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Comparison of the Rose Bengal and ELISA Tests with the Standard Tube Agglutination Method in the Diagnosis of Brucellosis

Bruselloz tanısında Rose Bengal ve ELISA testlerinin Standart Tüp Aglütinasyon Yöntemiyle Karşılaştırılması

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Abstract

Brucellosis, an infectious bacterial zoonotic disease caused by bacteria of the *Brucella* genus, can cause high morbidity and is rarely associated with mortality. In order to guide clinicians in the rapid and accurate diagnosis of brucellosis, it is of great importance to use easy-to-apply and highly sensitive and specific screening tests in microbiology laboratories. This study included 1,709 patients that presented to our hospital with various complaints (mainly joint complaints, fever, and fatigue) compatible with brucellosis and underwent the Rose Bengal test (RBT), Brucella ELISA IgM and IgG tests, standard tube agglutination (STA) test, and Coombs agglutination tests between January 2020 and December 2020. All the laboratory tests were performed in standard laboratory conditions in accordance with the manufacturer's recommendations. The department to which the patients presented, their test results, regions of birth, age and gender data were reviewed retrospectively. The first three clinics to which the patients presented were rheumatology (27.8%), infectious diseases (18.4%), and internal medicine (11.1%). We followed two different paths when comparing the test results. In the first approach, we considered patients with at least one positive test result as seropositive and analyzed the results of the remaining tests in the same patients. In the second approach, we compared the results of the RBT and ELISA tests with the reference tests of STA and Coombs agglutination. Of a total of 3,365 tests evaluated for 1,709 patients, 7.43% (127/1,709) were seropositive according to at least one method. According to this initial approach, the sensitivities of all the serological tests ranged from 20.4% to 49.3%, and their specificities ranged from 93.8% to 100%. In the second approach, based on confirmatory tests, the sensitivities of the screening tests were 100%, 100% and 84.6% for RBT, ELISA IgG and ELISA IgM, respectively, while their specificities were 90.6%, 81% and 63.8%, respectively. These findings indicate that serological methods can be significantly misleading in the diagnosis of brucellosis if the test results are not combined. Although the number of seropositive cases was low, we obtained results consistent with the regional prevalence data when we distributed the patients according to their place of birth. In conclusion, since there is no gold standard test for the serological diagnosis of brucellosis, the test results should be combined based on the advantages and disadvantages of each test. It is important to evaluate the laboratory diagnosis together with the patient symptoms and complaints consistent with the disease and to consider epidemiological details in this process.

Keywords: Agglutination, Brucella, Diagnosis, ELISA, Epidemiology, Turkey.

