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The Prevalence of *Brucella* Biotypes Isolated From Sterile Body Fluids of Patients With Brucellosis in Kashan, Iran in 2013

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Abstract

Background: *Brucella* species are classified based on their pathogenic and genetic properties and hosts. Considering the significance of identifying different biotypes of *Brucella* from the epidemiological point of view and lack of such information in the city of Kashan, Iran.

Objectives: This study was designed to determine the biotypes and strains of *Brucella* isolated from patients with brucellosis.

Methods: This was a descriptive study of 206 samples obtained from patients with suspected brucellosis in 2013 in Kashan. BACTEC 9050 culture media was employed to test the samples. Suspected colonies of *Brucella* were identified through morphology, staining, and biochemical tests. The biotypes were identified by the Razi Research Institute. Lysis tests with the Tbilisi (Tb) phage were performed, the need for CO2, SH2 production, sensitivity to basic fuchsin and thionin stains, and the reaction of all the samples to specific antiserum A and M (monospecific) were tested.

Results: Fifty (24.3%) of the 206 samples were culture positive. SH3 production was not detected in any of the isolates, and none of the isolated strains required CO2. The results of the sensitivity test to basic fuchsin and thionin staining and specific agglutination and phage lysis (phage typing) tests indicated that all the isolated strains were biotype 1 *B. melitansis*.

Conclusions: The cause of human brucellosis in Kashan and its suburbs was biotype 1 *B. melitensis*. The identification of various biotypes of *Brucella* is important. Similar studies should be performed to detect the presence of new biotypes originating from neighboring countries.

Keywords: *Brucella*, Biotype, Prevalence

1. Background

Brucellosis, which is one of the most important and widespread zoonoses in the world, is endemic throughout Iran. Brucellosis is caused by *Brucella* species, which are small, gram negative, unencapsulated, nonsporulating coccobacilli. The bacteria grow aerobically, although some require supplemental CO2 for primary isolation. All strains are catalase positive, but oxidase and urease activities and the production of H2S vary among species. Major species and their biovars are differentiated based on metabolic tests, growth on media containing dyes, and lysis by specific bacteriophages. Based on their pathogenic and genetic characteristics and hosts, *B. abortus*, *B. canis*, *B. ovis*, *B. melitensis*, *B. suis*, and *B. neotoma* are recognized. *B. melitensis* and *B. aboratus* are the most prevalent types isolated from humans and cattle. There are three biotypes of *B. melitensis*. These are differentiated as M (monospecific) and A according to their reaction to specific antiserum. *B. melitensis* is the most frequently reported cause of human brucellosis worldwide.

The usual reservoirs of *Brucella* are goats and sheep, but camels can act as reservoirs in some areas (1). In a study in Iran of 568 blood samples, 121 (25%) isolates were type 1 *B. melitensis* (2). Typing of samples collected from 1107 cases of *Brucella* during 10 years in Iran revealed that the majority of the cases were biotype 1 and that this biotype was responsible for brucellosis in the provinces of Isfahan, Khorasan, Gilan, Khuzestan, Yazd, and Eastern Azerbaijan (3). In a study performed in Keeriakali in central Anatoly, all the isolates were *B. melitensis*, and 45 of the species isolated were biotype 3 (4). Baykam et al. used the BACTEC method to test the blood of brucellosis patients and isolated 42 strains, 37 of which were *B. melitensis* and 5 of which were *B. abortus* strains (5).

The identification of biotypes of different *Brucella* bacteria is epidemiologically important. Given that the disease spreads via the transfer of animals and their products,

the most prevalent strains in each region need to be identified to locate the source of primary infection. In addition, due to the high prevalence of brucellosis in the region and lack of sufficient information on the strain and serotype of *Brucella* causing the disease in the city of Kashan.

2. Objectives

This research was conducted to isolate and identify the species and types of *Brucella* isolated from patients in Kashan, a hot spot for brucellosis, to aid health policy making.

3. Methods

This was a descriptive study of 206 blood samples obtained from patients with suspected brucellosis in Kashan, Iran in 2013. Of the 206 samples, 50 (24.3%) were positive. All the samples were collected from sterile body fluids. The sample size was determined based on previous research.

