



# EVALUATION AND IMPORTANCE OF SELECTED MICROBIOLOGICAL METHODS IN THE DIAGNOSIS OF HUMAN BRUCELLOSIS

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## ABSTRACT

Brucellosis is an important public health problem in Bosnia and Herzegovina. The diagnosis of brucellosis in the country without any experiences with this kind of infection may be very difficult. The aim of this study was to evaluate diagnostic methods: Rose Bengal test, blood cultures and ELISA IgM and IgG in the patients with brucellosis. The study included 91 brucellosis patients in the period 2004 to 2007. All the patients were treated at the Clinic for Infectious Diseases, University of Sarajevo Clinics Centre. Blood cultures were positive in 28/91 (30, 8%) patients. This method often needs a long period of incubation and specimens need to be obtained early. These limitations make serology the most useful tool for the laboratory diagnosis of *Brucella* infection. Rose Bengal is a rapid plate agglutination test, very sensitive irrespective of the stage of the disease. In our study, Rose Bengal test was positive in all patients 91/91 (100, 0%). *Brucella* IgM antibodies with ELISA were positive in 59/91 (64, 8%). *Brucella* IgG antibodies with ELISA were positive in 51/91 (56%). In order to determine the diagnostic value of the different tests, we compared the sensitivity among test-methods: Rose Bengal test-100.0%, blood culture-30.8%, ELISA IgM-64.8% and ELISA IgG-56.1%. Sensitivity of test methods was different in the different stages of illness. It is necessary to use combination of different tests such are blood culture, Rose Bengal test and ELISA in order to ensure the diagnosis. Rose Bengal test is excellent for the screening. Blood culture is a method of choice for the diagnosis acute infection. ELISA is a very good method for the diagnostic chronic disease and relapse.

KEY WORDS: *Brucella spp.*, brucellosis, laboratory diagnosis.

## INTRODUCTION

Brucellosis is worldwide zoonosis with a high degree of morbidity in humans. The disease remains endemic in many countries, particularly around the Mediterranean basin and in the Middle East, India, Mexico, and Central and South America, and thereby represents an important public health problem. According to WHO data about 500.000 cases of this disease were registered in the world every year (1,2,3). In Bosnia and Herzegovina before the recent war (1992-1995) only one outbreak was reported on Manjača Mountain, in a military training camp during 1985/86. Brucellosis has become the increasing public health problem in Bosnia and Herzegovina since 2000 (4). Brucellosis is caused by bacteria from genus *Brucella*. It is Gram-negative facultative intracellular cocco-bacillus size of 0,5-0,7 x 0,5-1,5 µm. Although the six species can be differentiated by conventional phenotypic tests, these species display a high degree of DNA homology in DNA-DNA hybridization assays (>90% identity). Therefore, it has been proposed that the *Brucella* genus should comprise only one species – *Brucella melitensis* and that the other species should be considered as biovars (5,6,7). Brucellosis is primarily an animal disease, and in them it passes as an asymptomatic chronic infection. Infections of humans are followed by outbreak of brucellosis in animals. It is usually a professional disease of cattle-breeders, farmers, butchers and members of their family, veterinarians and laboratory workers because of transmission ways (direct contact, aerosol inhalation, food). In humans, brucellosis behaves as a systemic infection with a very heterogeneous clinical spectrum. The disease usually presents as fever with no apparent focus, although there are focal forms in 20-40% of cases. The diagnosis of brucellosis in the country without any experiences with this kind of infection may be very difficult. Early diagnostic of brucellosis and inclusion of adequate antibiotic therapy have a crucial importance for patients, especially for the protection development complications and appearance relapses of disease.

As the clinical picture in human's brucellosis is fairly non-specific, a definitive diagnosis requires isolation of causative organism, or the demonstration of high levels of specific antibodies, or seroconversion (8). The blood culture is method of choice, but specimens should be taken in the early stage of the disease. The incubation of the blood culture takes a long time, between five to ten days. The isolation of the bacterium is proceeding of the biological hazard for the laboratory staff. Brucellosis is one of the most common laboratory acquired infections (9). The polymerase chain reaction (PCR) based laboratory tests have been proposed in the last few years (5,6,7). PCR is a very expensive test and it can not be considered as a routine diagnostic method yet. These limitations make serology the most useful tool for the laboratory diagnosis of the *Brucella* infection. The antibody detection is not always sufficient to indicate the existence of active infection especially in the endemic areas in which equivocal serologic profiles among affected individuals are frequent (10,11,12,13). Several reports dealing with the significance of various laboratory tests in the diagnosis of brucellosis have been published (14, 15,16,17). The present study deals with usefulness and significance of blood culture and serology tests in the diagnosis of the human brucellosis. The main purpose of this work was to evaluate diagnostics methods: Rose Bengal test, blood culture and ELISA IgM and IgG in order to design diagnostic protocol applicable in hospitals.

