Molecular Typing of Human *Brucella melitensis* Isolated from Patients in Erbil, Iraq

Bushra K. Amin[1], Khulod I. Hassan[2]

[1]Department of Biology, College of Science, Salahaddin University, Erbil, Kurdistan Region - F.R. Iraq

[2]Department Food Science and Human Nutrition, College of Agriculture, University of Sulaimani, Kurdistan Region - F.R. Iraq

***Abstract*– Brucellosis is a reemerging infectious zoonotic disease of worldwide importance. In the Kurdistan Region of Iraq, it is a widely spread disease and remains a challenging health problem. This disease is mainly caused by *Brucella melitensis*, in human. For confirmation of these isolates, a study was performed, by isolation and molecular typing of *Brucella* Spp. from human patients in Rizgari Hospital at Erbil city (Iraq), between March 2014 and November 2016. One hundred sixty seven samples of blood collected from patients suspected for brucellosis, one hundred twenty one samples from these were recorded as genus of *Brucella*, using biochemical test and confirmed by applying polymerase chain reaction (PCR), using genus specific primer for *omp*31 gene which was specific for *B. melitensis*. These results support using molecular method that based on PCR as diagnostic test for the control of brucellosis in Erbil. Further studies are needed from different geographical areas of the country with different level of endemicity to plan and execute control strategies against human brucellosis.**

***Index Terms*—Brucellosis, Biochemical test, *Brucella melitensis*, Polymerase chain reaction.**

I. Introduction

Brucellosis, also known as Malta fever, is one of the most infectious zoonotic disease, with >500,000 new cases reported each year and becomes a major public health challenge in many countries (Seleem et al., 2010), especially developing countries, due to various sanitary and socioeconomic factors (Pappas and Memish, 2007). In humans, this highly diverse illness initially presents as a fever and may later develop into a chronic illness affecting various organs and tissues (Probert et al., 2004). Brucellosis in humans occurs as an acute, sub-acute, or chronic illness, and it is usually transmitted from animal reservoirs such as cattle, buffalo, camels, sheep, and goats, through consumption of unpasteurized milk or dairy products and undercooked meat products, inhalation of contaminated dust, and contact with infected animal body fluids or tissues (Greenfield et al., 2002). Brucellosis caused by microorganisms belonged to the genus *Brucella*, Gram-negative facultative intracellular bacteria (Navarro et.al., 2004). To date, 12 *Brucella* species have been reported and each species has a preference to certain group (Scholz et al., 2016). Five of the known *Brucella* species can infect humans, but the most pathogenic species for human is *Brucella melitensis* (Acha and Szyfre, 2003; Valdezate et al., 2007). *B. melitensis* with *Brucella suis* and *Brucella abortus* are listed as potential bioweapons by the Centers for Disease Control and Prevention in the USA due to the highly infectious nature of these species, as they can be readily aerosolized (Hoover and Friedlander, 2010). The intracellular location of the bacteria protects it from some of the basic mechanisms of the host's immune system and from antibiotic therapy; moreover, an outbreak of brucellosis would be difficult to detect because the initial symptoms are easily confused with those of influenza (Chain et al., 2005). Identification of *Brucella* species in developing countries still depends on culture isolation and biochemical test as there is no reliable, reproducible, and validated molecular tests for confirmation (Affi et al., 2011); moreover, several serological tests such as Rose Bengal plate test and enzyme-linked immunosorbent assay are also used for the diagnosis of human brucellosis (Mantur et al., 2010), and the major disadvantages with these serological tests are that they cannot differentiate between an acute and a chronic infection, besides cross reaction can occur with other Gram-negative bacteria such as *Escherichia coli*, *Yersinia enterocolitica,* and some *Salmonella* species, which have antigenic similarities with *Brucella* and can lead to false positive reactions (Nielsen et al., 2004). Hence, more reliable tests are needed, especially those which depend on using DNA techniques to overcome the problems associated with the traditional detection methods in terms of specificity and accuracy; among those, polymerase chain reaction (PCR) was the easiest and fastest (Ying et al., 2014). Several PCR-based assays have been developed and evaluated for identification of the genus *Brucella* (genus-specific PCR assay) based on a single unique locus that is highly conserved in all *Brucella* species including 16 s rRNA, 16–23 s intergenic transcribed

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Corresponding author's e-mail: bushra.amin@su.edu.krd

spacers, outer membrane proteins (*omp 2b, omp2a* and *omp31*) (Bricker et al., 2000, Gee et al., 2004, Imaoka et al., 2007). PCR have been described for identification of Brucella at the species level including *B. melitensis* (Baddour and Alkhalifa, 2008; Rees et al., 2009). Recently a real-time PCR based assay was used to identify *Brucella* genus in human sera (Shalini et al., 2018). The aim of this study was an attempt to evaluate a rapid and accurate technique for the detection of brucellosis in suspected patients in Erbil city of Iraq by PCR-based techniques using two kinds of primers; one of them was genus-specific primers and the other was species-specific primer for detect *B. melitensis*, the pathogenic species for human.

