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Agreement Among Rose Bengal, Complement Fixation Test, and iELISA in Diagnostic Discrimination of Sheep and Goat Brucellosis (*Brucella melitensis*)

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Abstract

Small ruminant (sheep and goats) brucellosis caused by *Brucella melitensis* remains widely recognized as a major zoonosis causing profound economic, animal production, and public health consequences. Even though definite diagnosis depends on the isolation of the bacterial agent from clinical and post-mortem specimens, a presumptive diagnostic based on the assessment of specific serological response is used in routine diagnostics for the purpose of disease control or animal trade. The Rose Bengal test, the complement fixation test, the enzyme-linked immunosorbent assay, or the fluorescence polarization assay are considered by the World Animal Health Organization—OIE as a suitable serological test for diagnosing *B. melitensis* infection on a herd and individual animal level. The aim of this study was to assess agreement among results of the Rose Bengal, complement fixation test, and indirect enzyme-linked immunosorbent assay using small ruminant sera samples collected through brucellosis surveillance program in Bosnia and Herzegovina. A subset of these samples from non-vaccinated animals (2250) was reused and tested on each test. Agreement among test results was assessed pair vise using Kappa statistical analysis with correspondent 95% CI. Additionally, Landis–Koch scale was used for the classification of observed agreement based on established Kappa. The highest agreement was found between the complement fixation test and the Rose Bengal test (0.643), while the lowest was between the enzyme-linked immunosorbent assay and the rose Bengal test (0.533). Choice of serologic tests and testing protocols used in brucellosis surveillance programs depends on the program aim, alongside specific epidemiological, animal production, economic, and cultural circumstances.

Keywords: Complement fixation test, iELISA, Rose Bengal, small ruminant brucellosis, test agreement

Introduction

Brucella melitensis is a host-specific *Brucella* species due to frequent transmission from its natural hosts (sheep and goats) to other susceptible species of animals and to humans (Moreno, 2014). Sheep are also a natural host for *B. ovis*; however, this is a "rough" *Brucella*, antigenically easily differentiated from "smooth" *B. melitensis* (Garin-Bastuji & Blasco, 2018). Brucellosis caused by *B. melitensis* remains widely recognized as a major zoonosis with profound economic, animal production, and public health consequences (Franc et al., 2018; Mandal et al., 2017). Direct diagnosis of *B. melitensis* infection is the isolation of the bacterial agent from host tissue samples. Molecular methods, such as variants of polymerase chain reaction, have not yet been proven useful as alternative direct diagnostics, although these methods are increasingly used in the detection and typing of *Brucella* spp. from culture samples (Garin-Bastuji & Blasco, 2018; Gupta et al., 2014; Yu & Nielsen, 2010). Routine diagnostics for the purpose of disease control or animal trade are made by serological tests since infection results not only in the development of primarily cellular but also strong humoral immune response (Ducrotoy et al., 2016; Garin-Bastuji et al., 2006). Classes of antibodies-produced post-infection as well as the timeline of their activity in small ruminant hosts are still topics of ongoing investigation particularly in consideration of vaccination and latency of infection in some hosts (Ducrotoy et al., 2016). Specificity of a serological test is hindered due to cross-reactivity of *Brucella* epitopes with other gram-negative bacteria (mainly *Yersinia enterocolitica* O:9) (Garin-Bastuji et al., 2006). The Rose Bengal test (RBT) and complement fixation test (CFT) are the most commonly used serological tests used (Garin-Bastuji & Blasco, 2018; Seria et al., 2020). The RBT is based on the agglutination of acidified antigen as well as indirect enzyme-linked immunosorbent assay (iELISA). Both are considered highly sensitive hence applied as screening tests (Sadhu et al., 2015). Complement fixation test is used for the confirmation of samples positive on screening

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tests (Ducrotoy et al., 2016; Garin-Bastuji & Blasco, 2018). Currently, all tests used for serodiagnosis of sheep and goat brucellosis are directly validated as tests for brucellosis caused by *B.abortus* provided close antigenically relation of these two "smooth" *Brucella* species (Blasco et al., 1994a; Ducrotoy et al., 2016; European Commission, 2001). This, together with the increased probability of false-positive results in vaccinated and animals from brucellosis endemic areas, may reduce the specificity of serological tests in field conditions (Blasco et al., 1994a; Mandal et al., 2017). The aim of this study was to assess agreement among results of the RBT, CFT, and iELISA using small ruminant (sheep and goats) sera samples collected through brucellosis surveillance program in Bosnia and Herzegovina.

