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Development and standardization of a western blotting test for detection of antibodies against *B. abortus*

[*Desenvolvimento e padronização do teste Western blotting para detecção de anticorpos contra* B. abortus]

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ABSTRACT

Brucellosis is an infectious disease caused by bacteria of the genus *Brucella* spp. with diagnosis based on use of serological techniques. The present study aimed to develop and standardize a western blotting (WB) test for detection of antibodies against *B. abortus*. Samples from two groups of cattle were analyzed: group I: 60 serum samples from true positive and true negative vaccinated animals (30 positive samples from infected animals according to rose bengal test (RBT), 2-mercaptoethanol, serum agglutination test (SAT) and complement fixation test (CFT) and 30 RBT negatives samples); group II: 383 field samples (90 positive and 293 CFT negative sera). The most reactive band in the western blotting, which properly identified and separated infected from non – infected had a molecular weight of ≤ 20kDa. The sensitivity, specificity and accuracy of the WB compared to RBT was 93%, 99%, 98%, respectively and *k*= 0.938. When compared to CFT, the sensitivity, specificity and accuracy of the WB was 97%, 98% and 97%, respectively and *k*= 0.929. The WB developed and standardized in the present study is a serological test with potential use as a confirmatory test for the diagnosis of bovine brucellosis.

Keywords: brucellosis, immunodiagnosis, proteomics, serology

RESUMO

A brucelose é uma doença infectocontagiosa, causada por bactérias do gênero Brucella *spp., com diagnóstico baseado no emprego de técnicas sorológicas. Objetivou-se neste estudo desenvolver e padronizar um teste Western blotting (WB) para detecção de anticorpos contra* B. abortus. *Foram analisados dois grupos de amostras bovinas: grupo I, com 60 amostras de animais verdadeiros positivos e verdadeiros negativos vacinados (30 amostras positivas de animais infectados e positivos nos testes de antígeno acidificado tamponado (AAT), 2 – mercaptoetanol (2 – ME), soroaglutinação lenta em tubos (SAT) e fixação do complemento e de 30 amostras negativas no AAT); grupo II, com 383 amostras de campo, sendo 90 soropositivas e 293 soronegativas no TFC. O resultado da análise do WB revelou peso molecular ≤20kDa como sendo a área mais reativa e característica para identificação e separação dos animais infectados dos não infectados. A sensibilidade, a especificidade e a acurácia do WB, quando este foi comparado com o AAT, foram, respectivamente, 93%, 99% e 98%, e k= 0,938. Quando comparadas com a TFC, a sensibilidade, a especificidade e a acurácia foram 97%, 98% e 97%, respectivamente, e k= 0,929. O WB padronizado neste estudo mostrou-se um teste sorológico com potencial uso como teste confirmatório no diagnóstico da brucelose bovina.*

Palavras-chave: brucelose, imunodiagnóstico, proteômica, sorologia

INTRODUCTION

Brucellosis is an infectious disease that affects cattle and buffaloes, among other animals, causing considerable economic losses. This disease is caused by *Brucella abortus*, a nonmotile gram-negative coccobacillus, with a smooth colony morphology (Teixeira *et al.*, 1998). Twenty species have been described in the genus *Brucella*, which are classified according to their biochemical characteristics,

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antigenic variation and main hosts (Godfroid *et al*., 2011; Whatmore *et al*., 2014; Scholtz *et al*., 2016).

Isolation of *Brucella abortus* is considered the most reliable diagnostic method of brucellosis. The samples of choice for isolation include secretions, purulent content, abomasal content of aborted fetuses, cerebrospinal fluid, pleural fluid, synovial fluid and ascitic fluid (Mantur *et al*., 2006; Bovine…, 2012). However, bacterial isolation is a complex and expensive technique making it difficult to be routinely used in a laboratory. Serological methods work better in the laboratory environment and several serological tests are used for the diagnosis of bovine brucellosis worldwide (Nielsen *et al*., 2000).

In Brazil, the most common tests used are the rose bengal test (RBT), the fluorescence polarization assay (FPA), the serum agglutination test (SAT), the 2-mercaptoethanol (2-ME) and the complement fixation test (CFT) as golden standards for the diagnosis of brucellosis (Brasil, 2006; Brasil, 2017). RBT and FPA are highly sensitive; thus, in Brazil, they are used for screening individual animals and herds, respectively. Confirmatory tests such as SAT, 2-ME and CFT are highly specific; therefore, they are used to confirm the diagnosis of animals that were positive in screening tests. CFT is the method of choice for purposes of international trade (Nielsen *et al*., 2005; Brasil, 2006; Bovine…, 2012).

