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Original Article

Comparison of PCR and Conventional Serological Methods for Detection of *Brucella spp.* in Ovine and Caprine Blood Serum

Ali Ahmadi, M[1], Saadati, D[1 *], Najimi, M[2], Ganjali, H[3], Shah Karami, F[4]

1. Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran
2. Department of Pathobiology, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran
3. Zabol Veterinary Office, Sistan and Baluchestan Provincial Veterinary Service, Iranian Veterinary Organization, Zabol, Iran
4. Stuff in Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran

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Corresponding Author: saadatdariush@uoz.ac.ir

Abstract

Brucellosis is an anthropozoonotic disease. Infection of livestock with *Brucella* is endemic in most parts of Iran. Sistan-Baluchestan is bordered on the east by the countries of Afghanistan and Pakistan. The high prevalence of brucellosis in livestock in the eastern neighboring countries results in transmission of the disease to this province. The present research aimed to determine the prevalence of brucellosis in small ruminants in the Sistan region of Iran and to compare serological and molecular tests for the detection of brucellosis. Blood samples were taken from 150 randomly selected sheep and goats, and sera were separated. All sera were analyzed by serological (Wright and 2-ME) and molecular (Polymerase Chain Reaction (PCR)) tests. Serological tests were carried out according to the instructions of the Iranian Veterinary Organization The degree of agreement between serological tests and PCR was determined by kappa value. In this study, 17 cases (11.3%) were identified as positive by the PCR method. Wright and 2-ME tests had the highest agreement with PCR in titers ≥2/80 and ≥2/40, respectively. The results of this study show that the brucellosis in sheep and goats has a greater prevalence in the Sistan region than in most other parts of Iran, and this is important in terms of public health. It is suggested that brucellosis vaccination coverage in livestock be increased in this area and that the people in Sistan region must be notified about methods for preventing brucellosis. Also, further studies to compare conventional serologic tests with the gold standard test are recommended.

Keywords: *Brucella*, PCR, serological tests, Sistan

Comparaison de la RPC et des Méthodes Sérologiques Conventionnelles Pour la Détection de *Brucella spp.* dans le Sérum Sanguin des Ovins et des Caprins

Résumé: La brucellose est une infection anthropozoonotique. L'infection du bétail par *Brucella* est endémique dans la plupart des régions d'Iran. Le Sistan-Balûchestân est bordé à l'est par les pays de l'Afghanistan et du Pakistan. La forte prévalence de la brucellose chez le bétail dans les pays voisins de l'est entraîne la transmission de la maladie à cette province. La présente recherche visait à déterminer la prévalence de la brucellose chez les petits ruminants dans la région du Sistan en Iran et à comparer les tests sérologiques et moléculaires pour la détection de la brucellose. Des échantillons de sang ont été prélevés sur 150 moutons et chèvres sélectionnés au hasard, et les sérums ont été séparés. Tous les sérums ont été analysés par des tests sérologiques (Wright et 2-ME) et moléculaires (Réaction de polymérisation en chaîne (RPC)). Les tests sérologiques ont été effectués

conformément aux instructions de l'Organisation vétérinaire iranienne. Le degré de concordance entre les tests sérologiques et la RPC a été déterminé par la valeur de kappa. Dans cette étude, 17 cas (11.3%) ont été identifiés comme positifs par la méthode RPC. Les tests Wright et 2-ME présentaient la plus grande concordance avec la RPC pour les titres ≥2/80 et ≥2/40, respectivement. Les résultats de cette étude montrent que la brucellose chez les moutons et les chèvres a une prévalence plus élevée dans la région du Sistan que dans la plupart des autres régions d'Iran, et cela est important en termes de santé publique. Il est suggéré que la couverture vaccinale contre la brucellose chez le bétail soit augmentée dans cette zone et que les habitants de la région du Sistan soient informés des méthodes de prévention de la brucellose. De plus, d'autres études pour comparer les tests sérologiques conventionnels avec le test de référence sont recommandées.
Mots-clés: *Brucella*, RPC, tests sérologiques, Sistan

