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Full Research Article

DEVELOPMENT AND EVALUATION OF TWO ELISA TESTS FOR DIAGNOSIS OF CANINE BRUCELLOSIS

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Abstract

Canine brucellosis is a disease that in most cases causes symptoms ranging from mild to severe reproductive disorders. In most infected animals though, the disease is inapparent, making clinical diagnosis very difficult. The only method by which a diagnosis can be made with certainty is the isolation of *Brucella canis*. When serological tests are performed, a large number of false positive results occur due to the presence of antigenic surface determinants common to *B. canis* and other bacteria.

Blood sera from 225 dogs were tested using the 2-ME TAT method and in-house ELISA tests using antigen extracts obtained by thermal extraction and by ultrasonication. Electrophoretic analysis and densitometric quantification of the antigen showed that in the thermally-extracted antigen, the most abundant molecular mass fraction was 10.95 kDa with a proportion of 43.12 %, which corresponded to the R-LPS of *Brucella*. The same fraction was present in the ultrasonicated extract, but accounted for 11.56 % of the protein mass, i.e., 3.7 times lower than in the thermal extraction. The protein composition of the ultrasonicated antigen was significantly richer than in the thermal extraction, and the quantitative composition was dominated by proteins with molecular masses of 30.5, 24.5, 38.0 and 22.0 kDa, which belonged to the outer membrane proteins.

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In the ELISA test with the thermally-derived antigen, 44 (19.55 %) of 225 sera tested positive, while in the ELISA test with the ultrasonicated antigen, 37 (16.44 %) were positive. In addition to the 2-ME TAT, the use of an indirect ELISA test performed on ultrasonically extracted antigen is recommended for serological testing in order to make an as accurate as possible diagnosis.

Key Words: antigen, *B. canis*, brucellosis, dog, ELISA, ROC

INTRODUCTION

Canine brucellosis is an infectious disease of carnivores caused by *Brucella canis*, a Gram-negative, facultatively intracellular coccobacillus that is virulent when it exhibits the rough (R) form of colony growth, which distinguishes it from most other *Brucella* species (Carmichael and Green, 1990). Although it belongs to the group of classical anthropozoonoses, it is not of major public health significance and human infections are rare and usually mild. The first data on the occurrence of brucellosis in dogs date back to 1963, when an epizootic of abortions in kennels was described in the United States. In 1966, Professor Carmichael of Cornell University in the US isolated the pathogen, *B. canis*, from dog tissue and vaginal discharge (Carmichael, 1966). *B. canis* can be carried asymptomatically by susceptible animals. However, when the organism causes disease in canids, symptoms include abortion in bitches, orchitis in males and sterility in both sexes. Reservoirs for *B. canis* are domestic and wild animals of the *Canidae* family, and stray dogs are an important reservoir for *B. canis* (Buhmann et al., 2019, Carmichael, 1979).

Canine brucellosis is widespread worldwide, with the exception of Australia and New Zealand (Wanke, 2004). In the Americas, canine brucellosis is an enzootic disease (Carmichael, 1990). Sweden was a *B. canis*-free country until 2012, when the first case was described in a dog imported from Poland (Holst et al., 2012). The first case of the disease in Hungary was described in 2011 (Gyuranecz et al., 2011), in Austria in 2012 (Hofer et al., 2012) and in Italy in 2020 (De Massis et al, 2021). In Serbia, Radojičić et al. described the first case of *B. canis* infection in dogs in 1999 (Radojičić et al., 1999).

