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Single-Gene Phylogeny of Brucella Melitensis in Milk Samples of Ewes in Kalar District, Iraqi Kurdistan Region

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Abstract

The present study aimed to investigate the frequency rate of ovine brucellosis in Kalar district, Iraqi Kurdistan Region and to identify Brucella species in the milk of seropositive ewes via DNA sequencing and phylogenetic analyses. Blood samples were collected from 300 ewes and subjected to the Rose Bengal Plate Test (RBPT), and milk samples of the seropositive animals were collected and underwent a PCR assay using genus- and species-specific primers. Eventually, the positive PCR products were processed for nucleotides sequencing and single-gene phylogenetic analyses. The RBPT revealed a high percentage (11.33%) of seropositive ewes (34 out of 300), and the PCR assay revealed 35.3% (12/34) Brucella genus-positive DNA templates. At the species level, 58.3% (7/12) of templates were positive for the melitensis-specific primers and negative for the abortus and ovis - specific primers, whereas the other five were negative for all species-specific primers used in this study (abortus, melitensis and ovis). The nucleotides sequence of Brucella strains detected in this study showed a 99-100% homology with eight GenBank-published B. melitensis strains, and the single gene phylogenetic analysis showed a close relationship between these strains and the B. melitensis biovar 3 strain NI, China. The high percentage of seropositive ewes showed that brucellosis represents a major obstacle to the animals' production improvement and a major risk to public health in the Iraqi Kurdistan Region and the single gene phylogenetic analysis confirms its transboundary spread and attracts attention for more strict control programs.

Keywards: Brucellosis, PCR, Rose Bengal Test, Single-Gene Phylogeny.

التحليل الوراثي الجيني المفرد لجراثيم *Brucella melitensis* في عينات حليب النعاج في منطقة كلار، إقليم كردستان العراق

الخلاصة

هدفت الدراسة الحالية إلى دراسة معدل تكرار الإصابة بداء البروسيلات في النعاج في منطقة كلار، إقليم كردستان العراق وتحديد أنواع البروسيلا في حليب النعاج إيجابية المصل من خلال تسلسل الحمض النووي والتحليل الوراثي الجيني المفرد (single gene phylogenetic analysis). تم جمع عينات الدم من 300 نعجة وإخضاعها لاختبار الروزبنگال (RBPT) ومن ثم جمعت عينات حليب من الحيوانات التي أظهرت نتائج إيجابية لهذا الفحص وأخضعت لتفاعل البلمرة المتسلسل (PCR) باستخدام بادئات خاصة بجنس وأنواع البروسيلا وبعد ذلك تم فحص تسلسل نيوكليوتايدات (nucleotides sequencing) لمنتجات تفاعل البلمرة المتسلسل PCR products وإجراء التحليل الوراثي الجيني المفرد). أظهر اختبار الروزبنگال نسبة عالية نسبيا (11.33%) من النعاج المصابة (34 من أصل 300) وكشف اختبار تفاعل البلمرة المتسلسل أن 12 نموذج DNA من مجموع الـ 34 نموذجاً المستخلصة من عينات حليب النعاج التي أظهرت نتائج إيجابية لاختبار اختبار الروزبنگال كانت إيجابية لجنس البروسيلا. وعلى مستوى الأنواع، أظهرت 7 نماذج DNA نتائج إيجابية للبادئات الخاصة بالـ *B. melitensis* في حين أظهرت النماذج الخمسة الأخرى نتائج سلبية لجميع البادئات الخاصة بالأنواع المستخدمة في هذه الدراسة (*B. abortus*, *B. melitensis* and *B. ovis*) . وقد أظهر تسلسل النيوكليوتيدات لسلالات البروسيلا المسجلة في هذه الدراسة تماثلًا بنسبة 99-100٪ مع 8 من سلالات *B. melitensis* المسجلة في بنك الجينات وأظهر التحليل الوراثي الجيني المفرد وجود علاقة وثيقة بين السلالات المسجلة في هذه الدراسة مع سلالة *B. melitensis* biovar 3 NI الصين. إن النسبة العالية نسبيا من الاصابة بداء البروسيلات المسجلة في هذه الدراسة تظهر ان هذا المرض يمثل عائقًا كبيرًا أمام تحسين الإنتاج الحيواني وخطرًا كبيرًا على الصحة العامة في إقليم كردستان العراق، ويؤكد التحليل الوراثي الجيني المفرد انتشاره عبر الحدود وضرورة تطبيق برامج أكثر صرامة للسيطرة عليه.

