Genotyping of *Brucella melitensis* isolated from human and sheep in Iraq

Khetam Qaid M. AL-Hamdawee
Coll. of Vet. Med./Univ. of Al-Qadisiyah
Email: Khetam.alhamdawee@qu.edu.iq
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Abstract

Brucellosis is a widespread endemic zoonotic disease as well as significant impact on human health together with ruminant's manifests as abortions or other reproductive problems in different animal's species. A specific sensitive PCR and DNA sequencing technique employed in this study to provide the first Iraqi profile about *B. melitensis* in Genebank to overcome the determinates posed by the others accurate diagnostic methods like isolation and serotyping. In Women's Maternity and Children Hospital, and Gynecology Outdoor Patient (OPD) in the city One hundred twenty two (122) samples (107 serum and 15 aborted fetus) collected from a women have a history of abortion and either aborted fetus, serum tested directly with rose Bengal while aborted fetus submitted to culturing. Seventy four (74) blood samples collected from different ewes with abortion history tested with Rose Bengal test conducted the positive cases to PCR then DNA sequencing. Out of 196 samples 6 samples (2 human and 4 sheep) were positive for PCR technique, while only 3 partial gene sequenced samples were identified as *B. melitensis* revealed three different biovars available under accession number (KX793714.1, KX793715.1, and KX793716.1) in Genebank A1, A2 strains isolated from sheep and A3 human strain. *B. melitensis* was the only species detected, ensuring its highest zoonotic potential in Brucella genus. A1 and A2 Sheep isolate were shown closed related to NCBI-Blast *Brucella melitensis* biovar 3 (DQ086122.1). Whereas, the *Brucella* spp. A3 Human isolate was shown closed relation to NCBI-Blast *Brucella melitensis* biovar 1 (DQ086119.1) and (DQ086121.1).

Key words: rpoB gene, DNA sequencing, *B. melitensis*, biovar.

Introduction:

Brucellosis (undulant fever or Malta fever) is one of the most reported zoonosis worldwide that can cause economic changes and healthcare losses (1). In Iraq, brucellosis is endemic since 1937 (2,3). It is attacked the human and animal simultaneously in all governorates until this days (3). And *Brucella melitensis* is the most frequent cause of brucellosis with high pathogenicity (10,11). Brucella *spp.* are environmentally stable gram-negative coccobacilli, facultative, intracellular bacteria that infect a wide range of animals and human, transmitted through numerous routes including direct animal or environmental contact, consumption of raw or poorly cooked animal products, and aerosol (4,5). *Brucella* genus has ten distinguished species depending on host distention and phenotypic variation with more than 90% DNA identity (6-8). Based on DNA-DNA hybridization, Brucella have previously been proposed to comprise a single species, with a series of biovars (9). Depending upon polymorphism specialty, rpoB gene used to identify all *Brucella* species and most of the biovars (10). All *Brucella spp*. grow slowly, and culture techniques can present risks to laboratory personnel (12). The genotyping of *Brucella* spp. still unclear in Iraq. My current efforts are aimed to establish certainly and differentiation of *Brucella*. spp and biovars in women and ewes. To investigate genotype relationships among regional groups of *Brucella*.

Materials and Methods:

Samples collections:

A. Blood samples: aseptically, about 3-5ml of blood collected from (107) women have a history of abortion and conducted 5 ml of (74) jugular veins blood samples from aborted ewes during the study course 2015

submitted to Rose Bengal test (12), the serum with positive results has been stored by freezing (-20 Cº) until sent to PCR approach.

B. Fetal samples: Only 15 aborted fetus samples obtained in bacterial culturing during June 2015. Approximately 1 ml of fetal stomach contents was collected by aspiration using a sterile syringe. Moreover, small pieces from internal fetus organs, which were liver, spleen, lung, kidney, and placenta have been propagated and handled over the surface of the medium. Drops of stomach content cultured on Brucella agar without selective supplement (Oxoid, CM169). The inoculated plates were incubated at 37ºC in presence of CO2 (5%-10%) within 5 days. After the incubation, the fishy colonies were examined as *Brucella* sp. All isolates has been detected by using standard microbiology procedures, Gram stain, oxidase, catalase, urease test, H_2S production.(13, 14).

Genomic DNA extraction: DNA was extracted from human fetus gut and ewes blood samples by (AccuPrep® DNA Extraction Kit, Bioneer. Korea) usage. The extraction was done according to supplier instructions. The extracted gDNA was checked by nanodrop spectrophotometer, stored at -20^0C at refrigerator until performance by PCR.

