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ORIGINAL ARTICLE

Serological and bacterial prevalence of *Brucella* spp. in suspected patients: a risk factor analysis in North Khorasan, Iran

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ABSTRACT

Background and Objectives: Brucellosis, a zoonotic bacterial disease caused by *Brucella*, affects humans and domestic animals, leading to significant economic loss. This study examined suspected cases in North Khorasan, Iran, to understand the prevalence of infection and its characteristics in this region.
Materials and Methods: Blood specimens were collected from 200 patients suspected of brucellosis after obtaining informed consent. Serum samples were tested using RBPT, Wright, and 2-ME agglutination tests. Blood samples were cultured on Brucella agar, and positive cultures underwent biotyping and PCR assays. A questionnaire identified correlated risk factors.
Results: RBPT, Wright, and 2-ME tests showed 25% brucellosis seroprevalence in symptomatic patients. In contrast, the prevalence was 2.5% among those with positive blood cultures. Notably, all culture-positive patients were also serologically positive, with titers exceeding 1:320 in Wright and 2-ME tests. Most positive cases were in people in their 30s, with *B. melitensis* biovar 1 identified as the causative agent, and the results were confirmed by multiplex PCR. Significant risk factors include contact with livestock and consumption of raw milk (P < 0.0001).
Conclusion: The findings highlighted the importance of comprehensive diagnostic approaches for accurate identification of brucellosis. Furthermore, education regarding close contact with animals and pasteurization of dairy products is essential for controlling human brucellosis.

Keywords: Brucellosis; Humans; Multiplex polymerase chain reaction; Blood culture; Risk factors

INTRODUCTION

Human brucellosis is the most prevalent zoonotic disease, with over 500,000 cases diagnosed annually. Beyond its impact on humans, brucellosis is markedly prevalent in livestock populations (1). It is induced by *Brucella*-genus bacteria. *Brucella* belongs to the family of proteobacteria. These Gram-negative coc-

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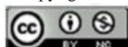
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cobacilli are characterized by their aerobic nature and facultative intracellular pathogenicity (2). *Brucella* species are classified into thirteen groups according to the biochemical reactions and animal reservoir type (3). Nonetheless, humans can be infected by *B. melitensis, B. abortus, B. canis, B. suis,* and some *Brucella* species found in marine mammals (4), through the respiratory system, non-intact skin, and the gastrointestinal tract (5). Moreover, human-to-human transmission can occur through blood transfusion, organ transplants, or vertical transfer from the mother to the growing embryo (6, 7). The bacteria spread throughout the body via the bloodstream and lymphatic system. Brucellosis typically manifests with symptoms such as flu-like symptoms, fever, debility, headache, fatigue, perspiration, loss of body mass, and musculoskeletal discomfort (8). Various complications, such as those affecting the bones and joints, heart, brain system, testicles, eyes, and have been associated with more severe cases (9).

Several researchers from across the world have recently examined the global distribution of brucellosis in humans (4). However, since national data are mostly passively reported, the true disease burden is often greatly underestimated. The underestimating of brucellosis can be attributed to the limited availability of high-quality healthcare in numerous areas affected by the disease, the non-specific symptoms of the disease, and its frequent prevalence (10). Human brucellosis cases in Iran are reported annually, with the majority being caused by *B. melitensis* (11). Human brucellosis is linked to variable risk factors, which arise from the extensive range of animals acting as reservoirs and the cultural practices that expose individuals to the disease (12). Brucellosis in the United States has been linked to the slaughter of pigs (13). In Chad, it is associated with the handling of cow umbilical cords (14), while in Yemen, it is connected to the consumption of camel milk (15). On the other hands, disease control in endemic areas involves identifying risk factors, implementing measures to reduce infection spread, and using accurate diagnostic tests at the right time to prevent misdiagnosis and incorrect treatment. However, little information is available about the strains that are now circulating in Iran's northeast and the causes and activities linked to human brucellosis. This study aimed to assess the incidence of *Brucella* infection, focusing on gathering comprehensive epidemiological data with a specific emphasis on identifying risk factors associated with the disease. Additionally, we evaluated serological tests and blood culture data from endemic areas to enhance diagnostic accuracy. Our study also aimed to identify the circulating *Brucella* species among individuals displaying symptoms suggestive of brucellosis.