Western blotting (WB) is an immunoproteomic assay widely used for the detection of small amounts of antibodies (Lee, 2007). This method identifies antigen-antibody interaction and, consequently, detects immunospecific proteins. WB has become a decision-making and interpretation tool due to its high sensitivity and specificity (Kim *et al*., 2014). Manat *et al*. (2016) identified the outer membrane protein 28 (OMP28) as a major immunodominant antigen and a potential antigen for developing serodiagnosis of bovine brucellosis.

The present study aimed to develop and standardize a western blotting test for detection of antibodies against *B. abortus* using the *B. abortus* 1119-3 strain and to determine the method sensitivity and specificity compared to serological techniques routinely used for the diagnosis of bovine brucellosis in Brazil.

MATERIAL AND METHODS

A saline suspension (0.85% NaCl) of *B. abortus* 1119-3 strain, acquired from experimentally infected animal by *B. abortus* provided by the National Agricultural Laboratory of Pernambuco (LANAGRO – PE, Brazil), was used for the extraction of whole-cell proteins. The strain was resuspended in phosphate buffered saline (PBS) to an initial concentration of 4.5 x 10^8cells/mL estimated at 600nm on a spectrophotometer. The cell suspension was concentrated to 9.8 x 10^9cells/mL in PBS and used for antigen preparation. Approximately 10mL of the cell suspension was centrifuged at 4000 x g for 10min at 4°C and the supernatant was discarded. Soluble components were removed by three consecutive centrifugation and washing steps with 9mL PBS. After the third washing, 3mL of formaldehyde (Sigma Aldrich, EUA) was added for bacteria inactivation and degradation as well as 6mL of PBS. Then, the cell suspension was incubated on an orbital shaker overnight at room temperature and the washing and degradation processes were repeated two more times. The cell suspension was washed once again with PBS and 3mL of PBS was added to it. Finally, Laemmli Sample Buffer (Sigma Aldrich, EUA) was added to the cell suspension in a 1:2 ratio resulting in the antigen suspension, which was stored at -20°C for later use.

The antigen suspension and a 250 – 10kDa molecular weight marker were loaded on a 3%-15% Bis-Tris gel (Invitrogen, EUA) and separated at a constant voltage of 200V for 1 hour using a vertical electrophoresis unit (Invitrogen, EUA) and 20X nupage mops SDS running buffer (Invitrogen, EUA). The gel was stained with SilverXpress kit (Invitrogen, EUA) (Figure 1) and bands with a molecular weight equal or less than 20kDa, which are considered specific markers of *B.* abortus (Pajuaba *et al*., 2012; Kim *et al*., 2014), were used to define positive results.

The antigen was transferred from the gel to a 0.45µm nitrocellulose membrane (Invitrogen, EUA) by blotting at constant 30V for 1 hour using a vertical electrophoresis unit (Invitrogen, EUA) and 20X nupage transfer buffer

(Invitrogen, EUA). After transfer, the lane containing the molecular weight marker was cut from the membrane, which was blocked overnight in Blocking Solution (Candor, Germany). After three washing steps with Washing Solution (Candor, Germany) (1:20 dilution) for 20min, the membrane was cut into strips of approximately 3mm. Some strips were used for membrane quality test and the remaining strips were stored in Falcon tubes at -20°C until use (Elschner *et al*., 2011).