Polymerase Chain Reaction (PCR): was carried out for detection *Brucella* spp. based on DNA-dependent RNA polymerase beta chain (rpoB) gene encodes the β subunit of bacterial RNA polymerase to amplify a 710 bp product. Specific primer which was designated according to (GenBank: AB848993.1) in this study, rpoB Forward primer (GATCGTTTCGCAGATGCACC) and rpoB Reverse primer (CCATAGTAGGCGGTTCACCC) were supplied by (Bioneer company. Korea). (AccuPower® PCR PreMix kit. Bioneer. Korea) PCR master mix reaction was prepared in 20ul total volume by added 5uL of purified genomic DNA and 1.5ul of 10pmole of forward primer and 1.5ul of 10pmole of reverse primer, then complete the size by deionizer PCR water into 20ul and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was performed in a thermocycler (Mygene Bioneer. Korea) by set up the following thermocycler conditions; initial denaturation temperature of 95 °C for 5 min; followed by 30 cycles at denaturation 95 °C for 1min, annealing 60°C for 1min, and extension 72 °C for 1 min and then final extension at 72 °C for 10 min. The 710bp PCR products were examined by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized under U.V trans-illuminator.

Sequencing of DNA: The 710bp PCR product was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). The purified rpoB gene PCR product samples were sent to Korea in company for test DNA sequencing by (AB DNA sequencing system) obtained on rpoB forward primer. Sequencing method was done for genotyping of Brucella biovar based phylogenetic tree analysis of rpoB gene using Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version).

Results:

Out of 15 human cultured aborted fetus 2 samples showed colonies in media, and only one of them showed a band in agarose gel after PCR reaction. 107 Women serum samples was negative to Rose Bengal test. Whereas only 4/74 ewes serum give a positive results to (RBT), only 2/4 samples detected as *Brucella* ssp. by PCR, amplicon size was 710 bp level, As expected showed in (figure1).The draft genome sequence of Iraqi isolates filed in GenBank with three accession numbers, query length 685 as cleared in (table1).

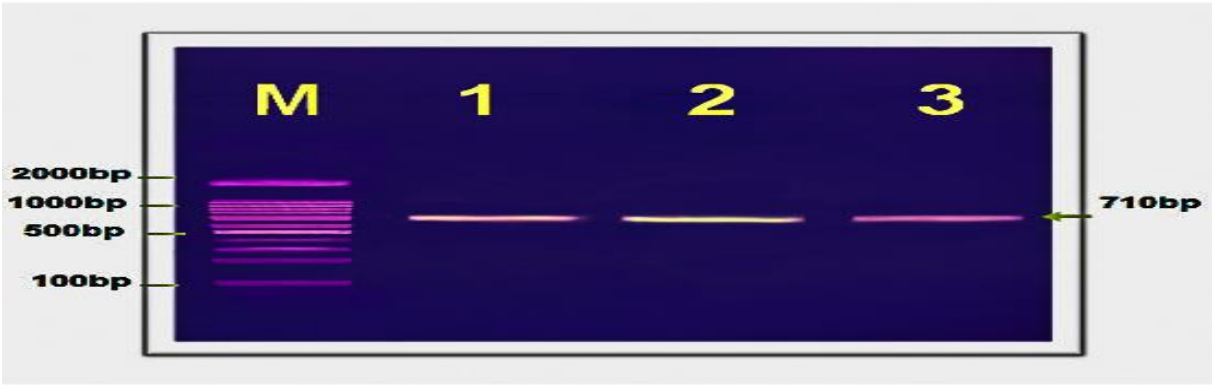

Fig. (1): Agarose gel electrophoresis image that show the PCR product of rpoB gene in Brucella isolates at 1% agarose gel. M: Marker (2000-100bp), Lane (1-3) positive DNA isolates at 710bp and submitted to sequencing technique.

Table (1): The Iraqi isolate accession numbers and genotypes as recorded in NCBI.

Isolate name in NCBI	Source of samples	Biovars	Accession No.	Protein
B. melitensis A1 Sheep isolate	Blood sample	3	KX793714.1	ARS01283.1
B. melitensis A2 Sheep isolate	Blood sample	3	KX793715.1	ARS01284.1
B. melitensis A3 Human isolate	Aborted fetus	1	KX793716.1	ARS01285.1

Table (2): The *Brucella melitensis* isolates that showed identity with the Iraqi isolates with accession numbers as recorded in NCBI.

