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Investigation of the Prevalence of Brucellosis Infection in Blood Donors in Rural Areas of Ardabil Province, Iran, 2017-2018

Parisa Eslami[1], Mehdi Fakhar[2], Ahmed Dheyaa[1], Soheila Ahanjan[3], Mohammad Ahanjan[4*]

1. Student Research Center, Mazandaran University of Medical Sciences, Sari, Iran.
2. Toxoplasma Research Center, Mazandaran University of Medical Sciences, Sari, Iran.
3. Student Research Center, Iran University of Medical Sciences, Tehran, Iran.
4. Pediatric Infectious Diseases Research Center, Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran.

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ABSTRACT

Background: Brucellosis is a zoonotic infectious disease with global distribution. This disease has been prevalent in various parts of Iran since ancient times and is significant due to its specific medical complications. Given the endemic nature of the disease in the country, this study aimed to investigate the prevalence of brucellosis infection in blood donors using serological and molecular methods in rural areas of Ardabil Province, Iran, during 2017-2018.

Materials and Methods: In a descriptive-cross-sectional study, anti-*Brucella* antibodies were investigated in the serum of 500 blood donors who referred to the Ardabil Province blood transfusion center. First, all blood samples were qualitatively tested by the Rose Bengal test. Then, on the Rose Bengal-positive samples, the Wright, 2-mercaptoethanol (2-ME), and polymerase chain reaction (PCR) molecular method tests were performed. Data were analyzed using SPSS software (version 18) and the chi-square statistical test.

Results: Out of 500 blood samples from individuals referring to the Ardabil Province blood transfusion center, 37 individuals had a positive Rose Bengal test, 27 had a Wright test titer of ≥1/80, and 18 had a 2-mercaptoethanol (2-ME) test titer of ≥1/40. No significant relationship was found between the seroprevalence of brucellosis and the gender of the blood donors, but a significant relationship was observed with age ($P<0.05$). All 37 individuals who had a positive Rose Bengal test were reported negative by the polymerase chain reaction (PCR) method.

Conclusion: If we consider the Wright test titer as the criterion for *Brucella* infection, the contamination with *Brucella* in the donors from Ardabil Province is not negligible.

*** Corresponding Author:**
Mohammad Ahanjan, PhD.
Address: *Pediatric Infectious Diseases Research Center, Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran.*
E-mail: *ahanjan2007@gmail.com*

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Introduction

Brucellosis, or Malta fever, is a zoonotic disease. Its causative agent is a gram-negative, aerobic coccobacillus. Brucellosis is found worldwide, especially in developing countries, and is an endemic disease in Iran. Annually, 500,000 new human brucellosis cases are reported worldwide [1]. According to the report of the Ministry of Health, Treatment, and Medical Education, the provinces of Iran are classified into four types based on the prevalence of human *Brucella* infection. According to this classification, Ardabil Province, Iran, with a prevalence of 11-20 cases of *Brucella* infection per 100,000 people, is among the provinces with medium prevalence.

This disease is specific to various animals, including livestock, and humans are its accidental host [2]. The primary routes of transmission to humans are direct contact with infected animals and consumption of unpasteurized milk and dairy products from infected animals, as well as inhalation of infectious aerosols. However, the possibility of transmission of this organism through infected blood donors from one person to another among humans, especially in endemic areas, also exists and should be screened for and identified [1, 3]. Furthermore, it can be considered an occupational hazard in jobs involving direct contact with livestock and their products (agriculture, animal husbandry, butchery, veterinary medicine, and laboratory personnel) [3, 4]. In animals, brucellosis is known as a reproductive disease causing abortion in the middle or late stages of pregnancy, infertility, and stillbirth. In humans, acute brucellosis is characterized by flu-like symptoms, including undulant fever, fatigue, headache, and body aches [5].

However, the disease can be asymptomatic or present in subacute or chronic forms. In the chronic state, symptoms, such as night sweats, weight loss, hepatosplenomegaly, lymphadenitis, inflammation of the spine and sacroiliac joints, neurobrucellosis, and endocarditis are observed [1]. In fact, it is a systemic disease involving various body organs, and the severity of the disease depends on the site of infection [6].

