Applicability of a brucellin skin test in seropositive cattle

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Abstract

Brucellosis is an infectious disease that affects livestock and may be transmitted to humans. Cattle may become infected with *Brucella* spp. by various routes, and the pathogens induce both humoral and cellular immune responses in the host organism. The aim of this study was to determine the characteristics of the cellular immune response by using a brucellin allergen in serologically positive cows, and to differentiate cross-reactions from true positive animals, and to contribute to improvement of the overall diagnostics of bovine brucellosis in Bosnia and Herzegovina. Using the Rose Bengal Test (RBT) and Complement Fixation Test (CFT) as a combined reference standard (CRS), seropositive (*n*=15) and seronegative (*n*=14) groups were defined. Cows from both groups were subjected to the Brucellin Skin Test (BST). By comparing CRS and BST results, we estimated the relative sensitivity and specificity at 93.3% and 100% for BST, respectively. The ROC analysis indicated a good accuracy score for BST of 0.9, while the calculated kappa statistic of 0.94 indicated excellent diagnostic agreement between BST and CRS. The importance of BST application may be found in the increased efficacy of diagnostics of latent brucellosis in cow populations in the country and improving the discrimination of cross-reactions caused by microorganisms with a similar antigen response in host organisms.

Key words: *Brucellin; brucellosis; Brucella skin test*

Introduction

Brucellosis is a zoonotic infectious disease that affects domestic and wild animals. Brucellosis caused by *Brucella abortus*, *B. melitensis* and *B. suis* and is recognised as a professional disease in enzootic regions that causes severe economic losses due to abortions and declining milk production (Alton et al., 1988).

Cattle are usually infected via the consumption of feed contaminated with abortion material, by transconjunctival route and inhalation, or by artificial

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insemination with contaminated semen (Nicoletti, 2010). Spread of the disease among animals is even possible before its laboratory confirmation due to certain characteristics of brucellosis in cattle, such as latent clinical infection, long incubation period, infected newborn calves, and occurrence of abortions before seroconversion, hindering surveillance and eradication of the disease (Nyanhongoet al., 2017).

Brucella spp. induces both a humoral and cell mediated immune response in infected animals. The humoral immune response is based on the production of specific antibodies against the smooth lipopolysaccharide (S-LPS) proteins in the bacterial cell membrane (Benet et al., 1991). Using classical serological methods for *Brucella* detection, antigenic cross reactivity to other bacterial species like *Escherichia coli* 0:157, *Yersinia enterocolitica* 0:9, *Salmonela urban*, *Pseudomonas malthopilia* and *Pasteurellae* can occur (Corbel, 1985; Kittelberger et al., 1995).

A cell mediated immune response is provoked by the use of purified and standardised antigens (brucellin) from which LPS has been removed, and as such does not induce a humoral immune response (Bercovich et al., 1992). Based on late phase allergic reaction of the skin, a brucellin skin test (BST) was the diagnostic test of choice in this study.

The objective of this study was to determine the characteristics of the cellular immune response using the brucellin skin test allergen in seropositive cows, to differentiate animals with cross reactivity, and to improve the overall diagnostics of bovine brucellosis in Bosnia and Herzegovina.

Materials and methods

Identification of animals

The study was conducted in two groups of cattle: positive and negative. The positive group consisted of cattle positive to specific antibodies against brucellosis determined by the Rose Bengal Test (RBT) and Complement Fixation Test (CFT) (n=15), while the negative group included cows (n=14) originating from farms with brucellosis-free status for at least five years. Blood specimens for serological testing were collected as part of the annual control programme of infectious diseases in the Federation of Bosnia and Herzegovina in 2017. Samples were delivered for serological testing to the Laboratory for Virology and Serology, Faculty of Veterinary Medicine, University of Sarajevo, which is also the National Reference Laboratory for Brucellosis of Bosnia and Herzegovina.