The inclusion criterion was a suspected diagnosis of brucellosis by an infectious specialist according to clinical manifestations and epidemiology. The exclusion criterion was a history of treatment for brucellosis. All outpatients or hospitalized patients who showed signs of a fever, shivering, headache, myalgia, bone pain, night sweats, weakness, and lack of appetite and those with a family history of brucellosis were identified by an infectious specialist as suspected cases of brucellosis and referred to the laboratory for sample collection. All the procedures were explained to the patients, and they all signed a consent form.

This study was approved by the ethics committee of Kashan University of Medical Sciences (No. B/29/5/1/3534). Samples of sterile body fluids were collected from the patients. In the majority of the cases, only a blood sample was obtained and tested. In a few cases, other samples, including cerebrospinal fluid (CSF), synovial fluid, and bone marrow, were obtained and tested. Approximately 8-10 cc of the blood samples were injected into BACTEC media culture in an aseptic environment. For other cases, the fluid from joints and cerebrospinal fluid, the amount of injection was within the possible quantity. For serology testing, an additional 10 cc of blood was drawn for CRP, Wright, Coombs-Wright, and 2ME tests. An ELISA was performed to detect IgG and IgM antibodies to *Brucella*. Hematology tests included a complete blood count and analysis of the erythrocyte sedimentation rate.

3.1. Laboratory Tests

Reference strains of *B. abortus* 544, *B. melitensis* 16M, and *B. suis* 1330 were provided by the Central Veterinary Laboratory, Weybridge and stored as freeze-dried cultures. Reference phages, Tbilisi (Tb), Weybridge (Wb), Firenze (Fi), Berkely (Bk2), and Izatnagar (Izl) were propagated for use in *Brucella* typing. Monospecific anti-A and anti-M sera were prepared in rabbits. The species of *Brucella* were mostly isolated from suspected specimens obtained from animal fetuses, placenta, vaginal swabs, lymph nodes, milk, human blood cultures, and bone marrow cultures. Six hundred-eighteen strains of *B. abortus* and 2413 strains of *B. melitensis* were subjected to identification procedures. All cultures were performed according to the method recommended by. To initiate growth, 10% CO2 was supplied. The production of H2S was evaluated using lead acetate paper in tubes of *Brucella* agar medium. Smooth and rough colonies of strains that grew were distinguished using acriflavin and crystal violet tests. When sufficient cultures were obtained, their sensitivity to dyes (thionin: 1/25000, 1/50000, and 1/100000; basic fuchsin: 1/50000 and1/100000) and reagents incorporated in *Brucella* agar medium was examined in parallel with control cultures. The lysis abilities of Tb and Wb phages was evaluated using a routine test dilution (RTD) and RTD of *10. Fi, BK2, and Izl phages were used in the RTD. Agglutination with antisera was carried out with monospecific anti-A and anti-M sera (2).

3.1.1. Washing Method

All the activities were carried out in a biosafety cabinet. All the specimens were washed with double distilled water (DDW). They were then mixed with 5 ml of DDW and centrifuged for 30 minutes. The pellet was then injected into the BACTEC culture with aerobic /F and placed in a BACTEC 9050 (U.S.). Based on the type of medium culture, the incubation time was 7 days. After this period, the negative cases were reported and eliminated from the study. The positive cases were passaged in a Chocolate agar environment in two series for 48 hours at 37°C. One was incubated with 5% - 10% CO2, and the other one was kept in atmosphere condition. The shape, gram staining, and urease, catalase, and oxidase activity of the colonies that grew were examined. The samples with the greatest likelihood of being positive were then transferred to *Brucella* agar medium for the final stage of diagnosis and biotyping. The 48 - 72 hours colonies in cold chain condition were transported to the Razi Institute in Karaj. In this laboratory, the reaction of the colonies to specific antiserum (monospecific) M and A, sensitivity to basic fuchsin and thionin stains, SH2 production, and phage lysis (phage typing) were examined (2).

3.1.2. Dye Sensitivity Test

From every isolated bacteria culture after 72 hours, a 0.5 McFarland standard was prepared, and 10 mL of the

standard were injected into the staining resistance environment of each of the sets containing three plates of BHI agar and different concentrations of thionin stain (1/100, 1/50, and 1/25) and two plates containing BHI agar with different concentrations of fuchsin stain (1/100 and 1/50). One plate without any stain was employed as a control. These cultures were then incubated in an atmosphere of 7% CO2 or in an oxygen atmosphere for 96 hour. The results were checked daily.