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Özet

Brucella cinsindeki bakterilerin neden olduğu bulaşıcı bakteriyel bir zoonotik bir hastalık olan bruselloz yüksek morbidite ve nadiren de mortalite ile ilişkili bir hastalıktır. Hızlı ve doğru bruselloz teşhisinde klinisyenlere rehberlik etmek için, mikrobiyoloji laboratuvarlarında uygulaması kolay, duyarlılık ve özgüllüğü yüksek tarama testlerinin kullanılması büyük önem taşımaktadır. Bu çalışmaya Ocak 2020 - Aralık 2020 tarihleri arasında bruselloz ile uyumlu çeşitli şikayetlerle (başlıca eklem şikayetleri, ateş, halsizlik) hastanemize başvuran ve Rose Bengal test (RBT), ELISA Brucella IgM ve IgG, standart tüp aglütinasyon (STA) ve Coombs aglütinasyon test istemi yapılan 1.709 hasta dahil edildi. Tüm laboratuvar testleri standart laboratuvar koşullarında üreticinin tavsiyelerine uygun olarak gerçekleştirildi. Her hasta için başvurulan birim, test sonuçları, doğum yeri, yaş ve cinsiyet verileri retrospektif olarak gözden geçirildi. Hastaların başvurduğu ilk üç klinik romatoloji (%27.8), enfeksiyon hastalıkları (%18.4) ve iç hastalıkları (%11.1) idi. Test sonuçları arasında karşılaştırma yaparken iki farklı yol izledik. Birinci yaklaşımda en az bir pozitif test sonucu olan hastaları seropozitif olarak kabul edip aynı hastalarda diğer testlerin sonuçlarını inceledik. İkinci seçenekte STA ve Coombs aglütinasyon testlerini baz alarak RBT ve ELISA test sonuçlarını bu testler ile karşılaştırdık. 1.709 hasta için toplam 3.365 test çalışılmıştı ve hastaların %7.43'ü (127/1.709) en az bir yöntemde seropozitif idi. Bu ilk yaklaşıma göre tüm serolojik testlerin duyarlılıkları %20.4 ile %49.3 arasında ve özgüllükleri %93.8 ile %100 arasında değişmekte idi. Doğrulama testlerinin baz alındığı ikinci yaklaşımda ise tarama testlerinin duyarlılıkları RBT, ELISA IgG, ELISA IgM için sırasıyla %100, %100 ve %84.6 iken, özgüllükleri sırasıyla %90.6, %81 ve %63.8 idi. Bu bulgular test sonuçlarının kombine edilmemesi durumunda serolojik yöntemlerin bruselloz tanısında önemli derecede yanıltıcı olacağına işaret etmektedir. Seropozitif olgu sayısı az olmakla beraber hastalar doğum yerlerine göre dağıtıldığında bölgesel prevalans verileri ile uyumlu sonuçlara ulaşıldı. Sonuç olarak bruselloz tanısında altın standart bir serolojik test bulunmadığı için her bir testin sahip olduğu avantaj ve dezavantajlar bilinerek test sonuçlarının kombine edilmesi ve hastalıkla uyumlu belirti ve şikayetler ile birlikte kronik yönü de olan bu hastalığın epidemiyolojik edinim yönünün de akılda tutulması gerektiğine işaret etmektedir.

Anahtar Kelimeler: Aglütinasyon, Brucella, ELISA, Epidemiyoloji, Tanı, Türkiye.

Introduction

Brucellosis is an infectious bacterial zoonosis caused by small gram-negative coccobacilli (rod-shaped), facultative intracellular bacteria in the *Brucella* genus [1,2]. *Brucella* spp. were first reported in Malta in 1886 by military personnel named Sir David Bruce, who was an Australian-born British pathologist and microbiologist [3,4]. The human brucellosis is known by many other names, such as Malta fever, Mediterranean fever, goat fever, remitting fever, and undulant fever [5,6].

Brucellosis is prevalent or endemic in many regions of the world, including Southern Europe, Western Asia, Middle East, Mediterranean countries, Africa, and Latin America [5,7]. More than 500,000 new cases of infection are reported annually worldwide [8]. Contact of the skin, wound or mucous membrane with infected animals or animal waste and consumption of contaminated raw or undercooked animal products (such as milk and meat) are the major reasons of transmission brucellosis [9,10]. Aerosol inhalation is another recognized route of transmission and considered to be occupational hazard for veterinarians, farmers, and laboratory personnel [7,9].

In brucellosis, the clinical presentation or course of infection is not specific; it may be acute (initial, 2 months), sub-acute (2–12 months), or chronic (more than 12 months), while asymptomatic human infections have also been reported in some cases [7,11,12]. In symptomatic cases, the most common symptoms are high fever, restlessness, loss of appetite, sweating, and muscle and joint pain [13]. Acute brucellosis may progress to a chronic infection with the relapse or development of the subacute phase with mild symptoms (e.g., fatigue, headache, and myalgia) and localized symptoms (e.g. ,epididymitis, orchitis and osteoarticular complications) [7,14]. Mortality due to brucellosis is rare and caused by the infection of the brain or heart [14,15]. Brucella endocarditis due to the

destruction of valve structures is observed in only 1-2% of cases but is associated with mortality at a rate of up to 80%cases [15,16].

