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Serological prevalence of Brucellosis in horses in the suburb of Tabriz, Iran

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Abstract

Brucellosis is a significant zoonotic disease that poses both health and economic risks. It is endemic in many developing countries, such as Iran. This study aimed to evaluate the prevalence of *Brucella abortus* infection in native horses in the suburbs of Tabriz, Iran, using serological methods. Blood samples were collected from 141 apparently healthy horses, 45 of which were less than five years old, and 96 were more than five years old. Additionally, 35 were female and 106 were male. The Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT), 2-Mercaptoetanole (2-ME) test, and indirect ELISA (i-ELISA) were used to detect the presence of *Brucella abortus* infection in serum samples. The results showed that 4.96%, 3.54%, and 9.2% of the samples were positive for RBPT, STAT, and i-ELISA tests, respectively. The 2-ME test indicated the presence of IgG in the five serum samples. Of the thirteen positive samples in the i-ELISA test, eight cases (61.5%) were more than five years old, and the remaining five cases (38.5%) were under five years old. Out of thirteen positive cases, four cases (30.8%) were females, and nine cases (69.2%) were males. No significant relationship was found between *Brucella abortus*-infected cases and the age and gender of the horses. The findings suggest the presence of *Brucella abortus* contamination in native horses in the study area, emphasizing the importance of considering these animals in control and eradication programs for Brucellosis in the region.

Introduction

Brucellosis is a zoonotic disease caused by the bacteria of the genus *Brucella*. It poses a significant threat to public health, particularly among vulnerable populations in rural areas, with substantial economic impacts (1). This disease spreads to humans during milking through direct contact with infected animals as well as the consumption of raw milk (2). Brucellosis has been reported all over the world, but it has been effectively eliminated in many developed countries. However, it is still considered a significant disease in developing countries, and clinical cases of the disease are reported (3). In

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Iran, like other developing countries, Brucellosis is an endemic disease that causes great economic damage to the industry of livestock (4). Brucellosis in horses is caused mainly by *Brucella abortus* and *Brucella suis* (5). The disease is not clinically common in horses but still has been reported as the cause of some diseases such as septic supraspinous bursitis (fistulous withers), atlantal bursitis (poll evil), olecranon bursitis, carpal hygroma, epididymitis, and abortion (6, 7).

Serological tests, such as RBPT, STAT, i-ELISA, and 2-ME, are commonly used to evaluate the prevalence of brucellosis in horses (8-10). Although extensive studies exist on the disease in ruminants (11-14), there are limited studies on the prevalence of the disease in horses. The individual seroprevalence of brucellosis in horses in Northeast Iran was 2.5% (15). Studies have shown varying rates of seroprevalence in different regions, with rates ranging from 0% to 12% in Iran and 13.29% to 14.4% in Turkey (9, 10, 16, 17). The above-mentioned studies utilized various serological techniques, such as RBPT, STAT, 2-ME, and i-ELISA, to detect the presence of Brucellosis. Considering that native horses are kept alongside ruminants in traditional animal husbandry systems, there is a possibility of disease transmission from them to other animals and humans (16). To control the disease and especially eradicate it, horses should also be considered in disease monitoring programs. Therefore, the epidemiological study of the disease in horses is of particular importance. For this purpose, the current study was conducted to assess the seroprevalence of *Brucella abortus* in native horses in Northwest Iran, using serologic methods including RBPT, STAT, 2-ME, and i-ELISA.

Materials and methods

Sampling

This was a cross-sectional study in some villages around the center of East Azarbaijan province, Northwest of Iran. The following formula was used to calculate the sample size (18):

$$n = \frac{Z_{1-\alpha/2}{}^2 p(1-p)}{d^2}$$

In this formula, "n" was the sample size, "$Z_{1-\alpha/2}$" was considered 1.96, with a confidence interval of 95% and $p<0.05$, "d" was an absolute error, which was considered 0.05 in this study. Using the above formula, the sample size was calculated as 138 horses. Then, 141 horses were sampled from the villages of cities, including Tabriz, Sofian, Khosroshah, Osku, and Bostan-abad. Thirty-five out of 141 horses were female, 106 were male. Also, 45 were under five years old, and 96 were over five years old. The blood samples were collected in simple tubes without anticoagulant (Mediplus, free additive tube, Sunphoria Co., Ltd, China), and transferred to the laboratory using ice. The samples were centrifuged at 5000 rpm for 5 min and the sera were separated, and stored at -20 °C for serum testing.