The standard definitive diagnostic method for *Brucella* is bacterial culture and isolation. However, a major limitation is that this bacterium is slow-growing and requires up to three weeks for growth, as well as experienced personnel and a biosafety level 3 laboratory for isolation. Other diagnostic methods include serological tests, such as the Rose Bengal test, Wright tube agglutination test, and 2-mercaptoethanol (2-ME) test. The diagnostic basis of these tests is the identification of antibodies against the bacterial outer membrane lipopolysaccharide in the patients' serum. The Rose Bengal test is a screening test that must be confirmed by the Wright and 2-ME tests [7]. In serological tests, false positives are also observed due to cross-reactivity of *Brucella* with other gram-negative bacteria, such as *Salmonella urbana* O:30, *Escherichia coli* O:157, *Yersinia enterocolitica* O:9, *Vibrio cholerae*, *Francisella tularensis*, etc. [8].

Consequently, confirmatory diagnostic methods for human brucellosis are molecular methods, such as polymerase chain reaction (PCR), which is a rapid and reliable method. This disease is uncommon in industrial countries and the United States but remains a major zoonotic disease in some countries. Major endemic areas include the Mediterranean countries, the Persian Gulf, India, parts of Mexico, and Central and South America [9]. Brucellosis is an important socio-economic health problem. Since it is rare in developed countries and not extensively covered in reference textbooks, research on it in Iran, which is known as an endemic area, seems necessary [6]. On the other hand, given the geographical situation of Ardabil Province and its proximity to traditional livestock breeding centers, the disease prevalence is high, and no recent research has been conducted in this regard. Therefore, this study aimed to investigate the prevalence of *Brucella* infection in blood donors in the endemic area of Ardabil Province using serological and PCR methods.

Materials and Methods

The study population consisted of blood donors referred to the Ardabil Provincial Blood Transfusion Center, including both males and females with an age range of 18-60 years, who were included in the study after approval for donation. The sampling method was non-random convenience sampling; based on previous studies and sampling limitations, 500 blood donors referred to the Ardabil Provincial Blood Transfusion Center were enrolled consecutively in the study. The study type was descriptive-cross-sectional, and the data collection tool was interviews. The background variables in this study were age and sex.

The procedure was as follows: Among healthy individuals referred to the Ardabil Blood Transfusion Center, 500 people were selected consecutively, and after obtaining consent, 4 mL of blood was drawn from the volunteer blood donors. 2 mL of the blood was gently emptied from the wall of the tube containing anticoag-

ulant for DNA extraction, and the remaining 2 mL of blood was transferred to tubes without anticoagulant for serum separation. The blood and serum samples were then stored at -20 ˚C until used for the relevant tests at the appropriate time.

Serological tests included the Rose Bengal test, standard tube agglutination test (Wright), and 2-ME test. The Wright, Rose Bengal, and 2-ME tests were performed using kits prepared by the Pasteur Institute of Iran.

For PCR, all Rose Bengal test-positive samples and 10% of the Rose Bengal test-negative samples were subjected to PCR for the detection of *Brucella* DNA. In this study, the phenol-chloroform method was used for total DNA extraction.

The main PCR reaction mixture with a final volume of 25 microliters included 1 microliter of the DNA sample placed in a PCR microtube, to which 24 microliters of the mixture (12.5 µL master mix + 0.5 µL reverse primer + 0.5 µL forward primer + 10.5 µL distilled water) was added.

The thermocycler program included an initial denaturation step at 95 ˚C for 5 minutes, followed by 30 cycles. The denaturation step was at 95 ˚C for 30 seconds, the annealing step at 54 ˚C for 90 seconds, the extension step at 72 ˚C for 90 seconds, and the final extension step at 72 ˚C for 6 minutes. The primers used in this research were prepared by ordering from the Takapo Zist Company. Table 1 presents information related to these primers.

To describe quantitative variables, central tendency and dispersion indices, such as Mean±SD, were used, and for describing qualitative variables, frequency and percentage were used. Before performing statistical analyses, the normality of the distribution of quantitative variables was assessed using the Kolmogorov-Smirnov test. If the data distribution was normal, the independent t-test was used to compare the means of quantitative variables between two groups; otherwise, its non-parametric equivalent, the Mann-Whitney U test, was used. The chi-square test and Fisher's exact tests were used to assess the relationship between qualitative variables. All analyses were performed using SPSS software, version 18, at a significance level of 0.05.

