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EXAMINATION OF SENSITIVITY AND SPECIFICITY OF SOME SEROLOGICAL TESTS IN DIAGNOSTICS OF BOVINE BRUCELLOSIS

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The most reliable diagnosis of an infectuous disease is confirmed by isolation of its pathogen. When it comes to brucellosis, it is important to know that brucella isolation is rarely successful; it is not only very complicated but is as well hazardous for laboratory workers. Due to the above mentioned reasons, it is reasonable to use serological tests for routine diagnosis of this zoonose. This paper deals with examination of bovine sera samples with the aim to detect the titer of specific antibodies against brucellosis. In order to choose and evaluate properly the best test in terms of applicability, speed of performance, and provision of correct results, five serological tests were assayed: rapid serum plate agglutination (Rose Bengal test), Brucella abortus bovis test (RB, BAB test); serum agglutination test (titration) – by Wright, as micro method (mSAT); reaction of complement fixation, and also as micro method (mCF); indirect imunoenzyime test (iELISA) and competitive imunoenzyme test (cELISA). This paper includes 630 samples of bovine blood sera, as well as positive and negative international antibrucella serum as the mandatory control. The presence of specific antibodies against brucella was determined in 125 samples of bovine blood sera. Based on the analysis of the results obtained, evaluation of sensitivity and specificity of these tests was conducted. iELISA and RB test proved to be the most sensitive, while the highest specificity was determined in mCF, and less specific were mSAT and iELISA. RB test had the lowest specificity.

Key words: brucella, serological tests, sensitivity, specificity

INTRODUCTION

Brucellosis, a contagious disease which affects a large number of domestic and wild animals and people, represents a significant large-scale zooantroposis (Godfroid and Kasbohrer, 2002). Due to a chronic case history which is common among animals and an atypical clinical picture, it has always been difficult to

control this disease. Its control is particularly significant in the countries of the Mediterranean endemic region to which Serbia belongs.

Given the fact that brucellas are optionally intracellular microorganisms, it is difficult to isolate the pathogen of the disease, it is time-consuming, requires particular conditions and is always hazardous for laboratory workers. Having these facts in mind, immunodiagnostic methods which provide analysis of a larger number of samples are used for diagnosis and epizootic research of this disease. Due to the antigenic similarity between brucella and gram negative bacteria, cross reactions occur in serological diagnostics of brucellosis. This cross reaction is particularly common in diagnosis of bovine brucellosis. This can lead to making a false diagnosis which causes severe economic damage since brucellosis is wiped out by strict veterinary-sanitary measures (Kittelberger *et al*., 1997).

Due to possible mistakes in the diagnosis of this disease, and severe direct and indirect damages which are the result of it, diagnostic procedures for detection, prevention and eradication of brucellosis are constantly developed and advanced. In 1976. Morgan *et al*. and Nikolleti studied the characteristics and applicability of the rapid Card test (Rose Bengal test), comparing it with serum agglutination test in titration and mCF method. Morgan *et al*. also dealt with the same topic in 1969. In 1976. Carlsson *et al.* evaluated the quality and characteristics of immunoenzym (ELISA) test in diagnosis of brucellosis comparing it with classical methods of serological diagnosis. Then, in 1979. Byrd *et al*. also dealt with this subject. Some time later, in 1895., Rylatt *et al*. described the competitive immunoenzyme test (cELISA) as a more selective test for detection of and differentiation between infected and uninfected animals in comparison to all other serological tests, including iELISA. In 1996., Nielsen *et al*., applied fluorescent polarization test (FPA) in the diagnosis of brucellosis, which can even be used out of a laboratory and represents one of the alternative tests in serological diagnosis of bovine brucellosis.

