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Comparative Evaluation of Serological, ELISA and Molecular Tests in Diagnosis of *Brucella abortus* in Human Serum

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ABSTRACT

Background: Brucellosis is one of the most prevalent and common diseases between humans and animals.Prompt diagnosis and timely treatment of this disease can prevent many complications. In this regard, this study aims to comparatively evaluate ELISA, PCR and serological methods to identify *Brucella abortus*.

Methods: In this study, the serum of 100 patients referred to Tonekabon private laboratory from July 2020 to January 2021 was examined by PCR, ELISA and Wright, Coombs Wright, and 2ME methods for the detection of *Brucella abortus*.

Results: In this study, the mean age of the sample was 43.3 ± 18.2 of which 21% were infected with *Brucella abortus* according to the above serological methods. According to ELISA test, 22% of the samples were IgM, 6% of the samples were IgG and 16% were PCR positive. Kappa agreement coefficient in Wright and Coombs Wright test and 2me were significant (P <0.001). Serological diagnostic indices and ELISA sensitivity were 68.75% and 68.75%, respectively. The lowest prediction rate of *Brucella abortus* among diagnostic methods was related to Elisa (IgM). Based on Fisher's exact test, there was no significant relationship between the percentage of *Brucella abortus* positive PCR cases and age, sex, previous history of infection with *Brucella*, fever, body aches and dairy consumption.

Conclusion: Based on the results of our study, the accuracy of all methods is comparative and the lowest accuracy is related to Elisa (IgM) which has a lower level of predictability than other methods. The highest level of prediction belonged to Wright and 2me tests.

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Introduction

Brucellosis is a common disease that affects both humans and animals. This disease is prevalent in many parts of the world, including the Middle East, Latin America, the Mediterranean Basin, Africa, and Asia (1-2). It is caused by small gram-negative coccobacillus without spores that has different strains. *Brucella melitis* is found mainly in sheep, goats, and camels. *Brucella abortus* is found in cattle, Suis in soil and canines in dogs. *Brucella melitensis* is the most common strain that affects humans (3). It is considered as an endemic disease in our country, especially in the northern region (4). According to WHO, over half a million new cases of infection are diagnosed annually all over the world. *Brucella* can transmit to human body through several methods such as Inhalation of microorganisms, consumption of non-pasteurized diary and also through the skin. There are various methods for diagnosing *Brucella* in humans, because the clinical symptoms of brucellosis are different in humans and a positive evaluation in a laboratory test is the nonspecific diagnosis of brucellosis. Currently, Molecular, serological and microbiological tests are widely used for this purpose. Blood culture is a gold standard method for diagnosing *Brucella* which is time consuming. It increases the risk of transmitting the disease to humans and has only 15 to 70% sensitivity to the acute phase (5-6). PCR is a sensitive and specific method to detect the organism which is based on copying the DNA or RNA sequence of the sample, based on which various diseases can be diagnosed (7). Recently, ELISA method is being used for serology along with Wright test. This test is fast and its kits have a longer shelf life (8). Rose Bengal and Wright usually react earlier than other tests due to the involvement of the two G and M immunoglobulins. While, immunoglobulin G intervenes in the 2me test and the Coombs Wright test is valuable in cases where agglutination is not evident due to blockade antibodies (10-11). Different methods have been developed for the diagnosis, treatment and follow-up treatment due to the significance of this disease and it is highly important to identify a method with high sensitivity.

Therefore, this study aims to compare the effectiveness of ELISA, Wright, and PCR methods for detection of *Brucella abortus* in human serum samples.

Materials and Methods

Study area, sampling, and data collection

The current study was carried out in Tonekabon. In this cross-sectional study, totally 100 consecutive patients suspected of brucellosis referred to the medical laboratory were examined. Suspected cases of brucellosis were registered in a prospective study from July 2020 to January 2021. According to WHO description, a clinically suspected brucellosis case is characterized by an acute or insidious onset followed by a continued, intermittent or irregular fever of variable duration with the following signs: fatigue, weight loss, profuse sweating, anorexia, headache, arthralgia, and generalized aching. Moreover, these suspected patients are epidemiologically linked to contaminated animal products or suspected/ confirmed animal cases. A questionnaire was prepared for each suspected patient during the course of sample collection to record appropriate information such as age, sex, fever, pain body and previous history of infection. Treatment by antibiotics during 3 months prior to sampling was considered as the exclusion criterion. We separated serum from each blood sample after centrifuging at 1300g for 10 minutes. The serum was separated and kept at 20 ºC until further analysis.