Introduction

Brucellosis is a major bacterial zoonotic disease that affects animals and humans all over the world, especially in developing countries (1). Human's greatest incidence of this disease is largely dependent on the animal reservoir and the high rates of infection reported in cattle, goats, sheep, pigs and to lesser extent dogs (2). It is transmitted mainly from infected animals to humans through the consumption of contaminated raw animal products or through direct contact with an animal's secretions or visceral organs (3). Brucellosis can be caused by six classical species: B. melitensis, B. abortus, B. ovis, B. suis, B. canis and B. neotomae (4), of which the first three can be responsible for inducing the disease in sheep (5), whereas the majority of reported human cases are caused by B. melitensis, B. abortus, B. suis, and B. canis in a descending rate of occurrence (6).

In Iraq, the disease is endemic and represents an actual risk for both human and livestock populations and real obstacle for the improvement of animal sector as it causes massive economic losses in farm animals due to the significant rates of abortion which usually occurs during the last third of pregnancy, in addition to the infertility and stillbirths that may associate the disease (7-9).

Molecular detection and identification of B. abortus and B. melitensis in human blood samples and in serum & milk samples of different farm animal species by the conventional and real time PCR assays showed significant rates of the infection in some of the north and middle Iraqi provinces (10-13) and the phylogenetic analysis of the 16S rRNA gene revealed that B. melitensis strains detected by the RT-PCR in blood samples of aborted ewes shared a common ancestor and were genetically related to B. melitensis strains reported in the USA, Greece, China, and Nigeria (13).

In Kalar district at the centre of Garmian Administration, Iraqi Kurdistan Region, which is 30 km from the Iranian border, only a few studies have been conducted on ovine brucellosis, thus this study, which represents a new supplement to the previous brucellosis studies in this region, aimed to determine the frequency rates of brucellosis in sheep in Kalar district using the RBPT and to detect and identify Brucella species in milk samples of seropositive cases by means of conventional PCR assay, DNA sequencing and molecular phylogenetic analysis.

Materials and Methods

Study area and samples

Blood and milk samples were collected from 300 randomly-selected ewes from 63 flocks in 22 villages in Kalar district, Iraqi Kurdistan Region.

Blood sampling and serum preparation

Blood samples were collected from the jugular vein. Approximately 8-10 ml of blood was collected from each animal. The samples were coded and transferred in an insulated ice box to the clinical pathology laboratory at Garmian Veterinary Directorate. For serological examination, sera were prepared according to Stockham and Scott (14) by centrifugation of the blood samples at 2500 rpm for 5-15 min. Following that, 1-2 ml of the serum were pipetted from each blood sample and stored in a sterile test tube at -20 ˚C until used in the RBPT.

Milk sampling

Milk samples were taken from the ewes according to Stockham and Scott (14). Approximately 10–30 ml of milk was obtained from the 2 quarters of each ewe and collected in a labeled, sterile, screw- capped vial. The milk

samples were directly stored in an insulated ice box and transferred to the laboratory where they were preserved at -20˚C until used for DNA extraction.

Serological detection of brucellosis using the rose bengal plate test (RBPT)

The RBPT was performed for the 300 serum samples according to the manufacturer's instruction (SPINREACT Company, Spain) as follows: Both the Rose Bengal reagent and serum samples were first brought to room temperature; one drop of the serum sample to be tested and one drop of both the positive and negative control sera were placed into separate circles on the test slide; thereafter, one drop of the reagent was added to each of the three circles. Each drop was mixed with the corresponding serum with a stick and spread over the entire surface of the circle. Different sticks were used for each circle. The slide was rotated manually and the results were noted by naked eyes immediately after four minutes.

Genomic DNA extraction from the milk samples

The genomic DNA was extracted from milk samples of the seropositive ewes (which gave a positive reaction to the RBPT) and from the live attenuated vaccine of B. melitensis strain Rev.1 "Jovac, Jordan Bio-Industries center" using a DNA extraction kit according to the manufacturer's instructions (Bioneer, South Korea).