MATERIALS AND METHODS

Samples of bovine blood sera were used as a material for examination of sensitivity and specificity of 5 selected serological tests. In regular, epizootic, clinical and/or previous serological, preliminary examinations, 330 samples of bovine blood sera were singled out (the samples in which the reaction of rapid agglutination was positive or negative) and 300 samples of blood sera in which the reaction of rapid agglutination did not occur, i.e. it was negative. The group containing 330 samples of bovine blood sera was examined by the following methods: RB, mSAT, mCF, iELISA and cELISA. Blood sera samples that had a negative reaction of rapid plate agglutination (300), were simultaneously examined by iELISA test. Blood sera samples that reacted positively in iELISA test and sera whose values were around cut-off, or suspicious were examined by cELISA test. As a control, standardized, international, positive or negative brucella anti-sera produced by Central Veterinary Laboratory (CVL) Weybridge, UK were used. For the reaction of rapid brucella agglutination (Rose Bengal - RB test) and

serum agglutination test in titration (reaction by Wright - SAT), antigens produced by Pourquier, France, were used.

Rose Bengal test was conducted according to the producer's instructions, and the results were recorded.

Instructions of previously mentioned producers were used for the reaction of serum agglutination test as a micro method (mSAT), complement fixation, and macro method iELISA and cELISA.

The results obtained by the above mentioned methods were interpreted according to the model for calculating sensitivity and specificity, shown in the Table 1.

Table 1. Calculation of sensitivity and specificity of serological tests used in diagnostics

Testing and results		Actual status of animal (Referent test)		Total results (Σ)
		Infected animal (Pos.(+) value)	Uninfected animal (Neg. (-) value)	
Results	Pos. (+)	TP	FP	TP+FP (Manifestation prevalence)
	Neg. (–)	FN	TN	TN+FN
Total results (Σ)		TP+FN (Actual prevalence)	TN+FP	N
Sensitivity (Se) Specificity (Sp)		Se=TP/(TP+FN)	Sp=TN/(TN+FP)	

Legend:
TP – True positive samples; FP – False positive samples; FN – False negative samples;
TN – True negative samples; N – Total number of samples processed from both tests;
Se – Test sensitivity; Sp – Test specificity

RESULTS

The results obtained after the examination of bovine blood sera are shown in Table 2.

Table 2. The results of examination of bovine blood sera by RB test and iELISA

Test	Number of sera examined	Positive	Negative	% of pos. samples
RB	630	265	365	42.06
iELISA	630	125	505	19.84

Out of 330 examined samples, a positive reaction of agglutination by RB test was determined in 265 samples, while brucella antibodies were found in 126 samples.

The presence of brucella antibodies examined by RB test was determined in only one out of 300 samples by iELISA test.

Lacking a golden standard, the obtained values of the examination by competitive immunoenzyme test were used for results processing as the most reliable. All the iELISA positive samples and a part of samples whose values were either suspicious or around cut-off were also examined by cELISA. By this confirmation test, the presence of specific antibodies against brucella was determined in 125 samples.

For the examination of validity of the tests used, their sensitivity and specificity, 125 samples of bovine blood sera were included and both ELISA tests showed that they were positive to specific antibodies. 125 bovine samples were taken from bovine sera that reacted positively according to RB test.

Sensitivity and specificity of RB test were determined on the basis of the results obtained by iELISA as the referent test:

Sensitivity, Se = 120/(5+120) = 120/125 = 0.96 x 100 = 96 %
Specificity, Sp = 360/(360+145) = 360/505 = 0.7128 x 100 = 71.28 %.

The group containing 330 bovine sera were examined by the reaction of serum agglutination test in titration (mSAT).

The animals in whose sera the titer of antibodies (agglutination was present) in sera dilution in the proportion of 1:40, i.e. the cattle that had 100 or more international units (IU) of agglutinin in a mL of the serum were classified as positive to brucellosis. The results of the examination of 330 samples of bovine blood sera, and their titers obtained by mSAT and iELISA method are shown in Table 3.