Serological assessements

In the first step, the diagnosis of brucellosis was performed before starting an antibiotic treatment by serological techniques including the Wright

standard tube agglutination test (STA) and 2-ME (2-Mercaptoethanol) test. STA was performed similarly to Coombs Wright, and all brucellosis-specific antibodies were identified. *Brucella* capture was provided by the manufacturer to specify that 50 µL of the diluted serum sample was placed in a U-shaped microtiter plate to which anti total human immunoglobulin was added, then 50 µL of the antigen suspension added Formaldehyde was added to all liquids. The plates are covered with adhesive tape and left in a dark, damp room at 24°C for 24 hours. The positive reaction at the bottom of the liquids was showed by agglutination, and the negative reaction was indicated by a plate at the center and bottom of the liquid. 2-ME (2-Mercaptoethanol) test was used as a complementary test (12). In the current study, patients with STA titers equal to or greater than 1:80 are considered to be infected with *Brucella* spp. Furthermore, values of 1:160 and above were considered positive (2-ME titer), according to the standard methods.

ELISA Tests

The manufacturer's instruction (IBL International GmbH, Germany) was followed in conducting the ELISA protocol. The presence of IgG and IgM antibodies against *Brucella* were investigated by analyzing all the samples. According to the protocol, in order to dilute patient serum sample in the ratio 1:101, 100 µL solution was used. 50 µl of the diluted serum sample was then added to the ELISA plate and incubated for 60 min at room temperature. After washing, each well was filled with 100 µL of the enzyme conjugate; then, it was incubated at room temperature for 30 min. TMB substrate solution (100 µL) was added and the plates were incubated for another 20 min at room temperature. The color action was read at 450 nm using an ELISA reader after adding stop solution to the reaction.

DNA extraction and PCR

DNA was extracted using the DNA extraction kit (High Pure PCR) Template Preparation Kit, Roche, Germany, Kat. No, 11769828001) according to the manufacturer's instruction. The sequence of primer pair for specific detection of *Brucella abortus* were 5'-TGGCTCGGTTGCCA ATATCAA–3' and 3'-CGCGCTTGCCTTTCAG GTCTG–5' (Table 1). A total volume of 50 µL was used for PCR reaction. The reaction mixture consisted of the following: (a) forward and reverse primers at a concentration of 0.5 µM each, (b) 0.5 U of Taq polymerase, (c) 0.2 mM each dNTP, (d) 1.5 mM MgCl2, (e) 10 µL of template DNA (150 ng/mL) and, (f) 1× PCR reaction buffer. A thermocycler (Eppendorf, Germany) was used to perform PCR reaction. PCR cycling conditions were as follow: primary denaturation of 95°C for 5 min followed by 40 cycles of 95°C for 1 min, 60°C for 30s, and 72°C for 30 min. Then a final extension step of 72°C for 5 min was performed. The presence or absence of the PCR product in 731 bop (10 µL from each reaction mixture) was determined by agarose gel electrophoresis (2% w/v) at 80 V for 45 min; gel was stained with ethidium bromide (0.5 µg/mL) and examined under a UV trans illuminator (Figure 1).

Statistical analysis

Necessary information about patients such as fever, gender, pain body and age was collected from database, and the data were inputted into SPSS software version 23 (SPSS Inc., Chicago, IL, USA). The prevalence of brucellosis among Research variables was analyzed using the Chie square test and Fisher's exact test. A P-value of ≤0.05 was considered to be statistically significant. Moreover, specificity, sensitivity, and positive and negative predictive values of the serological, Elisa and PCR assays were calculated.

Result

In this study, the mean age of the sample was 43.3 ± 18.2; the smallest sample was 12 years old while the largest was 90 years old. The majority were in the age group 30-60 years old (58%). The majority of samples were male (53%), while 47% were women. In the study of the disease, 8% of people had a history of infection with *Brucella abortus*. Also, 53% of patients had fever, 91% had body aches and 89% of patients had consumed dairy products. 19% had *Brucella* based on Wright method, 21% Coombs Wright, and 19% 2me, and generally 21% had *Brucella* according to the above serological methods. In the ELISA test, 22% of the samples were positive based on IgM, 6% based on IgG, and generally 23% based on ELISA. Also, according to molecular PCR method, 16% of the subjects were positive (Table 2). Kappa agreement coefficient was significant in Wright and Coombs Wright test and 2 me (P <0.001). However, IgM and IgG-based ELISA had a lower agreement coefficient in contrast. Serological diagnostic indicators: sensitivity 68.75%, specificity 88.10%, positive probability ratio 5.77%, negative probability ratio 0.35%, positive predictive value 52.38%, negative predictive value 93.67% and accuracy was 85% (Table 3). Also, ELISA diagnostic indices: 68.75% sensitivity, 85.71% specificity, positive probability ratio 4.81%, negative probability ratio 0.36%, positive predictive value 47.83%, negative predictive value 93.51% and accuracy 83 % (Table 4).