There are no standardized serological methods for the diagnosis of canine brucellosis. Serological methods are based on the use of antigens obtained by different techniques. Regardless of the choice of strain or antigen used, there is data in the literature showing that although specific antibodies to *B. canis* can be detected two weeks after infection, serological methods are not reliable in the first 12 weeks after infection (Zoha and Carmichael, 1982). The various diagnostic methods used in the diagnosis of canine brucellosis differ in their sensitivity and specificity. False positive and false negative results are common. The outer membrane lipopolysaccharide (LPS) is the main virulence factor of *Brucella*. The most pathogenic *Brucella* species (*Brucella abortus*, *Brucella suis*, *Brucella melitensis*) carry smooth LPS (S-LPS) involved in *Brucella* virulence. A feature that distinguishes *B. canis* and *Brucella ovis* from other members of the *Brucella* genus is the presence of rough LPS (R-LPS), which makes these bacteria less

pathogenic than other *Brucella* species. Classical methods for the detection of antibodies against S-LPS in *Brucella* spp. cannot be used to investigate the seroprevalence of infections with *B. canis*. Instead, the R-LPS structure in *B. canis* necessitates the use of a homotypic antigen in the serologic diagnosis of canine brucellosis.

Serological tests for the diagnosis of brucellosis in dogs are mostly used to diagnose infections or to check the health status of dogs before mating. Therefore, it is necessary to find the best test or a combination of the most reliable tests. Although canine brucellosis has been known for six decades, not much has been done in the field of diagnostics, so there is still no agreement on the most reliable test. Since there are neither standardised diagnostic protocols nor a general agreement on the most appropriate test, each laboratory sets its own criteria. This diversity of tests and the lack of clearly defined protocols leads to difficulties in interpreting the serological test results in different laboratories. For this reason, the aim of this study was to improve diagnostics through the use of new, in-house ELISA tests.

The aim of this study was to develop and evaluate indirect ELISA tests with antigens obtained by thermal extraction and ultrasonic disintegration of *B. canis* and to compare the results of the 2-mercaptoethanol tube agglutination test (2-ME TAT) with the results of the immunoenzymatic test (ELISA) in order to evaluate the sensitivity, specificity and reliability of the ELISA tests, as well as the possibility of their use in the routine diagnosis of canine brucellosis.

MATERIALS AND METHODS

The dogs included in this study were neutered at the Faculty of Veterinary Medicine, University of Belgrade, as part of the Programme for Control and Reduction of Stray Dog Population in the City of Belgrade. The study was approved by Decision No. 323-07-03455/2015-05/1 of the Veterinary Department of the Ministry of Agriculture and Environmental Protection of the Republic of Serbia.

The blood samples came from a total of 225 stray dogs. The blood was collected in sterile glass tubes in accordance with the asepsis principle. After spontaneous clotting, the blood was centrifuged at 2000 g for 15 minutes. The blood sera were then separated in Eppendorf microtubes and stored at -20 ºC until further processing.

The serum of a positive dog was used as a positive control (isolated *B. canis* was typed in 2004 by the reference laboratory in France, i.e., Agence Française de sécurité sanitaire des aliments (AFSSA)). The specific *B. canis* antibody titre of this serum was 1/3200. As negative controls, sera from 30 certainly healthy dogs before sexual maturity at the age of six months were taken and used to calculate the cut-off value for the ELISA tests.

The blood sera were tested with 2-ME TAT (Thermo Scientific) and ELISA, and were obtained from two differently prepared antigens.

Antigen preparation and 2-ME TAT

The reference strain *B. canis* RM 6/66 was used for the antigen preparation. The antigen required for the 2-ME TAT was prepared according to the procedure described (Alton et al., 1988). Any dilution of the serum in which the liquid in the test tube was completely clear was considered a positive result. According to the literature recommendations for this type of test, titres of 1/50 and 1/100 are considered suspicious, while a dilution of 1/200 is considered a sign of active infection (Alton et al., 1988).

Antigen preparation for the ELISA test

Brucella canis RM 6/66 was cultured on tryptose agar. After 24 hours of incubation at 37 ºC, the bacterial growth was washed from the medium with sterile saline solution. After washing, the bacterial suspension was washed three times with sterile physiological solution by centrifugation at 2000 g for 30 minutes. One part of the bacterial sediment was resuspended in sterile distilled water (2.1 g *B. canis* in 10 mL sterile distilled water) and autoclaved at a temperature of 121 ºC and a pressure of one atmosphere for 30 minutes to produce thermally extracted antigen. The second part of the bacterial sediment was resuspended in sterile distilled water (6.91 g *B. canis* in 21 mL sterile distilled water) and then sonicated for 30 minutes on ice using a Bandelin Sonoplus HD 2070 (power 70 W, capacity 70 %) to produce ultrasonicated antigen. Both antigens were then centrifuged at 10000 g for 30 minutes. The supernatant was then separated and dialyzed in distilled water for 48 hours at 4 ºC with constant stirring on a magnetic stirrer. Cellulose tubes with a pore size of 6000 to 8000 Da (ZelluTrans, Roth, Germany) were used for this process.