Rose Bengal Plate Test

All sera samples were screened for the anti-*Brucella* antibodies presence using the RBPT. Briefly, equal volumes (25-30 µL) of RBPT antigen (Pasteur Institute, Iran) and serum were poured into a slide and thoroughly mixed with the applicator. The plate was placed on the rotator for about 4 min to do the interactions of antigen and antibody and then assessed for agglutination (19).

Standard Tube Agglutination test

The STAT was conducted using the Wright tube antigen of *Brucella abortus* obtained from the Razi Vaccine and Serum Research Institute in Iran. A total of 19 tubes were used, with ten tubes in the first row and nine tubes in the second row. The tubes in the first row were used to prepare serial dilutions of serum samples from 1:20 to 1:2560, the last tube was chosen as a negative control. The second row contained the same number of serum tubes and was used for positive control samples. To prepare the tubes, 0.9 mL of normal saline was added to the first tube and 0.5 mL of normal saline to the subsequent tubes in the first row. In the negative control tube, 0.9 mL of normal saline was

added along with 0.1 mL of negative control serum. Next, 0.1 mL of the serum sample was added to the first tube and mixed, and 0.5 mL from the first tube was poured into the second tube and mixed. This process was continued until the last tube, from which 0.5 mL was discarded. The same procedure was followed for positive control tubes with positive control serum. Subsequently, 0.5 mL of Wright tube antigen was added to each tube, and they were incubated at 37 °C for 24 hours. The tubes were then examined for agglutination. The last tube in which agglutination was observed at the bottom was considered the test titer (9).

2-Mercaptoethanol test

For the 2-ME test, the procedure was similar to STAT, except that 2-ME buffer (0.1 M) was used instead of normal saline. The Wright tube antigen of *Brucella abortus* which was used for this test was obtained from the Razi Vaccine and Serum Research Institute in Iran**.**

Indirect ELISA

The ELISA test in this study was performed using a commercial i-ELISA kit according to the manufacturer's instructions (ID Screen Brucellosis Serum Indirect Multi-species kit, ID-Vet, France). Before starting the test, reagents and serum samples were allowed to thaw at room temperature (22˚C ± 4˚C), and 100 µL of diluted buffer were added to each well. Ten µL of positive control, negative control (included in the kit), and serum samples were poured into different wells of the plate. Each plate was sealed and manually homogenized gently. After incubation at room temperature for 45 min, each plate was washed three times with PBS-Tween, and 100 µL of multispecies horseradish peroxidase (HRP) conjugate was added to each well. Each plate was subsequently incubated for 30 min at room temperature and washed three times to eliminate the excess conjugate. After that, 100 µL of the substrate solution (tetramethylbenzidine in substrate buffer containing H_2O_2) was added to each well, and the plate was incubated for 15 min in the dark at room temperature. The reaction was stopped by adding 100 µL of 1 N hydrochloric acid (HCl). The optical density in each well was measured at 450 nm using a microplate photometer (Bio Tek ELX800 absorbance reader). The result for each tested sample was expressed using the following formula:

The mean value of the positive control ($O.D_{PC}$) O.D. is greater than 0.350 ($O.D_{PC} > 0.350$).

The ratio of the mean values of the positive and negative controls ($O.D_{PC}$ and $O.D_{NC}$) is more significant than 3 ($O.D_{PC}$/ $O.D_{NC}$). For each sample, the S/P percentage (S/P %) was calculated as follows using the sample and control values:

$S/P\ \% = OD_{sample} - OD_{NC} / OD_{PC} - OD_{NC} \times 100$

Finally, the results was considered as follows:

S/P % ≤ 110%: negative; 110% < S/P % < 120%: doubtful; and S/P % ≥ 120%: positive.