## MATERIALS AND METHODS

The study included 91 brucellosis patients in period 2004 to 2007. All patients were treated at the Clinic for Infectious Diseases, University of Sarajevo Clinics Centre. The average age of patients was 35,9 years of life- range from 0,6 to 70 (Table 1.). Most of the cases were between 41 and 50 years (Table 2.). Gender structure of patients was: 66 (72,5%) males and 25 (27,5%) females (Table 3). All the laboratories testing for brucellosis was performed at the Institute of microbiology, immunology and parasitology, University of Sarajevo Clinics

Statistics Age	
Arithmetic.middle	35,9451
Std. error	1,67925
Std. deviation	16,01899
Minimum	0,00
Maximum	70,00

TABLE 1. The average age of patients

	Age	
	N	%
<20 years	20	22,0
21-30 years	17	18,7
31-40 years	15	16,5
41-50 years	21	23,1
>50 years	18	19,8
Total	91	100,0

TABLE 2. Age structure of patients

	Gender	
	N	%
men	66	72,5
women	25	27,5
Total	91	100,0

TABLE 3. Gender structure of patients

Centre. The disease was diagnosed by positive blood-culture results and/or by positive relevant serological test results (ELISA, Rose-Bengal latex agglutination).

*Blood culture*

The blood culture was performed by inoculation 8-10 cm<sup>3</sup> of freshly collected blood into each of Plus aerobic/F BACTEC bottle and incubation for up to seven days in the „BACTEC 9120“ semi-automated systems (Becton-Dickinson Diagnostic Instruments Systems, Maryland, USA). Bottles were examined for the presence of growth on a 10-minute cycle by the measurement of CO<sub>2</sub>-induced fluorescence emitted by the sensor at the bottom of the culture. Bottles giving a positive growth index were Gram stained and subcultured to blood agar plates. Brucella isolates were identified by the conventional biochemical testing (catalase, oxidase and urease activity; glucose fermentation and production of H<sub>2</sub>S). *Brucella spp.* suspected isolates were confirmed by slide agglutination using type-specific antisera (Murex Diagnostics Dartford, United Kingdom). The Rose-Bengal latex agglutination (RB test) was performed according to standard procedures. Undiluted serum samples (30µL) were mixed with an equal volume of Rose Bengal Slide Screening Test antigen (bio Merieux, Marcy L Etoile/France) on a white agglutination card. Results were rated negative when agglutination was absent and 1+ to 4+ positive according to the strength of the agglutination. Brucella IgM and IgG enzyme-linked immunosorbent assays (ELISAs) were obtained from Genzyme Virotech GmbH/Germany. The test was performed and evaluated according to the kit procedure. The test result was read automatically by BEP 2000-Behring ELISA processor. For the evaluation of the results, standard statistical methods were used. Statistical analysis was performed by using a Chi-square test. Statistically significance was defined for p<0, 05. In our study we observed ethical principles outlined in World Medical Association Declaration of Helsinki.

**RESULTS**

Of the 91 brucellosis patients, the blood cultures were positive in 30, 8% (28/91) (Table 4).

Blood culture		
	N	%
neg.(-)	63	69,2
pos.(+)	28	30,8
<b>Total</b>	<b>91</b>	<b>100,0</b>

TABLE 4. Results of examination blood cultures

Rose Bengal			
	N	%	
pos.(+)	91	100,0	

TABLE 5. Results of Rose Bengal test

IgM		
	N	%
neg.(-)	32	35,2
pos.(+)	59	64,8

TABLE 6. The value of Immunoglobulin M (IgM)

Brucella IgM antibodies with ELISA were positive in 59/91 (64, 8%) and negative in 32/91 (35, 2%) patients (Table 6).

Brucella IgG antibodies with ELISA were positive in 51/91 (56%) and negative in 40/91 (44%) patients (Table 7).

IgG		
	N	%
neg.(-)	40	44,0
pos.(+)	51	56,0

TABLE 7. The value of Immunoglobulin G (IgG)

On Table 8. there was relationship between blood culture and IgM. Results of this methods corresponded at 54/91(59, 3%) patients: in 29/91(31, 8%) patients both methods were negative, and in 25/91(27, 5%) both methods were positive.  $\chi^2$  (Chi square test) demonstrates statistical significance coincidence among blood culture and IgM (p=0,001).

