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Serological and molecular detection of *Brucella* spp. infection in the equine population of Kerman, Iran

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Abstract
Brucellosis is a widespread disease that affects both humans and animals worldwide. It causes significant economic losses and is a public health concern. In horses, this disease often goes unnoticed but can lead to various health issues, such as fistula withers, pollevil, arthritis, synovitis, bursitis, and abortion. The aim of this study was to determine *Brucella* spp. using molecular and serological methods in apparently healthy horse populations in Kerman. A total of 100 blood samples were randomly collected from asymptomatic horse farms in Kerman province of Iran. The Rose Bengal Plate Test (RBPT) was performed to detect the presence of *Brucella*-specific antibodies. Additionally, conventional and real-time PCR techniques specifically targeting the *IS711* gene were used to detect the presence of *Brucella* spp. The detection of *Brucella spp.* in all three tests RBPT, conventional PCR, and real-time PCR was 3%. Also, the statistical analysis on the obtained data revealed no significant correlation between the incidence of equine brucellosis and various risk factors including age, sex, breed, exposure to other susceptible animals, and herd size. These findings confirm that horses can act as natural hosts for *Brucella*. Therefore, it is crucial to prioritize horses as reservoirs of infection and implement screening, control, and prevention programs to eradicate this disease effectively.

Introduction

Brucellosis is the most common zoonotic bacterial disease caused by the genus *Brucella*. This disease has a global spread, but it is more prevalent in the Mediterranean countries of West Asia and Latin America (1). Every year, there are approximately half a million new reports of human involvement with brucellosis (2). This disease affects a wide range of domesticated animals, particularly cattle and small ruminants, as well as wild animals and marine mammals (3–5). Brucellosis can have severe consequences on livestock, such as the loss of offspring, temporary or permanent infertility, and decreased milk production. Reproductive failure and abortion are major clinical manifestations observed in the animals. Consequently, it is a

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significant economic and public health concern worldwide (6). Although control measures (including vaccination, testing, and slaughtering) against this disease have been taken, the disease is still endemic in many parts of the world, including Iran. The incidence of brucellosis in the Iranian population varies from zero to 41 cases per 100,000 individuals in different cities (7).

Several species of *Brucella* can cause disease in animals; however, *Brucella abortus* and *Brucella melitensis* are the most significant (8). In horses, brucellosis is commonly caused by *B. abortus*, whereas *B. suis* has been isolated from horses suffering from infectious bursitis and aborted fetuses (9, 10). The disease in horses ordinarily occurs through ingestion of *Brucella*-contaminated feed, the respiratory system, and sometimes through skin wounds (9, 10). The organism usually localizes in the bursae, joints, and tendon sheaths (11). *Brucella* infection in horses typically does not produce any clinical signs, although serological testing may indicate the presence of the organism. This raises concerns about horses serving as carriers and potentially spreading the bacteria, making it an alarming subject. In horses that do develop clinical symptoms, fistulous withers, and poll evil, which are forms of septic supra-spinous and supra-atlantal bursitis respectively, are the most typical clinical signs associated with brucellosis (10). Rare cases of vertebral osteomyelitis, abortion, and infertility in stallions have been recorded (12). Equine infection can be diagnosed using direct or indirect methods. Direct methods involve microbiological methods (culture) or PCR-based DNA detection. Indirect methods include the Rose Bengal Plate Test (RBPT), tube agglutination test, milk ring test, 2-mercaptoethanol test, indirect ELISA, immunofluorescence assay, and complement fixation test, which are widely used because of their rapid function and low cost. However, serological methods have low sensitivity and specificity for diagnosis (13, 14). Among the disadvantages of the culture technique, we can mention the use of live bacteria and as a result the risk of infection of laboratory personnel (15). Isolation of *Brucella* using molecular methods such as polymerase chain reaction (PCR) and real-time PCR is one of the most reliable diagnostic methods. PCR is a sensitive technique that can detect low levels of *Brucella* DNA in contaminated tissues and fluids (16).

While most research on brucellosis in Iran has concentrated on cattle and other ruminants, less attention has been paid to horses (5). One of the most critical risk factors for equine brucellosis is the concurrent keeping of horses and other *Brucella*-sensitive animals, particularly cattle. Livestock farming in Kerman province is ordinarily done in a traditional or semi-industrial manner, where horses and other ruminants keep together, creating conditions that facilitate the transmission of *Brucella* bacteria between them (17). Therefore, the present study aimed to detect *Brucella* spp. infection in the horse population of Kerman using serological and molecular methods.