Some diagnostic tests, including the isolation of *Brucella* spp. from the culture samples, serological tests based on elevated antibody titers in body fluids (e.g., blood and cerebrospinal fluid), detection of Brucella antigens in blood or other clinical samples, and nucleic acid amplification assays used for the detection of Brucella genetic material in blood or other clinical samples (e.g., bone marrow) are used in the laboratory diagnosis of brucellosis [7,17,18]. Blood cultures for brucellosis are confirmatory; however, it is very difficult in clinical practice due to the exacting culture requirements and early tissue localization of the bacteria [19]. Additionally, blood cultures positivity is seen in only 10–90% of brucellosis cases, depending on the stage of the disease, bacterial species, cultivation conditions, and tested clinical specimens, and it is also necessary to obtain multiple blood culture samples to increase the sensitivity of the test [17,20,21]. As a result, due to the non-specificity of disease symptoms and signs and difficulties in isolating the microorganism, most cases are diagnosed serologically [18].

Since there are no gold standard tests for the serological diagnosis of brucellosis, a combination of serodiagnostic tests are used for the diagnostic evaluation of the disease [17]. Some studies have suggested the use of the standard serum tube agglutination (STA) test, which examines both IgM and IgG antibodies referenced as the gold standard diagnostic test in brucellosis [22]. A STA titer of ≥1:160 is considered to indicate active brucellosis in the presence of consistent clinical findings [17]. However, there are some concerns regarding the sensitivity of this test. The Rose Bengal test (RBT), enzyme-linked immunosorbent assay (ELISA), direct fluorescent antibody test, Coombs test, complement fixation test, IgG avidity ELISA, microagglutination tests, immunocapture agglutination tests, and lateral flow assay are other diagnostic tests for used initial screening, confirmation of diagnosis, or rapid diagnosis of acute cases in the management of brucellosis [17,22,23]. The current study aimed to compare the results of the widely used screening tests (RBT and ELISA IgG and IgM) with those of the confirmatory tests (STA and Coombs) and to draw attention to the clinical test request algorithms and the importance of epidemiological evidence.

Material and Method

Patients presenting to all clinics of Gulhane Training and Research Hospital (Ankara, Turkey) and tested using STA, RBT, and Brucella-IgM/IgG ELISA between January 1, 2020 and December 31, 2020 were included in the study. For each patient, clinical findings at the time of presentation, test results, geographic region of birth, and gender were retrospectively reviewed. Ninety-three patients with repeated tests were excluded from the study.

Serological tests

The serum sample (30 µl) to be tested was placed to a white glossy ceramic tile and mixed thoroughly with an equal volume of the Rose Bengal antigen. The plate was rocked gently for three minutes and observed. Reading was performed as previously described, and the sample was evaluated as positive (visible agglutination and/or the appearance of a typical rim) or negative [24]. The STA test (Seromed, Istanbul, Turkey) was performed as previously described [19]. Each serum sample (20 µl) and the controls were diluted with 1.98 µl of NaCl in six double dilutions from 1/20 to 1/640 titers, and the presence of agglutination was evaluated after 24 hours of incubation at 37 °C. The presence of ≥1:160 titers was considered as a positive result. The Brucella-IgM/IgG antibody levels were measured using the ELISA method (VIRCELL, Santa Fe, Granada, Spain), and an antibody index of <9, 9-11, and >11 was considered as a negative, equivocal (intermediate), and positive, respectively [25]. All the tests were carried out in accordance with the manufacturer's recommendations.

Statistical analysis

Sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and accuracy calculations, mean age, and standard deviation were determined using the standard statistical formulas.