MATERIALS AND METHODS

Ethical statements. The study, conducted with obtained consent at health centers in North Khorasan province, Iran, lasted from November 2022 to June 2023. The Ethics Committee for Health Research at the Razi Vaccine and Serum Research Institute granted approval for the research in June 2022 (Reference: IR.RVSRI.REC.1402.002).

Patients and sampling. The serological surveillance reports of brucellosis in Iran have led to the categorization of Iranian provinces into five groups based on incidence levels: very low, low, moderate, high, and very high (16). The study employed a weighted average formula, using North Khorasan as the sampled province due to its high brucellosis incidence. With a calculated sample size of 138, participants were individuals with clinical suspicions of brucellosis, documented by medical records, and data was collected using structured questionnaires on socio-demographic, epidemiological, and clinical factors. The questionnaire's topics were chosen based on previous studies (16-18). Blood samples (10 ml) were collected from all patients presenting with symptoms of fever, sweating, arthralgia, back pain and headache, and those who were suspected of having brucellosis. These samples were collected at health and medical centers of North Khorasan province between November 2022 and June 2023.

Laboratory procedure. After sampling, serum was harvested from blood samples and got preserved at -20°C until further analysis. The RBPT (Rose Bengal Plate Test), Wright, and 2-ME (2-mercaptoethanol) (Razi Vaccine and Serum Research Institute, Iran) were performed on the serum samples based on the standard procedures that has been described previously (19).

Bacterial culture and classical typing. Blood broth medium (Bahar Afshan, Iran) was used to inoculate 10 mL blood samples. The blood culture bottles

were incubated at a temperature of 37°C for 21 days and possibly even longer. After a certain time, samples from each bottle were cultivated weekly using selective methods. The agar plates were infused with specific antibacterial and antifungal agents (Thermo Scientific™ Oxoid™, UK). Traditional culture and phage-based methods were used to characterize the isolated bacteria on a selective medium that showed suspected *Brucella* morphological traits at the species and biovar levels by standard standards (19). The lysis reaction on strains was assessed by employing Tbilisi (Tb) phage at two dilutions of RTD and RTD×10^4 as well as Izatnagar (IZ) phage. A positive reaction was determined when complete lysis occurred after 48 hours of incubation at 37°C. Following that, the rough phases of colonies and the A and M antigens were assessed using a slide agglutination test with A, M mono-specific sera. The control strains utilized in this study were *B. melitensis* biovar 1 strain 16M, *B. abortus* biovar 1 strain 544, and vaccine strains of *B. melitenis* Rev.1 and *B. abortus* RB51.

Extraction of bacterial DNA. Genomic DNA was extracted using the Exgene Cell SV kit from Gene All (South Korea), and its concentration was measured at 260/280 nm with the ND-1000 Nanodrop spectrophotometer. The DNA integrity was evaluated on a 1% agarose gel. Bacterial DNA concentrations were determined with a NanoDrop Spectrophotometer and stored at -20°C.

Molecular identification of isolated bacteria. To identify the presence of *Brucella* species, the bacterial DNA was subjected to a PCR experiment using the IS711 marker (20). The thermal program of the AMOS PCR technique included an initial denaturation phase at a temperature of 95°C for five minutes. Subsequently, a series of 35 denaturation cycles were performed at a temperature of 95°C for thirty seconds, followed by annealing at 55°C for sixty seconds, extension at 72°C for three minutes, and a final extension at 72°C for ten minutes (20). The multiplex PCR experiment for species-level molecular characterization was conducted using eight primer sets (Bruce-ladder PCR) as described elsewhere (21). The thermal program for running was as follows: The setup for the experiment involved 15 minutes of first denaturation at 95°C, 35 cycles of 30 seconds at 95°C as second denaturation, 2 minutes at 60°C as annealing, and 1 minute at 72°C as first extension, and a final extension step of 5 minutes at 72°C. The visualization of PCR products was achieved through a 2% agarose gel in an electrophoresis process (Table 1).

