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Standardization of an Indirect ELISA for Diagnosis of Bovine Brucellosis

Padronização de um Teste ELISA Indireto para Diagnóstico da Brucelose Bovina

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Abstract

Bovine brucellosis, caused by the bacterium Brucella abortus, is a zoonotic disease of sanitary and economic importance, difficult to diagnose clinically, and associated with the One Health concept, classified as an occupational disease transmitted through food of animal origin, demanding greater attention to food safety. In Brazil, the National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) adopts official tests such as the Buffered Acidified Antigen (BAA) for screening and 2-Mercaptoethanol (2-ME) as a confirmatory test. However, the indirect ELISA test shows potential as an alternative screening method due to its high sensitivity, specificity, and large-scale processing capacity. This study aimed to standardize the indirect ELISA test for the diagnosis of Brucellosis in bovine serum samples from slaughtered animals in a meat processing plant. For standardization of the indirect ELISA technique, the commercial antigen used for the slow agglutination test was subjected to a study of optimal dilution. The cutoff point, sensitivity, and specificity were determined. For standardization, 90 serum samples were used, 45 from naturally infected animals and 45 from animals proven to be free of the disease. After standardization, the test was applied to serum samples collected at a slaughterhouse. The ELISA showed a cut-off of 0.2083, 100% sensitivity, and 97.8% specificity.

Keywords: Veterinary Serology. Immunodiagnosis. Bovine Reproductive Diseases. Serological Test Animal Health.

Resumo

A brucelose bovina, causada pela bactéria *Brucella abortus*, é uma zoonose de relevância sanitária e econômica, de difícil diagnóstico clínico que está associada ao conceito de Uma Só Saúde, classificada como uma doença ocupacional e transmitida por alimentos de origem animal, demandando maior atenção à segurança alimentar. No Brasil, o Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose (PNCEBT) adota testes oficiais como o Antígeno Acidificado Tamponado (AAT) para triagem e o 2-Mercaptoetanol (2-ME) como teste confirmatório. Entretanto, o teste ELISA indireto apresenta potencial como método alternativo de triagem, pela elevada sensibilidade, especificidade e capacidade de processamento em larga escala. Este estudo teve como objetivo padronizar o teste ELISA indireto para diagnóstico da Brucelose em amostras de soros bovinos abatidos em abatedouro frigorífico. Para padronização da técnica ELISA indireto, utilizou-se o antígeno comercial aplicado para realização do teste de soroaglutinação lenta submetido a estudo de melhor diluição Foi determinado o ponto de corte, sensibilidade e especificidade. Para padronização foram utilizadas 90 amostras de soros de animais, sendo 45 amostras de animais naturalmente infectados e 45 animais comprovadamente livre da doença. Após a padronização, o teste foi aplicado em amostras de soros coletadas em abatedouro. O ELISA apresentou cut-off de 0.2083, sensibilidade de 100% e especificidade de 97,8%.

Palavras-chave: Sorologia Veterinária. Imunodiagnóstico. Doenças Reprodutivas Bovinas. Teste Sorológico. Sanidade Animal.

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1 Introduction

Bovine brucellosis is an infectious disease caused by *Brucella abortus*, which compromises meat and milk production, resulting in significant economic costs and public health risks (Megid; Brasil, 2017; Mathias *et al*., 2016). In Brazil, the cattle herd is estimated at 238.6 million head, 13.2 million of which are located in the state of Bahia (IBGE, 2023). It is relevant in the context of the One Health Program as it is a zoonosis that is difficult to diagnose clinically, causes reproductive and systemic complications in animals, and can also infect humans through occupational contact or consumption of contaminated animal products (Santos *et al*., 2013).

Brucella species exhibit host preference. In the specific case of the bacterium *B. abortus*, cattle are the preferred reservoir, although the infection can affect various domestic and wild species, as well as humans (Gomes, 2013). The microorganism has two antigenic classifications — smooth and rough — which are determined by the presence or absence of the O-chain of the lipopolysaccharide, which gives the species the potential for virulence, with the smooth forms, such as B. abortus, being more virulent (Megid; Mathias, 2016; Quinn *et al*., 2007; Sola *et al*., 2014).

Transmission can occur directly or indirectly, with childbirth and abortion being the main forms of contamination. This occurs when there is a massive release of bacteria into the fetal membranes and fluids (Megid; Mathias, 2016; Quinn *et al*., 2007).

Pathogenesis begins with the penetration of the agent through the mucosa. After recognition of the bacteria, antigen-presenting phagocytic cells are activated, causing intracellular multiplication

and phagolysosomal disruption (Quinn *et al*., 2011; Sola *et al*., 2014).

