BRAZILIAN JOURNAL OF MICROBIOLOGY xxx (2018) xxx–xxx

BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Veterinary Microbiology

Evaluation of dot-blot test for serological diagnosis of bovine brucellosis

Carla Resende Bastos[a,*], *Luis Antonio Mathias*[a], *Márcia Mariza Gomes Jusi*[b], *Renata Ferreira dos Santos*[a], *Glaucenyra Cecília Pinheiro da Silva*[a], *Marcos Rogério André*[b], *Rosangela Zacarias Machado*[b], *Karina Paes Bürger*[a]

[a] *São Paulo State University (UNESP), School of Agricultural and Veterinarian Sciences, Jaboticabal, Department of Preventive Veterinary Medicine and Animal Reproduction, Jaboticabal, SP, Brazil*

[b] *São Paulo State University (UNESP), School of Agricultural and Veterinarian Sciences, Jaboticabal, Department of Veterinary Pathology, Jaboticabal, SP, Brazil*

ARTICLE INFO

Article history:
Received 3 March 2017
Accepted 30 October 2017
Available online xxx

Associate Editor: Miliane Souza

Keywords:
Brucella abortus
Animal health
Zoonoses
2-Mercaptoethanol
Complement fixation test

ABSTRACT

The objective of this study was to standardize and validate the dot-blot test for the serological diagnosis of bovine brucellosis, compare the results with those found in the 2-mercaptoethanol (2-ME) and complement fixation test (CF), and estimate the relative sensitivity and specificity of the dot-blot compared to these tests. Fifty bovine blood serum samples were used for the test standardization, and 1315 samples were used for evaluation and comparison between the tests; the results were compared using the *Kappa* indicator. At the end of standardization, it was established as optimal for the antigen obtained from *Brucella abortus* B19 after passing through a microorganism rupture process, the blood serum samples diluted at 1:100, and the conjugate at 1:30,000. The comparison of the dot-blot results with 2-ME showed *Kappa* index of 0.9939, sensitivity of 99.48%, and specificity 99.91%, with CF, *Kappa* index of 0.8226, sensitivity 100% and specificity 95.32%. Using the combination of the test results 2-ME and CF to establish the true condition of the animal, the dot-blot showed relative sensitivity of 100%, and relative specificity of 99.91%. The evaluated test proved to be effective and reliable, besides being easy to handle and interpret the results.

* *Corresponding author at*: São Paulo State University (UNESP), School of Agricultural and Veterinarian Sciences, Jaboticabal, Department of Preventive Veterinary Medicine and Animal Reproduction, Access route Prof. Paulo Donato Castellane, 14884-900 – Jaboticabal, SP – Brazil.
E-mail: carlarbvet@gmail.com (C.R. Bastos).
https://doi.org/10.1016/j.bjm.2017.10.002

Please cite this article in press as: Bastos CR, et al. Evaluation of dot-blot test for serological diagnosis of bovine brucellosis. *Braz J Microbiol.* (2018), https://doi.org/10.1016/j.bjm.2017.10.002

Introduction

Brucellosis is an infectious disease of chronic character that affects animals, causing great losses to livestock. It is also a public health problem, for being a zoonosis of occupational character, transmitted by contact with contaminated fetal membranes with the causative agent *Brucella abortus*, and foodborne by unpasteurized milk intake, fresh cheese and undercooked meat from animals with brucellosis.[1,2]

Due to the social and economic importance of this disease, the Ministry of Agriculture, Livestock and Supply of Brazil (MAPA) has set up a control and eradication program, which defined as official tests for the diagnosis of bovine and buffalo brucellosis: Milk ring test (MRT) used for monitoring the health condition and as a diagnostic tool in epidemiological surveillance systems, Rose Bengal test (RB) as a screening test and 2-mercaptoethanol (2-ME) and complement fixation (CF) as confirmatory tests in addition to the fluorescence polarization assay (FP).[3]

However, there are some difficulties concerning these tests, such as the need for highly trained staff, the use of labile reagents that need to be constantly prepared, and titrated, toxic reagents. These facts highlight the need to develop new diagnostic techniques in order to collaborate for the control and eradication of brucellosis.[4]

The aim of this study was to standardize and validation the dot-blot technique for the serological diagnosis of bovine brucellosis, and compare the results obtained by this technique with the ones found in the official tests: complement fixation and 2-mercaptoethanol, and also to estimate the relative sensitivity and relative specificity of dot-blot in comparison with official diagnostic tests used in the study.

