Molecular Detection of Outer Membrane Protein 2 Gene in Isolated Brucella from Sheep and Goats in Sharazar/Kurdistan Region of Iraq

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ABSTRACT

Brucellosis is an economically important zoonotic disease that is endemic in different parts of Iraq. An outbreak of abortion occurred in sheep and goats in Sharazar subdistrict, Sulaymaniyah, Iraq and Brucella infection was suspected to be the cause. Between November 1, 2014, and January 31, 2015, 47 tissue and vaginal swab samples were collected from 17 herds. Samples from each herd were mixed together to make 17 pooled samples. The Brucella genus-specific primer Bruc was used to amplify the 223 bp amplicon of bscp31 gene. Fifteen of the 17 samples (88%) were positive for Brucella genus. Thereafter, five samples were randomly selected to identify the species by designing four sets of Brucella-specific species primers. Sequencing of the Outer Membrane Protein 2 (omp2) gene was performed and the sequences were aligned with others in the GenBank databases. The results showed that the omp2 gene of the five isolates from Sharazar had 99-100% similarities with Brucella melitensis bv. 2 that was isolated from United States (accession number CP007789) and B. melitensis strain 20236 (accession # CP008750) isolated from human in China. The amino acid sequences of the omp2a gene in the five isolates were similar with other isolates in GenBank with the exception of B. suis bv. 2 (accession # CP007720). This study reported the occurrence of B. melitensis for the first time in Sharazar subdistrict, Sulaymaniyah, northern Iraq, using molecular techniques.

INTRODUCTION

Brucellosis is one of the most widespread zoonotic diseases infecting many animal species and humans. The causative agents are Gram-negative bacteria that belong to the genus Brucella (de Figueiredo et al., 2015). The most common pathogenic species worldwide are B. abortus in cattle, B. melitensis (mainly infecting sheep and goats), and B. suis in pigs. These species may cause abortion and result in huge economic losses in the infected animals (Seleem et al., 2010; Atluri et al., 2011).

A high level of homology among the currently recognized species of Brucella has been revealed by DNA-DNA hybridization analyses so that B. melitensis was suggested to be the only representative species of this genus. The other Brucella species were also proposed to be biovars of B. melitensis (Cloeckaert et al., 2002). However, the genus Brucella is still classified into six species, as this classification is dependent on factors such as the pathogenicity and host preference of each species (Cloeckaert et al., 2001; Scholz and Vergnaud, 2013).

Besides lipopolysaccharide, the cell wall of Brucella is mainly composed of outer membrane proteins (omp) (Vassen et al., 2019). The omp comprises two major fractions of molecular masses 36-38 and 25-27 kDa, designated group 2 and group 3, respectively (Wang et al., 2010). In contrast to the lipopolysaccharide component of the outer membrane of Brucella, the group 2 omp seem to be of little importance as antigens and immunogens during host infection. However, group 3 omp appear to exert high levels of immunogenicity in the course of B. ovis infection in rams (Cloeckaert et al., 2002).

The genes encoding the major omp in Brucella are designated as omp2a and omp2b for group 2 porin proteins, which are 36-38 kDa. The genes share 85% DNA identity, are separated by 900 bp on the
chromosome and oriented in opposite directions (Roussel et al., 2012).

Brucellosis is endemic in many parts of Iraq and several serological and molecular studies have revealed high incidences of the disease in small ruminants. For example, in a serological study by Al-Samarraee and Jabary (2015), blood samples were collected randomly from 160 sheep and 151 goats in Sulaymaniya governorate, located in the north-east of Iraq. The results revealed the presence of Brucella antibodies in 20.0% of the sheep and 8.6% of the goat samples. In another study by Alhamada et al. (2017), blood samples were collected randomly from 335 sheep and 97 goats in Duhok province in the north of Iraq. It was shown that 31.7% of the sheep and 34.0% of the goats had antibodies against Brucella.