The accuracy of all methods is close to each other and the lowest accuracy is related to ELISA (IgM). The lowest prediction of *Brucella abortus* by diagnostic methods was related to ELISA (IgM) with 0.724 and the highest predictive level was related to Wright and 2me tests with 0.918 (Table 5). Moreover, the values obtained by all methods are significant. So ELIZA (IgM) has a lower level of predictability than other methods. Based on Fisher's exact test, percentage of *Brucella abortus* positive PCR cases by age group was (P = .712), sex (P = 0.793), previous history of infection with *Brucella abortus* (P = 0.469), fever (P = 0.406), body aches (P = 0.675) and dairy consumption (P = 0.125) were obtained, none of which was significant (Table 6).

Discussion

Brucellosis is a common disease among humans and animals affecting both of them. Definitely, this infection may lead to a severe and long-term disease in humans. Therefore, It is necessary to identify the bacteria promptly and reliably in order to begin appropriate antibiotics-based treatment at the first opportunity. various alternative methods were used and introduced in order to overcome the limitations of the culture method used to identify the causative bacterium (13-14). It has been reported that molecular methods such as PCR can be a tool for rapid and sensitive detection of this bacterium (15). The most commonly reported symptoms in our study were fever, body aches and consuming dairy products by 89% of patients. In a study conducted in 2018, the symptoms of fever, fatigue and more severe body aches were reported and the majority of patients were consumer of dairy products (16).

Types of brucellosis serological tests include standard tests, tube agglutination, or Wright tests that assess IgG and IgM. Coombs Wright test that mainly shows IgG class antibodies, and Radioimmunoassay and ELISA has more sensitivity and better performance than standard test and complementary stabilization and show immunoglobulins M, G. Therefore, these tests can distinguish acute brucellosis from acute as well as an acute attack from a chronic one (18-17). In our study, 19% were positive in Wright's methods, 21% in Coombs Wright, 19% in 2me, and generally 21% had *Brucella* based on the above serological methods. In a study of 98 patients, 26.4% had been reported to be positive with Wright test and 85.7% with coombs wright test (19). In another study conducted in Mashhad, 312 cases were reported to

be positive with Wright test with a titer of 1:80 and above, and 324 cases with Coombs Wright test and 318 cases with 2me 21% (20).

Based on the results of our study, the rate of positive cases reported by PCR was 16%. In a study of 188 cases in 2017, 85 cases were reported positive based on PCR (21). In another study conducted on 113 suspected patients in 3 hospitals in Ardabil in 2020, 35% of cases were reported to be positive using PCR. Sensitivity and specificity of PCR were reported to be 94.1% and 100%, respectively (22). In a study of 446 samples in Pakistan in 2019, 206 serum samples of *Brucella abortus* were tested positive by PCR (23).

The ELISA test can detect defective antibodies commonly seen in patients with chronic brucellosis (24). In our study, 22% had IgM, 6% had IgG and generally 23% of the samples were positive based on ELISA test. (25).in a study on total 100 serum samples of suspected patients, 49 cases were reported positive by ELISA test. In another study performed on 1100 patients, 83.6% of cases were reported positive by ELISA, which were 29.1% and 58.2% for IgG and IgM, respectively (26). On the other hand, we evaluated the diagnostic value of IgG and IgM using the Roc curve by calculating the AUC, and obviously, the predictive level of ELISA IgM is much lower than the rest. According to a study conducted in 2020, the diagnostic value of EISA IgM is low which is consistent with the results of our study (27).