Results

Serum samples obtained from 1,709 different patients (mean age, 43.2 ± 17.9; median age, 43 years; range, 1-92 years) were tested in our laboratory for brucellosis serology using different tests. Of these patients, 58.2% (n = 995) were female (mean age, 44.5 ± 16.6; median age, 45 years; range, 1-92 years) and 41.8% (n = 714) were male (mean age, 41.4 ± 19.6 years; median age, 40 years; range, 1-91 years).

The rate of patients with at least one positive test result in the whole study group was 7.43% (127/1,709), of whom 67 (52.8%) were female (mean age, 46.4 ± 18.6 years; range, 12-86 years) and 60 (47.2%) were male (mean age, 42.2 ± 17 years; range, 17-82 years).

Table 1. All test requests and comparative results.

		Comparison with the RBT results			Comparison with the ELISA IgG test results				Comparison with the ELISA IgM test results				Comparison with the Wright-STA test results			Comparison with the Coombs test results		
	Total (n)	P	N	NT	P	N	E	NT	P	N	E	NT	P	N	NT	P	N	NT
RBT-positive	**47**				30	8	1	8	29	10	2	6	9	12	26	10	11	26
RBT-negative	**1433**				24	546	3	860	44	550	12	827	0	103	1,330	0	66	1,367
ELISA IgG-positive	**62**	30	24	8					29	31	2	0	8	12	42	11	13	38
ELISA IgG-negative	**710**	8	546	156					48	645	14	3	0	49	661	0	39	671
ELISA IgG-equivocal	**5**	1	3	1					1	3	0	1	0	0	5	0	1	4
ELISA IgM-positive	**80**	29	44	7	29	48	1	2					6	32	42	8	28	44
ELISA IgM-negative	**725**	10	550	165	31	645	3	46					1	50	674	2	50	673
ELISA IgM-equivocal	**18**	2	12	4	2	14	0	2					1	5	12	1	3	14
Wright-STA ≥1:160	**13**	9	0	4	8	0	0	5	6	1	1	5				5	0	8
Wright-STA ≤1:80	**172**	12	103	57	12	49	0	111	32	50	5	85				3	61	108
Coombs Test ≥1:160	**11**	10	0	1	11	0	0	0	8	2	1	0	5	3	3			
Coombs Test ≤1:80	**89**	11	66	12	13	39	1	36	28	50	3	8	0	61	28			

E: equivocal. N: negative. NT: not tested. P: positive. RBT: Rose Bengal test. STA: standard tube agglutination.

Table 2. Distribution of single and multiple test requests according to the clinics.

Patients (n) (%)	RBT	ELISA IgG	ELISA IgM	Wright-STA	Coombs test	H	ID	IM	N	PD	PT	Rh	other
791 (46.3)	×					113	77	64	33	58	19	225	202
522 (30.5)	×	×	×			42	177	83	8	12	0	157	43
158 (9.25)		×	×			46	2	34	28	17	0	12	19
49				×		11	1	1	0	10	5	9	12
3	×	×	×	×	×	5	20	1	1	1	0	8	0
34	×			×		0	1	0	1	3	1	14	14
28	×	×	×	×		2	16	3	0	0	0	2	5
23	×	×	×		×	1	17	1	0	0	0	3	1
19		×	×	×	×	0	0	0	0	0	0	19	0
49	*Other combinations (1 to 4)*					6	3	3	1	4	0	26	6
	Total (**n = 1709**)					226	314	190	72	105	25	475	302
	%					13.2	18.4	11.1		6.14		27.8	

E: equivocal. N: negative. NT: not tested. P: positive. RBT: Rose Bengal test. STA: standard tube agglutination.
H: hematology. ID: infectious diseases. IM: internal medicine. N: neurology. P: pediatric disease. PT: physical therapy. Rh: rheumatology.