Sharazor is one of the 10 districts of Sulaymaniya governorate and is located about 40 kilometers east of the latter. The district includes 49 villages and the sheep and goat population in the area is estimated to be around 84000. Different outbreaks of abortion in small ruminants have recently occurred in the area and the causative agent was reported to be Brucella melitensis. However, a definitive molecular characterization of the causative agent has not been conducted previously. In this study, we identified and characterized the gene encoding the omp2 and performed a phylogenetic analysis of the sequences with different isolates that were published in GenBank databases.

MATERIALS AND METHODS

Sample collection: The study was carried out in Sharazor region of Sulaymaniya governorate/Iraq, from November 1st, 2014 to January 31st, 2015. Seventeen mixed herds of sheep and goats from nine localities were included. The samples were taken from animals which had incidences of abortion.

Forty-seven tissues and vaginal swab samples were collected from aborted fetuses and dams in 17 vaccinated and nonvaccinated herds. The animals had a history of abortion in the previous 1 to 40 days. Samples taken from each herd were pooled together and numbered from 1 to 17. Sample number 17 was subdivided into 17a (from sheep) and 17b (from goat). Of the 47 samples, 45 were vaginal swabs and two specimens were taken from aborted fetal tissues (Table 2). The swabs and tissue specimens were stored in a cooled box and transported to the laboratory in less than four hours.

Extraction of DNA: DNA extraction from the collected samples was conducted immediately after arrival at the laboratory. PrimePrep genomic DNA isolation kits (GeNet Bio, Chungnam, South Korea) were used to isolate DNA from tissue (Cat. No. K-3000) and vaginal secretions (Cat. No. K-2000). The latter was also used to extract DNA from a vaccine containing Brucella melitensis strain Rev. 1 (Jovac, Jordan), which was used as the positive control.

Polymerase chain reaction: Four sets of primers encoding omp2 and one set encoding bsp31 genes, manufactured by Macrogen (Korea, Table 1), were used in the study (Baily et al., 1992).

The lyophilized primers were suspended in a volume of sterile nuclease-free distilled water, to prepare a 100 pmol stock solution, and stored at -20°C. Thereafter, a tenfold dilution was made to prepare 10 pmol/μL of primers for use in the PCR.

The PCR was done in 200 μL capacity AccuPower® PCR tubes using AccuPower®PCR PreMix (Bioneer/Korea). The PCR mix was prepared by adding 1 μL of each of the forward and reverse primers, 5 μL of the sample DNA, and 13 μL of double-distilled water. The PCR cycles included an initial denaturation for two minutes at 94°C, followed by 35 cycles of denaturation (30 seconds at 94°C), annealing (30 seconds at 57°C) and extension (80 seconds at 72°C). A final extension for five minutes at 72°C was also included.

Gel electrophoresis: The PCR product (7 μL) was stained with 3 μL SYBR green dye (Bio-Rad, USA) and was run on a 1% agarose gel. The electrophoresis system (Major Science, Taiwan) was run for 40 minutes using an electrical potential of 120 volts.

DNA bands were visualized under ultraviolet light using a molecular imager (Gel Doc XR System, Bio-Rad Laboratories, USA). The size of the amplified DNA strand was estimated by comparing with a 100 base pair DNA ladder (GenDirex, USA).

Sequencing of PCR products and phylogenetic construction: The Brucella omp2 genes of five selected Sharazor isolates (number 2, 4, 7, 9 and 17b) were sequenced from both ends. Sequencing was conducted by Bioneer Sequencing Service (Korea). Alignment of the sequences was performed using bl2seq program. The result was published in GenBank under accession numbers KP681853, KT896745, KT896746, KT896747, and KT896748 for samples 2, 4, 7, 9 and 17b, respectively.

The sequence identity of Brucella omp2 gene of Sharazor isolates was confirmed by using BLAST (Basic Local Alignment Search Tool) method in the NCBI (National Center for Biotechnology Information) homepage. Expasy translate tool software was used to translate the nucleotide sequences of omp2 of Sharazor isolates to sequence amino acids.

The omp2 gene sequences of samples 2, 4, 7, 9, and 17b were comparatively analyzed and a phylogenetic tree was constructed using the Clustal W Multiple Sequence Alignment Program (MEGA6 software).