Methods

Sera Samples

Blood samples were collected through the official brucellosis surveillance program by officially appointed veterinarians with a portion of the samples reused for the purpose of the study. Samples were collected through venipuncture, using a single-use vacutainer system. Once collected, samples were transported to the laboratory, where they were stored at 4°C for a maximum of 2 days. The extracted serum was separated from the blood clot in the tubes and, after testing with RBT, they are stored at −20°C. Frozen serum samples were thawed overnight in the refrigerator before further tests were conducted. The study included 2250 serum samples taken from adult animals (>1-year-old) from non-vaccinated herds. Sheep and goats were represented in the study sample proportionally to their respective population's size, while each administrative unit of the country contributed to the sample size equally.

Serological Tests

Serological testing of the serum samples was accomplished using RBT, CFT, and iELISA. Sample testing was done by the Serology laboratory within the Veterinary faculty of the University of Sarajevo.

The Rose Bengal Test Procedure

The RBT (Pourquier® Rose Bengale Ag IDEXX) was performed by mixing 25 μL of the serum and an equal volume of antigen on a white, shallow-welled enamel plate (Alton et al., 1988). The mixture was rocked gently for 4 minutes at room temperature and then observed. Any sign of agglutination was considered positive.

The Complement Fixation Test Procedure

The CFT (Viron Serion GmbH Wuryburg) was performed using standard 96-well mL plates. Test serum was diluted with CF test buffer 1:4 and then inactivated in a water bath (37°C) for 30 minutes. Volumes of 25 μL of diluted, inactivated test serum were placed in the wells of every odd-numbered row in the plate. The test was performed using the "warm" procedure described by Alton et al. (1988). The serum was considered positive if it showed at least 50% hemolysis at a given dilution (i.e. ≥20 International Complement Fixation Test Units (ICFTU)).

The ELISA Test Procedure

The ELISA test used was a commercial brucellosis serum enzyme immunoassay kit (IDEXX brucellosis Ovine/Caprine kit). The test was performed using microtiter plates coated with inactivated *Brucella* antigen, by the manufacturer's instructions. A positive and a negative control were conducted for each plate. The results were read using a photometer at a wavelength of 450 nm. Interpretation of the results was accomplished by calculating the percent optical density (OD) of samples in relation to the negative and positive control OD readings. All samples with ≥80% OD were considered positive.

Table 1

Landis Koch Scale in the Interpretation of Kappa Values

Kappa Value Range	Degree of Agreement
≤0.20	Poor
0.21–0.40	Fair
0.41–0.60	Moderate
0.61–0.80	Good
0.81–1.00	Very good

Statistical Analysis

Agreement between the applied serological tests for small ruminant brucellosis was assessed by calculating the Kappa statistic and associated 95% CI for each binary combination of tests (Kundel & Polansky, 2003). Additionally, Landis–Koch scale (Table 1) was used for the classification of observed agreement based on established Kappa (Landis & Koch, 1977).

Hypothetical true prevalence based on the proportion of positive on each test separately (considered as apparent prevalence) and reported sensitivities and specificities of the applied test by Minas et al. (2008) is calculated using Epitools epidemiological calculators (Sergeant, 2018).

Results

The proportion of positive samples found by applied tests and hypothetical true prevalence is shown in Figure 1. Complement fixation test yields the highest number of positive samples (307), followed by RBT (303) and iELISA (183), while 136 samples were classified as positive by all three tests.

Results of the applied tests concerning their agreement assessed via the calculation of each binary Kappa statistics and corresponding 95% CI are provided in Tables 2–4 [2 (RBT vs. CFT), 3 (iELISA vs. CFT), and 4 (RBT vs. iELISA)]. The highest agreement was established between the CFT and RBT results (0.643), while the lowest proportion of agreement, adjusted for chance occurred between the ELISA and RBT (0.533).

By the Landis–Koch scale, observed test agreement is good between RBT and CFT and moderate for combinations of tests RBT and iELISA and iELISA and CFT.