Of the patients in the study group, 1,660 were from 79 different provinces of Turkey and 49 were from 15 other countries (Afghanistan, Azerbaijan, Bulgaria, Ethiopia, France, Georgia, Germany, Iraq, Kyrgyzstan, Lebanon, Russia, Saudi Arabia, Singapore, Somalia, and Syria)

(Figure 1 and Figure 2). The distribution of a total of 3,365 tests evaluated in 1,709 patients and the comparative results are shown in Table 1. Single RBT test requests were 46.3% of the all-test requests. RBT and/or ELISA IgG and/or ELISA IgM test requests were 86.1% of the all-test requests. The two clinics receiving the highest number of patients were rheumatology (27.8%) and infectious diseases (18.4%). The distribution of all the test requests according to the different clinics is presented in Table 2.

The specificity, sensitivity, NPV, PPV and accuracy of these tests are shown in Table 3 (based on at least one positive test result) and Table 4 (based on the Wright-STA and Coombs tests as reference).

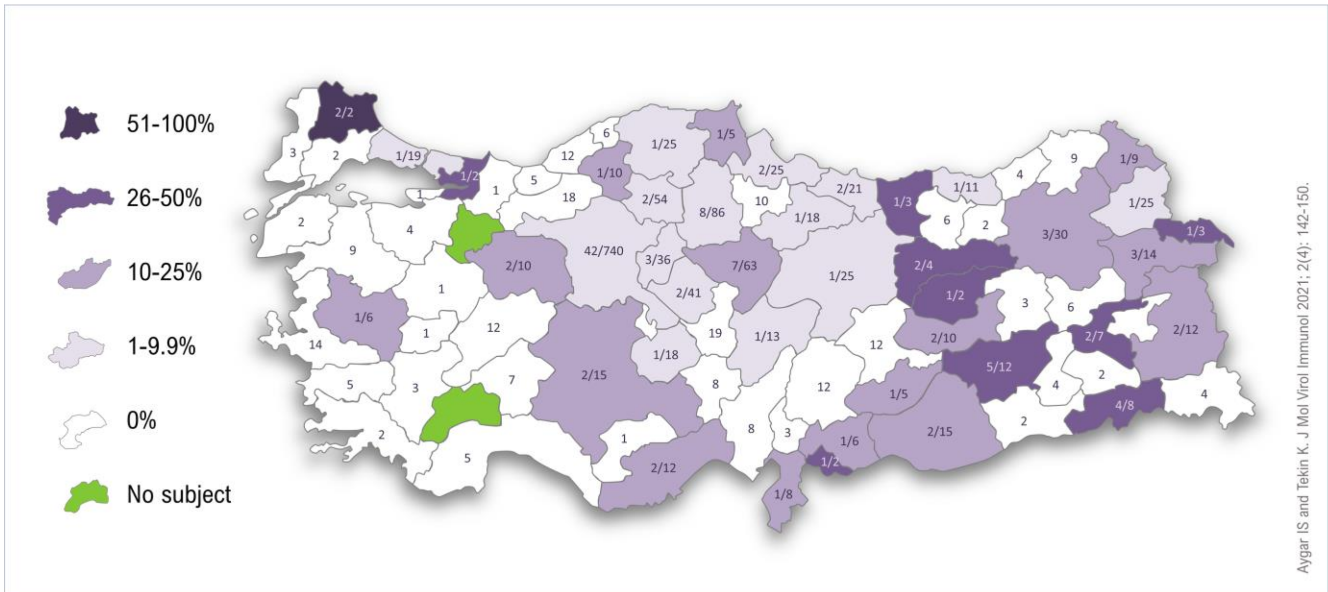

Figure 1. A map of Turkey showing the distribution of patients according to their province of birth and those with at least one positive result in any test method.

