows, revealed 18 samples were positive for the *Brucella* genus and the *Brucella abortus* species and were negative for *Brucella melitensis* and *Brucella suis* species. As a conclusion, the results of this study showed that brucellosis has been encountered in cows with or without a history of reproductive problems, and the RBPT followed by PCR assay for milk samples of the seropositive cows could provide more specific detection than performing either test alone and could be more useful for rapid screening of brucellosis in dairy cows.

DOI: 10.33899/ijvs.2021.127688.1520,

Introduction

Brucellosis is an animal disease with a significant zoonotic potential worldwide (1) and in Erbil (2). It causes considerable economic losses in the field of animal production due to abortion or the full-term birth of dead or weak neonates and due to the marked reduce in the levels of fertility and milk production (3). It is caused by gram-negative, non-motile, coccobacilli bacterial of the genus *Brucella* which includes *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae* (4). In addition, 2 more species have been reported in marine mammals including *B. cetaceae* in dolphins and whales and *B. pinnipediae* in seals (5). There are various serological tests used as screening tests for detection of brucellosis such as Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT), Enzyme Linked Immuno Sorbent Assay (ELISA) and several other serological tests (6). However, because of limitations of using these conventional serological tests for confirmatory detection of the fastidious *Brucella* pathogens, nucleic acid amplification techniques such as the polymerase chain reaction (PCR) offers a reliable diagnostic tool for the detection of brucellosis. This technique is characterized by high sensitivity and specificity, promptness and safety (7). Few studies were conducted on brucellosis in our region (Al-Sulaimaniyah Governorate, Iraq), therefore, the current study may represent a new addition to the information on

brucellosis in this region through the detection of brucellosis in dairy cows using sero-diagnostic tests (RBPT and iELISA) and identification of *Brucella* species in the milk of sero-positive cases using conventional PCR.

Materials and methods

Study areas and animals

Blood and milk samples were collected during the period extended from November 2014 to May 2015 from two groups of local breed dairy cows: group one included 50, 3-7 years old cows with a history of abortion, stillbirth, reduced milk production and/or reproductive problems from certain regions surrounding Al-Sulaimaniyah city including Garmk, Saidsadiq, Bngrd, Kalar and sharazur and group two included 50, 2-5 years old cows without such a history in 4 dairy farms in Tanjaro region. Cows of both groups were non-vaccinated against brucellosis.

Blood sampling and serum preparation

After disinfecting by 70% ethyl alcohol, 8-10 ml blood were collected from the jugular vein and poured slowly into a sterile test tube. The samples were coded and transferred to the laboratory with minimal delay in an insulated ice box. Sera of these blood samples were prepared after centrifugation at 2500 rpm for 10 min and 1-2 ml of each serum sample were collected by a disposable pipette, dispensed into two sterile Eppendorf tubes and stored at -20 ˚C for subsequent testing by the Rose Bengal Plate test and iELISA.

Milk sampling

Twenty-two milk samples were taken from the seropositive cows as recommended by the OIE Manual (8). Briefly: The whole udder was washed, dried, and the teats' tips were disinfected with swab of 70% ethyl alcohol and wiped to dry starting with teats on the far side of the udder. Following that, the milk samples were collected starting from the near side's teats and then moving to the far side's teats after discarding the first one or two milk streams. Approximately 10-20 ml of milk were collected from the 4 quarters of each cow into a labeled, sterile, screw capped vial and transferred to the laboratory by an insulated ice box. In the lab, the milk samples were stored at -20ºC in the freezer (Angelantoni, Italy) and subsequently used for DNA extraction.

Serological detection of Brucellosis by the rose bengal plate test

The Rose Bengal Plate test was performed for all of the 100 serum samples according to the manufacturer's instruction as follows: The serum samples to be tested and the Rose Bengal reagent were left for de-freeze at 18-26ºC for about 30 minutes; following that, the reactants were mixed by dispensing a 25 µl aliquot of each serum sample and a 25 µl aliquot of the Rose Bengal *Brucella* antigen on the kit's plate using a distinct pipette tip for each serum sample. The mixture was thoroughly mixed by inverting and swirling and the results were checked out by naked eyes after 4 minutes. The serum sample was considered positive if a distinctive agglutination was evident (IDEXX, USA).