Results

In this study, 500 healthy donors referred to the Ardabil Blood Transfusion Center were investigated and statistically analyzed for *Brucella* infection using serological methods (Rose Bengal, Wright, 2-ME) and the molecular method (PCR).

In the present research, the Rose Bengal test was first performed on 500 individuals, of whom 37 individuals (7.4%) had a positive Rose Bengal test. Subsequently, for a more accurate investigation, these 37 individuals with a positive Rose Bengal test were re-examined using the Wright, 2-ME, and PCR tests (Figure 1). Out of the 37 individuals under study, 16(43.2%) were male, and 21(56.8%) were female. The mean age of the studied individuals was 37.68±14.65 years, with the minimum age being 16 years and the maximum age being 60 years.

According to the results of the Wright test, 10 samples (27%) were negative, and 27 samples (73%) were positive. According to the results of the 2-ME test, 19 samples (51.4%) had a negative test result and 18 samples (48.6%) had a positive test result. Furthermore, the results of the PCR test showed that all 37 samples (100%) were negative (Figure 2).

According to the results of Fisher's exact test, no significant relationship was observed between the gender variable and the Wright test results ($P>0.05$). The results of the independent t-test showed a statistically significant difference between the mean age in the two groups with positive and negative Wright test results ($P<0.05$) (Table 2) (Figure 3).

Furthermore, according to the results of Fisher's exact test, no significant relationship was observed between the gender variable and the result of the 2-ME test ($P>0.05$). Before performing the statistical analysis, the normality of the distribution of variables in the two groups with positive and negative 2-ME test results was assessed using the Kolmogorov-Smirnov test. Based on its results, the age variable had a normal distribution in both groups ($P>0.05$). Therefore, the parametric independent t-test was used to compare the mean age in the two groups. The results of the independent t-test showed a statistically significant difference between the mean age in the two groups with positive and negative 2-ME test results ($P<0.05$).

Discussion

Despite control and prevention measures, brucellosis remains a significant socio-economic health problem in many countries. The prevalence of this disease is unknown in many areas due to inaccurate diagnostic methods, inefficient reporting systems, and inadequate follow-up. Iran is also considered an endemic region. Since there is a possibility of human-to-human transmission of the disease through blood transfusion, a high prevalence of brucellosis in a community can be an important risk

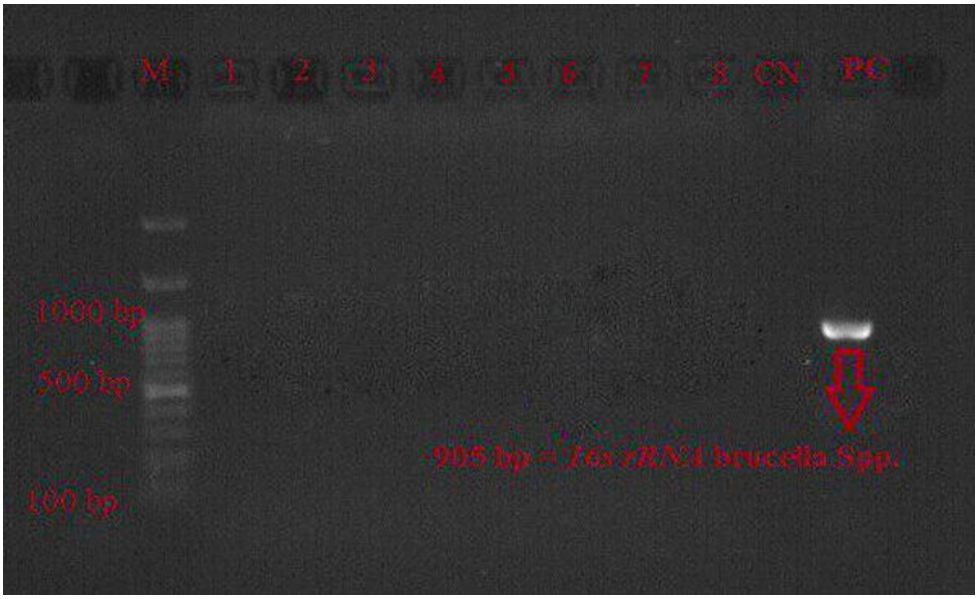


Figure 1. 16S rRNA *Brucella* - gene product electrophoresis
Abbreviations: M1: 1000 bp marker; PC: Positive control; NC: Negative control; 1,2,3,4,5,6,7,8: Test samples.