Serological testing

Cattle blood sera were tested using RBT according to the OIE procedure (OIE, 2009). Inconclusive and positive samples were retested for confirmation using the CFT (OIE, 2009). Interpretations of the results were based on the lysis of sensitized SRBC (Sheep red blood cells sensitized with haemolysin) for each dilution, and expressed in international CFT units (IU) in 1 mL blood serum. Findings of 20 IU and more are considered positive (OIE, 2009).

Brucellin skin test (BST)

The test was performed according to the manufacturer's instructions (Synbiotics, France), OIE Manual (2009) and Seagerman et al. (1999). Brucellin used in the experiment was an extract of *B. melitensis* B115 (Synbiotics Bruceller gene OCB, France). A surface of 10 cm^2 of healthy skin was trimmed and shaved on one side of the neck. A cutimeter (Hauptner, Germany) was used to measure skin thickness before and 72 hours after intradermal injection of 0.1 mL brucellin using a 4 mm needle. Successful injection was confirmed by palpation of a small, grain-size nodule at

the site of injection. The skin reaction was interpreted 72 hours after the injection by measuring increased skin thickness, where each increase of thickness greater than 1.0 mm was considered a positive reaction.

Statistical analysis

To determine the diagnostic performance of BST, the mean values of skin thickness at the injection site (in mm) for each tested animal were compared between the two groups (positive and negative). The cut-off value was determined based on good specificity (minimum 99%). The diagnostic potential of the test was further evaluated with receiver operation curve (ROC) analysis. Given the fact that cattle in Bosnia and Herzegovina is not vaccinated against brucellosis, the effect of the vaccine on BST performance was not taken in consideration.

To determine the reference cattle population, status (positive and negative) was defined by Composite Reference Standard (CRS). CRS is used for diseases such as brucellosis, where a single suitable reference standard is not available. Results of two assays with acceptable sensitivity (RBT and CFT) were combined and the individual status for each tested animal was determined by test agreement (Jacobson, 1998; Greiner and Gardner, 2000; TDR Diagnostic Evaluation Expert Panel, 2010). Agreement between the test results of BST and CRS was evaluated by calculation of the Cohen's kappa coefficient (Viera and Garret, 2005).

Results

Composite Reference Standard (CRS)

Based on results of serological testing of blood serum samples by the two CRS methods (RBT and CFT), 15 cows were assigned to the positive group. Among them, the presence of specific antibodies against causative agents of brucellosis was confirmed in 14 serum samples by both CRS methods, while one sample displayed a positive RBT result and negative CFT result (Table 1). Blood serum samples from all 14 brucellosis-free cows (negative group) showed negative results for both CRS methods.

Brucellin skin test (BST)

Of the 15 cows in the positive group, 14 showing positive results for RBT and CFT also displayed a positive BST reaction, *i.e.* increased skin thickness of more than 1.0 mm 72 hours after the application of brucellin. In contrast, the remaining cow having a positive RBT and negative CFT reaction displayed a negative BST reaction (skin thickness increase of 1.0 mm). Similarly, in the negative control group, skin thickness in

Table 1. Results of application of brucellin skin test (BST) in two serologically different groups of cows.

	RBT		CFT		BST	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive group (*n*=15)	15	0	14	1	14	1
Negative group (*n*=14)	0	14	0	14	0	14

RBT – Rose Bengal Test; CFT – Complement Fixation Test

Table 2. Determination of the cut-off value for the brucellin skin test (BST)

	Increase of skin thickness (mm) after BST				
	0.0-0.5	0.5-0.9	1.0-2.0	>2.1	Mean
Positive group (*n*=15)	0	1	4	11	4.7
Negative group (*n*=14)	7	7	0	0	

CUT- OFF

all 14 serologically negative bovines did not exceed 1.0 mm (Table 1).

Determination of cut-off value for BST

The cut-off value for BST was set up based on the comparison of quantitative results of BST for cows in the serologically positive and negative groups (Table 2). Increase of skin thickness of >1 mm was considered a positive BST result, and thus the optimal cut-off value for BST was set as a 1 mm increase of skin thickness with optimal specificity of 93.3%. Relative sensitivity of BST skin was 93%. Specificity of BST was 100%.