Materials and methods

Study population

A total of 100 blood samples were randomly collected from horses during the period between August and November 2021. Each collection site contained between one and twenty horses. Samples were taken from horse racing clubs and private horse owners in Kerman, Iran. In addition, a detailed questionnaire including age, breed, sex, contact with other animals, and herd size was collected from each horse owner to investigate the risk factors associated with the disease.

Sample collection

Ten mL of blood were collected from the jugular vein of each horse. Then, five mL of this blood were immediately transferred to an EDTA-containing tube for PCR tests, while the remaining five mL were transferred to a clot activator tube to isolate serum for serological tests. Following collection, the samples were promptly preserved and transported to the laboratory of the pathobiology department, Shahid Bahonar University of Kerman for subsequent analysis. The samples containing

clotted blood were centrifuged (5,000 RPM for 3 min) and the sera were isolated. Both the blood samples and sera were then stored at a temperature of -20°C until further analysis. All of the horses were clinically healthy and had no clinical symptoms of brucellosis at the blood collection time.

Rose Bengal Plate Test (RBPT)

Rose Bengal antigen (supplied by Pasteur Institute of Iran) and horse serum were kept at room temperature for about 20–30 minutes to reach the laboratory temperature. To perform the Rose Bengal plate test, 25 µL of each serum sample was mixed separately with 25 µL of the Rose Bengal antigen. Distilled water was used as a negative control. Serum with a high titer against brucellosis was used as a positive control. To ensure optimal binding between the antigen and antibody, the plate was placed on a shaker. After allowing the mixture to incubate for five minutes, the results were observed and recorded. Samples showing visible agglutination between the antigen and antibody were considered positive (4).

DNA extraction

DNA was extracted from each blood sample using a blood DNA extraction kit (Parstous, Iran) according to the manufacturer's instructions. The quality and quantity of DNA were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Also, DNA from the Rev1 vaccine (Razi Institute, Iran) was extracted as a positive control for the PCR technique. Then, the DNA samples were stored at -20°C until required for molecular analysis.

Conventional PCR

The PCR reaction was carried out in a final volume of 25 µL, containing 12.5 µL of PCR master mix (Ampliqon, Denmark), 1 µL of each primer IS711 (0.4 µM) (Pishgam biotech company, Iran), 8 µL of nuclease-free water and 2.5 µL of template DNA. The insertion sequence 711 (IS711) was used as a characteristic of all species of the *Brucella* genus. The information on primers is described in Table 1. PCR reactions were performed using a Thermal Cycler (Bio-Rad, USA). The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min, with final extension at 72°C for 10 min (18). Distilled water and Rev1 vaccine DNA were used as negative and positive controls, respectively. The PCR products were visualized on a 1% agarose gel stained with 10 µg DNA Green Viewer (Parstous, Iran) and the amplicon size was compared with a 100 bp DNA ladder (Ampliqon, Denmark).

Table 1. Primers used in the study

Primer name	Primer sequence	Product size (bp)	Reference
IS711-For	5′-GAGAAT AAAGCCAACACCCG-3′	317	(26)
IS711-Rev	5′-GATGGACGAAACCCACGAAT-3′		

Real-time PCR

A real-time PCR molecular technique was performed to confirm the positive conventional PCR samples more strongly. Real-time PCR was performed in a final volume of 20 µL. The components included 3.4 µl of distilled water, 10 µL of 2x qPCR Master Mix (Ampliqon, Denmark), 0.8 µl of each primer IS711 at concentrations of 0.4 µM (Pishgam biotech company, Iran) and 5 µL of DNA template. The reaction mixture was initially incubated at 95°C for 15 min. Amplification was then performed for 40 cycles, consisting of denaturation at 95°C for 15 s, followed by annealing and extension at 58°C and 72°C respectively for 20 s. Moreover, the melting stage was as follows: 95ºC for 10 s, 65ºC for 60 s, and 97ºC for 1 s. At the end of the real-time PCR run, the melting temperature (Tm) and cycle of threshold

(CT) number of each PCR product were analyzed using software on LightCycler 96® System (Roche, Germany) automatically.

Statistical analysis

The data was analyzed using SPSS software (version 19.8) with a confidence level of 95%. Also, chi-square analysis was conducted.