Table 3. The results of examination of the bovine blood sera samples obtained by mSAT i iELISA

Test	Examin.	Pos	Titers of antibodies							Neg.	% pos.
			40	80	160	320	640	1280	2560		
mSAT	330	81	32	22	6	3	5	9	4	249	24.54
iELISA	330	125								205	37.87

In the examination of sensitivity and specificity of mSAT as a referent value iELISA results were used. Out of 81 bovine blood sera samples that reacted positively when mSAT was applied, six of them reacted negatively in iELISA test. Out of the total number of 125 samples in which specific antibodies against brucellosis were determined by iELISA, 75 sera were determined as positive by the reaction of slow agglutination, which means that 50 bovine blood sera samples showed a false negative reaction by application of serum agglutination test in micro plates (mSAT).

Sensitivity, Se = 75/(50+75) = 75/125 = 0.60 x 100 = 60 %
Specificity, Sp = 199/(199+6) = 199/205 = 0.9707 x 100 = 97.07 %.
mCF method was performed by "cold" procedure.

The animals in whose sera the titer of antibodies at dilution 1:10 was determined, i.e. the cattle who had 20 or more international units (IU) of agglutinin in a ml of a serum were classified as positive to brucellosis.

The results of the examination of bovine blood sera samples and their titers by application of mCF and iELISA are shown in Table 4.

Table 4. The results of the examination of bovine blood sera samples by application of mCF and iELISA

Test	Examin.	Pos.	Titres of antibodies						Neg.	% posit.
			10	20	40	80	160	320		
mCF	330	57	8	6	6	5	9	23	273	17.27
iELISA	330	125							205	37.87

False-positive reaction in titer 1:10 was determined by mCF method in only one serum out of the total number of the examined sera. Anticomplementary activity was determined in 33 out of the remained 273 sera.

Based on the results obtained, specificity and sensitivity of mCF test was the following:

Sensitivity, Se = 56/(56+69) = 56/125 = 0.448 x 100 = 44.8 %
Specificity, Sp = 204/(204+1) = 204/205 = 0.9951 x 100 = 99.51 %

The total number of 44 bovine blood sera was examined by iELISA test of two different producers. All the sera samples whose values were about cut-off or their value corresponded to the suspicious reaction detected by iELISA, produced by VMRD Inc, were reexamined.

Table 5. The results of bovine sera examination obtained by iELISA from three different producers

Test name	Equal values in all three tests		Equal values in two tests		Different values in all three tests	
	Number of samples	% of samples	Number of samples	% of samples	Number of samples	% of samples
VMRD Inc. Pourquier Bommeli	32	72.72	9	20.45	3	6.82

The analysis of the results given in the Table 5. shows that the values obtained by all three tests were the same in 32 sera samples, which is 72.72%.

The values obtained for three blood sera samples (6.82%) were different (positive, negative, suspicious).

Sera in which specific antibodies against brucella were detected by iELISA (n=176) were examined by cELISA test. The results obtained are shown in Table 6.

Table 6. The results or blood sera samples examination obtained by iELISA and cELISA tests

Test	Number of sera examined	Positive	Negative	% of positive samples
iELISA	176	127	49	72.15
cELISA	176	125	51	71.02

The results obtained show that specific antibodies against brucella detected by iELISA were not found in two samples when cELISA was applied (one sample was RB negative).

The sensitivity and specificity of iELISA test was determined using cELISA as a referent test:

Sensitivity, Se = 125/(125+0) = 125/125 = 1 x 100 = 100 %
Specificity, Sp = 49/(49+2) = 49/51 = 0.9607 x 100 = 96.07 %

The results obtained by examination of 330 bovine blood sera samples are shown in Table 7 and 8 and in Figure 1 and 2.

Table 7. Positive results of bovine blood sera examination obtained by five serological tests

Type of a test	Number of sera examined	Positive
RB test	330	120
mSAT	330	75
mCF	330	56
iELISA	330	125
cELISA	176	125

Table 8. The results of specificity and sensitivity of the tests applied for brucellosis diagnosis

Type of a test	Sensitivity %	Specificity %
RB test	96	71.28
mSAT	60	97.07
mCF	44.8	99.51
iELISA	100	96.07

All the examined sera originated from cattle that cohabited either in facilities, yards or pastures in which sheep infected with *B. melitensis* were registered.