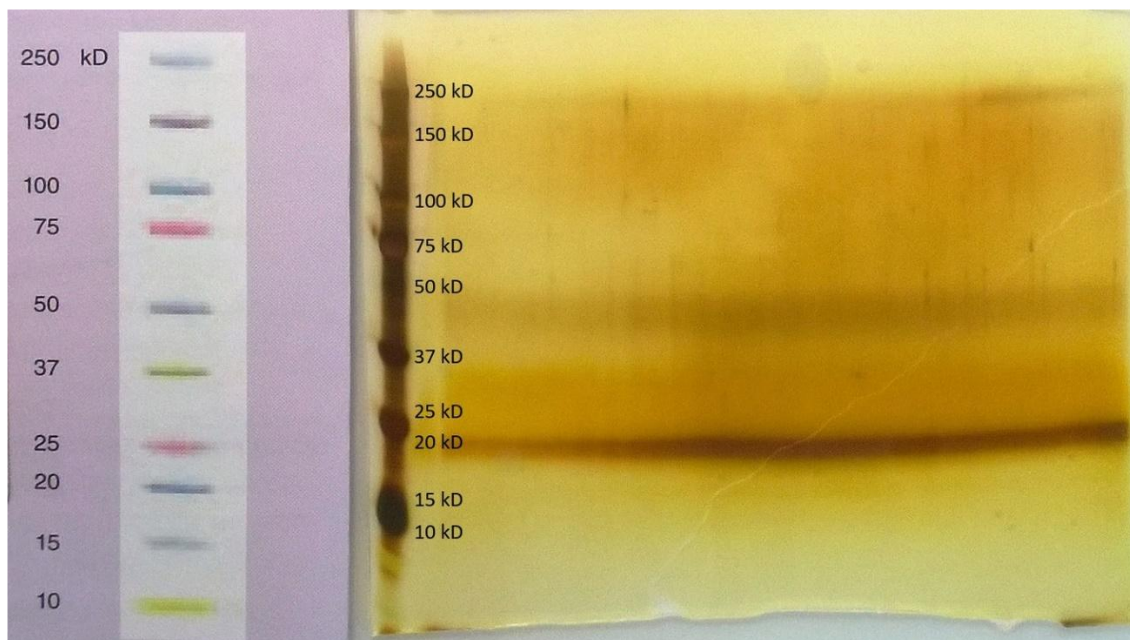

Figure 1. 3% - 15% Bis-Tris Gel silver stained showing the antigen and a 250 – 10kDa molecular weight marker.

Four hundred and forty three bovine serum samples were tested using the western blotting technique developed in the present study. The samples belonged to two groups, group I: 60 serum samples from 30 true positives animals with clinical signs of brucellosis, *B. abortus* isolated and positive results in RBT, 2-ME, SAT and CFT and 30 true negative vaccinated animals with negative results in RBT, 2-ME, SAT and CFT; group II: 383 field samples from CFT positive (n= 90) and negative (n= 293) animals.

All experimental procedures with animals were conducted in accordance with the ethical principles for animal testing adopted by the Ethic Committee for the use of Animal at the Federal Rural University of Pernambuco. Protocol Number: 102/2015.

All the serum samples from groups I and II were screened by RBT following the methodology recommended by chapter 2.4.3 of the manual of diagnostic tests and vaccines for terrestrial animals from the world organization for animal health (OIE) (Bovine…, 2012).

Sera from groups I and II were also tested by CFT following the methodology described in chapter 2.4.3 of the manual of diagnostic tests and vaccines for terrestrial animals from OIE (Principles…, 2013). CFT was performed using a whole cell antigen (Tecpar, Brazil) in 1:500 dilution and incubation at 37°C for one hour (Brasil, 2006).

The western blotting standardization followed the methodology described by Elschner *et al.* (2011).

The sera from all 443 animals were diluted in Low Cross buffer (Candor, Germany) at a 1:50 ratio and incubated with the membrane strips for 1.5h on an orbital shaker, and washed three times with washing solution. For antibody identification, the strips were incubated with anti-bovine IgG serum diluted in 1:100 Low

Cross Buffer for 1.5h on an orbital shaker. After three additional washing steps of 20min each, the strips were stained with a solution of 4-Nitro blue tetrazolium chloride (NBT) (Roche, Switzerland) (1:152) plus 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche, Switzerland) (1:303) in alkaline phosphatase solution (0.58g NaCl + 1.2g trisaminomethane + 101.65mg $MgCl_2.6H_2O$ in 10mL deionized water) pH 9.5±0.2 for 10min. The strips were individually analyzed by comparison with positive and negative controls.

The Kappa (*k*) coefficient was used to study the agreement between the tests used, considering the conventional interpretation of *k*-values as follows: 0.00 – 0.20= poor agreement; 0.21 – 0.40= regular; 0.41 – 0.60= moderate; 0.61 – 0.80= good; 0.81- 1.00= very good, negative values are interpreted as zero. The sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and accuracy (ACC) (Pereira, 2008) of the WB developed in the present study were calculated by comparing the WB results with the RBT and CFT results using 2x2 tables.

RESULTS

Considering the WB and RBT results for cattle of group I, WB showed 100% Se, Sp, PPV, PNV, ACC and *k*= 1. Similar results were observed when WB was compared to CFT for the same group of animals.

Of the 383 serum samples from group II, 99, 90, and 94 were positive for bovine brucellosis and 284, 293 and 289 were negative, according to the results of RBT, CFT, and WB, respectively. WB presented 97%, 98%, 93%, 99%, 97% and 0.929 of Se, Sp, PPV, PNV, ACC and *k*, respectively, when compared to CFT. On the other hand, when compared to RBT, the Se, Sp, PPV, PNV, ACC and *k* of the WB were 93%, 99%, 98%, 98%, 98%, 0.938, respectively (Table 1).

Table 1. Comparison between results of rose bengal test (RBT), complement fixation test (CFT) and western blotting (WB) for animals from group II

Total= 383		WB	
		Positive 94	Negative 289
RBT		Se	93%
		Sp	99%
		PPV	98%
		NPV	98%
Positive 99	Negative 284	ACC	98%
		K	0,938
CFT		Se	97%
		Sp	98%
		PPV	93%
		NPV	99%
Positive 90	Negative 283	ACC	97%
		K	0,929

DISCUSSION

In the present study, we developed and standardized a western blotting test as an alternative for the diagnosis of bovine brucellosis in Brazil. Despite the efforts of the official veterinary service (OVS) to eradicate bovine brucellosis, recent reports show the disease remains endemic and present in all regions from country (Brasil, 2017). Different regions and high prevalence in Brazil, could be causing great economic losses to the national livestock production (Brasil, 2006).

