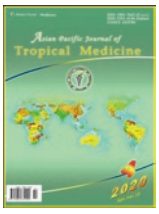

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Epidemiological, molecular characterization and risk factors of human brucellosis in Iran

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ABSTRACT

Objective: To determine epidemiological, molecular characterization, and potential risk factors of human brucellosis.

Methods: This descriptive study was carried out in the clinical setting in Iran between 2017 and 2018. A total of 297 participants enrolled in the study. The sample size was calculated based on the occurrence rate of brucellosis in different areas. Patients were assessed using serological tests and conventional culture methods. Phage and multiplex PCR methods typed all of *Brucella* isolates. Potential risk factors of disease were determined.

Results: A total of 141 of 297 (47.5%) *Brucella* strains were isolated and all of them were detected as *Brucella melitensis* biovar 1. Based on serologic titers, high culture positivity was recorded at 1/640 titer (P<0.006). The risk factors for brucellosis were patients older than 40 years (*OR*=2.23, 95%*CI*: 1.4-3.55, P=0.001), animal keeper (*OR*=7, 95%*CI*: 1.51-32.41, P=0.005), housewife (*OR*=8.76, 95%*CI*: 1.85-41.37, P=0.002), farmer (*OR*=6.42, 95%*CI*: 1.21-33.97, P=0.019), and contact with animal (*OR*=1.31, 95%*CI*: 0.60-2.85, P=0.005).

Conclusions: To the best of our knowledge, this is the first comprehensive report from Iran presenting the detection of *Brucella* species by the multiplex PCR. *Brucella melitensis* biovar 1 is still the dominant causative agent in Iran. The consumption of unpasteurized dairy products, living in rural areas, and animal contact were risk factors of brucellosis.

KEYWORDS: Brucellosis; Epidemiology; Molecular detection; Risk factors

1. Introduction

Brucellosis is an important and widespread zoonosis which is transmitted to humans through direct exposure with infected materials or not directly by the eating of contaminated animal products and by inhalation of airborne agents[1]. This disease has high morbidity both for humans and animals, and it is a significant cause of economic loss and a public health risk in many developing countries[2]. Endemic areas for brucellosis comprise countries of the Mediterranean basin, Middle East, Central Asia, China, the Indian subcontinent, sub-Saharan Africa, and parts of Mexico and Central and South America. In Iran cases of human brucellosis are described yearly; most of them are because of *Brucella (B.) melitensis*. Unpasteurized dairy products, such as fresh cheese are a significant source of infection.Brucellosis is an endemic disease illustrated by a high infection rate in both animals and humans in Iran[3]. Presently, there is limited information existing on the molecular epidemiology of circulating strains. Human infection is related to factors and activities associated with exposure to shedding animals and contaminated food products. Brucellosis transmission to humans is controlled through behavior change and food safety interventions that target milk production. For prevention measures, more information on risk factors is required.The aim of this study is to describe the molecular epidemiology of *Brucella* strains isolated in high-risk areas in species level and evaluate the risk factors of human brucellosis.

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2. Material and methods

2.1. Study areas

This cross-sectional study was performed in the clinical setting at the western, northwestern, northern, and central provinces of Iran from December 2017 to June 2018.

2.2. Sample size

Based on the recent serological-surveillance reports of brucellosis in Iran, provinces of Iran were categorized into five groups, including very high, high, moderate, low, and very low incidence areas[3]. The sample size was determined using a weighted average (weighted mean) formula as the prevalence rate of brucellosis in each province considered as its weight. Regarding the maximum incidence rate of 80-101 per 100 000 persons, minimum sample size at 5% level of precision, 95% confidence level, the calculated sample size was 138. One province selected from each province groups for sampling: Lorestan (very high incidence rate) located around 33° 29' 16" N and 48° 21' 21" E. Kermanshah (high incidence rate) found approximately 34° 18' 51" N and 47° 03' 54" E, East Azerbaijan (moderate incidence rate) located around 38° 04' N and 46° 18' E, Mazandaran (low incidence rate) found about 36° 33' 48" N and 53° 03' 36" E, and Isfahan (very low incidence rate) located around 32° 38' N and 51° 39' E. We have chosen 138 patients based on recent serological-surveillance brucellosis in Iran using a weighted average formula, considering the prevalence of brucellosis in each province. It distributed as follows in the five provinces: 105 (Lorestan), 80 (Kermanshah), 64 (Eastern Azerbaijan), 37 (Mazandaran), and 11(Isfahan) (Figure 1).