Blood culture and IgM					
		IgM		Total	
		neg(-)	pos(+)		
Blood culture	neg.(-)	N	29	34	63
		%	31,8	37,4	69,2
pos.(+)	N	3	25	28	
		%	3,3	27,5	30,8
Total	N	32	59	91	
		%	35,2	64,8	100,0

$\chi^2=10,605$  p=0,001 (p<0, 05) statistical significance

TABLE 8. Review of relationship between blood culture and IgM

The relationship between IgG and IgM is shown in Table 9.

IgG * IgM Crosstabulation					
		IgM		Total	
		neg(-)	pos(+)		
IgG	neg.(-)	N	0	40	40
		%	0,0	43,9	43,9
pos.(+)	N	32	19	51	
		%	35,2	20,9	56,1
Total	N	32	59	91	
		%	35,2	64,8	100,0

$\chi^2=38,711$  p=0, 0001 (p<0, 05) statistical significance

TABLE 9. Review of relationship between IgG and IgM

Results show that the values of IgG and IgM corresponded at 19/91 (20, 9%) patients.  $\chi^2$  (Chi square test) demonstrates statistical significance distinction inside number of patients with positive and negative value IgM in relation of value of IgG ( $p=0,0001$ ).

The sensitivity of the test-methods was: Rose Bengal test-100, 0%, blood cultures-30, 8%, ELISA IgM-64, 8% and ELISA IgG-56,1%. Positive predictive values were 100, 0% for all methods (Table 10.). Sensitivity of test methods was different in different stages of illness (Table 11.).

Total examination			
	Sensitivity	Accuracy	PPV
Blood cultures	43,8	73,8	100
Rose Bengal	100	100	100
IgM	64,8	64,8	100
IgG	56,1	56,1	100

PPV= Positive predictive value

TABLE 10. Sensitivity of test methods

1 month			
	Sensitivity	Accuracy	PPV
Blood cultures	63,2	82,5	100
Rose Bengal	100	100	100
IgM	71,3	72,5	100
IgG	58,6	58,6	100
1-3 months			
	Sensitivity	Accuracy	PPV
Blood cultures	52,6	70,2	100
Rose Bengal	100	100	100
IgM	62,5	61,5	100
IgG	54,3	52,8	100
3-6 months			
	Sensitivity	Accuracy	PPV
Blood cultures	43,6	62,3	100
Rose Bengal	100	100	100
IgM	44,3	56,2	100
IgG	38,2	48,3	100
6-12 months			
	Sensitivity	Accuracy	PPV
Blood cultures	23,6	53,2	100
Rose Bengal	100	100	100
IgM	25,3	56,8	100
IgG	19,4	42,6	100

TABLE 11. Sensitivity of test methods in different stages of illness

## DISCUSSION

Brucellosis represents a prevalent disease in humans and animals in Bosnia and Herzegovina in the last ten years. The clinical features and presentation of human brucellosis overlap with different infectious and autoimmune or neoplastic processes. Since the symptoms of brucello-

sis are non-specific, the clinical diagnosis of the disease is difficult. Therefore, its accurate diagnosis necessitates the use of specific tests, mainly culture and serologic tests (8). In this study we evaluated the diagnosis of brucellosis by culture, Rose Bengal test, and ELISA IgM and IgG. The study included 91 brucellosis patients in period 2004 to 2007. Blood cultures were positive in 28/91 (30, 8%) patients. In total 21/28 (75%) patients blood cultures were positive in the first month of infection. This fact suggested that it is method of choice in the acute phase of the disease. The examination of blood cultures gave possibility to identify species in genus *Brucella* and to perform antibiotic susceptibility testing. Although blood culture is the gold standard in the diagnosis of brucellosis, it may sometimes be abolished by the factors inherent to the microbe itself despite the strong laboratory measures undertaken. The isolation of the bacteria are hazardous and brucellosis is one of the most common laboratory acquired infection (9). Despite the important advances made in the diagnosis of human brucellosis following the general introduction of the new semi-automated methods for blood culture processing, the diagnosis of this disease is still based mostly on the demonstration of specific antibodies by means of different serological techniques. This is mainly because the greatest incidence of brucellosis is found in under-developed countries with poor technical resources and so they have not adequate equipment. Brucellosis tends to occur in rural communities. Blood culture demonstrated the high sensitivity in the primary infected patients, but expressed limitations as laboratory test in a rural area in which brucellosis is endemic (10,11,12,13). A large number of different tests have been used for the serological diagnosis of brucellosis, thus demonstrating the lack of an ideal technique. Rose Bengal is a rapid plate agglutination test that uses a suspension of *Brucella abortus* in an acid buffer. It has a high degree of sensitivity for diagnosis infection with *Brucella spp.*, irrespective of the stage of the disease. This high sensitivity, together with the fact that the technique is simple and rapid (4 min.), makes the Rose Bengal test ideal for the screening patients for human brucellosis. In our study, Rose Bengal test was positive in all patients 91/91 (100, 0%). Use of the Rose Bengal test as the sole diagnostic tool to establish treatment of brucellosis in endemic areas is not a reliable practice with individuals who are exposed repeatedly to the disease, or who have a recent history of the disease (14,15,16). Rose Bengal test continue to be the mainstay of the laboratory diagnosis, due to their simplicity, low cost, and ability (>90% sensitivity) in diag-