RESULTS

Detection of Brucella genus-specific gene: Forward and reverse Bruc primers were used to amplify a 223 bp amplicon on the Bsp31 gene, which is specific for the genus Brucella and encodes a 31 kilodalton cell surface protein. The results showed that 15 of the 17 pooled samples were positive for Brucella, of which, 13 were vaginal swabs and the other two were tissue specimens. Two herds of sheep (number 10 and 14) were negative for Brucella (Table 2). Four herds (number 1, 2, 9 and 10) were previously immunized with a B. melitensis (strain Rev. 1) vaccine. However, three of the four samples showed positive results for Brucella.
**Brucella species detection:** Five of the fifteen positive samples for *Brucella* genus (number 2, 4, 7, 9 and 17b) were randomly selected and subjected to further amplification using *Brucella* species-specific primers Bru1, Bru2, Bru3, and Bru4 (Table 1). Amplification was successful for all the primers in all the five PCR products.

**Sequence identity:** The nucleotide sequences of *omp2* gene of samples number 2, 4, 7, 9 (sheep) and 17b (goat) were subjected to comparing with those published in the GenBank databases (Fig. 2). A lower nucleotide similarity was recorded with the vaccine strain (accession # CP000708) and *B. suis* bv. 2 (accession # CP007720) showed nucleotide substitutions at G300A, T304A and C314G, so they were different from all Sharazor isolates and all other aligned *Brucella* species.

Sample number 4 showed a nucleotide substitution at G1029A, which was not present in the other samples. This substitution was also present in *Brucella* species isolates, with the exception of *B. suis* bv. 2 (accession # CP0007720) and *B. ovis* (accession # CP000708). Both isolates exhibited nucleotide substitutions at several positions such as V591, H60S, S75T, T77S and N80K.

**Amino acid alignment of *omp2a*:** The amino acid sequences of the *omp2a* gene result showed similar amino acid compositions in all the aligned *Brucella* isolates, with the exception of *B. suis*. The sequences of *B. ovis* (accession # CP000708) and *B. suis* bv. 2 (accession # CP007720) showed nucleotide substitutions at G300A, T304A and C314G, so they were different from all Sharazor isolates and all other aligned *Brucella* species.

Sample number 4 showed a nucleotide substitution at G1029A, which was not present in the other samples. This substitution was also present in *Brucella* species isolates, with the exception of *B. suis* bv. 2 (accession # CP0007720) and *B. ovis* (accession # CP000708). Both isolates exhibited amino acid substitutions at several positions such as V591, H60S, S75T, T77S and N80K.

**Amino Acid alignment of *omp2b*:** The amino acid sequence of sample number 4 was similar to the sequence of *B. melitensis* strain 20236 (accession # CP008750), *B. melitensis* M5-90 (accession # CP0007720) and *B. ovis* (accession # CP000708). Both isolates exhibited amino acid substitutions at several positions such as V591, H60S, S75T, T77S and N80K.

### Table 1: Sequences of the *Brucella* gene primers

| Gene     | Length | Primer | Forward primer | Reverse primer |
|----------|--------|--------|----------------|----------------|
| bcp31    | 223    | Bruc   | 5'-TGCGCTCGGTCCGCAATATCAA-3' | 5'-CGGCGTTGGCTTTTACGGTCTG-3' |
| omp2b    | 756    | Bru1   | 5'-GCGATCTTCGCCGACCCCTTAG-3' | 5'-CATACCTTCAGCAGGTAATGCTG-3' |
| omp2     | 818    | Bru2   | 5'-CTTGGCGGCTGGATAGGCAACA-3' | 5'-GTTAACATCGGTATATTGACGG-3' |
| omp2     | 873    | Bru3   | 5'-GTCGCTCATAGAAAGGGGAGT-3' | 5'-CGGCCCTTACGTCGGAACCCACG-3' |
| omp2b    | 921    | Bru4   | 5'-CGATCTGGATTAGAAAGCAAGC-3' | - |