Statistical analysis. The data was analyzed statistically using SPSS 21 (SPSS Inc, USA). The relationship between risk factors and brucellosis was assessed using the Chi-square test. For nonparametric, especially ordinal data, the Mann-Whitney U-Test was utilized. Statistical significance was set at a p-value of less than 0.05.

RESULTS

The study included 200 participants suspected of having brucellosis, with exhibiting clinical symptoms, who visited health and medical centers in North Khorasan province between November 2022 and June 2023. Participants were excluded if they did not provide informed consent, had a confirmed diagnosis of another infectious disease, or had severe complications or comorbid conditions that could interfere with the study outcomes. The average age of patients was 44.10 ± 9.78 years. Of the participants, 66.5% were males and 33.5% were females. Among the 200 individuals, 24.5% had direct contact with livestock, while 31.5% consumed unpasteurized dairy products. Of the 50 individuals with positive antibodies, 92% consumed unpasteurized dairy, and 82% had close contact with livestock. The human brucellosis prevalence was higher in urban areas (35.2%) compared to rural areas (24%), although the difference was not statistically significant. Furthermore, brucellosis seropositivity was more common in males (24%) than in females (26.8%), and it was higher in individuals aged 30 and above (28.5%) compared to those below 30, although the differences were not statistically significant. Additionally, 96% of seropositive individuals had education levels below high school (Table 2).

Laboratory finding. The commonly seen clinical features in suspected persons were fever (54.5%), headache (34%), arthralgia (21.5%), and adenopathy (5%). Nevertheless, 70% of individuals who tested positive for the brucellosis exhibited fever, while 52% experienced headaches. Additionally, 34% reported arthralgia, and 14% had adenopathy (Table 3). All clinical features in this study demonstrated a significant association with seropositivity in

Table 1. Specific particular primer sets and anticipated amplicon sizes for each *Brucella* species

Strains	Primer	Primer sequence (5-3)’	Target gene	Amplicon size	References
B. abortus	IS711	TGCCGATCACTTTCAAGGGCCTTCAT	IS711	489	(20)
	AB	GACGAACGGAATTTTTCCAATCCC			
B. melitensis	IS711	TGCCGATCACTTTCAAGGGCCTTCAT	IS711	731	(20)
	BM	AAATCGCGTCCTTGCTGGTCTGA			
B. abortus	BMEI0998f	ATCCTATTGCCCCGATAAGG	Glycosyltransferase,	1682	(21)
B. melitensis	BMEI0997r	GCTTCGCATTTTCACTGTAGC	gene wboA		
B. melitensis Rev.1					
B. abortus	BMEI0535f	GCG CATTCTTCGGTTATGAA	Immunodominant	450	(21)
B. melitensis	BMEI0536r	CGCAGGCGAAAACAGCTATAA	antigen, gene bp26		
B. melitensis Rev.					
B. abortus	BMEI1436f	ACGCAGACGACCTTCGGTAT	Polysaccharide	794	(21)
B. melitensis	BMEI1435r	TTTATCCATCGCCCTGTCAC	deacetylase		
B. melitensis Rev.1					
B. abortus	BMEII0428f	GCCGCTATTATGTGGACTGG	Erythritol catabolism,	587	(21)
B. melitensis	BMEII0428r	AATGACTTCACGGTCGTTCG	gene eryC		
B. melitensis Rev.1					
B. abortus	BMEII0987f	CGCAGACAGTGACCATCAAA	Transcriptional	152	(21)
B. melitensis	BMEII0987r	GTATTCAGCCCCCGTTACCT	regulator, CRP family		
B. melitensis Rev.1					

