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Evaluation of two rapid immunochromatographic tests for diagnosis of brucellosis infection in cattle

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Abstract
Brucellosis is a serious infectious disease that causes significant economic losses in the livestock industry. Its early diagnosis allows an adequate disease control in cattle. DAVIH Laboratories designed a lateral flow immunochromatographic assay using protein A-colloidal gold as a detector reagent (LFIA-PA). The objective of this work was to compare the performance of this assay using protein G-colloidal gold (LFIA-PG) with its performance using protein A-colloidal gold as the detector reagent. The assays were carried out with 20 µL of serum and 130 µL of running buffer. Interpretation of bands was by visual inspection with the naked eye at 15- 20 minutes after sample application. The tests were evaluated with 449 samples of bovine serum (111 positive and 338 negative). The diagnostic sensitivity and specificity, the positive and negative predictive values, and the efficacy of both assays were calculated, and their concordance was estimated by calculating the *kappa* (k) index. The estimated values of the parameters for LFIA-PG and LFPIA-PA were 100% and 95.2% of diagnostic sensitivity, 96.2% and 97.3% of diagnostic specificity, 89.5% and 92.3% for the positive predictive value, 100% and 98.5% for the negative predictive value, and 97.1% and 96.89% of efficacy, respectively. The concordance between both tests was very good ($k = 0.95$). It was shown the possibilities of developing a system with LFIA-PG capable of detecting antibodies against *Brucella* spp. The performance of the test makes possible its use as a screening method in the diagnosis of brucellosis.
Keywords: Bovine, *Brucella* spp., Brucellosis, Diagnosis, Rapid immunochromatographic test.

Introduction

Bovine brucellosis is caused mainly by *Brucella abortus* (Sbriglio *et al.*, 2007; Dongre and Maheshwari, 2013). This microorganism shows affinity for the reproductive tract; it is mainly characterized by the production of abortions, altering the reproduction. This infection has a worldwide distribution. Especially in dairy cattle, it has a great economic and social importance because of the huge losses it can cause in the livestock industry, the potential barriers it creates to the international trade of animals and animal products, and the occupational risk that it represents for the farmers, veterinarians, and butchers who handle either animals or animal consumption of contaminated products (Gasque, 2008; Moreno, 2014; Bamaiyi, 2016).

In cattle, the disease is transmitted through grazing in contaminated areas, consumption of water contaminated with secretions, as well as through infected fetal membranes and contact with aborted fetuses or infected neonates.

In humans, the disease is transmitted by ingestion, penetration through the conjunctiva, through the skin or by the udder contamination during milking. In addition, the infection also occurs through the consumption of raw products from infected animals (Gasque, 2008). This duality, where the infection can be perpetuated in both animals and humans, makes brucellosis to be observed with a new approach: "one health" (Sbriglio *et al.*, 2007; Gasque, 2008; Moreno, 2014).

In Cuba and worldwide, cattle is one of the most economically important species, hence the need to carry out a strict control of brucellosis in livestock (Sbriglio *et al.*, 2007; Gasque, 2008; Dongre and Maheshwari, 2013).

According to the World Organization for Animal Health (OIE), the Rose Bengal test, the standard tube agglutination, the complement fixation method, the slow agglutination test with 2- Mercaptoethanol, the determination of immunoglobulins G (IgG), A (IgA) and M (IgM) by ELISA-type immunoenzymatic assays, and the Fluorescent Polarization assay are the serological techniques commonly used for brucellosis diagnosis (NRAG 586, 1982; Dongre and Maheshwari, 2013; Galińska and Zagórski, 2013; OIE, 2016).

At present, bacterial DNA testing is also used, but its diffusion and availability are still scarce because of its requirements of qualified personnel, specific laboratory equipment, cold chain, and electric fluid. Thus, it is not feasible for the surveillance and diagnosis of

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brucellosis, especially under field conditions, where large numbers of animals are examined (Galińska and Zagórski, 2013; Moreno, 2014; OIE, 2016).

In the last 10 years, rapid lateral flow immunochromatographic test formats have been designed and evaluated in the screening and surveillance of brucellosis, both in herds and in humans.

These tests present a similar performance to the ELISA systems (Beesley, 1989; Díaz *et al*., 2015; Geresu and Kassa, 2016), but their use does not require highly trained personnel, specific materials and equipment, or expensive reagents.