### Table 2: Occurrence of *Brucella* in the studied farms

| Herd no. | Village name | Animal species | Type of sample | Number of samples per pool | PCR result |
|----------|--------------|----------------|----------------|---------------------------|------------|
| 1        | Greza        | Sheep          | Tissue         | 1                         | Yes, Positive |
| 2        | Kelakawa     | Sheep          | Vaginal swab   | 5                         | Yes, Positive |
| 3        | Qara         | Sheep          | Vaginal swab   | 5                         | No, Positive |
| 4        | Greza        | Sheep          | Tissue         | 1                         | No, Positive |
| 5        | Qumash       | Sheep          | Vaginal swab   | 3                         | No, Positive |
| 6        | Qumash       | Sheep          | Vaginal swab   | 1                         | No, Positive |
| 7        | Qumash       | Sheep          | Vaginal swab   | 2                         | No, Positive |
| 8        | Qumash       | Sheep          | Vaginal swab   | 2                         | Yes, Positive |
| 9        | Qumash       | Sheep          | Vaginal swab   | 1                         | Yes, Negative |
| 10       | Qumash       | Sheep          | Vaginal swab   | 4                         | No, Positive |
| 11       | Halabjay Taza| Sheep         | Vaginal swab   | 5                         | No, Positive |
| 12       | Halabjay Taza| Sheep         | Vaginal swab   | 2                         | No, Positive |
| 13       | Palhyro      | Sheep          | Vaginal swab   | 1                         | No, Negative |
| 14       | Bawakuzhyaw  | Sheep          | Vaginal swab   | 1                         | No, Positive |
| 15       | Bawakuzhyaw  | Sheep          | Vaginal swab   | 1                         | No, Positive |
| 16       | Bereshka     | Sheep          | Vaginal swab   | 3                         | No, Positive |
| 17a      | Kanipanka    | Sheep          | Vaginal swab   | 5                         | No, Positive |
| 17b      | Kanipanka    | Goat           | Vaginal swab   | 4                         | No, Positive |
Fig. 1: Phylogenetic tree constructed based on amino acids alignment of omp2b gene of Sharazor isolates (field strains) and Brucella melitensis strain Rev.1 (vaccine strain). This alignment shows the formation of three groups: samples 7 and 9, sample 4 and B. melitensis strain Rev.1, and samples number 2 (from sheep) and 17b (from goat).

Fig. 2: Phylogenetic tree constructed based on Nucleotide sequence alignment of omp2 gene of Sharazor isolates and Brucella species that submitted at GenBank. All the isolates from the current study are clustered in one group.

Fig. 3: Alignment of amino acids of omp2a gene from Sharazor isolates and Brucella species submitted at GenBank. The isolates show similar amino acid sequences with other Brucella isolates with the exception of B. suis bv. 2 (accession # CP007720) and B. ovis (accession # CP000708).
**Phylogenetic tree of the vaccine and field strains:** A phylogenetic tree was constructed, based on amino acids alignment of *omp2b*, between the five samples and the vaccine strain (*B. melitensis* strain Rev. 1). The results showed the construction of three groups: samples 7 and 9, sample 4 and *B. melitensis* strain Rev. 1 vaccine, and samples 2 (sheep) and 17b (goat). So, the vaccine strain was genetically closer to sample 4 than the other Sharazor isolates (Fig. 1).

**Phylogenetic tree based on nucleotide sequence alignment of the *omp2* gene:** The topology of the phylogenetic tree indicated that all the five isolates were clustered in one group together with *B. melitensis* bv. 2 (CP007789), *B. melitensis* strain 20236 (CP008750), *B. melitensis* M5-90 (CP001851.1), and *B. melitensis* strain Rev. 1 (KC847057). This phylogenetic tree revealed that the Sharazor isolates were more genetically related to *B. melitensis* bv. 2 (CP007789) (Fig. 2).

**Phylogenetic tree based on the alignment of amino acids in the *omp2a* protein:** The alignment of amino acid sequences of the *omp2a* protein showed little variation among the *Brucella* isolates as they were clustered together (Fig. 3), with the exception of *B. suis* bv. 2 (CP007720) and *B. ovis* ATCC 2840 (CP000708).

**Phylogenetic tree based on the alignment of amino acids in the *omp2b* protein:** The *omp2b* protein amino acid sequences showed the formation of different groups. Samples number 7 and 9 were clustered together while samples number 2 and 17b were clustered together and they were mostly related to *B. melitensis* strain 20236 (CP008750) from China (Fig. 4).