Table 2. Factors associated with human brucellosis seropositivity

Factor	Group	Total (%)	Positive (%)	Negative (%)	OR	CI	P value
Age group	≤ 30	21 (10.5)	6 (12)	15 (10)	1.22	0.44-3.35	0.69[b]
	>30	179 (89.5)	44 (88)	135 (90)			
Gender	Male	133 (66.5)	32 (64)	101 (67.3)	0.86	0.44-1.68	0.66[b]
	Female	67 (33.5)	18 (36)	49 (32.7)			
Residency	Rural	183 (91.5)	44 (88)	139 (92.7)	0.58	0.20-1.66	0.307[b]
	Urban	17 (8.5)	6 (12)	11 (7.3)			
Education	Below high school	195 (97.5)	48 (96)	147 (98)	0.49	0.07-3.01	0.43[b]
	Above below high school with university degree	5 (2.5)	2 (4)	3 (2)			
Unpasteurized dairy	Yes	63 (31.5)	46 (92)	17 (11.3)	89.97	26.78-281.20	<0.00[b*]
	No	137 (68.5)	4 (8)	133 (88.7)			
Livestock contact	Yes	49 (24.5)	41 (82)	8 (5.3)	80.86	29.34-222.85	<0.00[b*]
	No	151 (75.5)	9 (18)	142 (94.7)			

human brucellosis (P<0.05). The standard tube agglutination test yielded antibody titers ranging from 80 IU/mL to 2560 IU/mL in seropositive individuals. *Brucella* was detected in culture from 5 out of 200 blood samples, representing a prevalence of 2.5%. Our findings indicated that the culture-based method required a minimum of three days for detection, with a maximum duration of up to eight days in certain instances. All samples that tested positive in culturing also tested positive using PCR. The fifty seropositive cases exhibited antibody levels of 80 IU/mL (4%), 160 IU/mL (14%), 320 IU/mL (16%), 640 IU/mL (16%), 1280 IU/mL (48%), and 2560 IU/mL (2%) in the wright test. Furthermore, the 2-ME test results in patients with brucellosis showed the following titers: 80 IU/mL in 7 patients (14%), 160 IU/mL in 8 patients

Table 3. Correlation between clinical symptoms and human brucellosis seropositivity

Factor	Group	Total (%)	Positive (%)	Negative (%)	OR	CI	P value
Fever	Yes	109 (54.5)	35 (70)	74 (49.3)	2.39	1.20-4.75	0.01b*
	No	91 (45.5)	15 (30)	76 (50.7)			
Headache	Yes	68 (34)	26 (52)	42 (28)	2.78	1.44-5.38	0.002b*
	No	132 (66)	24 (48)	108 (72)			
Arthralgia	Yes	43 (21.5)	17 (34)	26 (17.3)	2.45	1.19-5.05	0.01b*
	No	157 (78.5)	33 (66)	124 (82.7)			
Adenopathy	Yes	10 (5)	7 (14)	3 (2)	7.97	1.97-32.17	0.001b*
	No	190 (95)	43 (86)	147 (98)			

(16%), 320 IU/mL in 8 patients (16%), 640 IU/mL in 25 patients (50%), and 1280 IU/mL in 2 patients (4%). All the strains isolated and described using the blood culture and phage typing technique were identified as *B. melitensis* biovar 1. According to serologic titers, a strong culture positive was seen at a level of 640 IU/mL in wright and 2-ME tests ($P<0.05$).

Molecular identification of isolated bacteria. The identity of all isolates was verified as wild-type *B. melitensis* using both Bruce-ladder PCR, which produced products of 1682, 1071, 794, 587, 450, and 152 base pairs, and AMOS-PCR, which produced a PCR product of 731 base pairs (Figs. 1 and 2). The efficacy of the multiplex PCR in identifying field *Brucella* vaccine and species strains was confirmed by testing *B. abortus* strain 544, *B. abortus* strain Rb51, *B. melitensis* strain 16M, and *B. melitensis* strain Rev.1.

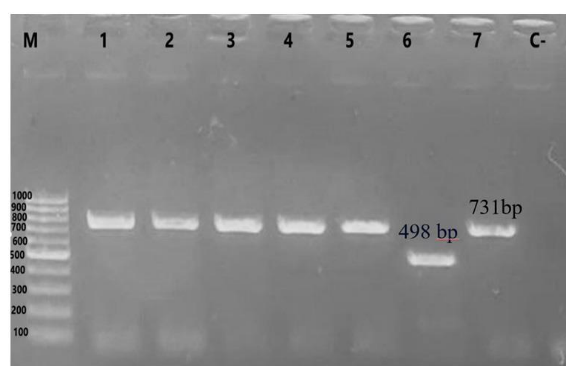

Fig. 1. Multiplex AMOS PCR. Lane M indicates molecular marker 100bp, lane 1-5: isolates of *Brucella melitensis* in this study (731 bp). Lane 6: *B. abortus* 544 reference strain (498 bp); Lane 7: *B. melitensis* 16M reference (731 bp); lane C- represents the negative control.