Kappa agreement coefficient was significant in Wright test, Coombs Wright, and 2me ($p < 0.001$). In contrast, IgM and IgG-based ELISA had a lower agreement coefficient. Serologic diagnostic indicators of sensitivity and specify reported to be 68.75% and 88.10%, respectively; in addiction, sensitivity and specify indicators of ELISA reported to be 68.75% and 85.71%, respectively. In a study of 59 patients with PCR, Wright, Elisa IgG, and IgM tests, 47%, 36%, 57%, and 38% of the patients tested positive, respectively. According to PCR test, positive predictive value and negative predictive value and overall accuracy of Wright test was calculated 75%, 95.45%, 60%, 63%, 63%, 94.73% and for IgM ELISA test was equal to 70,19%, 76,11%, 59%, 45%, 77,27%, 57.89% and for IgG ELISA was 85.45%, 61.22%, 71.21%, 78.94, 74% for IgG ELISA. Moreover, Wright test as higher sensitivity compared to ELISA test and IgG EISA is reported to be better than IgM ELISA (28). In another study of 89 samples, 44 cases were positive by ELISA and 21 cases were positive by PCR; a significant agreement was found between PCR and ELISA (29). According to the results from the reports of Kumar, Neha et al. it was found that *Brucella* is usually diagnosed based on serological and microbiological tests. Serological methods are not always sensitive or specific due to interaction with other bacterial antigens (30-31-32). A study was performed on serum samples of 102 patients in two native regions (Tehran and Lorestan provinces) showing excellent diagnostic performance of ELISA and 97.8% specificity and 95.7% sensitivity (33). In a study which aimed to compare the diagnosis of brucellosis in humans using PCR and serological methods, showed that PCR method has a higher sensitivity and specificity in the diagnosis of brucellosis than serological methods and it can be used in the diagnosis of human brucellosis (34). Another study by Rajeswari et al. In 2019 in India was carried out for serological and molecular analysis of brucellosis in pigs and showed PCR was a rapid, sensitive and accurate method for the diagnosis of *Brucella* (35). In a study by Metica et al., hey concluded that PCR was sufficiently effective in diagnosing acute as well as recurrent disease (36). Based on Fisher's exact test, percentage of *Brucella abortus* positive cases in PCR by age group ($P = .712$), sex ($P = 0.793$), previous history of *Brucella* ($P = 0.469$), fever ($P = 0.406$), body aches ($P = 0.675$) and dairy consumption ($P = 0.125$) was obtained and none of which were significant. In a study conducted in Egypt to identify brucellosis, the effect of age on the incidence was underestimated (37). A 2020 study on children found no significant relationship between age and sex and brucellosis (38). Another

Table 1. Primers used for DNA pattern and PCR synthesis.

Primer	Sequences
Forward	5'-TGGCTCGGTTGCCAATATCAA–3'
Reverse	3'-CGCGCTTGCCTTTCAGGTCTG–5'

Figure 1. Expected fragment based on designed primers was 731 bp in length. M represents the 100 bp marker, number 7 represents the positive control, number 2 represents the negative control, number 3 represents the person without infection with *Brucella abortus*, and numbers 6 and 5 represent the *Brucella abortus* infection with a bandwidth of 731 bp.

Table 2. Frequency distribution of *Brucella abortus* in patients based on studied laboratory test.

	Negative		**Positive**		**Total**	
	Number	Percent	Number	Percent	Number	Percent
Wright	81	81 %	19	19 %	100	100 %
Coombs Wright	79	79 %	21	21 %	100	100 %
2me	81	81 %	19	19 %	100	100 %
Wright Global	79	79 %	21	21 %	100	100 %
IgG	78	78 %	22	22 %	100	100 %
Igm	94	94 %	6	6 %	100	100 %
Elisa	77	77 %	23	23 %	100	100 %
PCR	84	84 %	16	16 %	100	100 %

Table 3. Serologic diagnostic indicators in the diagnosis of *Brucella abortus* with standard PCR method.

Diagnostic indicators	**All Serologic Methods**		**2ME**		**Coombs Wright**		**Wright**	
	95% CI	Value	95% CI	Value	95% CI	Value	95% CI	Value
Sensitivity	34.41% to 98.88%	68.85%	41.34 to 98/88%	68.75%	41.34 to 88.98%	68.75%	41.34 to 88.98%	68.75%
Specificity	19.79% to 14.94%	88.10%	95.8 to 82.09	90.48%	79.19 to 94.14	88.10%	82.09 to 95.8 %	90.48%
Positive probability ratio	96.2 to 27.11	5.77	15.09 to 3.45	7.22	2.96 to 11.27	5.77	3.45 to 15.09	7.22%
Negative probability ratio	0.17 to 0.19	0.35	0.17 to 0.72	0.35	0.17 to 0.72	0.35	0.17 to 0.72	0.35
Disease prevalence (*)	9.43 % to 68.24%	16%	9.43% to 24.68%	16%	9.43% to 24.68%	16 %	9.43% to 24.68%	16%
Positive predictive value (*)	04.36% to 23.68%	52.38%	39.68% to 74.19%	57.89 %	36.04% to 68.23%	52.38%	39.68% 74.19%	57.89%
Negative predictive value (*)	69.87% to 85.96%	93.67%	87.99% to 96.93%	93.83%	87.69 % to 96.85%	93.67%	87.99% to 96.93%	93.8.%
accuracy (*)	47.76% to 35.91%	85%	78.8 to 92.89	87%	76.47 to 91.35	85%	78.8 to 92.89%	87%

Table 4. ELISA diagnostic indicators in diagnosis of *Brucella abortus* based on standard PCR method.