Statistical analysis

The data were analyzed using the SPSS 21 statistical software (IBM SPSS Inc). The Chi-square test was used to analyze the relationship between seropositive horses (based on the results of the i-ELISA test) and age and gender. $p<0.05$ was considered significant.

Results

Rose Bengal Plate test

Agglutination was observed in seven out of 141 serum samples (4.96%). Subsequently, the sera that were positive in this screening test were used in the STAT (Table 1).

Standard Tube Agglutination test

Of the seven samples that were positive in RBPT, five (71.42%) were also positive in the STAT, and there were no reactions in two samples (28.58%), implying a false positive. The overall prevalence of Brucellosis in STAT was 3.54%. Three of the five positive samples in the STAT had a titer of 1/160, and two samples had a titer of 1/80 (Table 2).

2-Mercaptoethanol test

All of the five positive samples in the STAT had a titer of 1/20 in this test. According to the guidelines of the Veterinary Organization for the control of Brucellosis in Iran, if a sample had a titer of 1/160

or greater in the STAT, it is considered positive with any titer in the 2-ME test. In addition, if a sample had a titer of 1/10 to 1/20 in the STAT, it is considered positive in the 2-ME test with a titer of 1/10 and greater.

Indirect ELISA

As shown in Table 3, 13 (9.2%) out of 141 serums were positive in i-ELISA. All five positive samples in RBPT and STAT were also positive in i-ELISA. The eight positive serums in i-ELISA did not react positively in other serological tests. Out of 13 positive samples in i-ELISA, eight (61.5%) were more than five years old and five (38.5%) were under five years old. Four (30.8%) were female and nine (69.2%) were male. There was no significant relationship between *Brucella abortus* infection in horses and the age and gender in this study.

Table 1. Results of evaluation of serum contamination of horses in suburb of Tabriz to *B. abortus* using RBPT.

Variables		Positive numbers (%)	Negative numbers (%)	Total (%)
Age	<5 y	0 (0)	45 (100)	45 (100)
	>5 y	7 (7.3)	89 (92.7)	96 (100)
	Total	7 (4.96)	134 (95.04)	141 (100)
Gender	Female	1 (2.9)	34 (97.1)	35 (100)
	Male	6 (5.7)	100 (94.3)	106 (100)
	Total	7 (4.96)	134 (95.04)	141 (100)

Table 2. Results of evaluation of serum contamination of horses in suburb of Tabriz to *B.* a*bortus* using STAT.

Variables		Positive numbers (%)	Negative numbers (%)	Total (%)
Age	<5 y	0 (0)	45 (100)	45 (100)
	>5 y	5 (5.3)	91 (94.8)	96 (100)
	Total	5 (3.54)	136 (96.46)	141 (100)
Gender	Female	1 (2.9)	34 (97.1)	35 (100)
	Male	4 (3.8)	102 (96.2)	106 (100)
	Total	5 (3.54)	136 (96.46)	141 (100)

Table 3. Results of evaluation of serum contamination of horses in suburb of Tabriz to *B. abortus* using i-ELISA.

Variables		Positive numbers (%)	Negative numbers (%)	Total (%)	*p*-value	χ^2
Age	<5 y	5 (11.1)	40 (88.9)	45 (100)		
	>5 y	8 (8.3)	88 (91.7)	96 (100)	$p>0.05$	0.28
	Total	13 (9.2)	128 (9.8)	141 (100)		
Gender	Female	4 (11.4)	31 (88.6)	35 (100)		
	Male	9 (8.5)	97 (91.5)	106 (100)	$p>0.05$	0.27
	Total	13 (9.2)	128 (90.8)	141 (100)		

Discussion

The current study was conducted to assess the seroprevalence of *Brucella abortus* in native horses in some cities around Tabriz, Northwest Iran, using serologic methods of RBPT, STAT, 2-ME test, and i-ELISA. The seroprevalence of *Brucella* in the current study was 4.96%, 3.54%, and 9.2% using RBPT, STAT, and i-ELISA, respectively. Considering that any of the serological methods cannot help alone in diagnosing the disease, we used the collection of these methods.