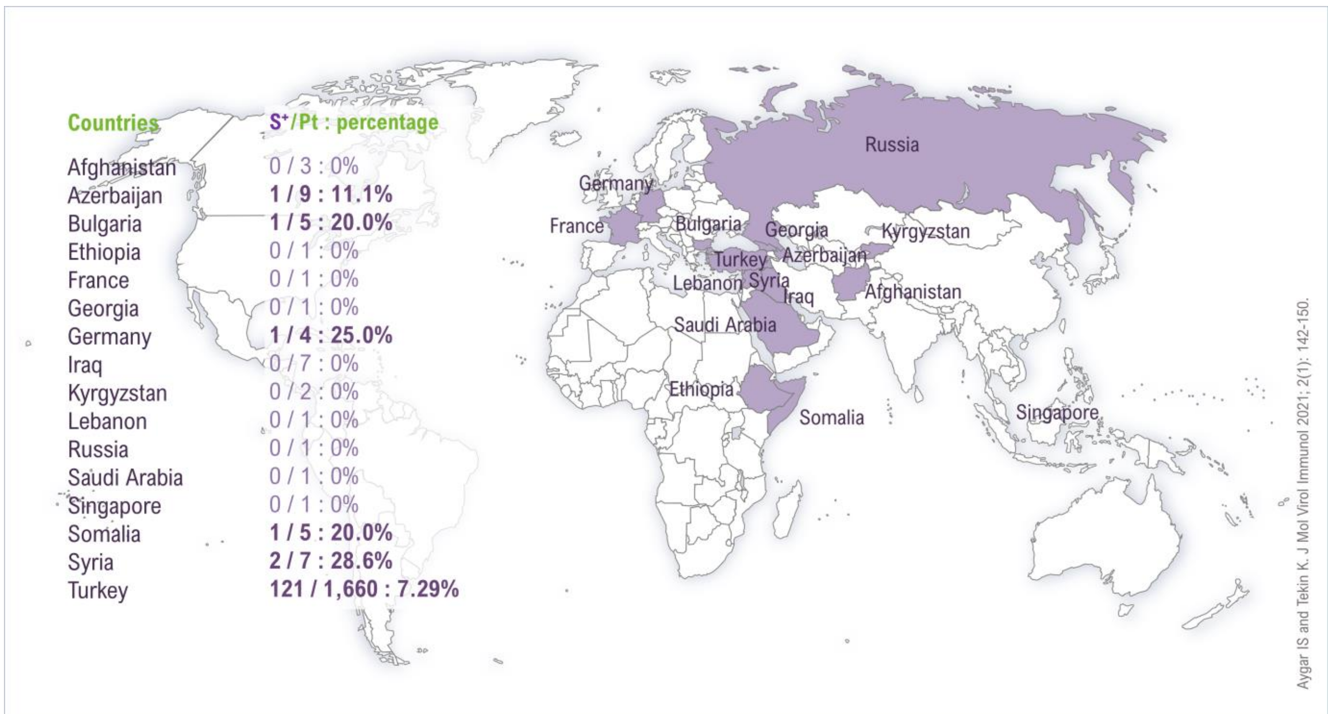

Figure 2. Distribution of patients according to their country of birth and rate of patients with at least one positive result in any test method.

Table 3. Statistical evaluation of the diagnostic tests performed in patients with at least one positive test result.

		RBT			ELISA IgG				ELISA IgM				Wright-STA			Coombs test		
	Total (n)	P	N	NT	P	N	E	NT	P	N	E	NT	P	N	NT	P	N	NT
Positive*	**127**	38	64	16	37	51	2	12	35	36	2	9	10	39	75	11	37	79
Negative**	**741**	5	582	154	25	658	3	80	45	682	16	43	0	87	654	0	52	689
Single test results***		4	787		0	1	0		0	7	0		3	46		0	0	
Sensitivity (%) (%95 CI)		37.25 (27.9-47.4)			42.05 (31.6-53.05)				49.3 (37.2-61.44)				20.4 (10.2-34.3)			22.9 (12.03-37.3)		
Specificity (%) (%95 CI)		99.15 (98.0-99.7)			96.3 (94.6-97.6)				93.8 (91.8-95.4)				100 (95.85-100)			100 (93.15-100)		
NPV (%) (%95 CI)		90.1 (88.7-91.35)			92.8 (91.5-93.9)				94.99 (93.8-95.98)				69.05 (65.9-72)			58.4 (54.64-62.1)		
PPV (%) (%95 CI)		88.4 (75.4-94.96)			59.7 (48.4-70.0)				43.75 (34.98-52.9)				100			100		
Accuracy (%) (%95 CI)		89.99 (87.5-92.1)			90.14 (87.8-92.15)				89.85 (87.5-91.9)				71.3 (62.95-78.75)			63 (52.76-72.4)		