PCR amplification of Brucella genus and Brucella species-specific DNA targets

The B4 and B5 primer pairs (BIONEER Inco., South Korea) were used in this study for amplification of the Brucella genus-specific gene BCSP31 (which encodes for a 31-kDa outer membrane protein conserved among all Brucella species) in milk samples of seropositive ewes (Table 1). The PCR assay was performed in 20 µl ready to use PCR tube that contains DNA polymerase, dNTPs, a tracking dye and reaction buffer in a premixed format, freeze-dried into a pellet (AccuPower PCR PreMix, BIONEER Inco., South Korea). Four µL template DNA, 1 µL of each of the forward and reverse primers (10 µM), and 14 µL of DNase-free, distilled water were added to PCR tube. DNA sample extracted from the live attenuated vaccine of Brucella abortus strain 19 (Vital, Turkey) was used as the positive-control, and a sterile, nuclease free water was used as the negative control. The PCR was carried out in a conventional thermocycler (BIONEER MyGenie96) as follows: initial denaturation at 95°C for 5 minutes, 45 cycles of denaturation at 95°C for 1 minute, annealing at 65°C for 30 seconds and extension at 72°C for 1 minute and a final extension at 72°C for 7 minutes.

DNA extracts of the milk samples that displayed positive results for the PCR detection of the BCSP31 gene of the Brucella genus were exposed to three more PCR assays using three species-specific primer pairs ((BIONEER Inco., South Korea) derived from specific insertion sequences of the IS711 gene for identification of the B. abortus, B. melitensis and B. ovis species (Table 1). The PCR assay was achieved in a total volume of 20 µL containing the same mixture used for detection of the Brucella genus. The amplification profiles consisted of an initial denaturation at 95°C for 3 minutes, 45 cycles of denaturation at 95°C for 90 seconds, annealing at 65°C for 1 minute, and a final extension at 72°C for 5 minutes. The amplified PCR products were analyzed by electrophoresis on an ethidium bromide-stained 1.5% agarose gel and photographed using a UV trans-illuminator (Clinx Science Instruments Co., Ltd., Shanghai).

Gene sequencing and phylogenetic analysis

The PCR products (amplicons) were purified using the QIAquick PCR Purification Kit (Qiagen) according to manufacturer instructions and about 40 µl of each amplicon together with the corresponding genus-specific or species-specific primer pairs were sent to Macrogen Inc, South Korea. The DNA sequencing was performed using the Sanger sequencing technology (17) and the obtained nucleotide sequences were aligned with each other and with the database of sequences using the BLAST of the National Centre for Biotechnology Information (NCBI) and the Clustal Omega software (18).

A single gene phylogenetic tree was performed based on the nucleotides sequence of the Brucella species-specific gene IS711 obtained in this study. The nearest match of the obtained nucleotide sequence was determined by the BLAST analysis and a phylogenetic tree was reconstructed by the neighbor joining method (19) using the molecular evolutionary genetics analysis (MEGA) version 7 (20).

Results and discussion

Serological detection of brucellosis

Serological detection is the main diagnostic means for brucellosis (21), and the RBPT has been specified in many countries as the standard serological test used for screening of the disease (22). The RBPT showed that 34 out of the 300 ewes involved in this study (11.33%) were seropositive for brucellosis (Table 2). This relatively high percentage aligns with the high prevalence of animal brucellosis in underdeveloped and developing countries (23,24) and in communities with poor socioeconomic status, especially in Middle Eastern countries (25-27) and it indicates that this disease represents a significant hindrance to improving farm animal production and poses a substantial risk to public health in the Kurdistan Region of Iraq.

PCR detection of the Brucella genus in milk samples of seropositive animals

Out of the 34 DNA templates extracted from milk samples of the seropositive ewes involved in this study, only 12 templates showed a positive result for the conventional PCR assay, which was achieved using the Brucella genus-specific primer pair (B4/B5) for amplification of the BCSP31 gene (Table 3, Figure 1). This finding indicates that some of the Brucella organisms in milk DNA extracts of seropositive ewes could not be detected by the PCR assay, is compatible with findings of Abd Al-Azeem et al, (28) and it can be attributed to the intermittent excretion of the Brucella organisms in the milk of infected animals (29,30), degradation of target DNA in the serum samples (31) or to the presence of few Brucella organisms below the detection limit of the conventional PCR assay (31,32).

PCR detection of Brucella species

The DNA extracts of the 12 milk samples that showed positive results for the genus-specific primers were additionally used as templates for identification of Brucella species (B. abortus, B. melitensis, and B.ovis) using species-specific primers derived from specific insertion sequences of the IS711 gene. Seven samples out of these 12 milk samples showed positive results with the B. melitensis-specific primers (Figure 2), whereas the remainder 5 samples displayed negative results with all the species-specific primers used in this study (B. abortus B. melitensis, and B. ovis). The non- successful amplification of the IS711 gene by these 5 milk samples which already showed positive results for the genus-specific primers can be attributed to the detection limit of the species-specific

PCR which is lower than the detection limit of genus-specific PCR. This finding is in agreement with that of Garshasbi et al. (32) who stated that the PCR sensitivity for the Brucella genus was 96% compared to 82% sensitivity for the species-specific PCR and it also in agreement with Moussa et al., Baddour and Al-Khalifa, Ghodasara et al. (31, 33, 34) who proved that the Brucella genus-specific primer pair B4/B5 is more sensitive than the species-specific primers in the detection of Brucella organisms.