1. Introduction

Brucellosis is an anthropozoonotic disease caused by Gram-negative coccobacilli bacteria of the genus *Brucella*. Infection with *Brucella* affects a wide range of mammals including cattle, goats, sheep, camels, pigs, and dogs. Brucellosis causes abortion and infertility in sheep and goats (1). Humans usually get *Brucella* by consuming non-pasteurized milk, cheese, or other dairy products. Occupational exposure to the aborted tissues of infected animals can also result in human infection with brucellosis (2). This disease causes a severe acute febrile illness in humans and can cause endocarditis, arthritis, epididymo-orchitis, encephalitis, and complications in other organs (3).

Sheep and goats predominantly comprise the livestock population in Iran (2). To date, the brucellosis control program has been more focused on cattle than sheep and goats. Several studies have indicated that the rate of brucellosis is higher in sheep and goat populations than in cattle populations in the country (4-6). It seems that there is a need for more attention on brucellosis control in small ruminants.

Serological tests such as the Rose Bengal, Wright, and 2-mercaptoethanol (2-ME) tests are most commonly used in the diagnosis of ruminant brucellosis in Iran. The Rose Bengal test is used as a screening test, and positive samples are confirmed by the Wright and 2-ME tests. Serological tests have false positive and false negative results. Nevertheless, isolation of *Brucella* or the detection of *Brucella* DNA by polymerase chain reaction (PCR) is the only method that allows certainty of diagnosis (7).

The Sistan region is located in the north of Sistan-Baluchestan Province. Sistan-Baluchestan, the largest province of Iran, is bordered on the east by Afghanistan and Pakistan countries. The high prevalence of brucellosis in the eastern neighboring countries (8-10) can cause transmission of the disease to this region. This research aimed to determine the prevalence of brucellosis in small ruminants in the Sistan region and the degree of agreement between serological tests and PCR results.

2. Material and Methods

2.1. Sampling

The current cross-sectional study was carried out in January and February 2017. In total, 150 sheep (92 cases) and goats (58 cases) were randomly selected. Jugular blood samples were collected from all animals without an anticoagulant. Sera were separated from clotted blood samples and stored at –20 °C for subsequent *Brucella* serological and molecular tests.

2.2. Serological Tests

All sera were analyzed by the serum tube agglutination test (known as the Wright test) and the 2-mercaptoethanol test (2-ME) carried out according to the instructions of the Iranian Veterinary Organization (11). Wright and 2-ME antigens were obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran). Serological tests were conducted in seven test tubes. The antigen was mixed with serum from an

animal at different concentrations. The concentrations of serum from tube 1 to tube 7 were 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640, respectively. The tubes were closed with parafilm and incubated at 37 ºC for 24 hours. Control standard tubes were prepared corresponding to 0%, 25%, 50%, 75%, and 100% agglutination which were scored respectively as 1+, 2+, 3+, and 4+. Afterwards, the titer and degree of agglutination in test tubes (tube 1 to tube 7) were assessed as compared with the control tubes (12). Seroprevalence of brucellosis was determined based on the guidelines of the Iranian Veterinary Organization (IVO) for interpretation of the Wright and 2-ME results. IVO recommends that animals with a titer ≥1/20 (1+ agglutination at 1:20 serum dilution) in 2-ME be defined as affected. Moreover, animals with any value of titer in 2-ME are defined as affected if they have a titer of ≥4/40 (4+ agglutination at 1:40 serum dilution) in the Wright test. Other titers were considered as negative results (11).

2.3. Molecular Tests

All sera were tested by PCR. The boiling method was used to extract DNA from the sera samples (13). DNA extractions were stored at -20 °C until PCR analysis. Primers were synthesized by Pishgam Co. (Table 1).