They are a novel alternative for low-income countries and hard-to-reach populations (Abdoel *et al*., 2008; OIE, 2016), where an effective diagnosis of this infection at the herd level and an adequate control of the disease in cattle can be attained (Díaz *et al*., 2015; Geresu and Kassa, 2016; OIE, 2016).

DAVIH Laboratories (Mayabeque, Cuba) developed a lateral flow immunochromatographic assay with a colloidal protein A-gold conjugate (LFIA-PA) (Díaz *et al*., 2015). The evaluation of the assay with this detector reagent showed an adequate performance. However, high levels of sensitivity reached by this type of assay were reported when protein G-colloidal gold were used as the detector reagent, attributed to its high affinity for bovine IgG immunoglobulins (Hermanson, 1996; Geresu and Kassa, 2016). The objective of this work was to compare the performance of a lateral flow immunochromatographic assay using protein G-colloidal gold as a detector reagent with its performance using protein A-colloidal gold.

Materials and Methods

Animals

A total number of 449 bovine sera (111 positive and 338 negative) selected from the reference panel of DAVIH Laboratories (Mayabeque, Cuba) were used for the test evaluation. The samples included in the study were taken from different herds in both brucellosis-free and brucellosis-affected areas in Cuba. The samples were from the provinces Camagüey (61 positive and 69 negative), Pinar del Río (50 positive and 155 negative), and Mayabeque (114 negative). They were characterized by conventional serological tests (Rose Bengal, Standard Agglutination Test, Complement Fixation and 2-Mercaptoethanol Test) (NRAG 586, 1982; Galińska and Zagórski, 2013; OIE, 2016).

The biological reagents necessary for the development of these techniques were produced in Cuba by the Biological Pharmaceutical Laboratories (LABIOFAM), and the methodology and interpretation criteria used for the evaluation of the results were those included in the Resolution 586 of the Institute of Veterinary Medicine of the Republic of Cuba: 1982, Veterinary Diagnosis, Brucellosis, Test methods (NRAG 586, 1982).

The Rose Bengal test was performed as follows: 30 µl of test serum was added to 30 µl of commercial Rose Bengal antigen on a white porcelain plate and mixed thoroughly with a clean toothpick to produce an area of approximately 2 cm in diameter. The plate was shaken slowly for 3 min. The test was read and scored as positive if any degree of agglutination was observed (NRAG 586, 1982; OIE, 2016).

The Standard Agglutination Test was performed with a *Brucella abortus* strain 99 antigen, diluted to 5% in 0.85% saline solution, with 0.5% phenol. Four glass tubes were used per sample, each tube containing 0.08 mL, 0.04 mL, 0.02 mL and 0.01 mL of serum. Two mL of the diluted antigen was added to each tube. The samples were incubated at 37 ° C for 16-24 hours. Those samples with a film at the bottom of the tube and a clear supernatant from the 1: 100 dilution were considered positive; those samples with no films and associated with cloudy supernatant were considered negative (NRAG 586, 1982; Galińska and Zagórski, 2013).

The Complement fixation test (CFT) was carried out in a microtitre format by hot fixation with two units of complement. Test sera and positive and negative controls were diluted two fold in Veronal Buffer (VB), starting from 1:5 dilutions. Diluted serum samples (1:5) were inactivated at 58°C for 50 min. The minimum haemolytic dose (MHD) was estimated for each test set-up using 2% sensitized sheep red blood cell (SRBC) in VB. Two MHD units were used throughout the test. The end point titre was taken as the first well showing approximately 50% lysis of SRBC. Serum dilutions of 1:5 or higher giving a titre equivalent to 20 international CFT unit (ICFTU)/ml or more were considered as positive for the CFT (NRAG 586, 1982; OIE, 2016).

The 2-Mercaptoethanol (2-ME) test was performed with a *Brucella abortus* strain 99 antigen, diluted to 10% in saline buffer at 0.85%. One mL of 0.1M 2-ME solution was added to each of four glass tubes, each containing 0.08 mL, 0.04 mL, 0.02 mL and 0.01 mL of the sera to be analyzed. The tubes were incubated at 25 °C for 1 hour, and then, 1mL of the diluted antigen was added to each tube. Then, the tubes were incubated at 37 °C for 16-24 hours. The reading of the reactions followed the same standard observed for the SAL, but, in this case, the samples were considered positive from the 1:25 dilution (NRAG 586, 1982; OIE, 2016).