II. Materials and Methods

A. Sampling and Isolation of Brucella Spp.

From March 2014 to November 2016, 167 samples of bloods were collected from patients' suspects of Brucellosis from Rizgari Hospital at Erbil (Iraq), and all samples were collected aseptically in sterile tubes containing anticoagulant and send under refrigeration to the laboratory of Microbiology Division of Salahaddin University for analysis. The samples were inoculated on sterile plates of *Brucella* selective agar media with hemin and Vitamin k1 media (Hi-Media) and incubated at 37°C for 48h. The plates were observed at every 24h for the development of growth. After the growth, the colonies suspected for *Brucella* on the basis of cultural characteristics were picked up and streaked to another *Brucella* selective agar with hemin and Vitamin k1 plates and incubated at 37°C for 2 days to obtain pure culture. The pure cultures of the isolates examined by morphological tests, and some biochemical tests including: catalase, oxidase and urease production, hydrogen sulfate production, growth on media containing thionin and basic fuchsine (20 µg/ml).

B. DNA Extraction

DNA was extracted from the cell following cetyl trimethylammonium bromide (CTAB) method described by Wilson (1990) with slight modifications. Three loops of growth from pure *Brucella* culture grown on selective *Brucella* medium were transferred to a microfuge tube containing 400 µl of ×1 TE buffer. The cells were killed at 80°C for 20 min in a water bath followed by cooling at room temperature. Bacterial cell membranes were then disrupted by adding 70 µl of 10% sodium dodecyl sulfate solution and 5 µl of 10 mg/ml proteinase-K followed by incubation at 65°C for 10 min after brief vortexing. Following incubation, 100 µl of each of 5M NaCl and pre-warmed CTAB-NaCl solution was added. The mixture was vortexed until the liquid became milky white and incubated at 65°C for 10 min. Subsequently, 750 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed briefly, and then centrifuged for 8 min at 11,000× *g*. The aqueous phase containing DNA was carefully transferred to a fresh microfuge tube and the DNA was precipitated by addition of 0.6 volume of isopropanol. The tubes were then kept in –20ºC for 30 min followed by centrifugation for 15 min at ×11,000 g. The supernatant was discarded, leaving about 20 µl above the pellet, which was then washed with 1 ml of cold 70% ethanol and centrifuged for 5 min at ×11,000 g. After discarding the supernatant, the pellet was subjected to drying at room temperature for 15–30 min and finally dissolved in 20–30µl of ×1 TE buffer and was stored at –20ºC until further use.

C. Application of PCR Assay

Two sets of PCR primers were used in this study; the first set was used to screen the *Brucella* as a genus, by amplification of genus-specific primers targeting the gene coding for 16 s rRNA as described earlier by Unver et al. (2006) with the following sequence: Fwd (5'-TGACAGACTTTTTCGCCGAA-3') and Rev (5'-TATGGATTGCAGCACCG-3'), and the second set was used for the detection of *B. melitensis* by amplification of *omp*31 genes for the confirmatory identification of *B. melitensis* which was previously described by Bricker (2002) with the sequences: Fwd 5'-TGCCGATCACTTAAGGGCCTTCAT-3' and Rev: 5'-AAATCGCGT C CTTGCTGGTCTGA-3'. PCR reaction was performed in a total volume of 25 µl containing 2 µl of DNA sample, 0.5 mM MgCl2, 0.2 mM dNTP Mix,1 U/reaction of *Taq* DNA polymerase, 1µL forward primer (10pmol/µL), and 1µL reverse primer (10pmol/µL); and the final volume completed with nuclease-free water. The amplification of 16S rRNA gene was conducted with initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 54°C for 1.5min, extension at 72°C for 1.5 min, and finally, the final extension at 72°C for 10 min. The *omp*31 gene amplification was performed with initial denaturation at 95°C for 5min, denaturation at 95°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, and finally the final extension at 72°C for 10 min. The PCR amplified products were examined by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide (10 mg/mL) which was prepared to a final concentration of 0.5µg/mL. The PCR product (8µL) was mixed with 2µL of loading dye in gel apparatus and run at 70–80 volt/cm for 40–50min until the dye reached the half of the gel. The gel was photographed under the UV transilluminator.