To estimate diagnostic accuracy of BST relative to the CRS methods performed (RBT and CFT), a ROC analysis was performed, yielding a resultant area under the curve of 0.9 (Figure 1).

The kappa coefficient between BST and CRS, and the coefficient between BST and RBT was estimated at 0.94, while the coefficient between BST and CFT was slightly lower (0.91).

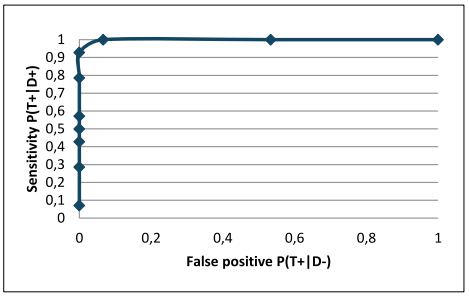

Figure 1. Receiver operating characteristic curve (ROC) analysis

Discussion

Principles of serology tests such as RBT and CFT are based on antibody detection for the smooth lipopolysaccharide (S-LPS) protein found in *B. abortus*, *B. melitensis* and *B. suis* (Diaz and Morion, 1989; Moreno and Moriyon, 2002). An almost identical lipopolysaccharide is present in other bacterial species, like *Y. enterocolitica* serotype O:9, creating significant problems in monitoring and conclusive diagnosis of brucellosis in many countries worldwide (Caroff et al., 1984a,b; Jungersen et al., 2006). The quantity and types of detectable antibodies in body fluids vary, while in a mild infection with *Brucella*, the immune response may even be absent (Ray et al., 1988). The first antibodies to appear in an infected organism are IgM antibodies, while IgG1 antibodies may appear simultaneously. The Rose Bengal Test is capable of detecting specific IgM and IgG antibodies, with higher efficiency for the type IgG1, and less for IgM and IgG2 (Levieux, 1974). It has been described that the Complement Fixation Test, as one of most important diagnostic tools for brucellosis, successfully detects specific IgM and IgG1 antibodies (Hill, 1963), while IgG2 may disturb the complement fixation reaction (Levieux,

1974). This fact indicates that none of the available serological tests can precisely detect all stages of brucellosis (Mylrea and Fraser, 1976; Nielsen, 2002). Therefore, evaluation of a diagnostic test, such as a skin allergy test, should include its comparison to a "golden standard" diagnostic test, which, in the case of brucellosis, is isolation and molecular identification of the pathogen. However, even this test is not capable of detecting every stage of the disease. As it is impractical to perform bacteriological examination of all diagnostic samples in most field cases, we accepted the Composite Reference Standards RBT and CFT as the optimal diagnostic standard.

The relative sensitivity of the BST skin of 93% in the present study corroborates the results of other studies involving experimentally infected animals, which described that sensitivity decreases with time past after the infection (Sagerman et al., 1999), and may indicate a lower sensitivity of the test in chronic infections. Nyanhongo et al. (2017) performed a similar study that found relatively low sensitivity of the test if herd screening is performed only with BST. However, the high specificity estimated in the present study results from the fact that we validated the test in seropositive animals. Using the test to confirm brucellosis in seropositive animals can compensate for the test's low sensitivity.

The specificity of BST estimated at 100% in our study corresponds with the results of other similar studies (Saegerman et al., 1999), indicating a high positive predictive value of the test. Under these circumstances, it is possible that a cow with a negative CFT test, but positive to RBT and BRT could present an error of the CRS (RB + CFT) used, and that the animal is truly infected (Nyanhongo et al., 2017). This may be explained by the bias of our standard due to the absence of humoral immunity in chronically infected animals and the consequent inability of serological test methods to detect specific antibodies, while, on the other hand, BST is mediated through cellular immunity.

The estimated area under the ROC curve of 0.9 is a good accuracy score for BST. In addition to high test specificity and accuracy, Nyanhongo et al. (2017) found a low to medium accuracy score in infected herds, which they explained as an error of the used reference standard due to humoral immunity, since they applied an iELISA test with lower specificity. Due to the absence of a bovine vaccination programme in Bosnia and Herzegovina, this factor did not affect our CRS.