Sample collection

An approximately 10 mL blood sample was collected from the jugular vein of each animal. The sera were obtained after blood centrifugation at 600 x g for 5 min. They were kept at – 20 °C and thawed at room temperature before being analyzed (Geresu and Kassa, 2016).

Assays

The rapid immunochromatographic assay tested for bovine brucellosis diagnosis was the LFIA-PG manufactured according to the methodology developed by Díaz *et al*. (2015) (Fig. 1). The assay consists of a nitrocellulose (15 µm) detection strip flanked at one end by a reagent pad and at the other end by an absorption pad. A sample application pad flanks the reagent pad in turn. The composite strip is contained in a plastic assay device. The detection strip contains *Brucella* lipopolysaccharide (LPS) and is applied by using a BioDotQuanti 2000 BioJet, England.

A mixture of human immunoglobulin G (IgG h), purified by protein A-Sepharose affinity chromatography, and poly L-lysine (Sigma -Aldrich, SL, USA) was applied in a second line to function as a reagent control in the assay. The detector reagent (protein G-colloidal gold conjugate) was obtained by conjugating protein G (Sigma-Aldrich, SL, USA) to 40 nm diameter colloidal gold particles (British Biocell International, England), following the protocol described by Beesley (1989) and Hermanson (1996). The conjugate obtained was adjusted to an optical density of 0.220, at a wavelength of 520 nm, determined on a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific, USA).

It was then diluted in a migration buffer and sprayed onto the conjugate pads with the air jet aerosol dispenser device of the BioDotQuanti 2000_BioJet equipment. The conjugate pads were dried at 37°C for 30 minutes. The amounts of conjugate applied to the test strips were optimized using positive and negative reference samples. The detection reagents were applied to the conjugate pad by using a BioDotQuanti 2000 BioJet, England apparatus. The strip tests were assembled by mounting the sample application pad, reagent pad, detection strip, and absorption pad onto a rigid support. The strips were placed in a plastic assay device with a round sample well positioned above the detection strip. Finally, they were sealed in moisture-resistant foil envelopes containing silica gel bags.

The rapid immunochromatographic lateral flow test using protein G-colloidal gold (LFIA-PG), developed in this study, and the test developed by Díaz *et al*. (2015), using protein A-colloidal gold (LFIA-PA) as the detection reagent, were evaluated for detection of antibodies against *Brucella* spp. by the following process: Twenty µl of serum were added on the sample pad, followed by 130 µL of running buffer composed of saline solution - Phosphate (pH 7.6) with 1.67% albumin (Sigma-Aldrich, SL, USA) and 3% Tween 20 (Merck, KGaA, Germany). Both tests were evaluated in parallel with each of the reference panel samples.

The interpretation of the results was by visual inspection with the naked eye at 15- 20 minutes after the samples were applied.

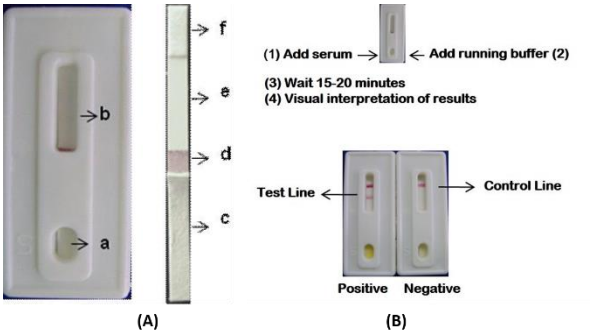

Fig. 1. Immunochromatographic lateral flow assay (ILFA) for brucellosis diagnosis: presentation, components and procedure. **(A):** Plastic assay device (left) containing a composite assay strip (right). (a): Sample application well; (b): Test and control window; (c): Sample application pad; (d): Conjugate pad; (e): Nitrocellulose membrane; (f): Absorption pad. **(B):** Procedure and result of the assay.

The test was considered valid when the control line appeared in all the cases. It was considered negative when only the control line appeared and positive when the two lines appeared.