The protein concentrations in the antigen solutions obtained by thermal extraction and ultrasonic disintegration of *B. canis* were determined using the spectrophotometric method according to Lowry (Lowry et al., 1951).

Electrophoretic analysis of antigens

Electrophoretic separation of proteins was performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 12.5 % polyacrylamide gel in TRIS-glycine buffer in the presence of SDS (Laemmli, 1970) (MINI VE HOEFFER, LKB, 2117, Bromma, Uppsala Sweden). Electrophoresis was carried out at 80 V for 30 minutes and then at 200 V for 60 minutes. After electrophoresis, the protein bands were fixed with trichloroacetic acid and sulfosalicylic acid, and the gels were then stained with Coomassie Brilliant blue R250 (Sigma-Aldrich, USA) (Simon, 2001). Densitometry of the protein bands was performed using the software packages TotalLab Tl 120 and Sci Image 7.0, Origin 6.0. The results are presented as a percentage of the total protein concentration determined according to Lowry (Lowry et al., 1951).

To determine the molecular masses of the individual protein fractions, a logarithmic representation of the dependence of the logarithm of the molecular masses (logMr) of protein markers (standard, 10 to 250 kDa) (Thermo Scientific, USA) and their mobility on polyacrylamide gel was created. The calculation was based on the linear equation: y=2.11747-1.15413x, r=-0.99304.

Preparation of the samples for electrophoresis

The antigen solutions obtained by thermal extraction and ultrasonic disintegration of *B. canis* were prepared by adding sample buffer at a ratio of 1:1 v/v and the mixtures were heated at 100 ºC for 3 minutes.

ELISA

The optimal antigen concentration and the optimal dilutions of the serum and the peroxidase-conjugated anti-dog antibodies (anti-dog IgG whole-molecule peroxidase antibodies, prepared in rabbit, Sigma Aldrich, USA) were determined by checkerboard titration. Antigen concentrations of 10 μg/mL, 1 μg/mL, 0.1 μg/mL and 0.01 μg/mL, positive serum dilutions of 1/200, 1/400, 1/800 and 1/1600 and conjugated antibody dilutions of 1/20000 and 1/25000 were tested. The dilution with the highest optical density was used as the final value.

The antigen was applied to NUNC (Nunc-Immuno Plate MaxiSorp Surface, Denmark) flat-bottomed polystyrene plates with 96 wells. The antigen was diluted in carbonate-bicarbonate buffer pH 9.6 (Sigma-Aldrich, USA) and applied to the plate at a rate of 100 μL per well. The plates with the added antigen were incubated overnight at 4 ºC. At the end of the incubation period, the plates were washed with phosphate-buffered saline (PBS) pH 7.2 containing 0.05 % Tween 20 (PBS-T). The amount of wash buffer was 300 μL per well, and the washing procedure was repeated five times. Then the unbound sites in the wells were blocked with 5 % bovine serum albumin (BSA, Sigma Aldrich, USA) in PBS-T at a volume of 300 μL per well overnight at 4 ºC. After five washes with PBS-T, the test sera were added. Prior to testing, the sera were treated with 0.7 % 2-ME overnight at 4 ºC. Sera were diluted 1/200 in 1 % BSA in PBS-T, and 100 μL volumes of diluted sera were added to the plate wells. All sera were tested in triplicate. The plates containing the sera were incubated at 37 ºC for 1 hour. They were then washed five times with PBS-T. Peroxidase-labelled anti-dog antibodies were added at a dilution of 1/25000 in PBS-T and a volume of 100 μL per well. After 1 hour incubation at 37 ºC and washing, TMB substrate (50 μL per well) was applied to the plates. After 15 minutes of light-protected incubation at room temperature, the reaction was blocked by adding 2M H_2SO_4 at a volume of 50 μl per well. Absorbance was measured at 450 nm using the Multiskan Thermo Scientific microplate photometer (Thermo Fisher Scientific, USA).