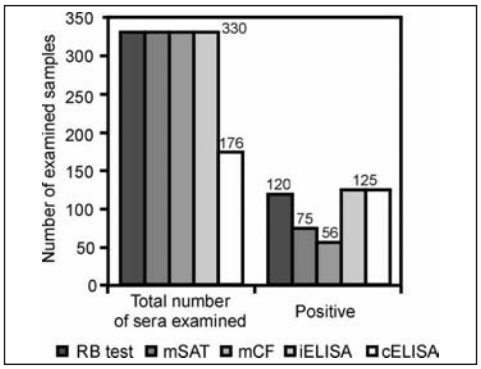

Figure 1. Positive results of bovine blood sera examination obtained by five serologiacal tests

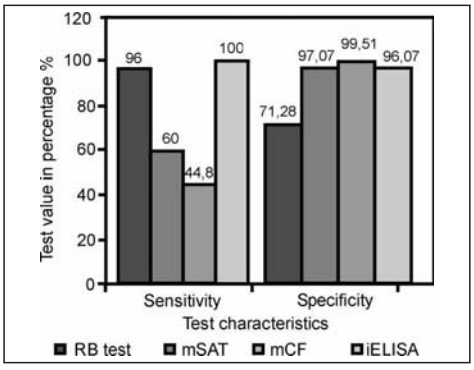

Figure 2. The results of specificity and sensitivity of the tests applied for brucellosis diagnosis

DISCUSSION

It is well known that the clinical picture of brucellosis is characterized by a great diversity of both the intensity of clinical manifestations and the place of their origin. Reproduction and dissemination of the pathogen in an organism is accompanied by cellular immunological response which creates new problems in serological diagnosis of this disease. All of this also depends on the type of an infected organism (animal, human). Particular problems for the final diagnosis are inadequate data on case history, course of the disease, chronic period, infections caused by microorganisms which are alike in terms of antigens, and also eventual treatment with antibiotics. Due to a number of subjective and objective problems which are the result of pathogen isolation, which is not even possible in the most of the cases, it takes a lot of time to isolate the pathogens even when some of the modern microbiologic methods are applied. If all of this is taken into consideration, immunological methods in brucellosis diagnosis are obligatory with a good reason (Emmerzaal *et al*., 2002).

During the examination of the validity of these five methods, experience and methodology of a number of authors was used (Lako, 1992; Gall and Nielsen, 1994; Saravi *et al*., 1995; Uzal *et al*., 1995; Weynants *et al*., 1996; Mathias and Pinto, 1996; Dohoo *et al*., 1998; Forbes, 1998; Gall *et al*., 1998; Omer *et al*., 2000; Radojičić *et al*., 2001; Paweska *et al*., 2002; McGiven *et al*., 2003; Samartino *et al*., 2003).

Sensitivity and specificity of RB test is lower in comparison to iELISA (Saravi *et al*., 1995; Stemshorn *et al*., 1998; Stryszak, 2002; Samartino *et al*., 2003).

The presence of agglutinin was not detected in five samples of bovine blood sera by the method of rapid agglutination (RB test), while they reacted positively in iELISA test, which is the result of lower test sensitivity, i.e. the fact that the infection is in the beginning stage and that the level of immunoglobulin is low. All of this was confirmed by subsequent repeated sampling and analysis.

In further diagnosis of brucellosis, the method of serum agglutination test in titration by Wright was applied. Comparative analysis of the results obtained by serum agglutination test and iELISA showed that six bovine blood sera samples were false-positive, which is most probaly the result of cross reaction, which other authors also noted (Lako, 1992; Kittelberger *et al*., 1997).

Further analysis shows that in 50 bovine sera samples (40%) titer of antibodies was insufficent to provoke the reaction of serum agglutination test which indicates a significantly lower sensitivity of the mentioned test in comparison to iELISA.