Analysis of Results

The results obtained in each test were organized in contingency tables (Silva *et al*., 2006; Ochoa, 2013) for analysis. The diagnostic sensitivity and specificity, positive and negative predictive values, and efficacy of each test and the concordance between them were estimated. Contingency table 2 x 2 for the calculation of quality indicators:

Test to evaluate	Reference		
	Positive	Negative	Total
Positive	a	b	a+b
Negative	c	d	c+d
Total	a+c	b+d	n

Sensitivity: a / (a + c) x 100
a: True positive values
c: False negative values
Specificity: d (b + d) x 100
b: False positive values
d: True negative values
n: Total samples in the study
Positive predictive value (PPV): a / (a + b) x 100
Negative predictive value (NPV): d / (c + d) x 100
Efficiency: [(a + d) / n] x 100

For the Concordance study, the *kappa* index *(k)*was determined. It was based on the comparison of the expected concordance index (pe) with the observed concordance (po) indexes, and it was calculated as follows:

k = po-pe / 1-pe where:
po: a + d / n
pe: (P + N) / n
Concordance of the positive values (P):
[(a + b) / n x (a + c) / n] n
Concordance of negative values (N):
(c + d) - {(a + c) - P}

Results

The evaluation of the immunochromatographic assays (LFIA-PG and LFIA-PA) with a panel of bovine reference samples from DAVIH Laboratories (Mayabeque, Cuba) are shown in Tables 1 and 2.

Table 3 shows the results of the performance parameters of the LFIA-PG and LFIA-PA systems with the bovine samples studied, and Table 4 shows the concordance results between them.

Discussion

Diagnostic systems are essential for control and monitoring of diseases. In the case of the veterinary profile, an easy access to the target livestock to be controlled is necessary to give an early analysis of the infection and take control and eradication measures, particularly when the disease is a reemerging zoonosis, such as in the case of brucellosis (Saegerman *et al*., 2004; Abdoel *et al*., 2008; Shome *et al*., 2015). At present, several institutions are working on the design of those immunoassays that offer greater advantages in the veterinary diagnosis. An example of this is the development and application of lateral flow immunochromatographic tests (Abdoel *et al*., 2008; Díaz *et al*., 2015; Shome *et al*., 2015).

There is not a serological test appropriated for all the epidemiological situations; therefore, all the factors influencing in the relevance of the analytical method and the test results for a particular diagnostic interpretation or application must be taken into account. In the case of the rapid lateral flow immunochromatographic tests, even when they are simple and fast running systems, reliable and accurate results are reported (Smits *et al*., 2003; Genç *et al*., 2012; Díaz *et al*., 2015), which, together with the possibility of carrying them to the fields where the herds are (as field tests), make them an alternative to the conventional serological diagnosis of brucellosis (Smits *et al*., 2003; Nielsen *et al*., 2004).

In the development of the LFIA-PG system, the appropriate concentration of the protein G conjugate was obtained for printing on the pads, and the migration buffer and drying time at 37 °C were effective. With the application of this conjugate, IgG antibodies were detected, since protein G had the ability to bind specifically and with high affinity to the Fc fragment of IgG (Saegerman *et al*., 2004; Díaz *et al*., 2015). Genç *et al.* (2012) also used a G-gold colloidal conjugate for diagnosis of brucellosis in cattle and sheep with reliable results. . In addition, Saegerman *et al.* (2004) developed an ELISA-type immunoenzymatic assay for diagnosis of bovine brucellosis using monoclonal antibodies as capture elements and a protein G conjugate as detector reagent that, on previous studies, showed a high affinity of this protein for the IgG. Tables 1 and 2 show the results of the evaluation of LFIA-PG and LFIA-PA variants against 449 reference bovine serum samples.

Table 1. Results of the evaluation of the LFIA-PG system with bovine reference panel.

LFIA-PG	Reference Panel		
	Reactive Samples	Non-Reactive Samples	Total
Positive	111	13	124
Negative	0	325	325
Total	111	338	449

Table 2. Results of the evaluation of the LFIA-PA system with a bovine reference panel.

LFIA-PA	Reference Panel		
	Reactive Samples	Non-Reactive Samples	Total
Positive	106	9	115
Negative	5	329	334
Total	111	338	449

Table 3. Results of the performance parameters of the LFIA-PG and LFIA-PA systems according the bovine samples studied.