Discussion, Conclusion and Recommendations

Proven tools in addressing the occurrence, spread, and negative consequences of small ruminant brucellosis are the identification and culling of infected animals and/or herds and vaccination (Franc et al., 2018; Mandal et al., 2017; Moreno, 2014). Ongoing efforts in developing new diagnostic tests for the detection of sheep and goat brucellosis caused by *B.melitensis* emphasize the inexistence of the perfect test, while the utility of tests available is affected by technical issues and complex biological, epidemiological, and socioeconomic factors (Ducrotoy et al., 2016;

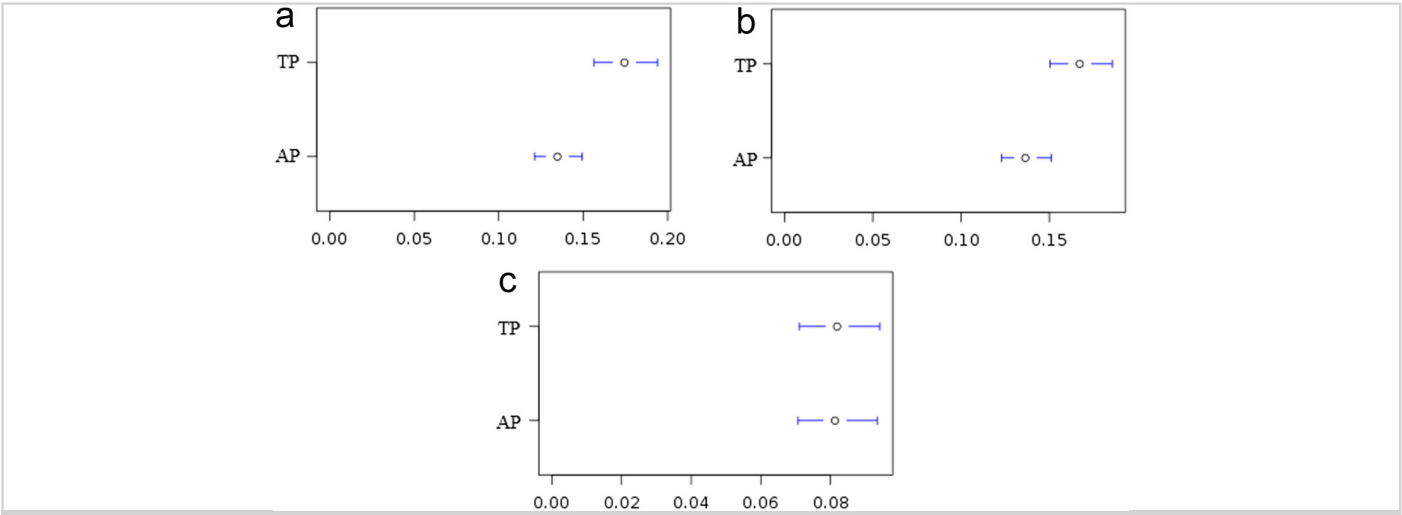

Figure 1
Proportion of Positive Samples with 95% CI Based on Classification by RBT (A), CFT (B) and iELISA (C) Hypothetically Considered as Apparent Prevalence — AP with Resulting TP Assuming Specificity and Sensitivity of the Tests Reported by Minas et al. (2008). RBT, Rose Bengal Test; CFT, Complement Fixation Test; iELISA, Indirect Enzyme-linked Immunosorbent Assay; TP, True Prevalence.

Garin-Bastuji et al., 2006; Gusi et al., 2019; Sadhu et al., 2015). Rose Bengal test and CFT are the most commonly used serological tests for official diagnostics of brucellosis in the EU, United States, China, Russia, and other countries (Blasco et al., 1994a; Ducrotoy et al., 2016; Minas et al., 2008; Ren & Peng, 2021). Antigen component in both tests is whole *B.abortus* 19 or 1119-3 cells, while iELISA uses smooth lipopolysaccharide *Brucella* antigen (Minas et al., 2008; Garin-Bastuji et al., 2006). Indirect ELISA has demonstrated high sensitivity in the detection of small ruminant brucellosis and represents a very good alternative for RBT as a screening test (Blasco et al., 1994b; Díaz-Aparicio et al., 1994; Gusi et al., 2019; Tittarelli et al., 2005). This study established moderate to good agreement between combinations of tests evaluated. While there is cross-reactivity with a few gram-negative bacteria with all three assays, the removal of the purified and/or synthetic antigen component and low-avidity antibodies used in iELISAs greatly reduces false-positive cross-reactions. (Ducrotoy et al., 2016; Mandal et al., 2017; McGiven et al., 2015). This may explain the lower number of positive samples found by ELISA compared to the number of positives by either RBT or CFT test alone. In addition, the reported sensitivity of ELISA, defined as the probability to correctly identify infected animals, is higher than the sensitivity of RBT and CFT (Blasco et al., 1994b; Gusi et al., 2019; Minas et al., 2008; Tittarelli et al., 2005). Several studies have confirmed the high reactivity of RBT and CFT applied on animals found negative in microbiological isolation originating from herds or areas considered endemic for small ruminant brucellosis (Blasco et al., 1994a; Díaz-Aparicio et al., 1994, Ferreira et al., 2003). Sadhu et al. (2015) reported that proportions of positive small ruminants by RBT are almost always higher than the proportion of positives by ELISA.