**DISCUSSION**

Despite the fact that the incidence of abortion due to brucellosis has declined in the surrounding areas, *Brucella* is still a major cause of abortion in small ruminants in Sharazor. The high prevalence of brucellosis is probably due to the poor management and husbandry practices by the farmers, such as grazing of different flocks of sheep on the same pasture and sharing of rams for mating between different herds.

The genus-specific primers of *Brucella* used in the current study (Bruc), which encode the 31 kilodalton surface protein, revealed that 88% of the tested samples were infected by the pathogen. The high rate of positive samples indicates that brucellosis is endemic in the region and that the vaccination programs regularly conducted by the Veterinary Public Services did not reduce the prevalence rate of the disease.

The DNA-DNA hybridization assays revealed that there were more than 90% homologies among the *Brucella* isolates, which is an indication that the isolates were different biovars of *B. melitensis*. The molecular genotyping techniques used for the differentiation of *Brucella* species showed a significant DNA polymorphism. One of these loci is the *omp2* gene, which encodes the 36 kDa outer membrane protein 2 of *Brucella* species (Yousefi et al., 2016; Yousefi et al., 2018). This gene reveals diversities among different isolates and special primers have been designed for the identification of *Brucella* species, biosvars, and strains (Cloeckaert et al., 2002; He, 2012; Kaden et al., 2017).

The *omp2* sequences of Sharazor and ten other previously mentioned *Brucella* isolates were analyzed by using Clustal Omega Multiple Sequence Alignment program. The results revealed a 99% nucleotide and amino acid sequences similarities with *B. melitensis* biovar 2 (GenBank accession number CP007789). This outcome indicates that the Sharazor isolates belong to *B. melitensis* biovar 2. This result coincides with other molecular assay outcomes which were conducted in Turkey (Büyükçangaz et al., 2009; Ica et al., 2012), Iran (Behrozikhah et al., 2012), and southern Iraq (Altememy et al., 2013), as the isolated species in these studies were also reported to be *B. melitensis* biovar 2. The amino acid sequence alignment of *omp2a* in Sharazor isolates with other GenBank isolates showed a high degree of similarity among the aligned sequences, with the exception of *B. suis* biovar 2 (accession number CP007720) and *B. ovis* (accession number CP000708). In contrast, a higher degree of polymorphism was detected when the amino acid sequences of the *omp2b* gene were aligned together. The same outcome has been previously reported by other researchers (Di et al., 2016).

The *omp2* gene was used to differentiate the vaccine strain (*B. melitensis* strain Rev. 1, GenBank accession number KC847057) from the field strains. This locus was widely used in other molecular research as a target for the differentiation of field strain from vaccine strain of...
**Brucella species** (Noutsios et al., 2012; Saeedzadeh et al., 2013). The results showed a unique nucleotide substitution at G83A. Therefore, the vaccine strain was different from the Sharazor and all other aligned Brucella isolates. However, the phylogenetic tree that was constructed based on the amino acid sequence of Omp2b gene showed clustering of sample 4 with B. melitensis strain Rev. 1 in one group and showed the same ancestor and genetic relationship. These results approved that the vaccine strain used in the immunization of sheep and goats in the Sharazor region is a suitable candidate, in particular with respect to Omp2 gene, for the control of Brucellosis in this region.

**Conclusions:** Despite the regular vaccination campaigns conducted by the Veterinary Public Services, brucellosis is still the main cause of abortion in small ruminants in Sharazor subdistrict of Sulaymaniyah governorate. Failure of the vaccination program is mainly due to the improper management and husbandry practices followed by the farmers. The causative agent of brucellosis in the area is *B. melitensis* and it seems that there are no strain variations among the different isolates in the region. The four sets of primers for the Omp2 gene, which were used in this study, are suitable for the identification of *Brucella* from sheep and goats. *Brucella melitensis* strain Rev. 1 is a suitable vaccine to be used in the immunization of sheep and goats in Sharazor.

**Author contribution:** All the authors equally contributed to the study.

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