The cut-off value was determined using the following formula:

cut-off = $\overline{X}$ + 3SD, where $\overline{X}$ is the mean of the optical density of 30 negative sera and SD is the standard deviation.

The coefficient of variation was calculated on the basis of a negative control serum tested in seven ELISA tests, each in three replicates. The coefficient of variation (CV) was calculated as follows:

$CV = \frac{SD}{\overline{X}} \times 100$, where $\overline{X}$ is the mean value of the negative serum tested in seven ELISA tests (seven replicates). All samples that had an optical density value in the interval of the cut-off ± CV were retested.

The results were statistically analyzed using the IBM SPSS Statistics 21 program. The kappa index of concordance and the McNemar test for the 95 % confidence interval were used to examine the agreement of the diagnostic tests used and to determine the specificity and sensitivity of the tests. The in-house indirect ELISA tests with two differently prepared antigens were analysed using the ROC (Receiver Operating Characteristic Curve) analysis and by determining the area under the curve (AUC).

RESULTS

Using the 2-ME TAT, 225 blood sera from dogs were analysed. The blood sera were tested at dilutions of 1/50, 1/100 and 1/200. Of the 225 canine blood serum samples, 33 (14.67 %) were positive in the slow serum agglutination test with 2-ME. A specific *B. canis* antibody titre of 1/200 was found in 12 blood sera (5.33 %). Eight blood sera (3.55 %) had a titre of 1/100, and 13 blood sera (5.78 %) had a titre of 1/50.

The protein concentration of the antigen obtained by heating was determined spectrophotometrically according to the Lowry method to be 0.606 mg/mL and that of the ultrasonicated antigen to be 0.561 mg/mL.

The antigens obtained by thermal extraction and ultrasonic disruption of *B. canis* were analyzed using SDS-PAGE (Figure 1, Figure 2).

In the densitometric analysis of the antigen solution obtained by ultrasonic disintegration, 23 proteins with different molecular masses were detected (range from 9.41 to 93.77 kDa), while nine proteins were detected in the antigen solution obtained by thermal extraction (range from 10.95 to 76.00 kDa). In the ultrasonicated extract, proteins of 10.95 kDa (11.56 %) and 30.5 (10.62 %) were the most abundant, followed by proteins of 24.50 kDa (9.20 %), 38.00 kDa (7.97 %) and 22.00 kDa (7.85 %). The protein composition of the thermal extract was slightly different: the largest percentage, 43.12 %, was a protein of 10.95 kDa, while proteins with molecular masses of 14.00 and 15.50 kDa each accounted for 6.50 % of the protein mass. In the thermal extract, a large molecular weight protein of 76.00 kDa was isolated, accounting for 7.41 % of the protein mass. The 10.95 kDa protein (presumed to be the same in both extracts) accounted for 11.56 % of the mass fraction in the ultrasonic extract, which was 3.7

times less than in the thermal extract. Compared with the thermal extract, the protein composition of the ultrasonic extract was significantly richer, and proteins with molecular masses of 30.5, 24.5, 38.00 and 22.00 kDa dominated in terms of quantity.

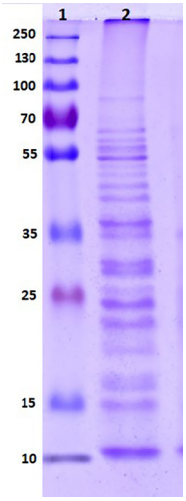

Figure 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5 % polyacrylamide gel, stained with Coomassie Brilliant blue: **1.** Molecular mass standard expressed in kDa; **2.** Antigen obtained by ultrasonic disintegration of *B. canis*.