Serological detection of Brucellosis by the iELISA

The iELISA test was achieved to detect the IgG in the sera of the cows using a ready to use kit according to the manufacturer's instruction (IDEXX, USA). The reagents, serum samples and positive and negative serum controls were brought to 18-26 °C before use. The optical density values of the samples and controls were measured using 96-well Microtiter plate ELISA reader equipped with a 450 nm filter (Biotech Company, USA). The obtained results were recorded, calculated and interpreted according to the manufacturer's instruction.

Genomic DNA extraction from milk samples

DNA extraction from the milk samples of the RBPT positive cows and from two positive control *Brucella* strains, *B. abortus* vaccine strain 19 (Vital, Turkey) and *B. melitensis* vaccine strain Rev.1 (Jovac, Jordan Bio-industries center) was performed using the Geneaid DNA extraction kit according to the manufacturer instructions (Geneaid, South Korea). The obtained DNA extracts were checked out by agarose gel electrophoresis and stored at -20°C until PCR technique use.

Conventional PCR technique

Four pairs of primers (*Accupower*®Bioneer, South Korea) were specifically used for DNA amplification (Table 1). The PCR mixture comprised 2 µl 10X PCR buffer (2.5 U *Taq* DNA polymerase, 2 mM $MgCl_2$, dNTPs (200 µM each)), 10 pmol (1 µl) of each of the forward and reserved primers, 10 ng (4 µl) of the template DNA and up to 20 µl nuclease-free de-ionized water. The amplification was carried out in a thermal cycler throughout an initial denaturation at 95°C for 5 min; 35 thermal cycles of denaturation at 90°C for 40 seconds, annealing at 60°C for 20 seconds for the genus specific primers and 58°C for 30 seconds for the species specific primers, and extension at 72°C for 40 seconds; and a final extension at 72°C for 7 min. Following that, the PCR products were processed for electrophoresis in 1% agarose gel stained with Green DNA dyes and visualized by a UV transilluminator at 320 nm.

Statistical analysis

Statistical analysis of the results was performed using the SPSS software version 19.0 (9), the Chi square test for independence (10) and Z-test for proportions (11). P values less than 0.05 were considered significant.

Table 1: List of primer pairs used in the PCR assay

Primers	Gen name	Target gene	Sequence 5' -3'	size (bp)	References
Brucella genus (general)	B4 (F) B5 (R)	BCSP 31	TGG-CTC-GGT-TGC-CAA-TAT-CAA CGC-GCT-TGC-CTT-TCA-GGT-CTG	223	(12)
B. abortus	F R	IS711	CATGCGCTATGTCTGGTTAC GGCTTTTCTATCACGGTATTC	113	(13)
B. melitensis	F R	IS711	CATGCGCTATGTCTGGTTAC AGTGTTTCGGCTCAGAATAATC	252	(13)
B. suis	F R	IS711	GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT	285	(14)

Results

Serological detection of Brucellosis by RBPT and iELISA

The results of the current study showed that the RBPT was significantly (P<0.05) different compared to the iELISA in serological detection of brucellosis. In group one (cows with a history of abortion, reproductive problems and/or decreased milk production), the RBPT showed that 18 out of 50 cows 36% were carriers for the *Brucella* antibodies in comparison with the iELISA test which revealed only 9 positive cases %18. In group two (only 4 positive cases 8% out of 50 cows were evident by the RBPT compared to only one positive case 2% was evident by the iELISA (Table 2, Figures 1 and 2).

Table 2: Serological detection of brucellosis by the RBPT and iELISA

Animal groups	No. of samples	RBPT positive	ELISA positive
Group one cows *	50	18 (36%) [a]	9 (18%) [b]
Group two cows **	50	4 (8%) [a]	1 (2%) [a]
Total	100	22 (22%) [a]	10 (10%) [b]

* Cows with a history of abortion, reproductive problems and/or decreased milk production. ** Cows without a history of abortion, reproductive problems and/or decreased milk production. Within a row, the positive RBPT and iELISA results that do not have similar small letter superscripts ([a] and [b]) vary from each other (P<0.05).