Isolate name in NCBI	Source of samples	Biovars	Accession No.	Identity rate
Italian	Unknown	3	DQ086122.1	100%
Italian	Unknown	3	DQ086121.1	100%
Italian	Unknown	1	DQ086119.1	100%

The phenogram unit (figure2) classified into two nodes (65 and 33) through the comparison between DNA-dependent RNA polymerase beta chain (rpoB) gene partial sequence available in NCBI the results suggests that *B. melitensis* biovar 1 in human strain A3 KX793716.1 (node 12) closely related to strain (DQ086119.1). whereas ewes strains A1 KX793714.1 and A2 KX793715.1 showed the same rpoB sequence with *Brucella* sp. at (node 5) and closed to *B. melitensis* biovar 3 (DQ086122.1) and B. melitensis biovar 2 (DQ086121.1). At unit 33 *B. suis* biovar 2, 5 closed to each other (node 14) and *B. suis* biovar 4 and *B. canis* matching respectively at (node 42), *B. ovis*, *B. abortus* biovar 4, junction with (node 14). *B. abortus* biovars 1, 2, 3, and 7 matching together at (node 49 and 20) respectively. And the identity were arranged between (99%-100%) (figure.1,2)

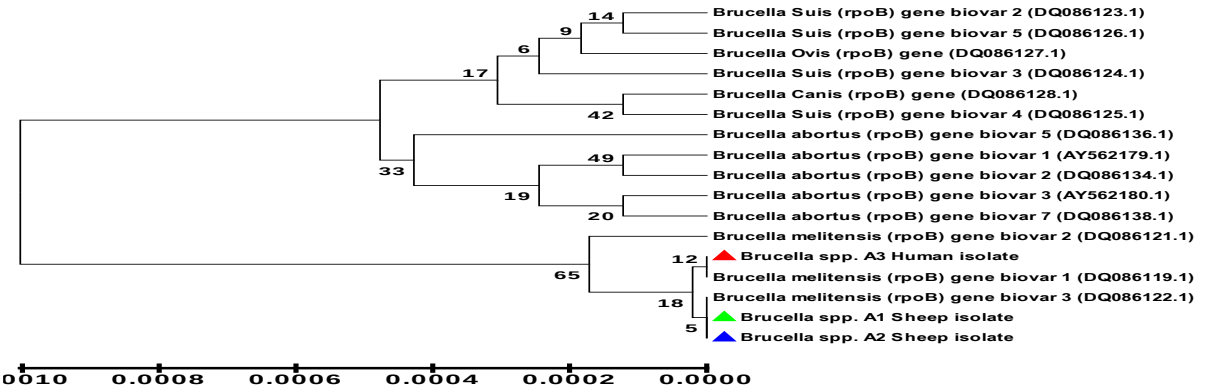

Fig. (2): Phylogenetic tree analysis based on DNA-dependent RNA polymerase (rpoB) gene partial sequence in *Brucella* spp. isolates from human and sheep samples. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version).

Fig.(3a) multiple sequenced alignment by clustalw2 online program http://www.ebi.ac.uk/Tools/services/rest/clustalo/result/clustalo-I20170731-214430-0255-37330503-es/aln-clustal_num

- ***Refer to similarity in letters.**
- **:, . Refer to similarity in each 3 letters.**
-