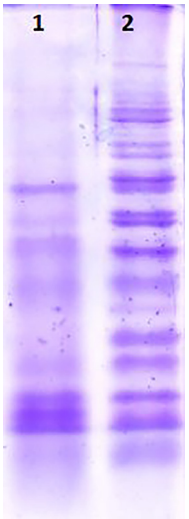

Figure 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5 % polyacrylamide gel, stained with Coomassie Brilliant blue: **1.** Antigen obtained by thermal extraction of *B. canis*; **2.** Antigen obtained by ultrasonic disintegration of *B. canis*.

The optimal antigen concentration was determined by checkerboard titration, whereby four different concentrations were tested: 10 μg/mL, 1 μg/mL, 0.1 μg/mL and 0.01 μg/mL. Checkerboard titration was performed with the serum of the positive control.

The serum dilutions tested were 1/200, 1/400, 1/800 and 1/1600, and the dilutions of the conjugated antibodies were 1/20000 and 1/25000. The dilutions with the highest optical density were used as final values. The reference serum was tested in three replicates for each antigen dilution and concentration. Checkerboard titration revealed that the optimal concentration of antigens obtained by both methods (thermal extraction and ultrasonic disintegration of *B. canis*) for use in the ELISA assay was 1 μg/mL. The optimal dilution of the positive serum was 1/200 and the optimal dilution of the conjugated antibodies was 1/25000.

The cut-off value of the ELISA test with the antigen obtained by thermal extraction was 0.382. The cut-off value obtained was 0.382±0.18, i.e., it ranged from 0.202 to 0.562. Sera with an optical density > 0.382 were marked as positive. Of the 225 sera tested, 44 (19.55 %) were positive.

The cut-off value of the ELISA test with sonicated antigen was 0.614. The cut-off value obtained was 0.614±0.30, i.e., in the interval from 0.314 to 0.914. Sera with optical densities in this interval were retested. Sera with optical density values > 0.614 were marked as positive. Of the 225 sera tested, 37 (16.44 %) were positive.

Statistical kappa analysis was used to determine the agreement between the applied diagnostic tests. The tube agglutination test with 2-ME (titre 1/200) was used as a reference test.

The sensitivity of the ELISA test with the antigen obtained by thermal extraction was 66.7 %, and the specificity was 83.1 %. The kappa value of 2-ME TAT and ELISA with antigen obtained by thermal extraction was 0.220, which according to the criteria published by Viera and Garrett (2005) is a sufficient, average agreement. Using the McNemar test, however, a statistically significant difference was found between these diagnostic tests ($p<0.05$).

The sensitivity of the ELISA test with the antigen obtained by ultrasound disintegration was 66.7 %, and the specificity was 86.4 %. The kappa value of the 2-ME TAT and the ELISA test with the antigen obtained by ultrasonic disintegration was 0.268, which according to the criteria published by Viera and Garrett (2005) means sufficient, average agreement. Using the McNemar test, again, a statistically significant difference was found between the two diagnostic tests ($p<0.05$).

The results of the ROC curve analysis are shown in Figures 3 and 4. From the analysis of Figures 3 and 4, it can be seen that the ELISA with the antigen obtained by ultrasonic disintegration (Figure 4) was a more discriminatory method than the ELISA with the antigen obtained by thermal extraction (Figure 3). Specifically, the curve in Figure 4 is closer to the left and upper line of the graph than the curve in Figure 3.

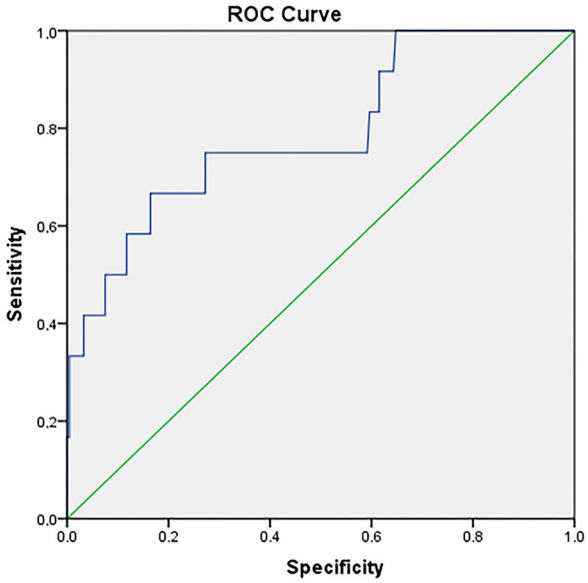

Figure 3. Receiver operating characteristic (ROC) curve for the ELISA test with thermally-extracted antigen