We also observed discordance between the results of RBT and CFT. It has been reported that a high proportion of animals from endemic areas can be found negative on RBT but CFT positive (Blasco et al., 1994a; Ferreira et al., 2003). It is likely that such results occur when the host immune response is not complete or lacking due to early stage, latent or chronic infection at sampling. Several studies even suggested that modified RBT procedure (3:1 volume of serum vs. antigen) increases RBT sensitivity, under the assumption that the levels of antibodies produced to main *Brucella* epitopes might be lower in infected sheep and goats than in cattle (Blasco et al., 1994a; Díaz-Aparicio et al., 1994; Gusi et al., 2019). Thus, in small ruminant brucellosis endemic areas such as Bosnia and Herzegovina, RBT results have greater value when interpreted on herd rather than individual animal level (Blasco et al., 1994a, b; Ramirez-Pfeiffer et al., 2007).

The CFT showed a comparable level of sensitivity as RBT and iELISA when applied on goat serum samples originating from *B.melitensis* culture-positive animals (Díaz-Aparicio et al., 1994). However, CFT in

Table 2
Agreement (Kappa Values with the Corresponding 95% CI) Between RBT and CFT Observed by Testing Samples (n = 2250) on Each Test

	CFT+	CFT−	Observed Agreement (%)	Kappa	95% CI for Kappa
RBT+	211	92	91.64	0.643	0.597–0.690
RBT−	96	1851			

Note: RBT = Rose Bengal test; CFT = complement fixation test.

Table 3
Agreement (Kappa Values with the Corresponding 95% CI) Between iELISA and CFT Observed by Testing Samples (n = 2250) on Each Test

	CFT+	CFT−	Observed Agreement (%)	Kappa	95% CI for Kappa
ELISA+	152	31	91.57	0.571	0.517–0.625
ELISA−	155	1912			

Note: CFT = complement fixation test; iELISA, indirect enzyme-linked immunosorbent assay.

Table 4

Agreement (Kappa Values with the Corresponding 95% CI) Between RBT and iELISA Observed by Testing Samples (n = 2250) on Each Test

	ELISA+	ELISA−	Observed Agreement (%)	Kappa	95% CI for Kappa
RBT+	141	162	90.93	0.533	0.477–0.589
RBT−	42	1905			

Note: RBT = Rose Bengal test; iELISA = indirect enzyme-linked immunosorbent assay.

comparison with RBT and iELISA lacks sensitivity for the detection of early infections, since it relies on attained steady antibodies levels (1–2 months after experimental infection) (Blasco et al., 1994b; Ducrotoy et al., 2016; Tittarelli et al., 2005). Also, very high titers of antibody in sera may yield (false) negative CFT reaction due to prozone effect (MacMillan, 1990).

In spite of the differences in reactivity to different classes and levels of antibodies produced post-infection, both RBT and CFT can detect immunoglobulin G1 and immunoglobulin M efficiently, which explains good agreement between tests as found in our study (Ducrotoy et al., 2016; Minas et al., 2008). However, both tests correlate to less extent with iELISA either due to differences in antigen components or probability of false positives in relation to test specificity and cross-reactivity (Ducrotoy et al., 2016; Minas et al., 2008; Sadhu et al., 2015).

The study showed that all three tests combinations detect brucellosis in sheep and goat sera in a relatively comparable agreement. However, the choice of serological tests especially since the common practice is to use more than one serological test in parallel or series in routine diagnostics depends mostly on the aim of the surveillance program. Both false positive and false-negative results may be expected with probability related to the performance of tests themselves but also concerning the applied vaccination, epidemiological provenience of samples, and timing of the sampling. Hence, a test or combination of tests, yielding fewer false-positive results, is preferable in demonstrating disease absence. However, if the surveillance aim is to reduce disease prevalence, common screening tests such as RBT or iELISA should be complemented with CFT particularly when the "test and slaughter" policy is applied.

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