Materials and methods

Fifty blood sera samples from cattle of various breeds, male and female, from different properties in the North, Northeast and Southeast regions of Brazil were used for test standardization, samples previously tested and with the same results in all official tests and more 1315 samples were used for validation of the tests 2-ME, CF and dot-blot.

Standardization of dot-blot

From an immunological point of view, Brucella antigens can be divided into two major groups: proteins and the LPS.[5] With the intention of evaluate what is the best antigen for the present study were tested the two antigens, the *Brucella abortus* sample B19 obtained from the commercial vaccine sold in Brazil after undergoing a microorganism rupture process, by the method of freezing for 5 min in liquid nitrogen and thawing for 5 min in water bath at 37 °C, twenty times in a row, and quantified at 3.1 μg/μL of protein, the reference antigen for dot-blot tests. The second antigen tested was lipopolysaccharide extract obtained from the *Brucella abortus* strain S99[6] quantified at 0.52 μg/μL, the LPS was tested for being one of the most important antigen combated by immunoglobulins, it is extremely immunogenic and commonly detected in serological tests.[3]

In order to standardize and establish the ideal amount of antigen used, tests were performed with 0.5 μg, 1 μg, 1.5 μg and 2 μg of the antigens tested. The quantity that showed the best results for the two antigens tested was 2 μg, concentration which allowed the visibility of the color reaction with excellent sharpness, and was the value established for the membrane sensitization.

For the technique standardization, 50 control samples of bovine blood serum were used, 17 positive with different titrations (weak, medium and strong), and 33 negative in the 3 tests: Rose Bengal test, 2-ME and CF.

The technique was developed according to the previously described methodology.[7] The test started by cutting the nitrocellulose membrane (code N-9888, Sigma-Aldrich, St. Louis, MO, USA) in two formats, square and circle, with the interest to establish the shape that would enable better solution homogenization, and economy of material when making the cuts. To perform the membrane cutting, scissors were used for the square format, and a hole punch for the circle format, both properly sanitized and handled with gloves. To make the cuts, materials of easy acquisition and manipulation were used, for the purpose of facilitating the technique.

Each nitrocellulose membrane was sensitized with 2 μg of antigen, manipulated with forceps and gloves to prevent contamination, and placed on a surface of hydrophobic material. As for the support on which to carry out the reactions, two types of plate were evaluated, the polyacrylamide plate with 96 wells, and the cell culture plate with 24 wells and a flat bottom.

The sensitized membranes were blocked for 12 h with 300 μL TBS (20 mM Tris, 500 mM NaCl, pH 7.5) with addition 0.05% Tween 20 and 5% powder milk into each well of the plate with 24 wells and 200 μL into each well of the plates, with 96 wells to minimize the occurrence of non-specific reactions,[8] leaving the plate stirring at 4 °C.

After incubation, the blocking solution was removed, and 500 μL of the serum to be tested, was pipetted into each well of the plate, with 24 wells and 200 μL into each well of the plate with 96 wells, diluted in the proportions 1:25, 1:50 and 1:100 in TBS 0.05% Tween 20 with powder milk at 5%. The material was then incubated for 2 h at room temperature under constant agitation.

After the incubation was finished, the wells were washed with TBS 0.05% Tween 20. Then, it was evaluated which was the optimal number of washes 1, 2 or 3 times, each wash lasting 5 min. Subsequently, 300 μL of conjugate IgG of rabbit anti-total bovine IgG linked to alkaline phosphatase (code n. A0705, Sigma-Aldrich, St. Louis, MO, USA) was pipetted into each well of the plate with 24 wells and 200 μL into each well of the plate with 96 wells, in the dilutions 1:4000; 1:10,000; and 1:30,000.

The material was incubated for 1 h at room temperature. Conjugate was removed and three washes, 5 min each, were performed using TBS 0.05% Tween 20. The bands were visualized by the addition of the enzyme substrate 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium chloride, following the manufacture's recommendations (code n. 170-6432, NBT-BCIP, Bio-Rad, Hercules, CA, USA).

Please cite this article in press as: Bastos CR, et al. Evaluation of dot-blot test for serological diagnosis of bovine brucellosis. *Braz J Microbiol.* (2018), https://doi.org/10.1016/j.bjm.2017.10.002