nosis acute brucellosis. However, this test suffers from high false-negative rates in complicated and chronic cases. ELISA which can reveal the classes and subclasses of immunoglobulins in sensitive is more successful assay because of the limitations of the Rose Bengal test. In our study, Brucella IgM antibodies with ELISA were positive in 59/91 (64, 8%). Brucella IgG antibodies with ELISA were positive in 51/91 (56%). Early in infection, antibodies of the IgM class predominate. The study reports that the IgM antibody may be detected during the first week following the entry of the organism. The peak level is reached four weeks later. IgG antibodies may be detected after 10 days. The peak level is reached after two months. The IgG antibody has delayed appearance, although it is found together with IgM four weeks after the initial antigenic stimulus; the IgM antibody level always exceeds the IgG antibody level in the acute stage of the disease. In our study, in few patients in acute phase of brucellosis with bacteremia, only IgM antibodies were detected. Several studies have shown that ELISA is the test of choice for the diagnosis of complicated and chronic cases, especially when other tests are negative (17,18,19). Gazapo et al, stated that ELISA IgM and IgG positivity are helpful for epidemiological evaluations, whereas some false positive results can be obtained in agglutination tests due to the cross reactivity between *Brucella spp.* and *Salmonella spp.*, *Vibrio cholera* and *Yersinia* bacteria

(20). In many studies performed with ELISA, it was determined that IgG positivity and the increasing of the antibody titers were considerably valuable in relapsed cases and in patients with chronic infections (21, 22). The sensitivity of test-methods is: Rose Bengal test-100, 0%, blood culture-30, 8%, ELISA IgM-64, 8% and ELISA IgG-56, 1%. The positive predictive values were 100, 0% for all the methods. The received diagnostic values for sensitivity were lower than values in the literature. The assumption was that this was caused by usage inadequate method for each stage of illness. This assumption was confirmed by evaluation of the sensitivity of applicable methods for different stages of illness. The sensitivity of test methods was different in different stages of illness and only combination of blood culture, Rose Bengal test and ELISA ensured the diagnosis. Rose Bengal test is excellent for the screening. The blood culture gave excellent sensitivity results in the patients with primary infections. ELISA performed equally well in the diagnosis patients at different stages of illness including patients with acute, subacute, or chronic disease and with relapse. A combination of those different Brucellosis tests would be considered appropriate for the practical diagnostic measurement, taking into account various factors including the effectiveness for the complementary purpose of the laboratory diagnosis, the different laboratory infrastructure and economic coverage capacity for laboratory reagents etc.

## CONCLUSION

Based on our results, the following conclusions can be drawn:

- Human brucellosis is an actual public health problem in Bosnia and Herzegovina.
- Rose Bengal test is excellent for screening.
- Blood culture is method of choice for acute stages of illness.
- ELISA (IgM, IgG) is method of choice for the diagnosis chronic disease and relapses
- Sensitivity of test methods was different: Rose Bengal test-100, 0%, blood culture-30, 8%, ELISA IgM-64, 8% and ELISA IgG-56, 1%.
- Sensitivity of the test methods was different in different stages of illness and only combination blood culture, Rose Bengal test and ELISA ensured the diagnosis
- Establishment Reference Laboratory for Brucellosis in Bosnia and Herzegovina would be necessary in order to support the strengthening of laboratory capacity and activities of Brucellosis diagnosis and surveillance and also for national brucellosis control strategies

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