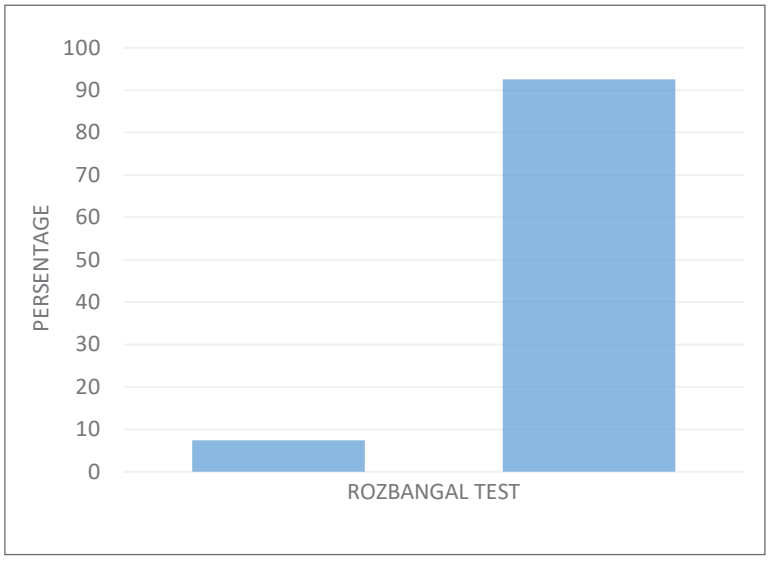


Figure 2. Percentage frequency of the Rose Bengal test result in the studied samples

factor in blood transfusion. Therefore, measuring the titer of anti-*Brucella* antibodies and identifying patients, especially in endemic areas, is necessary [10]. Hence, investigating the prevalence of this disease in areas, such as Ardabil Province, which has traditional livestock breeding centers and is located along the migration routes of infected animals from neighboring areas, is of particular importance. All exposed workers should undergo quantitative serological testing to detect an immune response to *Brucella* spp. Evidence suggests that seroconversion can occur shortly before symptoms appear, and therefore may be an earlier indicator of infection.

The common diagnosis of this disease is based on serological tests [11]. Although the best method is to isolate the organism, it has many limitations in practice. In the absence of positive *Brucella* cultures, diagnosis usually depends on the presence of antibodies in serum, milk, vaginal secretions, and semen [12].

Table 1. Specifications of the primers used in this research

Target Gene	Primer Sequence	Amplicon Size
16S rRNA	F-TCGAGCGCCCGCAAGGGG R-AACCATAGTGTCTCCACTAA	905 bp

Table 2. Comparison of the mean age in the two groups with negative and positive Wright test results

Variable	Mean±SD		Test Result
	Negative	Positive	
Age (y)	40.42±22.11	35.93±15.55	P=0.23

In the present study, the Rose Bengal test was first performed on 500 individuals, of whom 37 (7.4%) had a positive Rose Bengal test. Subsequently, for a more detailed investigation, these 37 individuals with a positive Rose Bengal test were re-examined using the Wright, 2-ME, and PCR tests. After performing the tests, 18 samples were positive in the 2-ME test, 27 samples were positive in the Wright test, and in the PCR test, none of the 37 samples were reported positive.

It is likely that in the present study, where all samples were negative by PCR, the reason is that the samples were not in the acute stage of the disease, and it can indeed be inferred that the studied individuals were not ill. In a study conducted by Torres-Padilla et al. in Mexico on 500 blood donor samples, 18 samples were positive for anti-*Brucella* antibodies or the Rose Bengal, standard tube agglutination (Wright), and 2-ME tests [13], which shows a nearly significant correlation with the results of the present study. In this study, 18 individuals from the studied population had a positive 2-ME titer, which is higher than that in studies conducted in Bushehr and Markazi provinces, where less than 0.1% of blood donors had a positive anti-*Brucella* antibody titer [14]. However, this statistic is somewhat consistent with the findings of Ghilian et al. in blood donors in Yazd, who reported the brucellosis infection rate as 0.6% [15]. The results of a study in India on blood donors also reported the prevalence of brucellosis disease as 0.62% among 321 blood donors, which is consistent with the findings of this research [16]. Other studies conducted in various parts of the country and neighboring countries, such as in Turkey with a seroprevalence of 0.63% [17] and Iraq with a seroprevalence of 5.29% [18], all indicate the endemicity of brucellosis in the Middle East region. Climatic conditions and geographical location, being on the route of nomadic pastoralists, traditional livestock breeding in the region, lack of hygiene, high contamination, and consumption of local dairy products could be reasons for the difference in disease prevalence. This study meticulously analyzes cases of brucellosis, revealing critical insights into its epidemiology, clinical manifestations, and treatment outcomes.