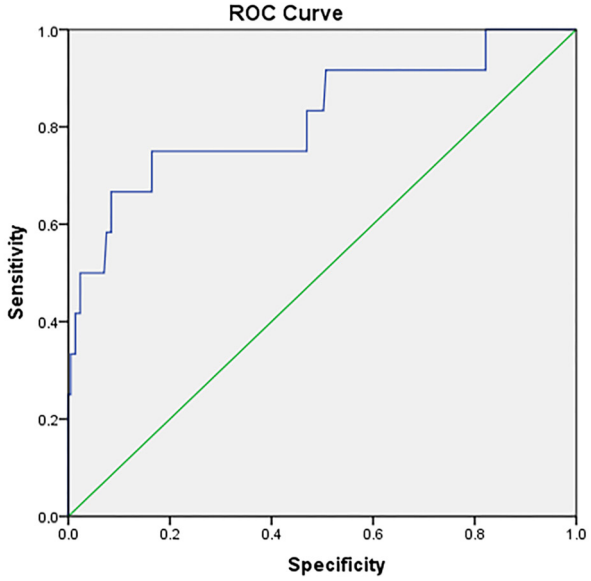

Figure 4. Receiver operating characteristic (ROC) curve for the ELISA test with ultrasonically-extracted antigen

The area under the curve in the ELISA with the ultrasonicated antigen was greater than the area under the curve in the ELISA with the thermally-extracted antigen. The area under the curve for the ultrasonicated antigen ELISA test was 0.820, which classified this ELISA as a good diagnostic test.

DISCUSSION

The results of the 2-ME TAT confirmed that *B. canis* is maintained in a group of stray dogs from the Belgrade city area. Our results (14.67 % seroprevalence in 225 stray dogs) matched the results of Hamdy et al. (2023), who reported an overall apparent *B. canis* seroprevalence of 15 % in the stray dog population. Although, based on the recommendations in the literature (Alton et al., 1988), rather high values of the titre of specific antibodies (1/200 when using 2-ME) are considered a sign of active infection, there is always a possibility that lower titres are a sign of the onset of infection, but also of chronic infection, which is followed by the maintenance of a low titre of antibodies in the circulation. The evidence for this assertion also lies in the false negative result found previously (Stević et al., 2017). In that study, one of the three dogs from which *B. canis* was isolated was serologically negative in the 2-ME TAT. Such findings certainly justify the testing of paired sera in cases where the antibody titre is 1/50 and 1/100.

It is known that dogs' blood haemolyses easily, so testing such sera is associated with numerous difficulties. A major problem in the development of ELISA is the frequent non-specific reactions which, due to their intensity, make it impossible to distinguish between positive and negative samples. In other words, it is impossible to determine a threshold value. For this reason, pre-treatment of the test sera is necessary. The M isotype of the antibody is mostly responsible for the occurrence of a high background effect, which makes it impossible to determine the actual threshold, so the elimination of this immunoglobulin class is most important. Although the anti-dog antibodies used for the development of various ELISA tests also have a partial affinity for IgM, ELISA tests mainly detect the G class of immunoglobulins. In the tests carried out, the sera were treated in different ways before the ELISA tests were performed; firstly, 5 % BSA was added as a non-specific blocker, secondly, the sera were inactivated at 56 ºC for 30 minutes and finally, 2-ME was added. It was found that the non-specific reactions were most markedly reduced by treating the serum with 2-ME (Mateu de Antonio et al., 1994), which, as a disulphide reducing agent, destroys the IgM molecules that are the most common cause of the non-specific reactions. Treatment of the serum with 0.7 % 2-ME at 4 ºC overnight reduced the number of non-specific reactions (data not shown). This conclusion is consistent with previously published data by other authors. Mateu de Antonio et al., (1994) stated that the problem of cross-reactivity is solved by treating the serum with 2-ME, which degrades IgM. Treatment of serum with 2-ME increases the specificity of the test used, but to some extent compromises sensitivity and increases the likelihood of false negative results. Corbel (1985) pointed out the