Deionized sterile water was added to lyophilized primers according to the manufacturer's instructions to obtain a concentration of 100 pmol/µl. PCR reactions were performed using 15 µl of PCR mix solution (including 1 µl of each forward and reverse primer, 3 µl of distilled deionized water, 8 µl of master mix (Pishgam Biotech Co.,© Iran), and 2 µl of DNA extract). Parameters used were initial denaturation at 94 °C for 5 minutes, followed by denaturing at 94 °C for 1 minute, annealing at 60 °C for 1 minute, and extension at 72 °C for 1 minute. This cycle was repeated 30 times and ended with a final extension of 72 °C for 7 minutes. Then PCR products were run on 1.5% agarose gel electrophoresis (80 V and 220 mA for 75 minutes), followed by staining with ethidium bromide and visualized under UV (Cambridge gel documentation). Positive control (*Brucella abortus* S19 strain) and negative controls (in which DNA sample was replaced by sterile water) were included in all reactions (Figure 1).

2. 4. Statistical Analysis

The prevalence of brucellosis by PCR and by serological tests was calculated based on the 95% confidence interval using binomial distribution. The degree of agreement between the serological tests and PCR was estimated by the kappa value. The molecular and serological prevalence rates of brucellosis were compared by McNemar's test based on respective cut-offs. SPSS software version 23 was used for statistical analysis. The significant level was considered to be $p<0.05$.

Table 1. Characterization of primers used in this study

Gene	Primer	Sequence (5'-3')	Length	Specific species	Source
Bcsp31	Forward	TGG CTC GGT TGC CAA TAT CAA	223 bp	*Brucella.* Spp.	(14)
	Reverse	CGC GCT TGC CTT TCA GGT CTG			

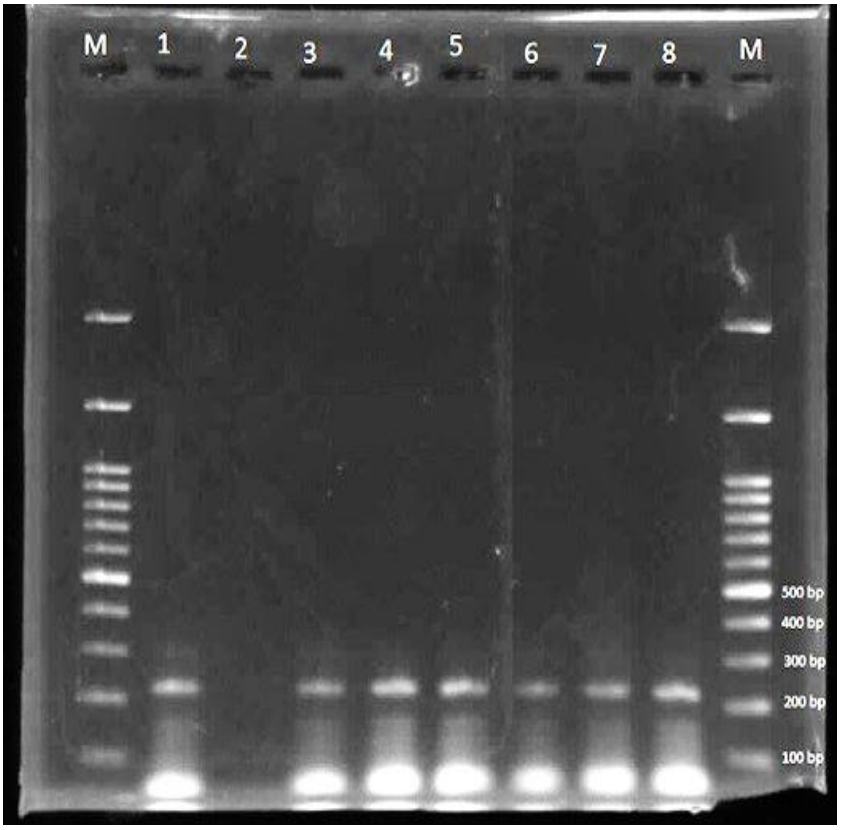

Figure 1. Gel electrophoresis of PCR products. The two lanes M: the 100 bp ladder markers, Lane 1: positive control, Lane 2: negative control, Lanes 3 to 8: positive samples characterized by identifying a gene fragment of 223 bp.