Gene sequencing and phylogenetic analysis

Based on sequence analysis of the genus-specific gene BCSP31 of the Brucella strains detected in this study, a 99-100% homology was found with B. melitensis biovar 2 strain 63/9 and B. melitensis biovar abortus strain 3196 (published in the GenBank under accession numbers CP007789.1 and CP007707.1 respectively). Similarly, the sequence analysis of the species-specific gene IS711 of the Brucella strains revealed a 99-100% homology (Figure 3) of the Brucella strains detected in the current study with six B. melitensis strains reported in China, USA, India and Iran (published in the GenBank under accession numbers CP002931.1, CP001851.1, CP001488.1, JF939171.1, JF939145.1, and DQ845343.1) which, apart from the USA, are considered endemic regions for brucellosis (35-37). In addition, the single gene phylogenetic analysis (Figure 3) of the nucleotide sequence of the species-specific gene IS711 of Brucella strains detected in this study (published in the GenBank under the accession number MT875166.1) showed that these strains are closely related (99-100%) to the B. melitensis biovar 3 strain NI, China (accession # CP002931.1). These findings confirm the transboundary nature of Brucella organisms spread (38-40), throughout China, India Iran and Iraqi, and they attract attention for the territorial, national, and international disease control programmes to apply strict biosecurity measures on animal transportation, particularly across international borders (41), and to improve the livestock owners knowledge regarding vaccination programmes, animal health management practices, and quarantine rules in order to reduce the vulnerability of their animals to the disease (42).

Conclusion

The high percentage of seropositive results observed in this study showed that brucellosis represents a major obstacle to the improvement of farm animals' production and a major threat to the public health at Kalar district in the Iraqi Kurdistan Region and the phylogenetic analysis confirms its transboundary spread and attracts the attention for more strict programmes to control the disease or at least to reduce its incidence among human and animals.

Acknowledgment

The authors would like to express their deepest appreciation and gratitude to the Directorate of Veterinary Hospital in Sulaimaniyah city in the Kurdistan Region of Iraq for their kind cooperation in providing laboratory facilities for performing the molecular part of this study.

Conflict of interest

The authors declare that they have no conflict of interest.

Table 1: List of the PCR primers pairs used in the present study

Primers	Target gene	Primer's name	Sequence 5' -3'	PCR product size (bp)	References
Brucella genus (general)	BCSP31	B4 (F)	5'-TGG-CTC-GGT-TGC-CAA-TAT-CAA-3'	223	(15)
		B5 (R)	5′-CGC-GCT-TGC-CTT-TCA-GGT-CTG-3′		
B. abortus	IS711	F	5′-GAC-GAA-CGG-AAT-TTT-TCC-AAT CCC-3′	498	(16)
		R	5′-TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-3′		
B. melitensis	IS711	F	5′-AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA-3′	731	(16)
		R	5′-TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-3′		
B. ovis	IS711	F	5′-CGG-GTT-CTG-GCA-CCA-TCG-TCG-3′	976	(16)
		R	5′-TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-3′		

Table 2: Serological and PCR assay findings of brucellosis in blood and milk samples

Type of sample	Test	No. of tested samples	No. of positives
Blood	RBPT*	300	34 (11.33%)
Milk	PCR**	34	12 (35.3 %)