Figure 1. General map of Iran showing the five provinces included in the study. The selection performed was based on seroprevalence rate of brucellosis. Lorestan: very high (81-100 per 100 000 persons) (n=105); Kermanshah: high (61-80 per 100 000 persons)(n= 80); East Azerbaijan: moderate (41-60 per 100 000 persons)(n=64); Mazandaran: low (21-40 per 100 000 persons)(n=37); Isfahan: very low (1-20 per 100 000 persons)(n=11).

2.3. Research ethics

Written informed consent was obtained from all adult contributors and the parents or legal guardians of minors. Kashan University of Medical Sciences Research Ethics Committee approved this study (IR.Kaums.REC.1395.156).

2.4. Inclusion criteria

The study contributors were patients of two years old and above with clinical doubt of brucellosis described by a stated history of fever and one or more of the following symptoms including chills, night sweats, headache, weight loss, fatigue, myalgia, arthralgia, malaise, weakness and arthritis. The identification of brucellosis was known according to the isolation of *Brucella* spp. in blood culture or other clinical samples. Noteworthy titers were Wright's agglutination titer of $\geqslant$1/160.

2.5. Questionnaire survey

Designed forms were used to gather data on socio-demographics (*e.g.*, gender, age, education, place of living and job), epidemiology (*e.g.*, ingesting of dairy foods and contacting with animals), as well as the clinical manifestation. The contents of the questionnaire selected based on other studies[3,4].

2.6. Serological tests

The Rose Bengal test for screening and the qualitative assay was used as a recommended procedure[4]. Also, the agglutination tube test was used as a quantitative assay for determining antibody titer. These assays were conducted based on the standard protocol.

2.7. Bacterial culture and phage typing

Ten mL blood samples were inoculated in brain heart infusion broth medium(Merck, Germany). The blood culture bottles were incubated at 37 ℃ for 21 d and a longer time. During the incubation period, samples of each bottle were weekly sub-cultured on selective *Brucella* Agar plates containing selected antifungal and antibacterial agents (Thermo Scientific™ Oxoid™). The grown strains on selective media with suspected morphological characteristics of *Brucella* were characterized by traditional culture and phage-based method at species and biovar levels according to the standard pressure. Lysis reaction on strains was determined using Tb phage at two dilutions, routine test dilution (RTD) and RTD×10^4. Complete lysis was considered a positive reaction after 48-hour incubation at 37 ℃. Subsequently, A and M epitopes and rough phases of colonies were evaluated by A, M, and R mono-specific sera using slide agglutination test. *Brucella* (*B.*) *abortus* biovar 1 strain 544 (ATCC No. 23448), *B. melitensis* biovar1 strain 16M (ATCC No. 23456), *Brucella* (*B.*) *suis* biovar 1 strain 1330 (ATCC No. 23444), *B. abortus* Rb51 (vaccine), and B. melitenis Rev.1 (vaccine) reference strains were used as control.

2.8. DNA extraction

Genomic DNA was obtained from the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbHCo, Germany). Briefly, 200 μL of bacterial suspension was centrifuged at 3 000 × *g* for 5 min, then the supernatant was discarded, and the bacterial pellet was suspended in 200 PBS buffer. After that, 5 μL lysozyme (10 mg/mL in 10 mM Tris-HCl, pH 8.0) was added and incubated for 15 min at 37 ℃, then, 200 μL binding buffer and 40 μL reconstituted proteinase K was added followed by incubating for 10 min at 70 ℃. A total of 100 μL isopropanol was increased and mixed well. After two washing steps, extracted DNA was eluted in TE solution (Tris 10 mM, EDTA 1mM, pH 8.0). The quality and quantity of DNA were measured using NanoDrop Spectrophotometer (NanoDrop® ND-1000 Technologies Inc., USA). It was then stored at -20 ℃ for further analysis.