problem of cross-reacting antibodies of the IgM class. For this reason, serological tests that detect the presence of IgM class antibodies, which are the first class of immunoglobulins to appear after infection, are not the best choice. IgG, cross-reacting antibodies produced against antigenically similar bacteria, may also be responsible for non-specific reactions. Since the sensitivity and specificity of diagnostic tests are inversely proportional, it is better to use the combination of tests that gives the best results. As a rule, in eradication campaigns of some diseases, highly sensitive tests are used at the very beginning, while specific tests are used at the end. Research by Barkha et al. (2011) supports this assertion, and their recommendation is that the initial serum screening should be done with an indirect ELISA test with sonicated antigens, which is highly sensitive.

In the densitometric analysis, the 10.95, 14.10, 15.59, 21.97 kDa proteins detected in both the thermal and ultrasonic extracts are likely the outer membrane minor proteins, according to Cloeckaert et al. (1990). The 24.50, 35.74, 38.19, 54.82, 76.01 kDa proteins, again detected in both extracts, probably are the outer membrane major proteins, as was described previously (Cloeckaert et al., 1990). According to Salhi et al. (2003), some of these proteins belong to porins. Salhi et al. (2003) reported that the major proteins of the outer membrane of Brucella are porins – group 2 proteins (36.00 to 38.00 kDa) and group 3 proteins (25.00 to 27.00 kDa and 31.00 to 34.00 kDa). The outer membrane proteins are exposed at the cell surface, they are surface antigens of the bacteria, and mostly belong to the rough lipopolysaccharide (R-LPS) (Wanke et al., 2002).

Our study showed that the indirect ELISA test using antigens obtained by thermal extraction is as sensitive (66.7 %) but less specific (83.1 %) than the ELISA test using antigens extracted by ultrasonic disintegration (sensitivity 66.7 %, specificity 86.4 %). Of the 225 samples tested, 44 (19.55 %) positive samples were detected in the ELISA test using antigen obtained by thermal extraction, while 37 (16.44) positive samples were found in the ELISA test using ultrasonicated antigen, which is more specific. Similar observations have been published by other authors (Barkha et al., 2011, Oliveira et al., 2011, Wanke et al., 2002). The use of the recommended 2-ME TAT test is difficult in all cases where the serum sample is haemolytic. Since the blood of dogs is easily haemolyzed, the indirect ELISA test with antigens obtained by ultrasonic disintegration should be an excellent substitute for agglutination tests.

Radojičić et al. (2001) examined 44 blood sera from dogs using a dot-ELISA test with two differently prepared antigens and found that the cytoplasmic proteins of *B. canis* are a reliable and highly specific marker for the rapid diagnosis of canine brucellosis. In that earlier study, sera were tested at a dilution of 1/100 without prior thermal or chemical treatment, and nitrocellulose was used as a carrier to which the tested antigens were applied. The immunoglobulins of isotypes G and M were detected using anti-dog antibodies, which provided good sensitivity and specificity of the test when using cytoplasmic antigens. In another study, cytoplasmic antigens obtained by ultrasonic disruption of bacteria were suitable for use in a highly sensitive, indirect

ELISA test (Barkha et al., 2011). Due to its high sensitivity, the authors recommended this particular test for the triage of serum samples, while the agar gel immunodiffusion assay (AGID) was recommended as a confirmatory test due to its high specificity (Barkha et al., 2011). The results obtained by Wanke et al. (2002) showed that ELISA tests using outer membrane proteins and cytoplasmic antigens of *B. canis* were highly specific for the diagnosis of canine brucellosis. Samples from dogs in the early stages of infection that are negative in agglutination tests can be detected with ELISA tests formulated in this way. Oliveira et al., (2011) developed an ELISA test using thermally-extracted antigens. This test showed a high sensitivity (91.18 %), indicating it could be used as a reliable and practical triage test as well as in epizootiology research.

