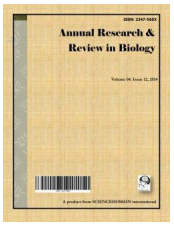

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Molecular Method for Direct Detection of *Brucella* spp. in Human Blood Samples

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Authors' contributions

This work was carried out in collaboration between all authors. Author AA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors HK, SK, FK, MJA, MR and EM managed the analyses of the study. Author PGD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aim: *Brucellosis* is a zoonosis of worldwide public health and economic importance, affects mainly domestic animals and it may be the genus *Brucella*, transmitted to humans by contact with infected domestic animals. Brucellosis control and eradication require serological tests and vaccines. The purpose of present study was to detection of *Brucella spp.* infection in human blood samples by molecular method.
Methodology: The number of 200 blood specimens (including 118 males and 82 females, between 10 and 70 years of age) were obtained from companion humans referred to the Polyclinic

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Laboratory in Kashani hospital, Shahrekord. Total DNA was extracted and the region encoded the *omp2a* gene was amplified by PCR using specific primers.
Results: 12 out of the 200 serum samples (6%) were found to be positive for *Brucella spp* (193 bp). 7 positive samples were in male group and 5 positive samples were in female group.
Conclusion: PCR was considered as the golden test for diagnosis of brucellosis. The eradication of brucellosis as a major zoonotic problem in animals is a necessary step to control the disease in human.

Keywords: Brucella spp; blood samples; omp2a gene; PCR; Iran.

1. INTRODUCTION

Brucellosis is a worldwide zoonosis caused by intracellular bacterial pathogens of the genus *Brucella* [1]. *Brucellae* are Gram-negative coccobacilli, small, facultative intracellular, non-capsulated, aerobic, non-spore-forming, non-motile [2,3]. *Brucella* members are aerobic, but some strains require an atmosphere containing 5 to 10% carbon dioxide added for growth, especially on primary isolation [4]. It has also been shown that the organism can survive for several months in blood stored at 4°C [5]. The optimum pH for growth varies from 6.6 to 7.4, and culture media should be adequately buffered near pH 6.8 for optimum growth. The optimum growth temperature is 36 to 38°C, but most strains can growth between 20 and 40°C [3]. *Brucella* can survive for longer periods in conditions of low temperatures, high humidity, no sunlight and in soil; and can remain viable for several months in water, aborted fetuses, and manure under appropriate conditions [6]. The genus *Brucella* comprises six species: *B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* [7]. *Brucella* species and their different biotypes are currently distinguished by differential tests based on serotyping, dye sensitivity, phage typing, CO_2 requirement, H2S production, and metabolic properties [6]. Most species of *Brucella* can infect animals other than their preferred hosts, when they come in close contact. *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis* are human pathogens. There are few different species of *Brucella*, each with slightly different host specificity: *B. melitensis* which infects goats and sheep, *B. abortus* which infects cattle, *B. suis* infects pigs, *B. ovis* infects sheep [8]. Brucellosis is a major public health concern in developing countries. The disease is found worldwide, but is more common in the Mediterranean countries, Indian subcontinent, the Arabian Peninsula and in parts of Mexico and Central and South America [9]. Iran has been the second country in the world for the prevalence of brucellosis [10]. The prevalence of brucellosis in Iran has been reported to be 0.5% to 10.9% in different provinces [11]. Brucellosis is endemic in Iran and many parts of the world and remains one of the major diseases in humans and domesticated animals. It emerged as a disease with the discovery of *B. melitensis* by Bruce in 1887 [12]. In Iran, the first *B. melitensis* isolation from an aborted sheep fetus was reported in 1950, and subsequently, its biovar 1 was isolated in different regions of the country from sheep and goats as well as cattle, camel, sheep, dogs, and humans [13]. Brucellosis in Iran represents a major health problem and is being reported with increasing frequency from various parts of the country. Brucellosis is quite a common disease throughout the country [14]. *Brucela* transmission, from human to human through blood transfusion, breast milk, needle sharing in drug users, bone marrow transplant, and also intrauterine transmission through the placenta is rare but possible [15]. The major outer membrane proteins (OMPs) of *Brucella* spp. Were initially identified in the early 1980s and classified according to their apparent molecular mass. The genes encoding the group 2 porin proteins consist of two genes, omp2a and omp2b, which are closely linked in the *Brucella* genome, and share a great degree of identity (>85%) [16]. Brucellosis is a zoonotic disease that used to get transmitted mainly by oral, cutaneous, respiratory, ocular and sexual routes [17]. This disease is generally transmitted to human through direct contact with an infectious animal, infectious meat, drinking unpasteurized milk or milk products, inhalation of dust containing the organism in the laboratory or place of animal husbandry and as a weapon of war (bioterrorism) [18]. Brucellosis is a multisystem disease with a broad spectrum of nonspecific symptoms that generally occur within 2 weeks (but sometimes up to 3 months) after inoculation [19]. Then it may spread through blood and the lymphatic system to any other organ where it infects the tissues and causes localized infection [20]. The global incidence of human brucellosis is estimated at more than