3.2. Statistical Analysis

Demographic information was collected from each participant and recorded in data collection forms. The result of the laboratory tests were added to the forms, and the data were analyzed using SPSS 16.0 software.

4. Results

This cross-sectional study consisted of 206 blood samples from patients with suspected brucellosis. The characteristics of the patients with a positive brucellosis culture are shown in Table 1. As shown by the analyses of the cultured samples, 50 cases were positive for *Brucella* infection, and 156 were negative. Among the positive cases, 54% were males. The majority of the patients were aged 30-39 years. The results of the analysis of the CBC indicated that 8.6% of the patients had a WBC higher than 10000 and that 91.4% had a normal WBC count. The ESR of 38.4% of the patients was increased, and it was normal in 61.6% of cases. In addition, 64.4% of the patients had negative CRP results, and 35.5% had positive results.

Of the 50 positive cultures, 40 were identified as positive on the fourth day. Three were identified as positive on the fifth day, three on the third day, two on the second day, and two on the sixth day. In addition to the isolation and identification of *Brucella* bacteria from the blood samples, one case was identified from bone marrow, two from CSF, and two from synovial fluid. Based on the clinical examination, two cases were identified as neurobrucellosis, and two were identified as *Brucella* arthritis.

Table 1. Characteristics of the Brucellosis Patients with Positive Cultures

	Number	Percent
Sex		
Male	27	54
Female	23	46
Job		
Cattle related	10	20
Noncattle related	40	80
Residency		
Village	11	32
City	39	78
Sample Type		
Blood	45	90
CSF	2	4
Synovial fluid	2	4
	1	2

Abbreviation: CSF; cerebrospinal fluid.

4.1. Results of Confirmatory Tests

Following the positive identification of the cases and observation of suspected *Brucella* colonies, confirmatory tests were performed. Upon the detection of tiny gram-negative coccobacilli, some of which were bipolar, as well in gram staining and positive results in catalase, oxidase, and urease tests, these colonies were isolated as *Brucella* (Table 2).

4.2. Determination of the Species Type

After isolating the bacteria, separate tests were performed to identify the species. The results revealed that none of the *Brucella* species in the 50 positive cases produced H2S or required CO2. The results of the cultures in various concentrations of thionin (1/100, 1/50, and 1/25) and fuchsin (1/100 and 1/50) also indicated that all 50 positive isolates grew in the various concentrations of stains in the culture environment.

4.3. Agglutination Results

All 50 *Brucella* isolates contained negative A and positive M, indicating that all the isolates were biotype 1 *B. melitensis*.

The phage employed in this research was Tbilisi. The sensitivity of this test in regular density was 104* (RTD, RTD × 104). The result of this test also showed that all the species were *B. melitensis* biotype 1. The *Brucella* isolated from CSF, synovial fluid, and bone marrow was also biotype 1 *B. melitensis*.

5. Discussion

This cross-sectional study consisted of 206 samples from patients with suspected brucellosis. of these samples, 50 were identified as positive for *Brucella*.

In a study of 906 blood samples, 139 (15.3%) of the samples were identified as positive using both BACTEC and

Table 2. Biochemical Characteristics of the *B. melitensis* Isolates

Diagnostic test of *Brucella* strain	SH2 Production	Catalysis	Oxidase	CO2 Need	Urease	Growth in Different Concentrations of Fuchsin	Growth in Different Concentrations of Thionin
B. melitansis	-	+	+	-	+	+	+

ordinary culture, whereas only 80 (8.8%) were identified as positive using the BACTEC system (6). Using BACTEC NR669, Yagupsky isolated 21 (78%) cases from 27 cases as *B. melitensis* in less than 7 days (7). In addition, Ruiz et al. isolated 16 out of 17 cases (94.1%) by using BACTEC within 7 days (8). In two separate studies that used the BACTEC method, 93% and 100% of isolates were identified during 5 days (7-9). Based on a study of positive blood cultures in 7 days and bone marrow cultures in 4 days, Ozturk et al. concluded that automated BACTEC culture systems were a fast and efficient method of isolating *Brucella* spp. (10). In the present study, the mean time for *Brucella* bacteria to grow in the BACTEC culture was 4 days. This length of time is in agreement with that reported (less than 7 days) by most researchers.