PCR analysis

Out of the 22 milk's DNA extracts of the RBPT-positive cows, 18 were shown to be positive for the *Brucella* genus-specific gene BCSP31 as indicated by amplification of the 223 bp DNA fragment (Table 3, Figure 3). These 18 milk's DNA extracts were also shown to be positive for the *B. abortus*-specific gene IS711 as indicated by amplification of the of the 113 bp DNA fragment (Figure 4). However, they were negative for the *B. melitensis* and *B. suis* specific genes. The remaining 4 milk's DNA extracts were shown to be negative for *Brucella* genus-specific gene BCSP31, *B. abortus*, *B. melitensis* and *B. suis* specific genes.

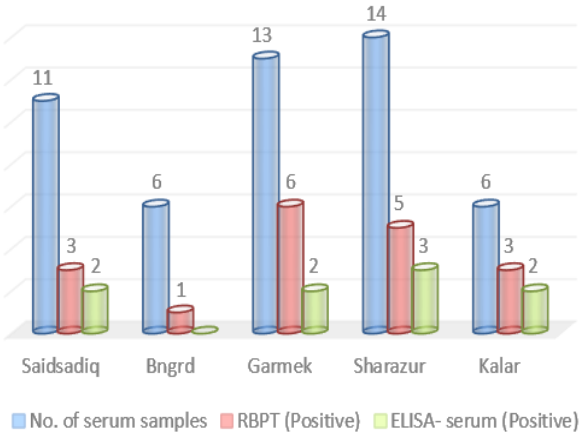

Figure 1: Serological detection of brucellosis by the RBPT and iELISA in cows of group one (cows with a history of abortion, reproductive problems and/or decreased milk production).

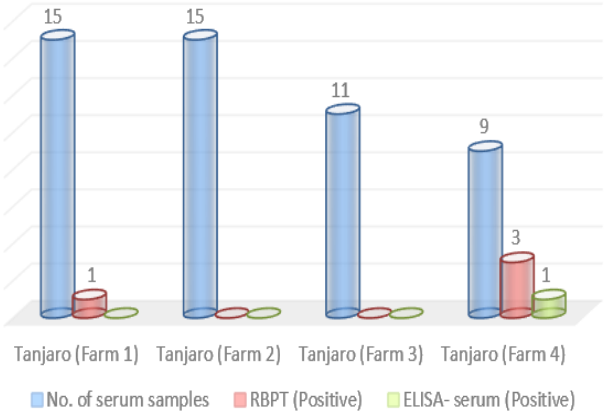

Figure 2: Serological detection of brucellosis by the RBPT and iELISA in cows of group two (cows without a history of abortion, reproductive problems and/or decreased milk production).

Table 3: PCR results of the milk's DNA extracts of the RBPT-positive cows

Animal groups	No. of seropositive cows (by RBPT)	No. of PCR positive cows
Group one cows *	18 [a]	14 [b]
Group two cows **	4 [a]	4 [a]
Total	22 [a]	18 [b]

* Fifty cows with a history of abortion, reproductive problems and/or decreased milk production. ** Fifty cows without a history of abortion, reproductive problems and/or decreased milk production. Within a row, the RBPT and PCR results that do not have similar small letter superscripts ([a] and [b]) vary from each other (P<0.05).

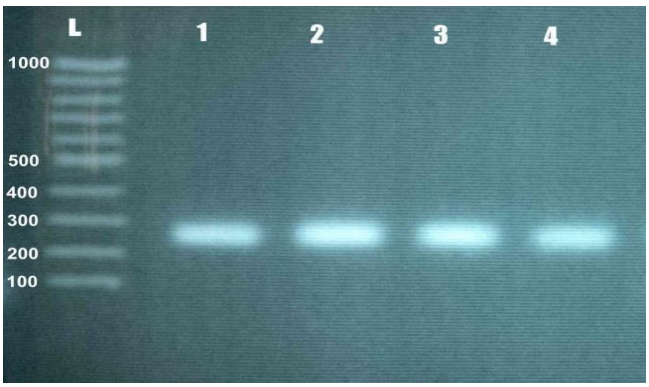

Figure 3: Agarose gel electrophoresis of the amplified milk's DNA extracts of the RBPT-positive cows. L: 100 bp DNA Ladder, Lane 1: Positive control (*B. abortus* S19), Lanes 2-4: Milk's DNA extracts positive for the 223 bp DNA fragment of the *Brucella* genus-specific gene BCSP31.