2.9. Multiplex PCR assay

The multiplex PCR assay was done by nine primer sets, as described previously (Table 1)[5]. Briefly, each PCR reaction consisted of 25 μL 2× QIAGEN Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 5 μL of 10× primer mix (100 pmol/μL), 2 μL template DNA (25 ng/μL) and up to 50 μL RNase-free water. The reactions were performed in GenAmp Eppendorf Thermal cycler (Germany). The running conditions were: 15 min for enzyme activation at 95 ℃ followed by 35 cycles of 30 s at 94 ℃, 2 min at 60 ℃, and 1 min at 72 ℃, and a final extension step of 5 min at 72 ℃. Amplified products were visualized by electrophoresis using 1.8% agarose gel and stained with ethidium bromide.

Table 1. Primer sets used for differentiation of *Brucella* strains in this study.

Primer	F/R	Sequence (5'-3')	Amplicon size (bp)
BMEI0998	F	ATC CTA TTG CCC CGA TAA GG	1 682
BMEI0997	R	GCT TCG CAT TTT CACTGT AGC	
BMEI0535	F	GCG CAT TCT TCG GTT ATG AA	450
BMEI0536	R	CGC AGG CGA AAA CAG CTA TAA	(1 320)
BMEII0843	F	TTT ACA CAG GCA ATC CAG CA	1 071
BMEII0844	R	GCG TCC AGT TGT TGTTGA TG	
BMEII0428	F	GCC GCT ATT ATG TGG ACT GG	587
BMEII0428	R	AAT GAC TTC ACG GTC GTT CG	
BMEII0428	F	AAT GAC TTC ACG GTC GTT CG	272
BR0953	R	GGA ACA CTA CGC CAC CTT GT	
BR0953	F	GAT GGA GCA AAC GCT GAA G	218
BMEI0752	R	CAG GCA AAC CCT CAG AAG C	
BMEII0987	F	CGC AGA CAG TGA CCA TCA AA	152
BMEII0987	R	GTA TTC AGC CCC CGT TAC CT	
BME2 0722	F	CCAACCGTATGTCCTCT CT	766
BME2 0722	R	TGCGGGAACTGGTGTTCGAC G	
BME1 r02	F	CTACTCAAGGACAACAG GTG	344
BME1 r02	R	TGTGTCGTTTAAGGCAATAG G	

2.10. Statistical analysis

Results from questionnaires and serological tests were stored and analyzed using SPSS version 16. *Chi*-square and Fisher's exact tests were figured to calculate the degree of association of the risk factors with brucellosis. $P<0.05$ was measured statistically significant. Odd ratios with 95% confidence intervals were calculated for determining the severity of association of all independent variables with culture test results. Multiple logistic regression models were applied to assess the effect of different factors on culture test results. In this model, the backward method was used and $P>0.1$ as excluding criteria was considered.

3. Results

An overall of 297 contributors participated in the study. The mean age of the patients was (41.4±17.4) years (range, 2 to 90 years). Of these participants, 164 (55.2%) were males, and 133 (44.8%) were females. A total of 268 out of 297 (90.2%) had contact with sheep and goats, 286 (96.3%) ingesting unpasteurized milk or soft cheese. The prevalence was higher (47.67, 133/297) in rural than urban (44.44%, 8/18) residents, but the difference was not significant.

3.1. Risk factors results

Table 2 presents the risk factors recognized. Older than 40 years (*OR*: 2.23, 95%*CI*: 1.40, 3.55), animal keeper (*OR*: 7, 95%*CI*: 1.51, 32.41), housewife (*OR*: 8.76, 95%*CI*: 1.85, 41.37), farmer (*OR*: 6.42, 95%*CI*: 1.21, 33.97), and contact with sheep and goats (*OR*: 1.31, 95%*CI*: 0.60, 2.85) increased the risk forbrucellosis. Multiple regression analysis revealed that variables including gender (male), education (under diploma), occupation (animal keepers, housewife), and animal contact were effective on culture test results (Table 3).