Brucellosis diagnosis is a constant challenge being restricted to serological tests and bacterial isolation (Alton *et al*., 1988). However, serological tests neither differentiate vaccinated animals from naturally infected animals, nor identify *Brucella* species nor avoid cross-reactions with other microorganisms because the LPS-O antigen present in the B19 vaccine is also

present in Gram-negative bacteria (Nielsen *et al*., 2004; Olsen and Stoffregen, 2005; Al Dahouk *et al*., 2006; Ko *et al*., 2012). Thus, the development of tests that do not require the use of LPS O-antigens is very promising because it would help reduce costs associated with vaccination, diagnosis and slaughter of positive animals (Abalos *et al*., 2000; Pajuaba *et al*., 2012).
In contrast to other applications of the western blotting test (Al Dahouk *et al*., 2006; Connolly *et al*., 2006; Elschner *et al*., 2011; Lee, 2007; Ko *et al*., 2012; Kim *et al*., 2014; Wareth *et al*., 2014), our study sought to adapt and standardize a technique developed for proteomic studies to serological diagnosis (Towbin *et al*., 1979), noting that there are no studies on the use of the western blotting test for detection of antibodies against *B. abortus*.

Studies have described *Brucella* sp. proteins that could be employed in WB. Pajuaba *et al*. (2012) and Kim *et al*. (2014) reported that proteins with ≤ 20kDa molecular weight are specific markers for animals naturally infected with *B. abortus*, differentiating them from animals vaccinated with the B19 vaccine. For this reason, we chose the ≤ 20kDa molecular weight as a limit between positive from negative animals in the WB developed herein (Figure 2).

In its "Principles and methods of validation of diagnostic assays for infectious diseases", the OIE states that in order to be considered validated, an assay must go through four stages of evaluation, namely, analytical characterization, diagnostic characterization, reproducibility and implementation also known as the "assay validation pathway". The first two stages (analytical and diagnostic characterization) are required for the standardization of an assay, while the latter two (reproducibility and implementation) are mandatory for validation. In the present study, we completed the first two stages recommended by the OIE, analytical and diagnostic characterization, so the WB developed herein could be considered a standardized test (Principles…, 2013). The analytical characterization corresponded to the screening of true positive and true negative samples by WB and methods routinely used in Brazil (RBT and CFT) with comparison of their results and calculation of analytical sensitivity and specificity. The diagnostic characterization was comprised of the screening of field samples for calculation of diagnostic sensitivity and specificity.

Figure 2. Membranes strips impregnated with NBT-BCIP. A and B, seropositives; C and D, seronegatives.

According to the OIE, an assay test could be considered validated if its sensitivity and specificity values are greater than 95% (Principles…, 2013). The WB standardized in our study showed excellent specificity (99%) but sensitivity (93%) below of limit set by OIE (Principles…, 2013) when compared to RBT; thus, WB should not be considered as an option to replace RBT for herd screening because it did not meet the OIE criteria of ≥ 95% sensitivity and specificity. When compared to CFT, WB had high sensitivity (97%) and specificity (98%); therefore, it could replace CFT as a confirmatory diagnostic tool (Stemshorn *et al*., 1985; Dohoo *et al*., 1986; Mac Millian *et al*., 1990; Nielsen *et al*., 1999; Pajuaba *et al*., 2012;).

The *k* coefficients for WB x CFT and WB x RBT were classified as very good when considering

the conventional interpretation of *k*-values (Pereira, 2008; OIE, 2013). corroborating with results obtained in previous studies on standardization of western blotting for the diagnosis of brucellosis (Pajuaba *et al*., 2012; Wareth *et al*., 2014). The use of new tests that use immunospecific proteins for the diagnosis of brucellosis adds greater confidence in the interpretation of the results; however, the WB developed in the present study has to be validated before it can be routinely used.

CONCLUSIONS

There is still a need for the development of serological tests using antigens capable of detecting species-specific against *B. abortus* antibodies in order to reduce cross-contamination with other microorganisms and, especially, to differentiate vaccinal immunity from acquired immunity, eliminating the false positive results caused by vaccination. In addition, easier and safer diagnostic methods are critically needed in the laboratory routine. The immunoproteomic technique could be used as a confirmatory diagnostic tool for bovine brucellosis in Brazil. It is quick to perform, less dependent on reagents and more reliable and with potential to be capable to differentiate vaccinated from naturally infected animals.

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