3. Results

Among the 150 selected animals, 17 cases (11.3%) (95% CI: 6.7% - 17.5%) were identified as positive based on the PCR method. The prevalence of brucellosis was 8.7% (8 cases) in sheep and 15.5% (9 cases) in goats as identified by the molecular method. The molecular prevalence of brucellosis between sheep and goats was not significantly different (p=0.119). The seroprevalence of brucellosis in sheep and goats in this study was 21.3% (95% CI: 15.1% - 28.8%) (32 cases). The agreement of the Wright and 2-ME tests with PCR was calculated at each cut-off point (Table 2 and Table 3).

According to Table 2, the highest agreement between Wright and PCR is seen when a cut-off titer of ≥2/80 for the Wright test is used. There was no significant difference in prevalence of brucellosis based on both Wright and PCR at this cut-off (McNemar's test p-value=0.146).

As shown in Table 3, the highest agreement between 2-ME and PCR is reached when a cut-off titer of ≥2/40 for 2-ME test is used. There was a statistically significant difference in prevalence of brucellosis based on both 2-ME and PCR at this cut-off (McNemar's test p-value=0.002).

Table 2. Indicators of the agreement between Wright and PCR at different cut-offs for Wright

Wright cut-off [a]	Negative in PCR		Positive in PCR		Kappa coefficient	McNemar's test *p*-value
	No. of negative samples in Wright	No. of positive samples in Wright	No. of negative samples in Wright	No. of positive samples in Wright		
$\geq \frac{1}{20}$ [b]	0	133	0	17	_	_
$\geq \frac{2}{20}$	46	87	0	17	0.107	<0.001
$\geq \frac{3}{20}$	62	71	0	17	0.165	<0.001
$\geq \frac{4}{20}$	80	53	0	17	0.255	<0.001
$\geq \frac{1}{40}$	81	52	0	17	0.261	<0.001
$\geq \frac{2}{40}$	103	30	0	17	0.438	<0.001
$\geq \frac{3}{40}$	109	24	0	17	0.507	<0.001
$\geq \frac{4}{40}$	120	13	2	15	0.612	0.007
$\geq \frac{1}{80}$	121	12	2	15	0.630	0.013
$\geq \frac{2}{80}$	124	9	3	14	0.655	0.146
$\geq \frac{3}{80}$	127	6	5	12	0.644	1.000
$\geq \frac{4}{80}$	130	3	9	8	0.530	0.146
$\geq \frac{1}{160}$	131	2	11	6	0.439	0.022
$\geq \frac{2}{160}$	131	2	14	3	0.233	0.004
$\geq \frac{3}{160}$	133	0	14	3	0.275	<0.001
$\geq \frac{4}{160}$	133	0	17	0	-	-

a. Minimum reactivity considered to be antibody positive.
b. Numerator and denominator represent degree of agglutination and final dilatation of serum, respectively.

Table 3. Indicators of agreement between 2-ME and PCR in different cut-offs for 2-ME

2-ME Cut-off [a]	Negative in PCR		Positive in PCR		Kappa coefficient	McNemar's test *p*-value
	No. of negative samples in 2ME	No. of positive samples in 2ME	No. of negative samples in 2ME	No. of positive samples in 2ME		
≥ 1/10 [b]	61	72	0	17	0.161	<0.001
≥ 2/10	113	20	0	17	0.562	<0.001
≥ 3/10	119	14	0	17	0.658	<0.001
≥ 1/40	120	13	0	17	0.677	<0.001
≥ 2/40	123	10	0	17	0.736	0.002
≥ 3/40	127	6	4	13	0.684	0.754
≥ 4/40	127	6	6	11	0.602	1.000
≥ 1/80	129	4	7	10	0.605	0.549
≥ 2/80	131	2	11	6	0.439	0.022
≥ 3/80	132	1	12	5	0.399	0.003
≥ 4/80	133	0	12	5	0.425	<0.001
≥ 1/160	133	0	15	2	0.191	<0.001
≥ 1/320	133	0	16	1	0.100	<0.001
≥ 1/640	133	0	17	0	-	-

a. Minimum reactivity considered to be antibody positive.
b. Numerator and denominator represent degree of agglutination and final dilatation of serum, respectively.