500,000 infections per year. But, the true incidence has been estimated to be 25 times higher than the reported incidence because of the lack of essential statistics, disease reporting and notification systems in many countries [21]. The main clinical signs of brucellosis are abortion, stillbirth, retained placenta, orchitis, arthritis in animals and undulant fever in humans. These signs are, however, common to several other diseases [22]. Several studies have been published on the detection of *Brucella* DNA by PCR, both from pure culture. Despite the importance of human brucellosis is areas where human are lived, few studies have been published on the diagnosis of brucellosis by PCR in human blood specimens [23,24]. The laboratory confirmation of human brucellosis is based on the microbiological, serological or molecular methods, each having its own advantages and disadvantages [25]. Bacteriologic culture, Polymerase chain reaction (PCR) and serologic tests such as tube agglutination test (TAT), agar gel immunodiffusion (AGID), rapid slide agglutination test (RSAT), rapid screening agglutination with 2-mercaptoethanol (2ME-RSAT) are often used for identifying the infection in suspect animals [26]. Real-time PCR systems have been developed that are faster and less prone to contamination and are thus more clinically useful [27]. PCR was considered as the golden test for diagnosis of brucellosis [16]. PCR is a very useful tool not only for the diagnosis of acute brucellosis but also as a predictive marker for the course of the disease and the post treatment follow-up, which is valuable for the early detection of relapses [16]. The disease is important from economic point of view it is one of the most devastating transboundary animal diseases and also a major barrier for trade. It also causes morbidity and considerable loss of productivity [20, 28]. The eradication of brucellosis as a major zoonotic problem in animals is a necessary step to control the harm amongst man [29]. Despite a significant reduction in incidence of brucellosis during the recent years, it is still a common infectious disease in rural areas [30]. The present study was performed to evaluate the specificity of a polymerase chain reaction assay for detection of *Brucella spp.* in human blood specimens.

2. MATERIALS AND METHODS

2.1 Sampling and DNA Extraction

A total of 200 blood specimens (including 118 males and 82 females, between 10 and 70 years of age) obtained from companion humans referred to the Polyclinic Laboratory in Kashani hospital, Shahrekord. Total DNA was extracted from the blood samples using a DNA extraction kit (QIAGEN Ltd., Crawley, UK) according to the manufacturer's procedure. The extracted DNA was stored at -20°C until needed for PCR amplification of part of *omp2a* gene.

2.2 PCR Amplification

The following primers: OmpF: 5′-GCGCTCAGGCTGCCGACGCAA-3′ and OmpR: 5′-ACCAGCCATTGCGGTCGGTA-3′ were used to amplify a 193 bp fragment from extracted human DNA, designed from the *omp2a* gene of *Brucella spp.* (Accession number: JF420977.1). PCR reactions were performed in a total volume of 25 µL, including 200 µM of each primer, 2.5 µl of 10X PCR buffer (50 mM Kcl, 10 mM Tris-HCl [pH 8.3], 0.1% Triton X-100), 1.5 mM MgCl, 200 mM dNTPs, 1 unit of *Taq* DNA and 2µl (1 ng) of DNA (CinnaGen, Iran). PCR were performed in a Gradient Palm Cycler (Corbett Research, Australia). The following condition was used for the amplification: hot start at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1min and extension at 72°C for 1 min. Then a final extension step of 5 min at 72°C was performed. 20 µl of the amplified product was analyzed by electrophoresis in 1% agarose gels in 1X TBE buffer. The 100bp ladder (Fermentas, Germany) was used as molecular weight marker and tubes containing distilled water free of template were used as negative control of the experiments. Gels were stained in a 0.5 µg/ml ethidium bromide solution for 15 min and photographed by UVIdoc gel documentation systems (UK). Constant voltage of 80 V for 30 min was used for products separation. In negative control tubes water was added instead of DNA sample and a positive control DNA from *Brucella* spp. ATCC 43642 strain were included in each amplification run.