3.2. Clinical features results

Common clinical features included fever (83.84%), sweats (83.50%), headache (77.78%), arthralgia (61.25%), backache (85.86%) and myalgia (60.2%). Antibody titers obtained in standard tube agglutination test ranged from 160 IU/mL to 5 120 IU/mL.

3.3. Laboratory finding

Our finding showed that the rate of brucellosis seropositivity was high in male participants (55.21%) is compared with the female (44.78%), as well as within the age group of 40 years old than other age groups. 141 out of 297 (47.5%) blood samples yielded *Brucella* in culture. Our results showed that the minimum time for detection by culture was three days, and in some cases, it was as long as seventy days. All samples positive cultures were positive by PCR too. All cases had antibodies levels of 160 IU/mL cut-off value in

Table 2. Potential risk factors for human brucellosis on the basis of univariate analysis.

Characteristics		Total population	Culture test		Statistical analysis		
		n (%)	Positive *n* (%)	Negative *n* (%)	*OR*[1]	95%*CI*	*P* value
Gender	Male	164 (55.21)	77 (46.95)	87 (53.05)	0.95	(0.60, 1.50)	0.840
	Female	133 (44.78)	64 (48.12)	69 (51.88)	-		
Age-group (years)	<40	146 (49.16)	84 (57.53)	62 (42.47)	-	(1.40, 3.55)	0.001
	⩾40	151 (50.84)	57 (37.75)	94 (62.25)	2.23		
Education	Under diploma	293 (98.65)	140 (47.78)	153 (52.22)	2.74	(0.28, 26.69)	0.360
	Graduated	4 (1.35)	1 (25.00)	3 (75.00)			
Region	Urban	18 (6.06)	8 (44.44)	10 (55.56)	0.87	(0.33, 2.29)	0.790
	Rural	279 (93.94)	133 (47.67)	146 (52.33)			
Occupation	Student[1]	14 (4.71)	12 (85.71)	2 (14.29)	-	-	-
	Animal keepers	143 (48.15)	66 (46.15)	77 (53.85)	7	(1.51, 32.41)	0.005
	Housewife	96 (32.32)	39 (40.62)	57 (59.38)	8.76	(1.85, 41.37)	0.002
	Farmer	29 (9.76)	14 (48.28)	15 (51.72)	6.42	(1.21, 33.97)	0.019
	Other	15 (5.05)	10 (66.67)	5 (33.33)	3	(0.47, 18.92)	0.231
Contact with sheep and goats	Yes	268 (90.24)	129 (48.13)	139 (51.87)	1.31	(0.60, 2.85)	0.489
	No	29 (9.76)	12 (41.38)	17 (58.62)			
Consumption of unpasteurized dairy products	Yes	286 (96.30)	136 (47.55)	150 (52.45)	1.08	(0.32, 3.64)	0.890
	No	11 (3.70)	5 (45.45)	6 (54.55)			

CI=confidence interval; *OR*=odds ratio; [1]student is baseline, *Chi*-square test.

Table 3. Multiple logistic regression model coefficients of factors affecting the results of culture test.

Variables	Parameter (beta)	S.E of beta	Wald statistics	Degree of freedom	*P* value	Adjusted *OR*
Gender	-0.896	0.438	4.181	1	0.041	0.408
Education	4.382	1.209	13.134	1	<0.001	80.038
Housewife	-2.824	0.821	11.829	1	<0.001	0.059
Husbandry	-2.110	0.791	7.118	1	0.008	0.121
Animal contact	1.073	1.073	3.571	1	0.049	0.049

Table 4. Results of the laboratory test of 297 participants in this study [*n*(%)].

Wright test titer	Culture positive	Culture negative	Total	*P* value
1/80 (160 IU)	24 (42.1)	33 (57.9)	57 (19.2)	0.006
1/160 (320 IU)	19 (29.7)	45 (70.3)	64 (21.5)	
1/320 (640 IU)	63 (56.8)	48 (43.2)	111 (37.4)	
1/640 (1 280 IU)	21 (61.8)	13 (38.2)	34 (11.4)	
1/1 280 (2 560 IU)	13 (43.3)	17 (56.7)	30 (10.1)	
1/2 560 (5 120 IU)	1 (100)	0 (0%)	1 (0.3)	

Brucella standard tube agglutination test, 48 (43.2%) patients having antibodies titers of 320 IU/mLwere negative by culture (Table 4). The results of the blood culture and phage typing system revealed that all isolated strains characterized as *Brucella melitensis* biovar 1. Based on serologic titers, high culture positivity recorded at ⩾1/640 titer (P<0.006). The results of the laboratory test of 297 participants in this study described in Table 4.