The preferred route in pregnant females is the uterus in the final third of gestation, which is prone to high erythritol concentrations, contributing to placental inflammation, necrosis, and abortion. In males, the bacteria act on the epididymis and testes, causing orchitis, testicular atrophy, and infertility (Radostits, 2007).

The National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT), established in 2001, determinates the Buffered Acidified Antigen test (AAT) as official diagnostic tools as screening and the 2-Mercaptoethanol (2-ME), Complement Fixation (FC), Fluorescent Polarization (FPA), and Milk Ring tests (TAL) as confirmatory (Brazil, 2017). Although not officially adopted by the program, the indirect ELISA test has emerged as a promising alternative due to its high sensitivity and specificity, objective reading, low cost, and the possibility of large-scale application (Putini *et al*., 2008; Maturino *et al*., 2014).

Therefore, this study aimed to standardize the indirect ELISA test, compare its performance with the official methods recommended by the Ministry of Agriculture and Livestock (MAPA), and evaluate the occurrence of bovine brucellosis in slaughtered cattle from the Recôncavo Baiano region, assessing the sensitivity, specificity, speed, and economic viability of the test, as well as the presence of B. abortus in animals inspected ante- and post-mortem in a state-inspected slaughterhouse.

2 Material and Methods

The protocol was standardized at the Infectious Diseases Laboratory of the Federal University of Recôncavo da Bahia (UFRB). The samples used were from the LDI sample bank. The antigen used was the same as that used in the slow agglutination test in tubes, with a cell concentration of 4.5%, without dye, standardized from the 1119-3 strain of *Brucella abortus*, heat-inactivated, and produced by the Federal Laboratory of Agricultural Defense (LFDA-MG), Paraná Institute of Technology (TECPAR). Batch: 01/22 and date of manufacture: Jul/22.

The indirect ELISA protocol was based on and adapted from the Maturino *et al*. (2014) protocol, which has a sensitivity of 23.33% and 100% specificity. The cut-off point for this technique is 0.181 (optical density). Therefore, a new standardization was performed with serial dilutions of the antigen. In a plate suitable for Indirect ELISA made of polystyrene material with 96 wells and a flat bottom (COSTAR), the following dilutions of the pure antigen were performed: 1:25, 1:50, 1:100, 1:200, 1:400, 1:800 (Frey; Di Canzio; Zurakowski, 1998).

Initially, the plate sensitization phase was performed, where the antigen was diluted in 0.05 M bicarbonate-carbonate buffer, pH 9.6, and distilled water. 2 mL of the dilution was placed in each tube along with the antigen at the concentrations mentioned above. Using a micropipette, 50 µL of

the dilution was added to each well of the even-numbered row and 100 µL of the dilution to each well of the odd-numbered row. After distributing the dilution throughout the plate, it was placed in a humid chamber where it remained overnight for 18 hours in the refrigerator at 4 to 8°C.

The plate was removed from the refrigerator and the wells were washed twice with a PBS-T20 buffer. Blocking began with 5% skim milk diluted in 20 mL of PBS-T20, adding 200 µl per well. The plate was returned to the humid chamber and incubated at 37°C for two hours, followed by washing the wells once with PBS-T20.

The 1% skim milk was diluted with 10 mL of PBS-T20, placing 2 mL of the dilution into four tubes (a blank, a proven negative control, a proven positive control, and a test serum). 10µl of the test serum was added to each tube; 50µl of the dilution was added to each well in the even-numbered row, and 100µl was added to each well in the odd-numbered row.

The plate was placed in an incubator inside a humid chamber for 1 hour. After this incubation period, the plate was washed five times with PBS-T20. The conjugate (rabbit anti-bovine IgG immunoglobulin labeled with peroxidase) was diluted in 10 mL of PBS-T20 at a concentration of 1:10,000 using 100 µl per well. The plate was left in the incubator at 37°C for 1 hour and washed five times with PBS-T20.

Then, the developing solution, 10 mL of citric buffer, 60 µl of 30-volume hydrogen peroxide (H_2O_2), and 60 µg of O-phenylenediamine dihydrochloride (OPD) (100 µl) were added to each well. The reaction was stopped with 20 µl of sulfuric acid (H_2SO_4) after 30 minutes and read on a spectrophotometer with a 492 nm filter.