3. RESULTS

Twelve out of the 200 serum samples (6%) were found to be positive for *Brucella* spp. Of the 200 studied samples, serological and molecular results demonstrated that 12 were infected with *Brucella* spp. 7 positive samples were in male group and 5 positive samples were in female group. Fig. 1 demonstrate a 193 bp PCR products from *omp2a* gene. This result showed

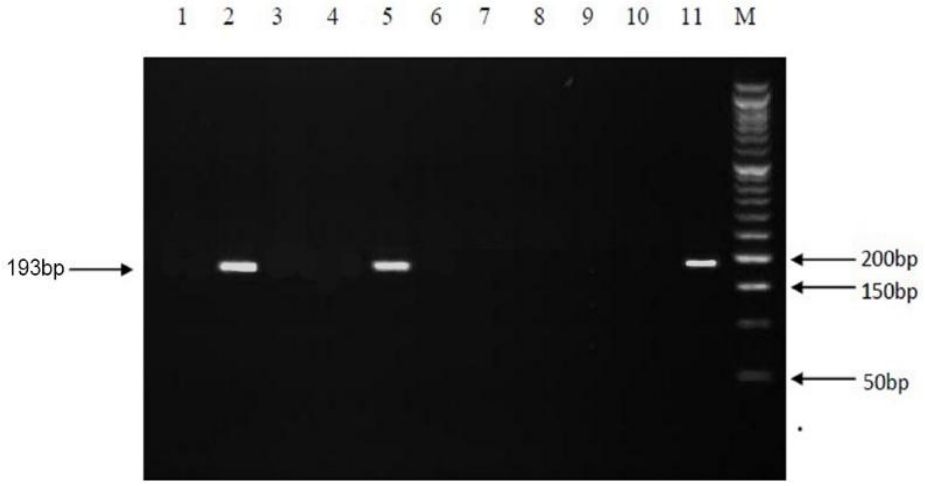

Fig. 1. PCR product of *omp2a* gene amplification, *Brucella spp.* samples and vaccines strains. Lanes 1, 3, 4, 6, 7, 8, 9 are negative samples; Lanes 2, 5 are positive samples; Lanes 10 and 11 are negative and positive control respectively and lane M shows 50 bp marker

an average frequency of *Brucella* spp. infection in humans in the southwest of Iran.

4. DISCUSSION

The most important aspects of *brucella* interaction with the human hosts were recognized previously and understanding of disease evolution has improved significantly in recent years through advances in molecular biology [31]. The difficulty in obtaining cheap, accurate and timely diagnostic tools for human brucellosis is a major problem in urban and rural settings in the developing world [32]. In several countries little is done to control the disease in man because of lack of financial and technical facilities. Identification of animals and control of animal movement represent a major obstacle in most of the countries [33]. Human brucellosis is widespread in the Middle East. Epidemiologic studies in such areas as southern Saudi Arabia have shown that 19.2% of that population had serologic evidence of exposure and 2.3% had active disease [19]. In our study the frequency of *Brucella* spp. pathogens was investigated in humans by PCR technique and *omp2a* gene of this infectious agent was amplified by species-specific primers [34]. *Brucella* was found in 12 out of 200 (6%) human blood specimens. In 2007, the prevalence of *B. melitensis* in aborted sheep fetuses in Turkey was 29.76% [35]. Swai et al showed that the overall *Brucella* antibodies seroprevalence in abattoir workers of Tanzania was 5.52% [36]. In a study in Bangladesh (2010), brucellosis seroprevalence determined 11.11% in veterinary personnel, 6.45% in dairy workers and 4.67% in animal farmers [37]. Nikokar et al. reviewed that the seroprevalence of brucellosis among slaughterhouse workers and the people living in rural areas were 9.8% and 5.5% respectively in North of Iran [38]. In a survey in Greek region endemic, a total of 78 blood samples 15% were found to be positive for *Brucella* spp. by PCR using specific primers [39]. Brucellosis continues to be an important public health problem in developing countries and remains endemic in Iran. The most common cause of mortality in brucellosis involves cardiac complications such as pericardial effusion [40].

5. CONCLUSION

From this study it can be concluded that there is a moderate prevalence of brucellosis in this part of the country; though there is still some threat belongs for the human population. Therefore, more investigations needed to be carried out in this region to make proper and effective control over here. In conclusion, our data suggest that the most rational approach for preventing human brucellosis is the control and elimination of the infection in animals. Pasteurization of milk can be another way of getting protection. Vaccination of cattle is recommended for controlling of bovine brucellosis in enzootic areas with high prevalence rates. Several detection methods have been developed and are widely used to detect, different classes of brucellosis. Also molecular methods have very high specificity and

sensitivity for effective identification of pathogens.

ETHICAL APPROVAL

For ethical approval in this study the protocol and informed consent forms were approved by the Islamic Azad University, Shahrekord Branch, Shahrekord, Iran. (Grant number: 17621105).

DECLARATION OF HUMAN RIGHTS

All applicable international, national, and institutional guidelines for the care of human were followed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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