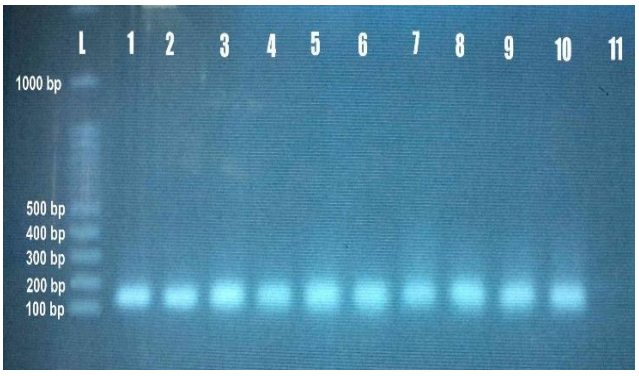

Figure 4: Agarose gel electrophoresis of the amplified milk's DNA extracts of the RBPT-positive cows. L: 100 bp DNA Ladder, Lanes 1-9: Milk's DNA extracts positive for the 113 bp DNA fragment of the *B. abortus*-specific gene IS711, Lane 10: positive control (*B. abortus* S19), Lane 11: negative control.

Discussion

The current study showed a lower sensitivity of the iELISA compared to the RBPT in detection of the seropositive cows is in agreement with the results of Zakaria (15) and it indicates that the RBPT could be more sensitive rather than more specific in comparison with the iELISA in serological detection of brucellosis (16) due to the higher possibility of false positive reaction that might take place as a result of concurrent or previous infection with some other gram-negative bacteria particularly *Yersinia enterocolitica* serotype O: 9; thus, it has been recommended that the serum samples that reveal a positive result by the RBPT should be certified by more specific tests (17). In addition, the higher sensitivity of RBPT compared to iELISA in the present study can be attributed to the fact that the RBPT is a screening test which qualitatively detects both the IgM and IgG antibodies whereas the iELISA used in this study is a quantitative test that specifically detects only IgG antibodies (18). Moreover, according to instructions of the iELISA kit manufacturer (IDEXX, USA), all of the serum samples were diluted to 1:100 resulting in a decrease in the concentration or quantity of antibodies in comparison with the RBPT procedure applied in this study which was performed without serum dilution.

The application of PCR-based assays for detection and identification of *Brucella* species has been increased due to their accuracy, sensitivity, speed and ability to work with DNA rather than the highly infectious live cultures (7). The PCR assays were proved to be a good means for rapid and accurate diagnosis especially for slow growing bacteria like *Brucella* (18), *Mycobacterium paratuberculosis* (19), *Shigella* (20), lactic acid bacteria (21), and the most proteolytic active bacteria as *Aeromonas hydrophila* (22) and to detect *Brucella* DNA from clinical specimens, thus the DNA detection of pathogenic organisms have been rendered biologically safe and reducing the risk of infection of laboratory workers (14).

In the present study, the PCR assay was used for detection of *Brucella* genus in the milk samples of the RBPT positive cows using the *Brucella* genus-specific primer pair B4/B5 which amplify the *BCSP31* gene 223bp that codes for a 31-kDa immunogenic outer membrane protein conserved among all *Brucella* species. The BCSP 31 gene based PCR assay is a highly sensitive and specific means widely used for detection of brucellosis in clinical samples (12). The PCR assay revealed that 18 out of the 22 milk samples of the RBPT positive cows were positive for the genus *Brucella*, whereas the remaining 4 milk samples were negative. This finding, which is in agreement with Moussa *et al*. (7) and El-Diasty *et al*. (23), can be attributed to the possibility of the periodic shedding of *Brucella* organisms in the milk of the infected animals and to the probability of false negative results that may encountered in the PCR assays particularly in the chronic cases due to presence of a number of *Brucella*

organisms below the threshold of detection by the specific primers (24). In addition, there are several other factors that can result in false negative results in the PCR assays such as degradation of target DNA in the sample, milk components such as Ca^{2+}, proteinase, fats, polysaccharides, and milk proteins which may act as inhibitors for the nucleic acid amplification by shielding DNA from polymerase access and/or presence of polymerase inhibitors such as hemoglobin, heparin, phenol, EDTA, and sodium dodecyl sulfate (25).