* RBPT: Rose Bengal Plate test

** PCR: Conventional PCR assay

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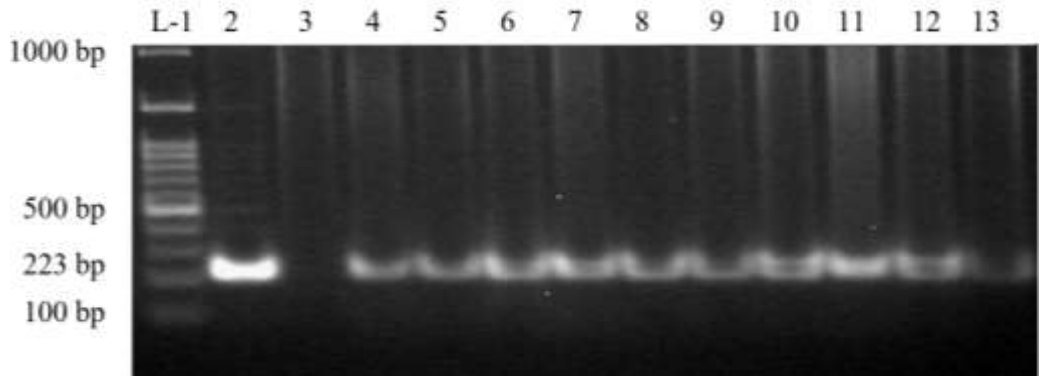

Figure 1: Agarose gel electrophoresis showing amplification of BCSP31 gene after using the genus-specific primers pair (B4/B5). Lane 1: DNA ladder, lane 2: positive control (DNA of the live attenuated vaccine of *B. abortus* strain 19), lane 3: negative control (DNases and RNases free water), lanes 4-13: DNA extracts obtained from milk samples of the seropositive ewes.

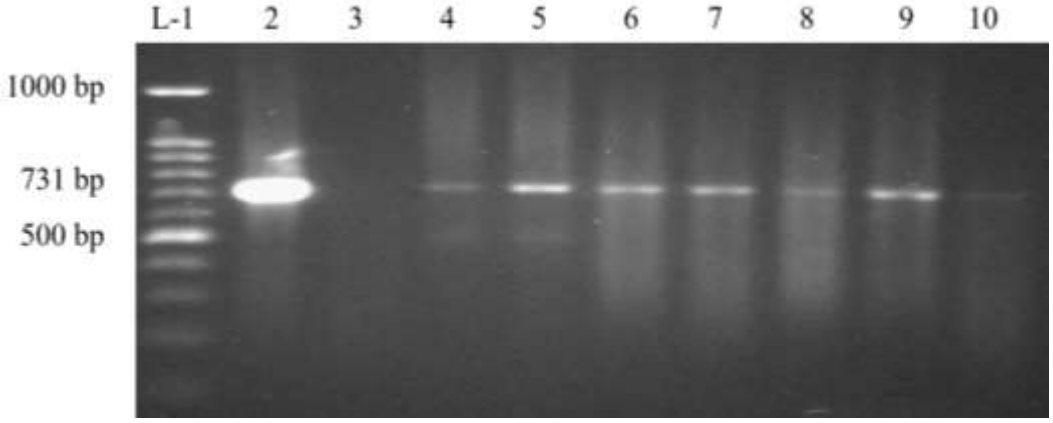

Figure 2: Agarose gel electrophoresis showing amplification of the IS711 gene after using a *B. melitensis* specific primers pair. Lane 1: DNA ladder, lane 2: positive control (DNA of the live attenuated vaccine of *Brucella melitensis* strain Rev 1), lane 3: negative control (DNases and RNases free water), lanes 4-10: test DNA extracts obtained from milk samples that displayed positive results for *Brucella* genus.

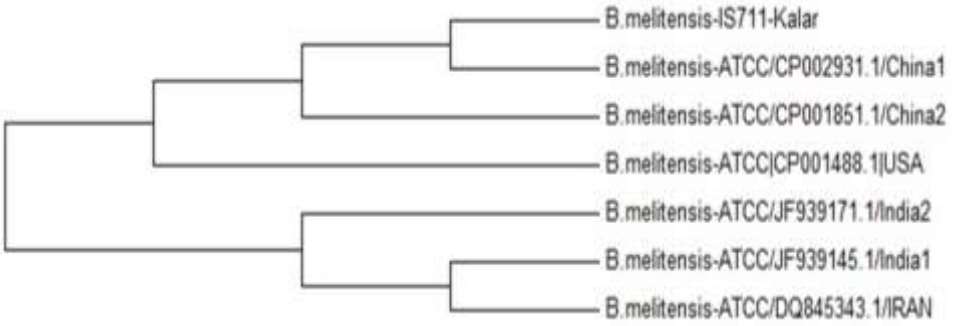

Figure 3: A dendrogram revealing 99-100% homology, based on the species-specific gene IS711 sequences, between *B. melitensis* strains (obtained in the current study from milk samples of ewes in Kalar district, Iraqi Kurdistan Region (accession # MT875166.1) and *B. melitensis* biovar 3 strain NI, China (accession # CP002931.1).

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