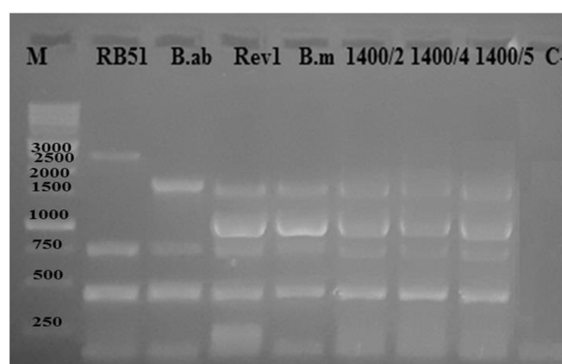

Fig. 2. Multiplex Bruce-ladder PCR. Lane M indicates molecular marker 1kb, RB51: *B. abortus* RB51 vaccine; lane; B.ab: *B. abortus* 544 reference strain (544), Rev.1: *B. melitensis* vaccinal strain, B.m: *B. melitensis* 16M reference strain, lanes 1400/2, 1400/4, and 1400/5 isolates of *B. melitensis* with the size of 1682, 1071, 794, 587, 450, and 152 base pairs; lane C- represents the negative control.

DISCUSSION

Brucellosis, a significant disease affecting both humans and animals globally, holds economic implications. Its impact varies across high-, middle-, and low-income nations. Africa and Asia are identified as major contributors to the global risk and cases of brucellosis, while specific regions in the Americas and Europe continue to face challenges (4). The study found that among 200 individuals with brucellosis symptoms, only 25% (50 individuals) tested positive for antibodies using the RBPT, Wright, and 2-ME methods. The complexity of serological test interpretation is influenced by factors such as the disease stages, the presence of different *Brucella* bacteria strains, and the performance characteristics of diag-

nostic methods. In this regard, bacterial culture is a crucial method for detecting brucellosis and provides several important advantages in the diagnostic process (19). Bacterial culture is a crucial method for directly isolating and identifying *Brucella* bacteria in clinical samples, aiding in the accurate diagnosis of brucellosis. This confirmation is essential for epidemiology and understanding infection sources. While differentiating between strains provides insights into disease severity, bacterial culture has limitations, including time-consuming results and lower sensitivity in early infection stages. Moreover, working with live *Brucella* cultures poses biohazard risks, necessitating specialized facilities and trained personnel (5, 22).

Our results indicated that the prevalence of human brucellosis among individuals with similar symptoms was 25% based on serological diagnosis and 2.5% with positive blood culture. The research indicates that 10% of patients with positive serological assessments of Wright and 2-ME also tested positive in bacterial cultures, with a minimum detection time of three days and, in some cases, up to eight days. All instances of positive culture were associated with a serological titer exceeding 1:320 in Wright and 2-ME. Approximately 70% of patients with negative culture results had a Wright and 2-ME titer of 1:80. The study establishes a significant association between positive culture and serological titer, contributing to our understanding of the diagnostic correlation in brucellosis. The findings also compare with other studies, revealing either alignment or deviation from reported trends in the rate of positive cultures among individuals with positive serological assessments. On the other hand, a higher seroprevalence rate (60.3%) during 2010 was reported in Mazandaran province, Iran (23). Two studies investigated brucellosis in individuals suspected of having the disease based on symptoms like psychological symptoms, headache, chills, sweating, weakness, back pain, lethargy, fever, and decreased appetite. The seroprevalence of brucellosis was examined in Guilan province, Iran, with a focus on rural residents and slaughterhouse workers. Results indicated a 5.5% seroprevalence rate among rural inhabitants and a 9.8% rate among slaughterhouse workers. Notably, these rates were lower than those observed in the referenced study (24). Other researchers have noted that the prevalence of human brucellosis varies across different countries worldwide, with differing rates, such as 12.6% in Iraq (18), 32.25% in Pakistan (25), 6.1% in Rwanda (26), 8.8% in Turkey (27), 16.7% in India (28). Different study methodologies, patient demographics, and locations may cause such variations. Only people with nonspecific symptoms were tested for serological brucellosis. Livestock brucellosis prevalence varies due to biological and socioeconomic factors (29, 30). This emphasizes the importance of identifying disease risk factors in each region. The article found significant infection risk variables. The findings highlight a strong link between illness, unpasteurized dairy consumption, and cattle interaction.