The results obtained show that the sensitivity of mSAT is lower in comparison to the reaction of iELISA which is confirmed by bibliographic data (Saravi *et al*., 1995; Stemshorn *et al*., 1998; Dohoo *et al*., 1998; Paweska *et al*., 2002; Samartino *et al*., 2003). Also, the comparison of results shows that mSAT specificity is higher than RB and iELISA specificity, which is seen in bibliographic data, too (Lord *et al*., 1989; Samartino *et al*., 2003). When smaller capacities (micro method - mCF) and cold procedure are applied, a more objective result is obtained (Lako, 1992). Serological examination of bovine sera samples, when mCF method was applied, showed that in this case the mentioned test provided high specificity, 99.51%, which is confirmed by many authors (Saravi *et al*., 1995; Stemshorn *et al*., 1998; Dohoo *et al*., 1998; Paweska *et al*., 2002). Low level of sensitivity of this test (44.8 %) can be explained by anticomplementary activity of the examined sera (Saravi *et al*., 1995). Using the products of various producers, almost identical results were obtained by comparative analysis of all sera samples (the total of 44), whose antibodies values were about cut-off or suspicios on iELISA test.

Comparing the results obtained on examination of 176 bovine blood sera samples by indirect and competitive immunoenzyme test, it is noted that immuneenzyme tests proved to be the most specific and sensitive. This is also confirmed by other authors (Rojans and Alonso, 1994; Saravi *et al*., 1995; Dohoo *et al*., 1998; Paweska *et al*., 2002; Samartino *et al*., 2003). Comparing the results of preliminary and final examinations, it can be concluded that a number of serologically positive animals to brucellosis differs from a number of actual positive animals. Based on the results obtained and the examination of bovine blood sera, by application of five serological tests, it was found that a number of animals positive to brucellosis is 125 (iELISA, cELISA). On the basis of the analysis of the results on sensitivity and specificity of the tests applied, it was concluded that the most sensitive were iELISA (100 %) and RB test (96 %).

The highest specificity was determined for mCF test (99.51 %), followed by mSAT (97.07 %), iELISA (96.07 %). RB test had the lowest specificity (71.28 %).

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ISPITIVANJE OSETLJIVOSTI I SPECIFIČNOSTI NEKIH SEROLOŠKIH TESTOVA U DIJAGNOSTICI BRUCELOZE KOD GOVEDA

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SADRŽAJ

Najsigurnija dijagnostika bolesti infektivnog karaktera vrši se izolacijom uzročnika. Kada je bruceloza u pitanju, mora da se ima u vidu, da je izolacija brucela uspešna u niskom procentu, veoma komplikovana i predstavlja opasnost po laboratorijske radnike. Zbog navedenih razloga, primena seroloških testova u rutinskoj dijagnostici ove zoonoze je opravdana. U ovom radu su vršena ispitivanja uzoraka seruma goveda radi određivanja prisustva titra specifičnih antitela protiv brucela, u svrhu dijagnostikovanja bruceloze. U nameri da se pravilno izbabere i proceni najbolji test u smislu aplikativnosti, brzine izvođenja i dobijanja pouzdanih rezultata, tokom ispitivanja je korišćeno pet seroloških testova: brza serumska aglutinacija na pločici – Rose Bengal test – *Brucella abortus bovis* test (RB, BAB test); spora aglutinacija (u titraciji) – metoda po Wrightu, kao mikrometoda (mSAT); reakcija vezivanja komplementa, takođe kao mikrometoda (mRVK); indirektni imunoenzimski test (iELISA) i kompetitivni imunoenzimski test (cELISA). Ispitivanjem je bilo obuhvaćeno 630 uzoraka krvnog seruma goveda kao i pozitivni i negativni internacionalni antibrucela serumi kao obavezne kontrole. Prisustvo specifičnih antitela protiv brucela vrsta ustanovljeno je u 125 uzoraka krvnog seruma goveda. Na osnovu analize dobijenih rezultata vršena je procena osetljivosti i specifičnosti navedenih testova. Najosetljivijim su se pokazali iELISA i BAB test, a najveća specifičnost je ustanovljena kod mRVK. Manje specifični bili su mSAT i iELISA, a najmanja specifičnost je ustanovljena kod BAB testa.