3.4. Multiplex PCR results

Our findings indicated that all 141 positive cultures, the detected *Brucella* species were *B. melitensis* by multiplex PCR. The ability of the multiplex PCR to detect *Brucella* species and vaccine strains was validated using *B. abortus* strain 544, *B. melitensis* strain 16M, *B. suis* strain 1330, *B. abortus* strain Rb51 and *B. melitensis* strain Rev.1 (Figure 2).

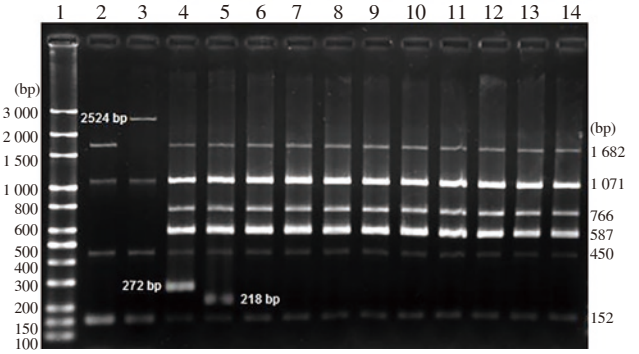

Figure 2. Multiplex PCR assay for typing of *Brucella* isolates. Lanes 1 and 15, mid rang DNA ladder (Invitrogen); Lane 2 *Brucella abortus* 544; Lane 3 *Brucella abortus* Rb51; Lane 4 *Brucella suis* 1330; Lane 5 *Brucella melitensis* Rev.1; Lane 6 *Brucella melitensis* 16M; Lane 7-14 isolates of *Brucella melitensis*.

4. Discussion

Results from our study showed that less than half percent of the patients who had been serologically diagnosed were culture positive for *Brucella*. Laboratory findings for brucellosis diagnosis interacted with contact history, job, clinical manifestations, and record of past infection[6,7]. Serological techniques, and various molecular approaches are used in identification of brucellosis[8–10]. The interpretation of serological tests is more complex, chiefly in the setting of chronic infections, re-infections, relapses, and in areas that brucellosis is endemic where the brucellosis antibodies have been found in a high percentage of the people[11]. PCR performed on blood or any body tissue samples[12]. However, using PCR-based tests due to the need for standardization of the procedures cannot be supposed usual diagnostic techniques[13]. Identification of the genus

and species of *Brucella* is achievable by several molecular tests[14,15]. Polymerase chain reaction-fragment length polymorphism (PCR-RFLP) is considered as an appropriate tool for the detection of *Brucella* spp. at the species and biovar level in epidemiological studies[16]. *Brucella* culture positive in other studies differ from 10%-90% according to sample type, phase of infection, previous usage of antibiotics, and by slow-growing character of *Brucella*[17]. In this survey, the results of culture for brucellosis suspicious cases were 141 out of 297 (47.5%) positive, which is higher than the previous report of 23.4% in Jordan[18] but was lower than the results of 74.1% positive reported from Kuwait[19]. Moreover, the most blood cultures will be positive at the 7th and 21st day[20]. Our results showed the minimum time of detection was three days, and in some cases, it was as long as seventy days. This study showed that 70.3% of patients with negative results in cultures had positive serology 1/160. Our results showed that a major of culture-positive individuals (61.8%) were in the titer group of 1/640. There was a close relation between the serology titer and the positive culture findings (P<0.006). Identification of species and biotyping of *Brucella* isolates play role as a helpful epidemiologic screening tool for surveillance studies of outbreaks and importation of *Brucella* strains in endemic territories[21,22]. All isolates from positive cases belonged to *B. melitensis* biovar 1, suggesting it as obviously the most common species/biovar implicated in the human disease in Iran. *B. abortus* biovar 3 is most isolated from Turkey, Egypt, India, Italy, and Africa, but in Iran, it is isolated just from domestic animals[22]. *B. melitensis* biovar 3 has been reported in the most human brucellosis cases in China[23]. *B. melitensis* biovar 3 showed the most frequently isolated biotype of *Brucella* from both animals and humans in Egypt[24]. Similarly, *B. melitensis* biovar 3 have revealed the causal agent in several epidemiologic studies in Turkey[25,26]. Zoghiet *et al.* reported *B. melitensis* biovar 1 as main agent for the disease in Isfahan, Khorasan, Guilan, Khuzestan, Yazd, and Kermanshah provinces in Iran. Still, in Tehran and Azerbaijan, biovars 1, 2, and 3 were the dominant ones[22]. The virulence of *B. melitensis* and *B. suis* for humans are more than *B. abortus* and/or *Brucella canis*[27-29]. At the present time the host preference and differential virulence between *B. melitensis* biovars have slight evidences.