Results

In the present study, three specimens of *Brucella* spp. (3%) were identified (Table 2) through laboratory testing using RBPT, conventional PCR (Figure 1), and real-time PCR. None of the positive samples exhibited any clinical symptoms of the disease. One of these positive samples was male and two were female. All three positive samples were from horses used for breeding. Two of these positive samples were *Dareshuri* whereas one was *Arabian*. Moreover, two of these positive samples had a history of contact with ruminants. Two positive samples were obtained from stables with 6-10 horses and one from a stable with less than five horses. The average age of the positive samples was 9 ± 2.64 years old (Table 2). Based on the results in Table 2, no significant association was observed between age, sex, type of use (Breeding or Racing), breed, contact with other animals, and herd size.

Table 2. Association between different risk factors and *Brucella* spp. infection in the studied horses

Variables	Positive	Negative
Age	9 ± 2.64	9.71 ± 4.31
Gender		
Male	1	64
Female	2	33
Type		
Breeding	3	79
Racing	0	3
Breeding and Racing	0	15
Contact		
Yes	2	42
No	1	55
Breed		
Arabian	1	8
Dareshuri	2	67
Kurd	0	1
Pony	0	3
Turkmen	0	18
Herd Size		
1 to 5	2	16
6 to 10	1	15
Bigger Than 10	0	66

Discussion

Brucellosis is a zoonotic bacterial disease with a global distribution that causes significant economic damage, particularly in developing countries (1). Although there are no host-specific *Brucella* species for horses, they can be affected by *B. abortus* and, rarely, *B. suis* through close contact with natural *Brucella* hosts (especially cattle and sheep) (5, 11, 19). *Brucella* infection in horses typically does not produce any clinical signs, although serological testing may indicate the presence of the organism. This raises concerns about horses serving as carriers and potentially spreading the bacteria, making it an alarming subject. There is no control program for equine brucellosis (17). Thus, the detection of *Brucella*

spp. infection in the horse population is important in eradicating this disease. So, the present study was performed to evaluate the molecular and serological detection of *Brucella* spp. infection in the horse population of Kerman.

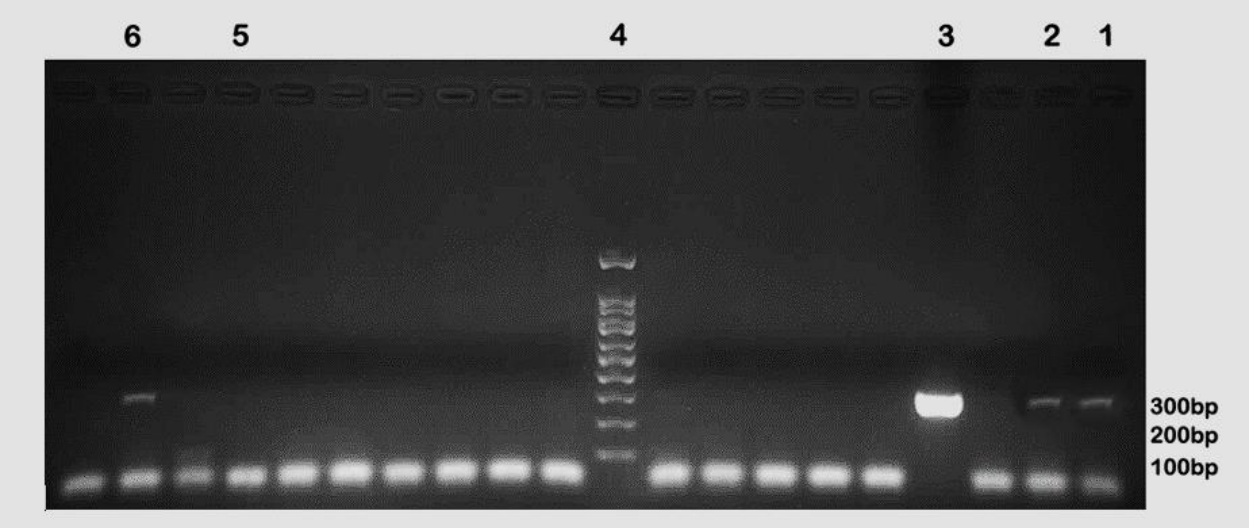

Figure 1. The electrophoresis of PCR products for the *IS711* gene of *Brucella* (317 bp); Number 1, 2, and 6 positive samples; No. 3, positive control *(Brucella melitensis* strain Rev.1 vaccine); No. 5, negative control (sterile distilled water); No. 4, 100bp DNA ladder (CinnaGen Co.); Unnumbered samples are negative samples.