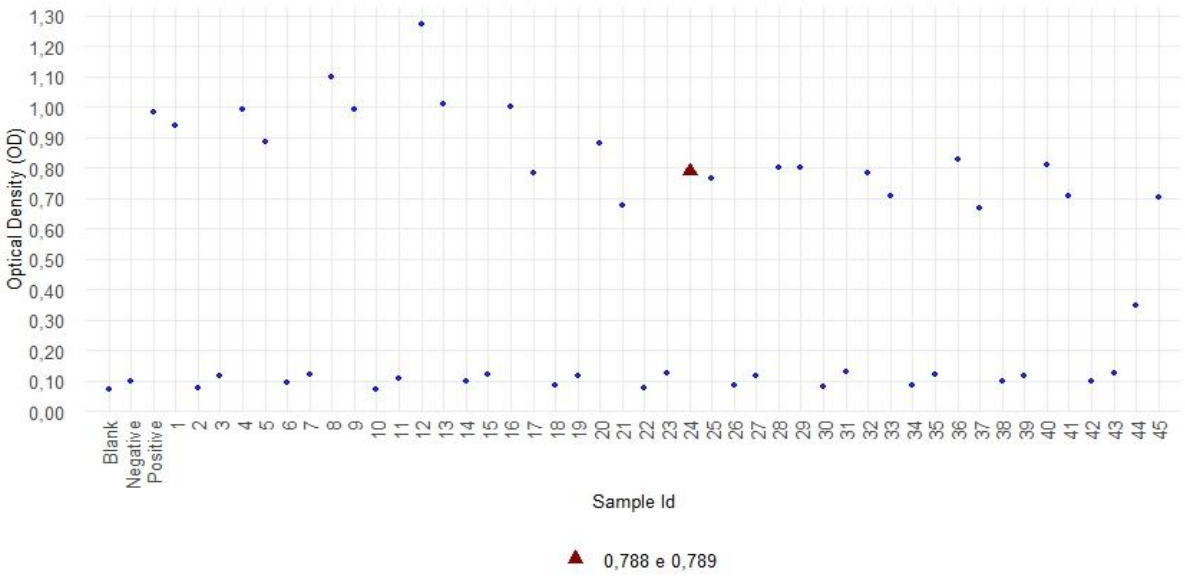
After defining the best standardization dilution, two plates were prepared: one containing proven positive sera and the other containing proven negative sera. This allowed calculating the cut-off point, sensitivity, and specificity of the test. To calculate the cut-off point, we used the following formula: mean optical density of non-reactive animals + 3 x the standard deviation, based on Frey; Di; Zurakowski (1998).

To study specificity, we used the formula: healthy individuals testing negative/total healthy individuals x 100 (p/negative plate); sensitivity: patients detected by the test/total patients x 100 (p/positive plate). The formulas recommended by Soares *et al.* (2013) were used to calculate the specificity.

3 Results and Discussion

The standardization of antigen dilution for the indirect ELISA assay defined the optimal dilution as 1:200 and 50 µL per well. (Figure 1).

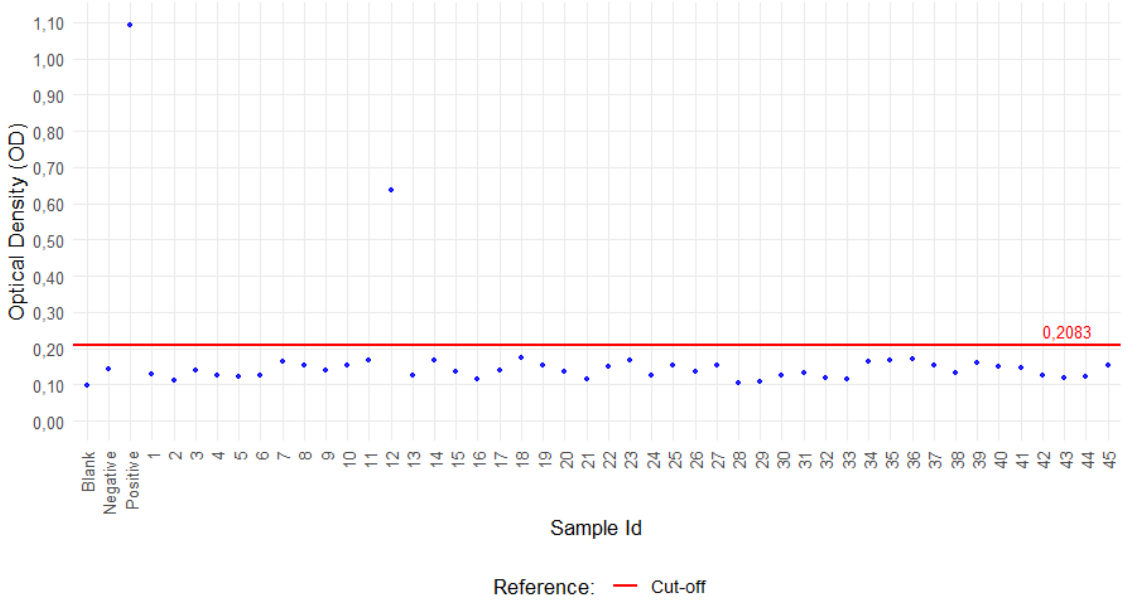
Figure 1 - Determination of antigen dilution and protocol for the indirect ELISA test for bovine brucellosis



Source: research data.

