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Molecular typing of *Brucella* species in human brucellosis cases from Eastern Türkiye

Tipificación molecular de especies de *Brucella* en casos de brucelosis humana en el este de Turquía

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ABSTRACT

Brucellosis remains a critical zoonotic infection with profound implications for public health across diverse regions, including the Middle East, Asia, the Arabian Peninsula, the Mediterranean, Africa, and South and Central American countries. This global threat necessitates ongoing investigation and surveillance. Accordingly, this study aimed to elucidate the presence and characteristics of *Brucella* spp. isolated from patients in a province of eastern Türkiye. A combination of conventional and molecular techniques was employed to achieve comprehensive species and biovar determination. A total of 189 human *Brucella* spp. strains isolated from blood cultures at Bitlis State Hospital between 2010 and 2020 were included in the study. Identification tests for the isolates comprised assessing serum requirement for growth, oxidase and urease production, as well as lysis testing with Tbilisi phage and R/C phage. Additional conventional biotyping tests involved evaluating hydrogen sulfide (H_2S) production, carbon dioxide (CO_2) requirement for growth, and growth in media containing thionin, basic fuchsin, and safranin. Furthermore, agglutination with Brucella A and M type monospecific antisera was performed. The isolates also underwent multiplex PCR, specifically the Bruce–Ladder PCR method, for biotyping. The results demonstrated the predominance of *Brucella melitensis* strains in human brucellosis cases, as identified by both conventional and molecular methods. Specifically, 185 isolates were classified as *B. melitensis* biovar 3, with the remaining 5 isolates classified as *B. melitensis* biovar 1. In conclusion, this distribution underscores the significant role of *B. melitensis* in the epidemiology of human brucellosis in the region. The current study highlights the efficacy of both conventional and molecular methods in *Brucella* spp. identification, with particular emphasis on the Bruce–Ladder PCR method's superiority in terms of rapidity and compatibility with traditional techniques. Continued research and surveillance efforts are imperative to deepen our understanding of the epidemiology and dynamics of this zoonotic disease.

Key words: Brucellosis; *Brucella melitensis*; multiplex polymerase chain reaction

RESUMEN

La brucelosis es una infección zoonótica crítica con profundas implicaciones para la salud pública en diversas regiones, incluido el Medio Oriente, Asia, la Península Arábiga, el Mediterráneo, África y países de América del Sur y Central. Esta amenaza global requiere investigación y vigilancia continúa. En consecuencia, este estudio tuvo como objetivo dilucidar la presencia y las características de las especies de *Brucella* aisladas de pacientes en una provincia del este de Turquía. Se empleó una combinación de técnicas convencionales y moleculares para lograr una determinación integral de especies y biovares. En el estudio se incluyeron un total de 189 cepas de *Brucella* humana aisladas de hemocultivos en el Hospital Estatal de Bitlis entre 2010 y 2020. Las pruebas de identificación para los aislados comprendieron la evaluación de los requisitos de suero para el crecimiento, la producción de oxidasa y ureasa, así como pruebas de lisis con fagos Tbilisi y fagos R/C. Otras pruebas de biotipado convencionales incluyeron la evaluación de la producción de sulfuro de hidrógeno (H_2S), los requisitos de dióxido de carbono (CO_2) para el crecimiento y el crecimiento en medios que contienen colorantes tionina, fucsina básica y safranina O. Además, se realizó aglutinación con antisueros monoespecíficos tipo Brucella A y M. Además, los aislados se sometieron a PCR múltiple, el método de PCR Bruce–Ladder para biotipado. Los resultados demostraron el predominio de cepas de *Brucella melitensis* en los casos de brucelosis humana, identificadas tanto por métodos convencionales como moleculares. Específicamente, 185 aislamientos se clasificaron como *B. melitensis* biovar 3, y los 5 aislamientos restantes se clasificaron como *B. melitensis* biovar 1. En conclusión, esta distribución subraya el importante papel de *B. melitensis* en la epidemiología de la brucelosis humana en la región. Este estudio destaca la eficacia de los métodos convencionales y moleculares en la identificación de especies de Brucella, haciendo hincapié en la superioridad del método Bruce-Ladder PCR en términos de rapidez y compatibilidad con las técnicas tradicionales. Es imprescindible realizar esfuerzos continuos de investigación y vigilancia para profundizar nuestra comprensión de la epidemiología y la dinámica de esta enfermedad zoonótica.