PCR assays based on three pairs of species-specific primers for *B. abortus*, *B. melitensis* and *B. suis* were also performed in the present study in order to identify the species of *Brucella* in milk samples of the RBPT positive cows. The results of these PCR assays revealed *B. abortus* as the only *Brucella* species identified in milk samples of the seropositive cows. This finding can be ascribed to the fact that the cows included in this study were raised separately from sheep and goat flocks. In addition, vaccination programs using the *B. melitensis* Rev-1 strain have been applied on sheep and goat flocks. These measures could result in restriction of *B. melitensis* transmission from sheep and goats to cattle and decrease the probability of pastures contamination and spread the disease to the other animal herds or areas (8,18).

Conclusion

Serological tests such as RBPT and iELISA followed by PCR assay for milk samples of the seropositive cows could provide more specific detection than performing either test alone and could be more useful for rapid screening of brucellosis in dairy cows.

Acknowledgments

We are grateful to the Directorate of Veterinary Hospital in Al-Sulaimaniyah governorate, Iraq for providing the laboratory facilities to perform the iELISA and PCR assays. We also thank Dr. Peshnyar Atta for providing the technical assistance and facilities to achieve the PCR assay.

Conflict of interest

The authors declare that there is no conflict of interest.

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التحري المصلي والجزيئي لداء البروسيلات في أبقار الحليب في مناطق معينة من محافظة السليمانية - العراق

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الخلاصة

كان الهدف من هذه الدراسة الكشف عن الأجسام المضادة لداء البروسيلات في مصل أبقار الحليب والتعرف على نوع جرثومة البروسيلا في حليب الأبقار المصابة. تم جمع ١٠٠ عينة مصل و ١٠٠ عينة حليب من مجموعتين من ٥٠ بقرة (المجموعة الأولى مع والمجموعة الثانية بدون تاريخ من المشاكل التناسلية أو انخفاض إنتاج الحليب). أستخدم فحص الروز بنغال والاليزا الغير مباشر للكشف عن الأجسام المضادة للمرض في عينات المصل، وبعد ذلك أجري فحص تفاعل البلمرة المتسلسل لعينات حليب الأبقار التي أظهرت مصولها نتائج موجبة باستخدام البادئات الخاصة بجنس البروسيلا و ٣ بادئات خاصة بالأنواع للتعرف على البروسيلا المجهضة و البروسيلا المالطية و البروسيلا الخنزيرية. أظهر فحص الروز بنغال أن ٢٢ بقرة كانت حاملة للأجسام المضادة للمرض، ١٨ في المجموعة الأولى و ٤ في المجموعة الثانية بينما أظهر فحص الاليزا أن ١٠ أبقار من أصل ٢٢ كانت حاملة للأجسام المضادة، ٩ في المجموعة الأولى وبقرة واحدة في المجموعة الثانية. أما فحص تفاعل البلمرة المتسلسل فقد أظهر أن ١٨ عينة حليب فقط كانت موجبة لجنس البروسيلا ولنوع البروسيلا المجهضة فيما كانت جميع العينات سالبة لنوعي البروسيلا المالطية والبروسيلا الخنزيرية. دلت نتائج هذه الدراسة على أن داء البروسيلات قد تم اكتشافه في الأبقار مع أو بدون تاريخ من المشاكل التناسلية، وأن فحص الروز بنغال متبوعًا بفحص تفاعل البلمرة المتسلسل لعينات حليب الأبقار إيجابية المصل يمكن أن يوفر فحصا أكثر دقة من إجراء أي منهما على حدة ويمكن أن يكون أكثر فائدة في التحري السريع عن مرض البروسيلا في أبقار الحليب.

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