Diagnostic indicators	**Elisa**		**IgM**		**IgG**	
	95% CI	Value	95% CI	Value	95% CI	Value
Sensitivity	41.34%	68.75%	7.27 to 52.38	25%	41.34%	68.75%
specificity	76.38% to 92.39%	85.71%	91.66% to 99.71%	97.62%	77.78% to 93.28%	86.9%
Positive probability ratio	2.59 to 8.94	4.81	2.10 to 52.58	10.50	2.76 to 9.98	5.25
Negative probability ratio	0.76 to 0.18	0.36	0.58 to 1.02	0.77	0.17 to 0.75	0.36
Disease prevalence (*)	9.43% to 24.68%	16%	9.43% to 24.68%	16%	9.43% to 24.68%	16%
Positive predictability value (*)	33.04 to 63%	47.83%	28.54% to 90.92 %	66.67%	34.47 to 65.53%	50%

Negative predictability value (*)	87.38% to 96.77%	93.51%	83.71% to 90.08%	87.23%	87.54% to 96.81%	93.59%
Accuracy (*)	74.18% to 89.77%	83%	77.63% to 92.13%	86%	75.32% to 90.57%	84%

Table 5. predictability level of ELISA and serologic test in diagnosis of *Brucella abortus* based on standard PCR method on ROC diagram.

Area Under the ROC Curve					
Test Result Variable(s)	Area	Std. Error	Asymptotic Sig.	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Wright	0.796	0.062	0	0.675	0.918
Coombs_Wright	0.784	0.062	0	0.662	0.907
2me	0.796	0.062	0	0.675	0.918
IgG	0.778	0.063	0	0.656	0.901
Igm	0.613	0.057	0.045	0.502	0.724
Serologic methods	0.784	0.062	0	0.662	0.907
Elisa	0.772	0.063	0	0.649	0.895

Table 6. Comparison of infection with *Brucella abortus* based on standard PCR method in terms of individual variables and symptoms.

		PCR						
		Positive		Negative		Total		
		Number	Percent	Number	Percent	Number	Percent	P
Age Group	Under 30	2	10%	18	90%	20	100%	712/0
	30 to 60	10	17.2	48	82.8%	58	100%	
	Above 30	4	18.2%	18	81.8%	22	100%	
	Total	16	16%	84	84%	100	100%	
Sex	Female	8	17%	339	83%	47	100%	793/0
	Male	8	15.1%	45	84.9%	53	100%	
	Total	16	16%	84	84%	100	100%	
History of Infection	No	14	15.2%	78	84.8%	92	100%	469/0
	Yes	2	25 %	6	75%	8	100%	
	Total	16	16%	84	84%	100	100%	
Fever	No	6	12.8%	41	87.2%	47	100%	406/0
	Yes	10	18.9%	43	81.1%	53	100%	
	Total	16	16%	84	84%	100	100%	
Body Ache	No	1	11.1%	8	88.9%	9	100%	675/0
	yes	15	16.5%	76	83.5%	91	100%	
	Total	15	16%	84	84%	100	100%	
Diary consumption	No	0	0%	11	100%	11	100%	125/0
	Yes	16	18%	73	82%	89	100%	
	Total	16	16%	84	84%	100	100%	

study conducted in Pakistan in 2021 examining the risk factors associated with *Brucella* reported age, sex, contact with animals, and milk consumption to be significant (39). In another study in 2015, no significant relationship was reported between age and sex and infection with *Brucella*, but the most common symptom of *Brucella* was reported to be fever (40).

Conclusion

There is general agreement that ELISA is a more sensitive method than traditional techniques used in diagnosis of brucellosis. The detection of specific immunoglobulins by a single, simple and rapid test is a major advantage of ELISA. The main problem with widespread use of ELISA in our country has been the lack of a definite cutoff value. The results of present study showed that PCR assay is a rapid and sensitive technique for diagnosis of brucellosis compared to ELISA. However it is more accurate when coupled with conventional methods. It is clear that more research is needed. Further studies should also be performed on a larger community of patient with *Brucella* infection in this geographical area.

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Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Also ethics committee of Azad University of Medical Sciences, Iran approved this study by (Code no: IR.IAU.CHALUS.REC.1400.003).

Conflict of interest

The authors declare no conflict of interest.

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