Palabras clave: Brucelosis; *Brucella melitensis*; reacción en cadena de la polimerasa multiplex

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INTRODUCTION

Brucellosis is one of the most common zoonotic infections all over the world. It is transmitted to humans by direct contact with infected animals or by ingestion of contaminated dairy products and raw milk [1, 2]. Brucellosis causes mild flu-like symptoms to serious clinical conditions such as meningitis, and is often accompanied by musculoskeletal system involvement [3]. As a result of successful intervention measures, including vaccination, the incidence of brucellosis has decreased in North America and Europe. On the other hand, it continues to be an important zoonotic infection affecting public health in the Middle East, Asia, Arabian Peninsula, Mediterranean, Africa, South and Central American countries [4]. The incidence of brucellosis in Turkey varies between regions, due to differences in climatic conditions, animal husbandry practices, methods of processing milk and dairy products, nutritional habits and socioeconomic status [5]. In countries where brucellosis is endemic, including Turkey, human brucellosis cases are significantly underreported; thus, it is believed that the incidence of brucellosis in the community is much higher than reported. Therefore, there is a need for a system for mandatory laboratory-based surveillance of the disease [6].

Blood culture is considered the gold standard in the diagnosis of brucellosis. However, it necessitates a lengthy duration, biosafety level 3 laboratory infrastructure, and experienced personnel, while also posing the potential risk of contamination to laboratory personnel during procedures [7, 8]. Molecular diagnostic tests are recommended by researchers for rapid and accurate disease diagnosis in the laboratory due to their high sensitivity, rapid results and safety from contamination [9, 10, 11].

It was aimed to identify *Brucella* spp. isolated from patients in the eastern region of Turkey, determine up to species and biovar levels using conventional and multiplex polymerase chain reaction (PCR) methods, and investigate the compatibility of these methods.

MATERIALS AND METHODS

Between 2010 and 2020, growth was detected in 1,701 of the blood cultures taken from 7,964 patients at Bitlis State Hospital. *Brucella* spp. grew in 189 (11.1%) of them. 103 of the patients were male and 85 were female, mean age of the patients was 27.3 years (0–75 years). In the same period, brucellosis standart tube agglutination (STA) tests were performed on 50,000 patients and was found to be positive at a titer of 1/160 and above in 3,954 (7.2%) patients.

Among 189 patients with *Brucella* growth in culture, the standard tube agglutination (STA) test was not performed in 3 patients. Of the remaining patients, 7 tested negative, while 11 exhibited a titer of 1/80. In 151 patients, STA titers were 1/160 or higher, with the following distribution: 17 patients at 1/160, 16 at 1/320, 26 at 1/640, 70 at 1/1280, 37 at 1/2560, and 1 patient at 1/5120. Of the patients, 167 presented to the hospital with brucellosis-related complaints (such as fever, muscle and bone pain), and 22 presented with non-specific complaints (such as menstrual irregularity, anemia, gastroenteritis).

Samples and quality control isolates

Blood samples were incubated at 37°C for 10 days (d) in automated blood culture system (Biomerieux, BacT/ALERT®, France) . Positive blood culture bottles were subcultured onto 5% sheep blood agar plates and incubated at 37°C for 5 d. Following Gram staining, Brucella-suspected colonies underwent catalase, oxidase, and urease tests. Bacterial colonies displaying the morphology of small Gram negative cocoid rods with positive catalase, oxidase, and urease tests were identified as *Brucella* spp. and were stored at -80°C freezer (Ildam, ILD-DF-720, Türkiye) until biotyping analysis.