In the present study, the sensitivity of the BACTEC system was 36%, its specificity was 96%, with a 93% positive predictive value and 46% negative predictive value. Maleknejad et al. conducted similar research, employing the BACTEC 9120 model for blood cultures. In their study, the sensitivity of the BACTEC culture was 42.2%, which was about 6% higher than that found in the present research (11). The reduced sensitivity (36%) of the BACTEC system found in the present study may be due to the sole use of the BACTEC culture environment, without employing a BACTEC set and automatic incubation. Another possible explanation for the discord between the findings of the present study and those of Maleknejad et al. is that some of the patients with suspected brucellosis may have had chronic disease. The isolation of organisms when patients are in the chronic phase of the disease is difficult. Furthermore, it is possible that the use of antibiotic medication decreased the likelihood of isolating the organism. In addition, the different findings may be attributed to the type of sets and automatic incubation used or the use of a different culture medium.

Brucella, the causative agent of brucellosis, includes various biotypes, which differ in many factors. These differences make it difficult to control and prevent the disease. Various studies conducted in Iran indicated that *B. melitensis* biotype 1 was endemic, biotype 2 was less common, and biotype 3 was rare. All three biotypes cause brucellosis and have been isolated from cattle and humans (3). In a study of 568 blood cultures obtained over a 10-year period in Iran, 121 (25%) of the bacteria identified were *B. melitensis* biotype 1 (2). Typing of 1170 *Brucella* bacteria obtained from patients in the provinces of Isfahan, Khorasan, Gilan, Khozestan, Yazd, and Eastern Azerbaijan in Iran in a 10-year period showed that the majority of the cases were biotype 1 (4). In a study conducted in 2008 of the isolation and identification of 3031 isolates (618 *B. abortus* and 2413 *B. melitensis*), *B. abortus* biovar 3 remained the dominant species in many regions of Iran, but *B. melitensis* biovar 1 was the most prevalent species (12). In a study of 45 cases in the Keeriakeli region in Anatoli center, all the cases were biotype 3 *B. melitensis* (4). Bodur et al. examined 41 species of *Brucella* isolated from the blood and CSF of patients hospitalized in Ankara, Turkey and reported that all the isolates were biotype 3 *B. melitensis* (13) The findings of the previous studies are in contrast to those of the present research, which found that all the blood isolates and CSF isolates were biotype 1 *B. melitensis*. Dokuzoguz et al. reported that *B. melitensis* was the most common subtype of *Brucella* infection in Turkey and that infections with *B. abortus* spp. were no less severe than infections with *B. melitensis* (14).

In the present study, there were two cases of neurobrucellosis and two cases of *Brucella*-related arthritis. In a similar study, Salehi employed a molecular method to identify the species and biotypes of isolated *Brucella* (15). In contrast, the present study employed biochemical and serology, in addition to lysis with the phage method. Although molecular methods are the preferred choice, the methods used in the present research are reliable, produce valid findings, and are more cost effective than molecular methods. As the Razi serum and vaccine producing research institute is a referral center and employs these methods for diagnosis, for this part of the identification procedure, isolated of species in cold chain condition in *Brucella* agar environment were sent to this institute. Due to the high prevalence of brucellosis in the region, patients with suspected disease who showed clinical signs were diagnosed as likely cases of brucellosis. We believe that the procedures used in this research were sufficient to identify the *Brucella* species.

5.1. Conclusions

The causative agent of human brucellosis in the Kashan region and suburbs area was *B. melitansis* biotype 1. No other biotype variations were identified in this study. As the identification of different biotypes of *Brucella* is important, other research is necessary in other regions to identify various biotypes transfer from one region to another region or country. Such information is necessary to identify the type of infection and apply prevention and treatment measures.

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Footnotes

Authors' Contribution: Mahzad Erami and Reza Razzaghi designed the study. Mahzad Erami and Saeed Alamian performed the study. Mansooreh Momen Heravi wrote the manuscript. All the authors read and approved the manuscript.

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