The high prevalence of brucellosis in the i-ELISA method indicates that the blocking antibodies were present in the studied sera and revealed that i-ELISA is more sensitive than other serological methods used in this study. In most of the epidemiological studies that investigated the status of Brucellosis in Iranian horses, the overall prevalence rate was less than 10%. The seroprevalence of *Brucella* infection in the horses of Mashhad using RBPT and STAT was 2.5% (15), which was lower than the current study. In another study in Mashhad, the prevalence of Brucellosis in native horses was 2.5% and 2.6% using RBPT and STAT, respectively (20). In a study of 200 horses in Hamedan in 2011, the prevalence of Brucellosis was very low using RBPT and STAT (0.005%), and only one of the 200 horses examined in both tests showed a positive response, which is very low compared to that of the current study (21). Similarly, a recent study indicated that all 495 horses from different equestrian clubs in Hamedan province were negative for Brucellosis in the RBPT (19). In a study of 312 club-race horses in southern Iran, the prevalence of Brucellosis was 9.9%, 8%, and 7% with RBPT, STAT, and 2-ME tests, respectively, which is consistent with the results of the present study (9). However, in some serological studies, no positive cases of serum infection with *Brucella abortus* were found; for instance, in a survey of 100 Turkmen horses in Iran using RBPT and STAT no positive reactions were observed (22).

The serological prevalence of brucellosis in horses has also been reported in Iran's neighboring countries. Hussain et al. (6) found that the seroprevalence of equine *Brucella abortus* in the Punjab Province of Pakistan was 21.4%, 3.56%, and 4.24% by RBPT, i-ELISA, and CFT, respectively. Results of a recent review study in Pakistan showed that the overall prevalence of equine Brucellosis was 20.7% by RBPT and 17.7% by STAT (23). In a study conducted in Turkey between 2008 and 2010, the prevalence of Brucellosis among 361 horses from 23 different villages in Ardehan and Kars Provinces was 13.29% and 14.40% as determined using RBPT and STAT, respectively (16).

The results of the present study showed that the serological prevalence of *Brucella abortus* in horses is relatively higher than those reported in most studies in Iran. It should be noted that the i-ELISA method was used in this study, which has not been used in previous studies in Iran except for a study on Arabian horses (10). As i-ELISA is more sensitive and specific than previous methods (24), the higher number of cases was seropositive on this method. Due to the higher specificity of STAT than RBPT in the diagnosis of Brucellosis, it can be concluded that two of the results of the RBPT in this study were false positives. In the present study, all five positive samples in the STAT had a titer of 1/20 in the 2-ME test, indicating the presence of IgG antibodies in the serum samples. Among 13 positive samples in i-ELISA, eight (61.5%) were more than five years old, and five (38.5%) were under five years old. In addition, of 13 positive horses, four cases (30.8%) were female, and nine cases (69.2%) were male. However, there was no significant relationship between age and gender and the seropositivity of horses. In agreement with the results of the present study, Ardo et al. (8) did not find a significant relationship between age and sex and the prevalence of the disease. According to the results of a review study in Pakistan, Brucellosis was observed more in female horses than in males. In addition, the prevalence of Brucellosis was significantly higher in horses over five years old, which is contrary to the results of the present study (23). The reason for this discrepancy can be due to the difference in the number of samples in each age and sex group. In the present study, the whole number of male horses was more than females, and horses over five years old were more than those under five years old. Therefore, it has led to a higher number of positive samples in males over five years old horses; however, there was no statistically significant

difference between age and gender and seropositivity.

Conclusion

The current study showed that there is a serum infection with *Brucella abortus* in horses in the suburb of Tabriz. Therefore, considering the impact of Brucellosis on the socio-economic status of vulnerable populations, the control and eradication of this disease should be considered as one of the public health goals.

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Conflict of interest statements

There is no conflict of interest.

Ethical approval

Our research protocol was approved by the Animal Research Ethics Committee of the University of Tabriz, and we conducted our research by their guidelines and standards.

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