Conventional biotyping of *Brucella spp.*

TSA (Tryptic soy agar) (Oxoid, United Kingdom) was used as the basal medium for conventional identification and biotyping processes. Initially, grown cultures were assessed for purity and colonial morphology. Smooth and rough isolates were differentiated by checking their colonial morphology using a stereomicroscope (Olympus, SZX10, Japan) and were tested for agglutination using 0.1% neutral acriflavin (Sigma, Australia). Any agglutination observed rendered the strain untypeable. Tests conducted to identify the species of the isolates included assessment of serum requirement for growth, oxidase and urease production, as well as lysis testing with Tbilisi phage at routine test dilution (RTD) and 104-fold RTD and R/C phage at RTD. For biotyping, further tests were conducted, including checking for the production of hydrogen sulfide (H_2S), carbon dioxide (CO_2) requirement for growth, growth in media containing thionine, basic fuchsin, and safranin O dyes. Agglutination with Brucella A and M type monospecific antisera was also investigated. To distinguish between field strains and vaccine strains, growth on media containing penicillin, streptomycin, thionine blue, and erythritol was tested [12, 13, 14].

Molecular typing of *Brucella* spp. by multiplex PCR (Bruce-ladder)

This assay was performed according to Anne Mayer-Scholl protocol [11, 15]. To extract the bacterial genomic Deoxyribonucleic Acid (DNA), a loopful of bacterial colonies was retrieved from the medium and suspended in 200 µL of sterile distilled water. Boiling method was used for DNA extraction. The amounts of isolated DNA were measured (ThermoScientific, NanoDrop ND-1000, USA) and 50 – 150 ng was used for each reaction tube. The test was conducted using a 25 µL reaction mixture comprising 2× Qiagen Multiplex Master Mix (Qiagen, Germany), 2 µM of each primer from a combination of nine primer sets, and 1µl of template DNA. Amplifications were astablished with the sample denaturation step (95°C, 15 min), followed by 25 cycles of template denaturation (94°C, 30 s), primer annealing (58°C, 90 s), and primer extension (72°C, 180 s) steps. Following the final cycle, samples were further incubated at 72°C for 10 min. (Rotor Gene, Qiagen, Germany). The amplified products were subsequently separated via electrophoresis (Orange, GRUN24H, India) on 1.5% agarose gels.

As quality control isolates, following strains were used: *Brucella melitensis* 16M (ATCC 23456), the reference strain for *B. melitensis* biovar (bv) 1; *B. melitensis* 63/9 (ATCC 23457), the reference strain for *B. melitensis* bv 2; and *B. melitensis Ether* (ATCC 23458), the reference strain for *B. melitensis* bv 3.

RESULTS AND DISCUSSION

All 189 isolates grew in media containing thionine, basic fuchsin and none of the isolates producted H_2S and required CO_2 for growth, therefore all isolates were identified as *B. melitensis* at the species level. All isolates tested negative for Tbilisi and R/C phage lysis, and the majority (184) were identified as bv 3 field strain due to agglutination with type A and M-type antisera. Agglutination was detected with only M-type antisera among 5 isolates (isolates number 11, 35, 64, 151, and 178), thus they were identified as *B. melitensis* bv 1 field strain. Using multiplex polymerase chain reaction (m-PCR), molecular identification

of the isolates was conducted at the species level (FIG. 1). It was observed that the results were in perfect concordance with those obtained through conventional culture-based methods.

The prevalence of brucellosis varies across countries, with the Mediterranean region being the most affected. Türkiye is one of the countries where brucellosis is endemic, particularly in the eastern Anatolia region. Bitlis is a city located in the eastern Anatolia region of Turkey and its main source of income is animal husbandry. Determining the species and subtypes of *Brucella*, as well as distinguishing between wild and vaccine strains, is crucial for detecting and controlling the source of the disease [16].