The findings of this study will help to understand the role of horses in brucellosis epidemiology. So, 100 blood samples were collected from clinically healthy horses of different breeds. Subsequently, the samples were analyzed using the Rose Bengal plate test as well as conventional and real-time PCR methods. In the present study, three specimens of *Brucella* spp. were identified, accounting for 3% of the total samples. Research similar to the current study has been conducted in Iran. In a 2012 study investigating the sero-epidemiology and molecular assessment of *Brucella* infection in Iranian horses, 312 serum samples were tested using the RBPT, SAT, and 2-mercaptoethanol methods. Most horses were asymptomatic. Blood samples from horses showing clinical signs of the disease were subjected to PCR testing. The results of this study demonstrated prevalence rates of 9.9%, 8%, and 7% for Rose Bengal, SAT, and 2-mercaptoethanol, respectively. In this study, three horses with clinical symptoms tested positive across all serological methods, but only one out of three samples tested positive in PCR (18). In a study conducted on 164 clinically healthy mares in Iran in 2020, no positive samples were found using the RBPT method for serum samples and the MRT method for milk samples. Additionally, PCR and bacterial culture were performed on the samples. Three milk samples tested positive by PCR, whereas no serum samples tested positive by PCR. In this study, specific primers were used to determine the *Brucella* species, and all three positive samples were identified as *B. abortus*. The bacterial culture of the three PCR-positive samples did not show any bacterial growth. This study attributes the lack of bacterial growth to the difficulty in isolating *Brucella* and the low sensitivity of this method in separating bacteria from milk (4).

As demonstrated by the results of the present study, the detection of equine brucellosis using all three methods (the RBPT, conventional PCR, and real-time PCR) was within the range of results obtained in previous studies (0-12%) conducted in Iran (4, 17–19, 21–24). Therefore, it can be concluded that despite the presence of various risk factors (such as diagnostic methods, sample size, and environment),

the prevalence of equine brucellosis in Iranian horses is relatively consistent and is expected to be less than 10%.

Previous studies on equine brucellosis in other countries have shown relatively diverse prevalence rates. The seroprevalence of brucellosis is reported to be as follows: 0% in Eritrea, 1-8.5% in Jordan, 3.6-4.9% in Sudan, 0.24% in Mexico, 0.25-60.6% in Turkey, 3.6-67.9% in Pakistan, 12.89% in India, 0.26-6.5% in Brazil, 8.3% in Mongolia, 6.5% in Costa Rica, and 0-100% in Nigeria (17). The overall seroprevalence of equine brucellosis is 1.92% globally (25). Differences in the prevalence of brucellosis across different countries and regions within a country can be attributed to various factors, including variations in animal husbandry practices, contact rates with both domestic and wild animals, population density, geographical location, climate, sample size, diagnostic methods, and host characteristics.

In the current study, the mean age of horses testing positive for brucellosis was 9 ± 2.64 years, and no statistically significant variance was observed between the different age groups of horses and the prevalence of brucellosis. Previous studies similarly found no notable distinction in the presence of anti-*Brucella* antibodies across various age groups (4, 17, 18). Safirollah et al. (2012) revealed a significantly higher prevalence of antibodies in older animals (5-11 years) compared to younger ones (20). In the present study, none of the horses exhibited the clinical symptoms of brucellosis. Most studies reported no clinical symptoms in horses that tested positive for brucellosis (4, 5, 17). However, other studies reported the presence of clinical symptoms (18). In the present study, no significant association was found between the prevalence of brucellosis and the coexistence of horses with other animals (such as cows, sheep, and goats), breed, sex, type of use, and herd size. This finding aligns with the results of the previous studies (4, 17, 18). In the present study, two out of the three horses were kept with cattle and sheep. Tahmtan et al. (2010), who investigated the prevalence of brucellosis in horses from Mashhad province, reported the highest prevalence of the disease in horses that cohabitated with cows, sheep, and goats (21).

Conclusions

In the present study, three specimens of *Brucella* spp. were identified, accounting for 3% of the samples. Therefore, according to the mentioned studies, it can be concluded that horses can have a notable impact on the epidemiology of the disease by acting as reservoirs or secondary hosts for the bacterium. Therefore, to prevent horse brucellosis, it is recommended to keep horses separate from other animals, especially cattle and sheep. Also, the results of this study will be beneficial for conducting additional epidemiological investigations and implementing control measures. However, more research is required to determine the prevalence of this disease across various regions of Iran and to understand the role of horses in transmitting it to both human and livestock populations.

Acknowledgments

Not applicable

Ethical approval

All implementation phases of this study were approved by the Animal Care Committee of the Veterinary College of the Shahid Bahonar University of Kerman.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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