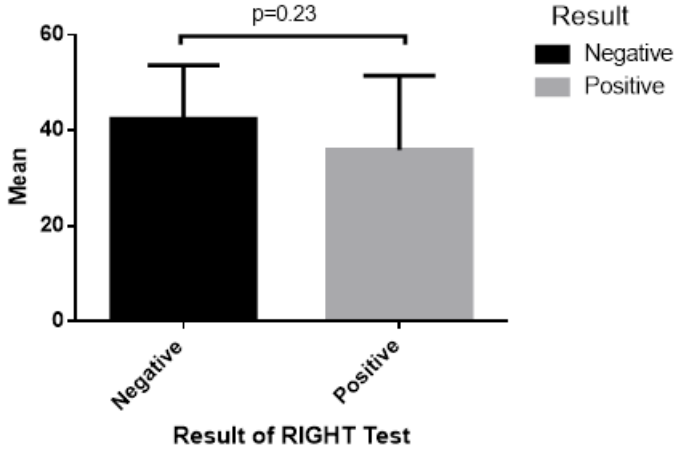


Figure 3. Comparison of the mean age in the two groups with negative and positive Wright test results

In this study, the seroprevalence of brucellosis did not show a significant difference between the two genders. This study, conducted on blood donors in Yazd City, Iran, also did not observe a significant difference between men and women [16]. However, some studies have reported a higher prevalence of brucellosis in men, while others have reported it is higher in women [17-18]. These differences indicate variations in animal husbandry practices, geographical location, local conditions, and the work culture of men and women in contact with livestock in different regions [19].

Regarding the age distribution of *Brucella* infection and disease in the studied population, the present study did not observe a significant difference between different age groups. The research conducted on blood donors in Yazd and nomadic pastoralists in Khuzestan also did not report a significant difference in *Brucella* infection among different age groups [4, 18]. Brucellosis is seen in all age groups. In the studies by Ghilian et al. and Abbaszadeh et al., the highest prevalence of infection was observed in the age group under 40 years [15, 19], a finding that was not confirmed in our study.

According to Almasi et al., quoting Azizi, in Iran and regions where brucellosis is endemic, significant difference in the incidence of infection was observed between adults and children [20].

Conclusion

Untreated brucellosis can lead to chronic disease, bone and joint involvement, liver and spleen involvement, neurobrucellosis, and, in rare cases, endocarditis. These complications are associated with persistent pain, functional limitations, and even life-threatening complications. In addition, the risk of repeated relapses and transmission to others through rare routes increases.

Given that this study was conducted on healthy blood donors with no clinical signs or symptoms, serological findings alone cannot prove infectivity. Certainly, more studies on brucellosis in this region would be helpful. Therefore, it is recommended that more consideration and precision be applied regarding blood donors, especially in Ardabil Province. These considerations should be particularly taken into account for individuals living in suburban or rural areas, and such individuals must be thoroughly investigated for a history of the disease or symptoms of brucellosis. Additionally, the Wright test should be performed randomly on blood donor samples from different areas throughout the year.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article. The participants were informed of the purpose of the research and its implementation stages. They were also assured about the confidentiality of their information and were free to leave the study whenever they wished, and if desired, the research results would be available to them.

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Authors contribution's

Investigation: Parisa Eslami; Data Collection: Mehdi Fakhar; Writing–original draft preparation: Ahmed Dheyaal; Writing–review & editing: Soheila Ahanjan; Supervision: Mohammad Ahanjan.

Conflict of interest

The authors declared no conflict of interest.

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