This study is the first biotyping study conducted with human brucellosis cases in eastern Turkey. There are limited studies on biotyping human isolates in Turkey. The current study was conducted using both traditional and molecular methods, revealing that the *Brucella spp.* isolated from patients in the region were predominantly *B. melitensis*, with the majority of isolates (185 out of 189) being bv 3, while 5 isolates were bv 1. The current study was found to be compatible with other studies conducted in Central Anatolia region of Türkiye. Bodur *et al.* [17] biotyped *Brucella* isolates obtained from human blood and cerebrospinal fluid samples in Ankara using conventional methods, which were the same as in current study. In total, 41 isolates were

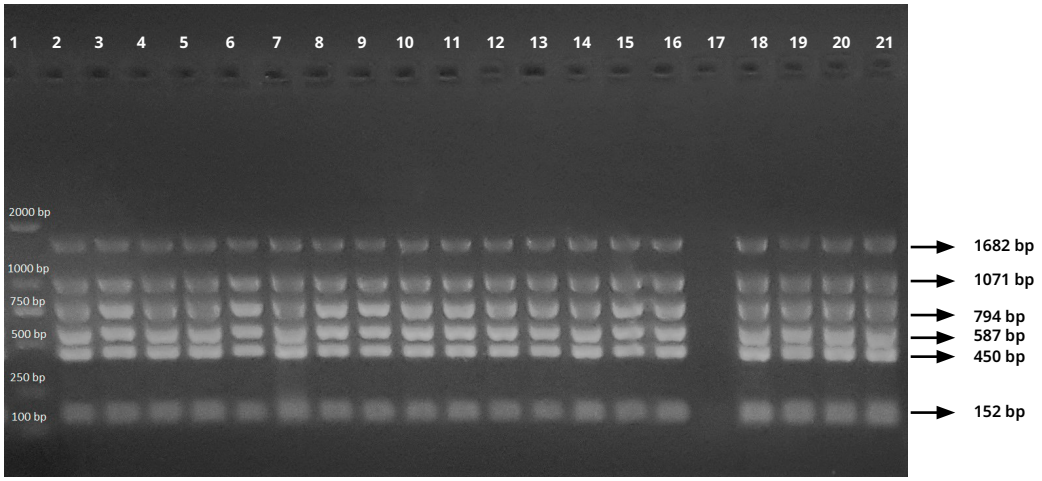

Figure 1: Multiplex PCR agarose gel image of the isolates. Lane 1: 2000 bp Mid Range Ladder (Qiagen), Lanes 2-16 and Lane 18: Isolates tested in the study, Lane 17: Negative Control (Water), Lane 19: Positive Control-1 (*Brucella melitensis* 16M), Lane 20: Positive Control-2 (*Brucella melitensis* 63/9), Lane 21: Positive Control-3 (*Brucella melitensis* Ether)

identified as *B. melitensis* at the species level, with 39 isolates typed as bv 3 and 2 isolates typed as bv 1 [17]. Similarly, in their 2004 study conducted in Ankara, Simsek *et al.* biotyped *Brucella spp.* isolated from human blood using conventional methods and identified 65 of the isolates as *B. melitensis bv 3* and 5 of the isolates as *B. melitensis* bv 1 [18]. In the study by Bolca *et al.* [19], in which they typed 26 human *Brucella spp.* isolates in 3 provinces from the Marmara and Central Anatolia regions of Türkiye, 22 isolates were identified as *B. melitensis* bv 3 and 4 isolates were identified as *B. melitensis* bv 1 [19].

The current study was also compatible with Karagul *et al.'s* [20] study of biovar distribution of livestock *Brucella* spp. isolates in Türkiye. A total of 5,203 *Brucella* spp. field livestock isolates from different regions of Türkiye were tested by conventional methods. In the period between 2010 and 2015, *B. abortus* bv 3 was found to be the most common cause of brucellosis in cattle, while *B. melitensis* bv 3 was the most common cause in sheep and goats. The study examined the percentage of biovars in different regions. The results showed that in Eastern Anatolia, the detection percentages of biovars were as follows: 94.68% for *B. abortus* bv 3, 3.52% for *B. melitensis* bv 3, 1.67% for *B. abortus* bv 1, and 0.06% for *B. melitensis* bv 1 [20].

Ica *et al.* [21] typed *Brucella* spp. isolated from human blood (50 samples) and animal abortion (17 cattle, 12 sheep) materials in Kayseri, a province in the Central Anatolia region of Türkiye, using both conventional methods and the Enhanced AMOS-ERY PCR method. The study revealed that all *Brucella* spp. isolated from cattle were typed as *B. abortus* bv 3b, while those isolated from sheep and humans were identified as *B. melitensis* bv 3 using both conventional and molecular methods .