*Positive result from at least one other test. **Negative (or equivocal) result from at least one other tests. ***Since only the result of the relevant test was available, a comparison could not be made and these data were excluded from the cross-evaluation.
E: equivocal. N: negative. NPV: negative predictive value. NT: not tested with the relevant method. P: positive. PPV: positive predictive value. RBT: Rose Bengal test. STA: standard tube agglutination.
Blue: true positive results. Green: true negative results. Purple: false positive results. Orange: false negative results.

Table 4. Statistical evaluation of the diagnostic tests compared with the Wright-STA and Coombs tests as reference.

		RBT			ELISA IgG				ELISA IgM				*** ELISA IgG, ELISA IgM, and RBT		
	Total (n)	P	N	NT	P	N	E	NT	P	N	E	NT	P	N	NT
Positive*	**19**	15	0	4	14	0	0	5	11	2	1	5	14	0	5
Negative**	**197**	13	125	59	15	64	1	117	38	67	5	87	14	56	127
Sensitivity (%) (%95 CI)		100 (78.2-100)			100 (76.8-100)				84.6 (54.55-98.1)				100 (76.84-100)		
Specificity (%) (%95 CI)		90.6 (84.4-94.9)			81 (70.6-88.97)				63.8 (53.85-72.96)				80 (68.7-88.6)		
NPV (%) (%95 CI)		100			100				97.1 (90.3-99.2)				100		
PPV (%) (%95 CI)		53.6 (40.75-65.9)			48.3 (37.2-59.5)				22.45 (17.03-28.99)				50 (38.5-61.5)		
Accuracy (%) (%95 CI)		91.5 (85.9-95.4)			83.9 (74.8-90.7)				66.1 (56.8-74.56)				83.3 (73.6-90.6)		

*Any positive result (≥1:160 titers) from the Wright-STA or Coombs test. **Negative result (≤1:80 titers) from the Wright-STA and/or Coombs test. *** Evaluation of patients with positivity or negativity in at least two of the screening tests (ELISA IgG-IgM and RBT).
E: equivocal. N: negative. NPV: negative predictive value. NT: not tested with the relevant method. P: positive. PPV: positive predictive value. RBT: Rose Bengal test. STA: standard tube agglutination.
Blue: true positive results. Green: true negative results. Purple: false positive results. Orange: false negative results.

Discussion

Despite recent scientific advances, it is still difficult to diagnose, treat and monitor brucellosis in endemic areas [4,13]. Our study was carried out in Ankara, one of the metropolitan cities of Turkey. When we examined the birthplaces of people tested for brucellosis, we observed many people from different national and international cultures living in Ankara for diplomatic, educational and economic reasons. The patients spread across almost all provinces of Turkey (Figure 1). Although their numbers were low, the distribution of brucellosis cases was high in the eastern provinces of the country, in accordance with previous epidemiological data [26,27]. This finding may be related to the previous acquisition of brucellosis, which also causes chronic disease or the patients' ongoing social relationships with their birthplaces. The positivity in patients from neighboring countries, such as Azerbaijan, Syria, and Bulgaria was also noteworthy (Figure 2). These data indicate that the epidemiology of infectious diseases in the globalizing world will need to be examined in detail with more complex parameters.