To validate the test, a plate containing proven negative sera was prepared (Figure 2), where the cut-off point and specificity were calculated. The cut-off point was calculated using the mean optical density of the non-reactive animals + 3 x the standard deviation = 0.2038. The test specificity was 97.8% and sensitivity was 100%.

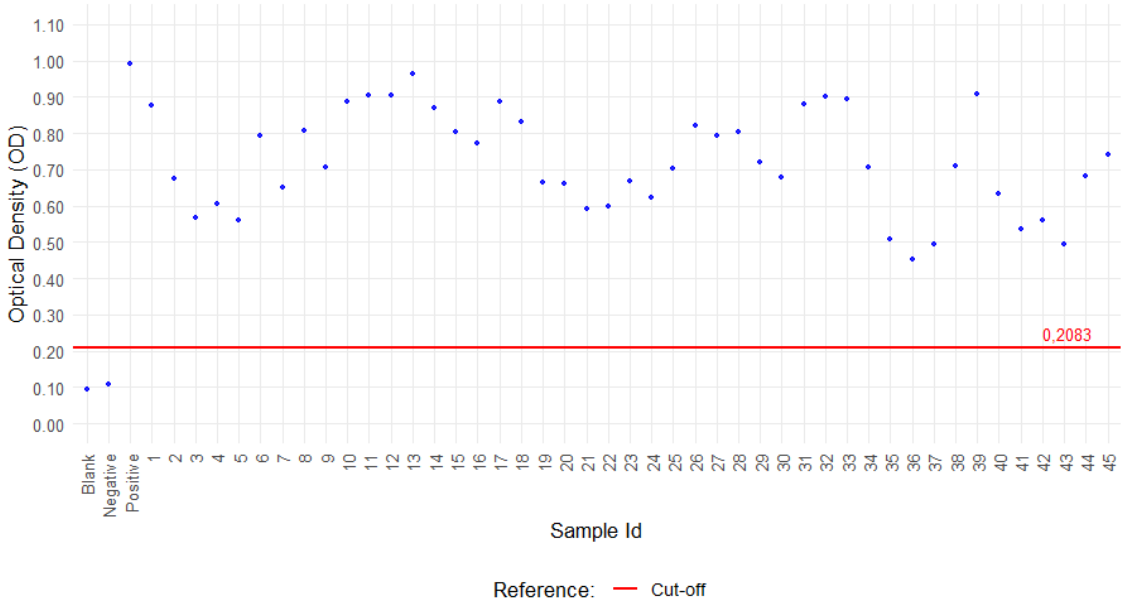
Figure 2 - Standardization of indirect ELISA for bovine brucellosis - Proven negative serum samples



Source: research data.

The plate with proven positive sera was used for calculation to determine the test sensitivity, which was 100% (Figure 3).

Figure 3 - Standardization of indirect ELISA for bovine brucellosis - Proven positive serum samples



Source: research data.

The standardization demonstrated greater sensitivity than specificity, indicating that the evaluated test outperformed both the detection of true positives and the exclusion of false positives compared to the Maturino *et al.* (2014) test. The high sensitivity of the test suggests that the standardized test is superior in correctly diagnosing individuals, thus reducing the chance of false-negative results. In both studies, specificity was high, indicating a lower likelihood of misidentifying healthy individuals as positive, minimizing false positives. Souza *et al*. (2019) demonstrated in their study the sensitivity and specificity of indirect ELISA, both at 97%, demonstrating the test's ability to detect both infected and uninfected animals.

Fernandes (2022) standardized an indirect ELISA test for canine distemper, achieving 95.5% sensitivity and 84.4% specificity, demonstrating that the test is effective in diagnosing truly positive animals, although with a higher rate of false positives compared to sensitivity. Despite the results obtained, the test is considered suitable for large-scale screening, allowing negative animals to be reliably monitored.

The results of Greve *et al*. (2007) are consistent with other standardizations, where an indirect ELISA test for sheep was standardized, achieving 100% sensitivity and 95.6% specificity, demonstrating satisfactory test performance in detecting brucellosis in sheep.

Indirect ELISA can be performed on multiple samples at once, thus facilitating herd monitoring, speeding up results and reducing costs, in addition to being able to detect low amounts of antibodies (Brasil, 2016; Baumgarten *et al*., 2016). Therefore, studies focused on standardizing and adapting the technique give the test greater reliability and efficiency.

4 Conclusion

The indirect ELISA test was standardized for the diagnosis of bovine brucellosis, with a cut-off point of 0.2083, 100% sensitivity, and 97.8% specificity. These values demonstrate high reliability in both detecting infected animals and excluding false positives, making it an efficient tool for serological screening.

As a result, this study demonstrated that the test can be implemented in routine veterinary practice as a diagnostic tool for cattle infected with brucellosis.

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