In a study conducted in Iran, the eastern neighbor of Türkiye, all 206 human isolates isolated in 2013 were identified as *B. melitensis* bv 1 using conventional methods [22]. In another study conducted in Iran in the same year, Mirnejad *et al.* [23] investigated the detection and typing of *Brucella* spp. from blood samples using the PCR-RFLP method. DNA belonging to the *Brucella* genus was detected by PCR in 52 out of a total of 160 blood samples. Biotype determination was performed using PCR-RFLP in 25 of the positive samples, with 14 samples (56%) identified as *B. melitensis* bv1, and the remaining isolates (44%) characterized as *B. abortus* biotypes (bv 3, 5, 6 and 9). Both studies indicated that *B. melitensis* bv 1 exhibited the highest prevalence in Iran [22, 24] .

In a study conducted in China in 2015, *Brucella* spp. isolates obtained from human samples in Shanxi Province between 2009 and 2011 were typed using traditional methods and confirmed by abortus-melitensis-ovis-suis (AMOS)-PCR method. All 81 tested *Brucella* strains were identified as *B. melitensis* bv 3 through conventional biotyping [24]. The majority of isolates in our study were also typed as bv 3.

Lucero *et al.* [3]. analyzed *Brucella* strains isolated from humans and animals from Latin American countries between 1968 and 2006. Their study covered two different periods; the first period included isolates between 1968 and 1991 (half of the isolates were human and half were animal isolates), and the second period included human isolates from Argentina between 1994 and 2006. In the first period, the main *Brucella* strain in Argentina was *B. suis,* while *B. melitensis* strains were dominant in Mexico and Peru. In Argentina, *B. suis* isolates were dominant in the first years of the study, and subsequently *B. melitensis* human isolates increased. In the second period, covering the years 1994–2006, of the 367 human isolates in Argentina, 145 were *B. melitensis*, 144 were *B.suis*, 75 were *B.abortus*, and three were *B.canis*. Biotyping of the isolates was as follows; Of the total 145 *B. melitensis* isolates, 135 (93.1%) were bv 1, 7 were bv1a (4.8%), 2 were bv 3 (1.4%) and 1 isolate was (0.7%) detected as bv 2a. The majority of the 75 *B. abortus* isolates (86%) were detected as bv1 [25].

In a review of childhood brucellosis cases by Mantur *et al.* [25]. in India, it was reported the majority of the isolates (number 43) were identified as *B. melitensis* bv 1 and 1 isolate was typed as *B. melitensis* bv 3 through microbiological, epidemiological and clinical evaluations. The current research employed the Bruce-ladder PCR technique, a multiplex PCR approach, for molecular typing of *Brucella* isolates. The main advantage of using the Bruce-ladder PCR method over previously used multiplex PCR assays is its ability to distinguish all *Brucella* species and vaccine strains in a single test. The Bruce-ladder PCR stands out in comparison to AMOS PCR, as it can identify DNA from various *Brucella* strains, including Brucella strains from marine mammals, *B. abortus* biovars (bv 3, 5, 6, 7, 9), *B. suis* biovars (bv 2–5), *B. neotomae* and *B. canis* strains. Additional advantages include the rapid yield of the test, the simplicity of sample preparation, and reduced risks of contamination. As a result, the Bruce-ladder PCR method is gaining recognition as an efficient way to identify Brucella strains in both animal and human sources. Furthermore, it can be used in any regular microbiology laboratory worldwide, rather than being limited to specialized facilities [26].

CONCLUSIONS

An investigation was carried out on 189 *Brucella spp*. isolates obtained from clinical cases in the Eastern Anatolia region of Türkiye. In our study, 184 out of 189 isolates were identified as *B. melitensis* bv 3 field strain, while the remaining 5 were identified as the *B. melitensis* bv 1 field strain. Traditional techniques were employed alongside Multiplex PCR Bruce-Ladder for typing, and it was observed that the Bruce-Ladder PCR method yielded results more rapidly compared to conventional microbiological standard tests. Additionally, there was 100% agreement between the two methods, with a kappa value of 1.

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Conflicts of interest

The authors declare no conflicts of interest.

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