4. Discussion

Brucellosis is endemic among sheep and goat populations in Iran. However, since 1963, the use of the REV1 vaccine has been efficient in decreasing the endemic rate of the disease (15). In the present study, the prevalence of brucellosis among sheep and goats was identified as 21.3% by the serological method. Sharifi, Tabatabaei (6) examined 1767 sheep and 1233 goats in 300 epidemiological units of Kerman Province (southeastern Iran). They found that 3.10% of the tested sheep and goats (93 cases) were *Brucella*-seropositive (6). In Sarab City (East Azarbaijan province) 740 sheep were tested and 4.18% were *Brucella*-seropositive (4). In Hamedan, a total of 3,250 blood samples from 2,550 sheep and 700 goats were collected randomly, and all samples were analyzed for the presence of *Brucella* antibodies. The seroprevalence rates of brucellosis were 4.6% in goats and 3% in sheep (5). There is a high prevalence of brucellosis in the Sistan region (16). In a study from Sistan, where 100 samples of cow's milk

were tested by PCR, 7 samples were infected with *Brucella* (17). In another study undertaken in the Sistan region, infection with *Brucella melitensis* was identified in 15 of 78 aborted fetuses (19.2%) (16). The high prevalence of brucellosis in Sistan is due to an abundance of rural and nomadic populations, a traditional livestock breeding system, and a poor vaccination rate among livestock in this region.

Isolation of *Brucella* spp. is the gold standard test for diagnosis of brucellosis in humans and animals (7, 18). Detection of *Brucella* in blood, bone marrow, or tissue cultures is irrefutable proof of the disease, especially in humans. Nevertheless, *brucella* is slow–growing; under optimum growing conditions, it is easily overgrown and crowded out by other organisms. Therefore, this approach is labor-intensive and delays confirmation of the disease (18, 19). The PCR method is a rapid and worthwhile diagnostic test. The DNA of *Brucella* bacteria is recognizable in serum, blood, pus, and tissue. Moreover, the process of DNA extraction is easier for serum than for blood and tissues (20).

In this research, kappa coefficient is used to evaluate the agreement between serological and PCR tests. Kappa ranges from zero (agreement is equal to that expected by chance) to one (complete agreement beyond chance) (21). Landis and Koch (22) suggest kappa>0.80 indicates almost perfect agreement, 0.80≥kappa>0.60 indicates substantial agreement, 0.60≥kappa>0.40 shows moderate agreement, 0.40≥kappa>20 shows fair agreement, and kappa ≤0.2 indicates slight agreement. According to Tables 2 and 3, the highest agreement was reached between serological tests and PCR when ≥2/80 and ≥2/40 were used as cut-off titers for Wright and 2-ME, respectively. The kappa results indicated that at these cut-off levels, there was a substantial agreement between serological and PCR tests.

In conclusion, the findings of this study showed that there is a high prevalence of brucellosis in the Sistan region. Moreover, this study determined the cut-off titers in which there is the highest agreement between the Wright and 2-ME and the PCR. It is recommended that further studies be done on determining the most appropriate cut-off value in serological brucellosis tests by comparing their results to the gold standard test. Furthermore, it is suggested that brucellosis vaccination coverage in livestock be increased in this area and the people in the Sistan region be notified about methods for preventing brucellosis.

Authors' Contribution

Study concept and design: D. S.
Acquisition of data: M. A. A. and H. G.
Analysis and interpretation of data: D. S.
Drafting of the manuscript: M. A. A., D. S. and M. N.
Critical revision of the manuscript for important intellectual content: D. S. and M. N.
Statistical analysis: D. S.
Administrative, technical, and material support: M. N., H. G. and F. Sh. K.

Ethics

All the procedures and animal handling were approved by the Animal Ethics Committee at the University of Zabol, Zabol, Iran under the project number of 2017-5885456-96.

Conflict of Interest

The authors declare that they have no conflict of interest.

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