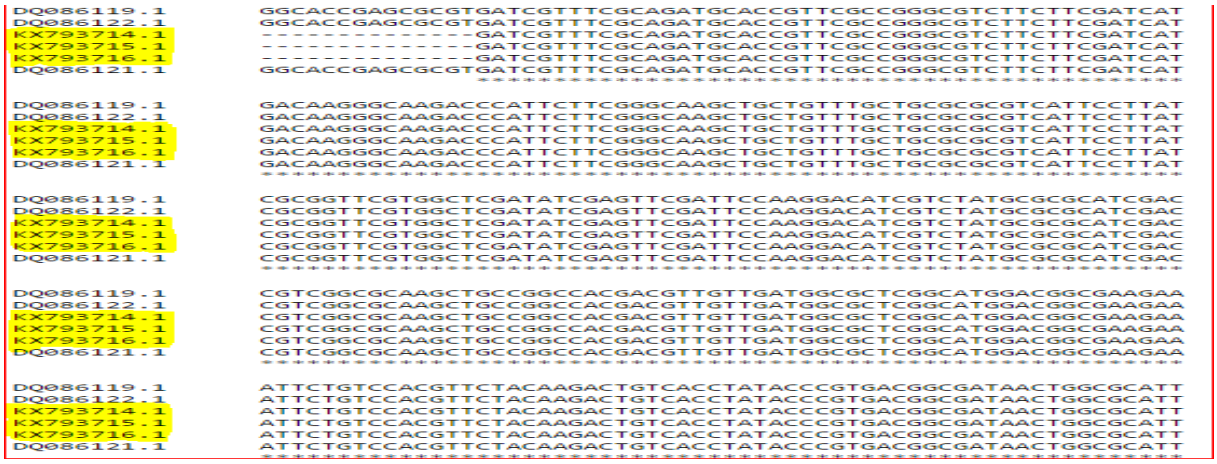

Fig.(3b) multiple sequenced alignment by clustalw online program http://www.ebi.ac.uk/Tools/services/rest/clustalo/result/clustalo-I20170731-214430-0255-37330503-es/aln-clustal_num

- ***Refer to similarity in letters.**
- **:, . Refer to similarity in each 3 letters.**

Discussion:

The results of this study ensured that Iraq one of the most endemic areas with Brucella in the world as a part of Asia (17-19). The recent study even with few numbers of samples it is consider the first explanation of Brucella situation in human and sheep genetically in Iraq. Molecular results of PCR approach more rapid, sensitive in diagnosis of *Brucella* ssp. Furthermore, it canceled the risks of handling with organism in laboratory in contrast with culturing method (20). Serum contain fewer inhibitors and fitness extraction in comparing with whole blood (21, 22), and for that reason women and ewes serum approved to DNA extraction whereas aborted human fetus has been cultured whereon very low sensitive method with difficulties in distinguish Brucella species and biovars (23,24). Recently distinction between Brucella species and biovars depending on phenotypic properties. PCR product in agarose gel at the level 710bp expressed sharp band without multiple bands, which were related to *B. melitensis* Rev.1 vaccine strain. PCR results confirmed by DNA sequencing analysis. Based on the genetic background, the use of a single gene for phylogenetic study be challenge to distinction the phylogenetic relationships between species (25), but it is possible to identify biovars level by sequencing rpoB gene of an unidentified *Brucella* isolate

where rpoB gene composed phylogenetically useful information (26) and showed specificity in detection of *B. melitensis* on the basis of the DNA polymorphism at the locus in *Brucella* spp and biovars (27). All DNA of the Iraqi isolates judged as *B. melitensis* with different genotypes 3, 3, and 1. In Iraq, the only one study concerned with *B. melitensis* biotypes exactly in Al-Najaf city. Depending on PCR technique the results revealed that biovar1 is the highest one in causing the abortion in ewes while 2 and 3 biovars came in last (16), And my study results agreed with that. Phylogenic analysis showed the identity of *B. melitensis* biovar 1 (KX793716.1) of women isolate with Italian isolate biovar 1(DQ086119.1) reached to 100% at same partial rpoB gene sequencing level with *B. melitensis abortus* (1,2,3, 5, and 7), *suis* (2,3, 4, and 5), *ovis, canis,* and *neotomae* with identity reached to 99% as showed in (table 2) This identity was not surprising because DNA-DNA hybridization genetic similarity grade reached to (98.5%) between *Brucella* spp. (28) (figures2,3a and 3b). Mismatching between Iraqi isolate and *B. suis* , *B. abortus* came from the few differences between genomic sequences especially of *B. melitensis, B. abortus* and *B. suis* (29). Furthermore, there is no profile or recorded infection by *B. suis* in Iraq because pig were not breeding there. Whereas the other species are *B. canis* and *B. ovis*, associated respectively with canine brucellosis (30). The Neighbor joining of phylogenetic tree of human and sheep isolate extremely showed different clusters similarities to *B. ovis* and *B. canis* and *B. neonate*. Despite a slight discordance between *Brucella* spp. and biovars can occur by single-nucleotide polymorphism analysis, host specifies, and virulence properties (31,32), even *B. melitensis , B. ovis, and B. canis* have similar behavior in pathogenesis, zoonotic potential.

Conclusions: In summary, this study identified different *B. melitensis* biovars as the etiological agent of brucellosis in both human and sheep. To create awareness for this potentially severe disease more information on the prevalence of the pathogen in different risk groups and in livestock in the Iraq is needed.

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