Although men are at a higher occupational risk for brucellosis, and the brucellosis prevalence is higher in males than in females, no difference has been reported in terms of gender in regions where the infection is endemic [19,26,28]. Although women constituted the majority of our study group (58.2%), the rates of seropositive

men and women was close to each other, being determined as 47.2% and 52.8%, respectively.

The reliable identification of *Brucella* is very important for initiating appropriate antibiotic treatment as early as possible [29]. Serological methods are frequently used in the diagnosis of brucellosis. In clinical practice, the most commonly used test for brucellosis screening is the RBT, which is a rapid and highly sensitive method [28]. The second most frequently used serological method to detect Brucella-IgM and IgG antibodies is ELISA tests [30]. STA is considered by some authors to be a standard test in the diagnosis of brucellosis [31].

In this study, the first three clinics to which the patients presented were rheumatology (27.8%), infectious diseases (18.4%), and internal medicine (11.1%). In our hospital, clinicians mainly (86.1%) preferred RBT and/or the ELISA IgG and ELISA IgM test as a brucellosis screening test to support clinical findings (Table 2). However, it is noteworthy that a confirmatory test was not applied in the majority of cases with positive results in RBT or the ELISA IgG or IgM test (Table 1).

In this study, based on the confirmatory tests (STA and Coombs) RBT had a higher sensitivity close to 100% and specificity of 90.6%. In a previous study, the sensitivity and specificity of RBT were found to be 96.9% and 62.5%, respectively compared with STA (≥1:160) as a reference [32], which is very similar to our result. However, as in other tests used in the diagnosis of brucellosis, the results of RBT can be affected by many factors. It has been shown that the sensitivity and specificity of RBT increase to 100% when a positive culture is taken as a reference, while they decrease even to 50% in certain patient groups with chronic, focal and complicated infections due to high rate of false-negative results [24,33].

Different kits of *Brucella* ELISA tests, which present as alternative screening or diagnosing tests for brucellosis, have varying sensitivity and specificity rates, with some being reported to be as low as 50% [23,25,34]. Our study showed that ELISA IgG or ELISA IgM seropositivity had low specificity when compared to the results of STA and Coombs tests, (81% and 63.8%, respectively) (Table 4). In addition, the sensitivity of ELISA IgM was found to be 84.6% when compared to the STA and Coombs tests. In active brucellosis cases, the IgM and IgG sensitivity rates are each reported as 80%; however, when evaluated together, the sensitivity of the test increases to 90%–100% [23,25,34]. Although we observed an increase in sensitivity and specificity when we combined the results of the three tests (ELISA IgG, ELISA IgM, and RBT), we were still not able to obtain very strong results. It is possible that this situation is related to the low number of our cases. However, false-negative results may be observed with STA due to several causes [31]. The results presented in Table 3 indicate that STA has a high specificity but very low sensitivity. Accordingly, in a recent study, it was shown that the STA test alone was not sufficient in the diagnosis of brucellosis, and the authors suggested using the STA test in combination with the Brucellacapt and/or ELISA tests [18]. We also observed that the statistical parameters (sensitivity, specificity, NPV, PPV, and accuracy) of the tests were significantly negatively affected when the evaluation was made based on patients with a positive result in at least one other test (Table 3).

Conclusion

It is of great importance to use screening tests with high sensitivity and specificity, which are easy to apply in microbiology laboratories in order to achieve a rapid and accurate diagnosis. When added to the standard STA method, the RBT and ELISA IgM/IgG tests, which are easier and faster, can increase the reliability of the results. In this study, except the infectious diseases clinic, it was observed that a significant portion of the patients were not referred to a confirmatory test, and the test results were not combined for the diagnostic evaluation of brucellosis. While emphasizing the importance of combining test results based on their advantages and disadvantages, we also consider it important to carefully evaluate the epidemiological histories of the patients in addition to their clinical findings.

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