The calculated kappa statistic of 0.94 indicates excellent diagnostic agreement between BST and CRS. Similarly, the calculated kappa statistics for individual comparison of BST with RBT and CFT also indicate almost perfect diagnostic agreement between the tests. The use of BST in addition to serological screening tests could enhance the detection of truly infected animals and improve overall diagnostic capacity for the monitoring and eradication of brucellosis. The test may be particularly useful as a confirmation test in cases of inconclusive results of two serological tests.

Efforts to eradicate brucellosis through vaccination programmes for small ruminants and "test and remove" programmes for cattle have shown positive results in decreasing the overall number of cases of human and bovine brucellosis in Bosnia and Herzegovina. Small ruminants in Bosnia and Herzegovina are considered reservoirs of brucellosis and are subjected to vaccination. Due to vaccination, small ruminants in Bosnia and Herzegovina are not included in the annual monitoring programme. Under such circumstances, any increase in the prevalence of human and bovine brucellosis may serve as an indicator of the status of the disease in small ruminant herds. Despite all efforts,

in 2017 a total of 811 RBT-positive bovine serum samples was sent to the National Reference Laboratory for Brucellosis of Bosnia and Herzegovina for confirmation, with 782 samples testing CFT-positive. Implementation of BST as an additional confirmation test could facilitate efforts to improve diagnosis of the disease in cases of inconclusive serological test results. Due to its high specificity, BST-positive animals should be considered infected. Also, the results of BST could help to clarify cross reactivity of serological tests in brucellosis-free areas (Nyanhongo et al., 2017).

Having in mind that there is no ideal serological test for the diagnosis of bovine brucellosis, introduction of BST as an additional method could increase overall capacity to correctly identify true individual cases of bovine brucellosis (Stemshorn, 1984; Bercovich et al., 1992; Nyanhongoet et al., 2017). Additional importance of BST application should also be seen in effective diagnostics of chronic (latent) bovine brucellosis, and in the exclusion of cross-reactions caused by microorganisms with a similar antigen. In addition to the applied immunological methods, it is mandatory to provide etiological diagnosis of brucellosis in herds in order to define the exact *Brucella* species causing the disease (Cvetnić et al., 2015). *B. melitensis* biovar 3 is the only *Brucella* spp. isolated from cattle, small ruminants and humans in Bosnia and Herzegovina to date, while *B. abortus* has never been isolated here (Velić, 2012).

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Primjena brucelinskog kožnog testa u seroloski pozitivnih goveda

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Bruceloza je zarazna bolest od koje obolijevaju i životinje i ljudi. Bolest se lako širi u stadu, a patogen u domaćinskom organizmu izaziva humoralni i stanični imunosni odgovor. Cilj ove studije bio je izazivanje stanične imunosti uporabom alergena u seropozitivnih životinja, otkrivanje lažno pozitivnih životinja i doprinos poboljšanju dijagnostike bruceloze goveda u Bosni i Hercegovini. Uporabom Rose Bengal Testa (RBT) i Reakcije vezanja komplementa (RVK) ujedinjenih kao udruženi referentni standard (URS) formirali smo dvije kontrolne grupe: seropozitivnu (n=15) i seronegativnu (n=14). Goveda obje kontrolne grupe ispitivane su brucelinskim kožnim testom (BKT). Usporedbom rezultata URS i BKT, ustanovili smo senzitivnost (93,3%) i specifičnost (100%) kožnog testa. Izračunata alergija ispod ROC krivulje za BST od 0,9 predstavlja dobar rezultat točnosti BKT, a kappa statistika (0,94) ukazuje na dobro podudaranje testova. Uvođenje ovog testa doprinijelo bi poboljšanju dijagnostike bruceloze poboljšanjem uspješnosti otkrivanja latentno inficiranih goveda i isključivanja lažno pozitivnih životinja kod seroloških unakrsnih reakcija sa sličnim antigenima.

Ključne riječi: *brucelin; bruceloza; brucelinski kožni test*