In addition to R-LPS, thermally-extracted antigens also contain other outer membrane proteins that are common to all *Brucella* (Bowden et al., 1995) and may be responsible for the lower specificity of tests using thermally-extracted antigens. It is possible that tests using cytoplasmic antigens are not suitable for the detection of animals in the early stages of infection, because several weeks must elapse after infection to synthesize specific antibodies against the cytoplasmic proteins of *Brucella*. Other authors also reported that R and S strains of *Brucella* species have common cytoplasmic antigens, while it was demonstrated that some cytoplasmic proteins in *Brucella* are specific only for this genus and that most of these proteins are common to all *Brucella* species (Carmichael et al., 1984).

CONCLUSION

Based on the results obtained in this study, we can conclude that for the *B. canis* ELISA test, using an antigen solution obtained by ultrasonic disintegration resulted in better test characteristics than using an antigen solution obtained by thermal extraction. For the accurate diagnosis of canine brucellosis, in addition to 2-ME TAT, we recommend using an indirect ELISA test prepared with antigen obtained by ultrasonic disintegration of *B. canis*. If the results of serological tests are unclear, paired serum tests and bacteriological examinations of available clinical samples are mandatory, as false-negative results can occur.

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Authors' contributions

NS interpreted the results, drafted and wrote the manuscript, NS and DB collected the samples and carried out the serological tests together with SR, SR conceived and designed the study. SB participated in the design of the study, biochemical analysis and interpretation of results.

Competing interests

The authors declare that they have no competing interests.

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RAZVOJ I EVALUACIJA DVA ELISA TESTA ZA DIJAGNOSTIKU BRUCELOZE PASA

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Kratak sadržaj

Bruceloza pasa je oboljenje koje najčešće izaziva simptome koji mogu varirati od blagih do teških reproduktivnih poremećaja. Kod većine inficiranih životinja bolest protiče inaparentno, što dodatno otežava postavljanje kliničke dijagnoze. Jedina metoda pomoću koje se sa sigurnošću može postaviti dijagnoza je izolacija *B. canis*. Prilikom izvođenja seroloških reakcija javlja se veliki broj lažno pozitivnih rezultata koji nastaju kao posledica postojanja površinskih antigenskih determinanti koje su zajedničke *B. canis* i drugim bakterijama. Testirani su krvni serumi 225 pasa metodom 2-ME TAT i sopstveno pripremljenim ELISA testovima u kojima su korišćeni antigen dobijen toplotnom ekstrakcijom i antigen dobijen ultrazvučnom dezintegracijom *B. canis*. Elektroforetska analiza i denzitometrijska kvantifikacija antigena su pokazale da je u antigenu dobijenom toplotom, najzastupljenija frakcija molekulske mase 10,95 kDa sa učešćem od čak 43,12 % koja odgovara R-LPS-u brucela. Ista frakcija je u antigenu dobijenom ultrazvukom bila zastupljena sa 11,56 %, odnosno u količini koja je bila 3,7x manja. Proteinski sastav antigena dobijenog primenom ultrazvuka bio je značajno bogatiji, a po kvantitativnom sastavu su dominirali proteini molekulskih masa od 30,5, 24,5, 38 i 22 kDa koji pripadaju proteinima spoljašnje membrane. U ELISA testu koji je formulisan sa antigenom dobijenim toplotom, od 225 ispitanih seruma, 44 odnosno 19,55 % je bilo pozitivno, dok je u ELISA testu sa antigenom dobijenim ultrazvukom 37 (16,44 %) bilo pozitivno. U cilju postavljanja tačne dijagnoze, za serološko ispitivanje, pored 2-ME TAT, preporučuje se i upotreba indirektnog ELISA testa formulisanog sa antigenom dobijenim ultrazvučnom dezintegracijom bakterija.

Ključne reči: antigen, *B. canis*, bruceloza, pas, ELISA, ROC