Therefore, a supplementary investigation on *B. melitensis* isolates is needed to elucidate at the biovar level. Several molecular tests are available to clarify the genus and species of *Brucella*. For example, using 16S rRNA gene sequencing works to identify at genus level, but it is not useful at the species level[30]. Resolution to species level is possible through ribotyping, amplified fragment length polymorphism analysis, DNA sequencing of omp2, and omp25, as well as PCR assays targeting species-specific insertions of IS711 or IS650 elements[31]. The multiplex PCR assays that have been described by several authors can identify *Brucella* important species, including *B. melitensis*, *B. abortus*, and *B. suis*, simultaneously. Hence, various primer sets targeting bcsp31, omp2b, omp2a, and omp31 have been described to identify the major causative agents of human brucellosis in a single PCR reaction[32-34]. Our data showed that the multiplex PCR approach could discriminate *Brucella* species and vaccine strains. The multiplex PCR approaches could be used to the eradication in resource-limited laboratories of developing and underdeveloped countries[35]. Depending on the disease phase, clinical manifestations of infections with *B. melitensis* are variable[36]. Our findings revealed that fever, backache, and sweating were the most prominent symptoms in patients. Fever, arthralgia, and malaise were the most complaints of pediatric brucellosis in Iran[37]. Several risk factors have described for human brucellosis[38,39]. Univariate analysis showed that history of using unpasteurized dairy products, living in rural area, and contact with animals were major risk factors of brucellosis. These findings are agreed with other studies from Iran, Palestine, Lebanon, and Saudi-Arabia[40-42]. We found evidence of a relationship between brucellosis and sheep and goat contact. This is in agreement with other studies that have concerned cattle as asignificant reservoir and it shows a possible role for them in the epidemiology of brucellosis in Iran. Our results that brucellosis is related with animal contact and with using unpasteurized dairy products may suggest that *B. melitensis* is circulating in Iran.

Performing multiplex PCR only on specimens with *Brucella* antibody titers $\geqslant$160 IU/mL, which determined by standard tube agglutination test, instead of all 297 collected samples due to lack of resources, was one of the restrictions of this study. We recommended that species-level identifications could be done directly from blood samples with a high meticulous diagnostic test through direct multiplex PCR method.

To our information, this is the first inclusive report from Iran presenting the identification of *Brucella* species by the multiplex PCR. Although the identification of *Brucella* strains remains a considerable laboratory challenge, particularly in the developing and endemic countries, the multiplex PCR approaches are considered safe and rapid diagnostic methods for *Brucella* species. *B. melitensis* biovar 1 was dominant in Lorestan, Kermanshah, Eastern Azerbaijan, Mazandaran, and Isfahan of Iran. The use of unpasteurized dairy foods, living in rural places, and close contact with animals were the significant risk factors of human brucellosis in Iran.

Conflict of interest statement

The authors declared there are no conflicts of interest.

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Authors' contributions

RM, AE, MS conceived and designed the research proposal. AE, AS, BAH performed sampling and experiments. HA performed the data analysis. RM and AE contributed to the final version of the manuscript.

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