III. Results and Discussion

A. Identification of Brucella Species using Biochemical Tests

In this study, both cultural isolation and identification were employed, as well as molecular detection by PCR of the causative agent of brucellosis. Of the 167 samples subjected to cultural isolation, 121 samples were recorded as *Brucella* species which initially identified as *Brucella* species based on gram staining and colony morphology, which observed on blood and *Brucella* selective agar plates after 4–5 days incubation at 37°C, and this is in coinciding with that recorded by Tille (2017); the colonies were round, convex, with smooth margin, translucent, honey-colored, glistening, and Gram-negative coccobacilli. Similar observations were also recorded by Habtamu et al. (2013). According to

biochemical reactions, *Brucella* organisms were found to be positive for catalase, oxidase, urease, and nitrate reduction tests and negative for methyl red, indole production, citrate utilization, and H2S production, The isolates were also able to grow in the presence of dyes, namely thionin, basic fuchsin, and safranin. Similar findings were reported by Erdenlig and Sen (2000).

B. Application of PCR

A PCR product of about 1412 bp was obtained from all the *Brucella* species isolates (Fig. 1) using genus-specific primer set for 16S rRNA as a target gene. Moreover, with a specific primer to an outer membrane protein (*omp*31), an amplified product size of about 731 bp (Fig. 2) was obtained from 97 isolates which was specific for *B. melitensis* with an overall isolation rate of 58%, and these results were in accordance with other studies who obtained similar size of amplified products for the detection of *B. melitensis* using the same pair of primers (Imaoka et al., 2007 and Al-Sanjary et al., 2014).

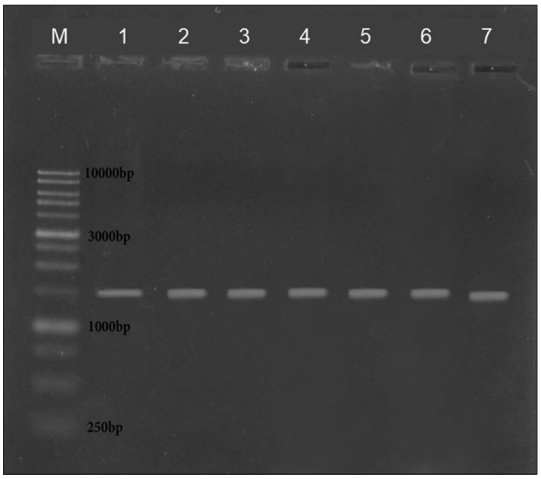

Fig. 1: DNA amplification of *Brucella* targeting 16 s gene using genus-specific primers, electrophorized on (1.2%) agarose gel, indicates positive sample that showed single band of 1412 bp.

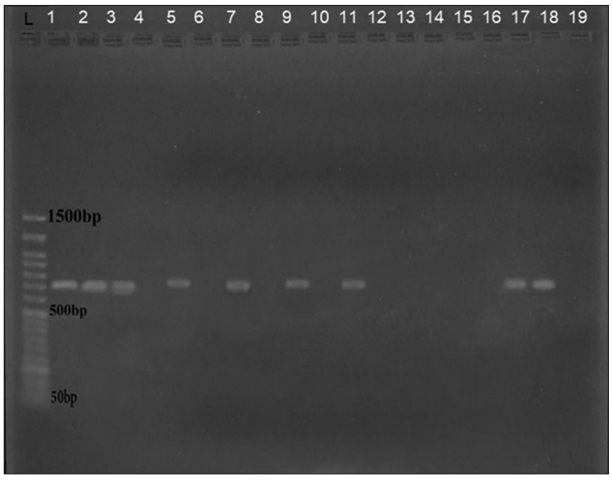

Fig. 2: DNA amplification of *B. melitensis* using species-specific primers targeting *omp*31 gene, electrophorized on 1.2 % agarose gel, indicates positive sample that showed single band of 731 bp.

The reason of selecting the gene *omp31*, that encode to the outer membrane proteins for detection of *B. melitensisis*, may due to that, *omp31* gene is highly diverse among *Brucella* species and strains therefore, it can be used to differentiate between them. Hence, molecular confirmation by PCR utilizing different gene targets has become the most common approach for confirmation of this pathogen (Shalini et al., 2018), due to that traditional methods are laborious, time consuming, and costly, and in addition, standard serological tests were used to detect *Brucella* lack sensitivity and specificity and are not able to distinguish between many species of *Brucella* (Elfaki et al., 2005). Obtaining high percentage of brucellosis in patients of Erbil city in Kurdistan region of Iraq agree with that reported by Gaff (2016) who mentioned to brucellosis as the most common bacterial zoonotic infections in Iraqi Kurdistan, which may due to insufficient preventive measures, the lack of adequate control programs, as well as, uncontrolled animal (as source of this bacteria) transportation through "open" borders, which increased the risk of spreading brucellosis in these regions, which remains a challenging health problem.

IV. Conclusion

The results of this study indicate that PCR technique efficiencies are higher than other methods used for species identification in terms of accuracy, specificity, sensitivity, and easy to perform, and hence, it has potential to be a promising tool for the diagnosis of acute disease; however, more studies from different geographical regions of the country on isolation of this important bacterial species are needed to understand the actual incidence of human brucellosis in the country, which will greatly help in the management of epidemiological studies.

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