According to our findings, people who eat unpasteurized dairy or work with livestock are more likely to get sick. Effective public health treatments and prevention efforts require an understanding of these major risk factors. Reducing exposure to unpasteurized dairy products and boosting livestock safety can reduce infection risk and improve public health (31). This finding aligns with prior studies that demonstrated a higher susceptibility to infection when consuming unpasteurized milk, whereas the consumption of boiled milk substantially decreased this risk (17, 18, 32, 33).

The findings align with a research conducted in Egypt, which showed a substantial association between direct interaction with livestock and the prevalence of human brucellosis (34). Implementing protective measures can provide valuable insights into identifying the specific livestock species acting as a reservoir for a particular strain of *Brucella* spp. Similarly, close results in this regard were observed both in Iran (35, 36) and Saudi Arabia (37).

Brucellosis can affect individuals of all age groups, although young adults face the greatest susceptibility (38). The findings of our study align with research conducted in Bangladesh and Uganda, which demonstrated an elevated risk of brucellosis infection among adults aged 40-80 years (17, 39, 40). A separate investigation conducted in the Northern Palestine revealed a positive correlation between age groups and the incidence of brucellosis infections (41). Nevertheless, a study conducted in Turkey revealed a rather high incidence of human brucellosis among the younger population. It is worth mentioning that cattle rearing in Turkey commences at young ages (42). The incidence of brucellosis among older individuals may be attributed to their consumption of unpasteurized dairy products (40). In our study,

male participants were more likely to be brucellosis-positive than females. In similar studies, it was observed that males exhibited a higher seropositivity rate for human brucellosis compared to females. This trend suggests a potential gender-related susceptibility or exposure difference to the *Brucella* bacteria (24, 43, 44). Males were more likely to farm domestic animals and handle their products in close contact with animals, which may explain their higher brucellosis seroprevalence. Another study found that females had a somewhat greater rate than males (40). *Brucella* isolate species identification aids epidemiological screening in surveillance investigations. They assess *Brucella* prevalence and imports in endemic areas. This study found *B. melitensis* biovar 1 in all positive samples. This confirms a prior Iranian study that found *B. melitensis* biovar 1 to be the most common strain in humans (11, 17, 45).

Numerous molecular methods can identify *Brucella* species. For instance, 16S rRNA gene sequencing may identify genus but not species. Ribotyping, AFLP, omp25, omp28 DNA sequencing, and species-specific IS711 (20) or Bruce-ladder PCR can resolve species (21). Our results also confirmed the genus and species of isolated *Brucella* spp. through the multiplex PCR. Another investigation detected *B. melitensis* and *B. abortus* using B4/B5 primers and AMOS PCR (35). The prevalence of each species of *Brucella* spp. in a country may be attributable to its traditional livestock (46). For example, *B. melitensis* has been identified as the leading cause of human brucellosis in Iran (45), Saudi Arabia (47), and, in Turkey (48, 49). The study results may influence future research directions, clinical practices, and public health strategies related to the diagnosis and management of brucellosis in affected populations.

CONCLUSION

The study provides valuable insights into human brucellosis diagnosis in North Khorasan province, Iran, revealing consistent isolation of *B. melitensis* biovar 1 in culture-positive samples, indicating its endemic nature. These findings emphasize the ongoing public health challenge in the region, stressing the importance of sustained surveillance, targeted interventions, and continued research for more effective disease management and control strategies.

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