Performance Parameters	LFIA-PG	LFIA-PA
Diagnostic Sensitivity	100%	95.5%
Diagnostic Specificity	96.2%	97.3%
Efficacy	97.1%	96.89%
Positive Predictive Value	89.5%	92.3%
Negative Predictive Value	100%	98.5%
Kappa	0.95	0.95

Table 4. Concordance results between LFIA-PG and LFIA-PA systems.

LFIA-PG	LFIA-PA		
	Positive	Negative	Total
Positive	117	6	123
Negative	6	320	326
Total	123	326	449

kappa: 0.95.

LFIA-PG was found to be more sensitive than LFIA-PA because it identified all the positive samples, while the sensitivity showed by LFIA-PA was 95.5% (Table 3).

The specificity level was 96.2% for LFIA-PG and 97.3% for LFIA-PA, being slightly higher in the LFIA-PA variant. Similar results of sensitivity and specificity were obtained by Montasser *et al*. (2012) when they evaluated an immunochromatographic test on latex particles for diagnosis of brucellosis in cattle, sheep and goats in Egypt.

Elshemey and Abd-Elrahman (2014) also obtained 94.4% sensitivity and 100% specificity in the

evaluation of these parameters in a rapid test for *Brucella abortus* in bovine sera. Similarly, Abdoel *et al*. (2008) reported a sensitivity over 95% in the evaluation of a lateral flow system for the detection of brucellosis in cattle. Díaz *et al*. (2015) evaluated the LFIA-PA variant functionality with 206 bovine samples and obtained sensitivity and specificity values of 98.3% and 100%, respectively.

Immunoglobulin binding proteins, such as protein A, G, or recombinant protein A / G, have been used as valuable tools in diagnostic systems for the detection of anti-Brucella antibodies in different animal species (Elshemey and Abd-Elrahman, 2014; Nicola *et al*., 2014; Shome *et al*., 2015).

Protein A reacts more specifically with the IgG 2 subclass, whereas protein G reacts with both subclasses of bovine IgG (IgG 1 and IgG 2), which allows detecting antibodies at any time of infection (Díaz *et al*., 2015; Shome *et al*., 2015). In comparative immunological studies on affinity and specificity of proteins A and G, protein G showed a greater affinity for G-type bovine immunoglobulins (Nielsen *et al*., 2004; Pajuaba *et al*., 2010; Genç *et al*., 2012).

The present study showed the greater affinity of protein G for bovine immunoglobulins compared with protein A, observed in the results of sensitivity and specificity obtained by the LFIA-PG system. Sensitivity and specificity are essential parameters that allow making inferences from the results of an assay (Genç *et al*., 2011; Ochoa, 2013). Both parameters define the effectiveness of a system since they ensure that the proportion of the infected animals will give positive results to the test; however, the animals still infected give a negative result. We are then in the presence of a false negative result and in a situation in which the proportion of uninfected animals will give negative results.

When uninfected animals give a positive result, we are in the presence of false positive results (Genç *et al*., 2011). The efficacy in both variants was similar; however, the effect of the false results was different in both variants. With LFIA-PG, there were no false-negative results, and 13 false positives were identified with an efficacy of 97.1%. With LFIA-PA, 9 false positives and 5 false negatives were identified for an efficacy of 96.89%. This element should also be taking into account to decide the variant to use.. As previously stated, the fact that no false-negative results were identified means that all the positive samples were correctly identified with the LFIA-PG variant, which makes it effective for the detection of antibodies against *Brucella* spp.

Table 3 shows the results of VPP and VPN. In both tests, high VPP and VPN results were achieved. The analysis of the predictive values showed that the LFIA-PG variant showed a VPN of 100%, which made it suitable for the research. It is evident that the tests with a high negative predictive value are preferred for brucellosis screening because their use minimizes the probability for an infected animal to be identified as false negative.

The systems with a high positive predictive value are very useful for disease confirmation because the probability of an uninfected animal being identified as false positive is low (Silva *et al.*, 2006; Al Dahouk *et al*., 2013).

VPP and VPN are measurable parameters in the performance of the diagnostic methods. They can be modified drastically according to the prevalence of the disease and with some immunological, genetic or biochemical markers studied in a population. The calculation of these predictive values guides the researcher about the possible utility of the diagnostic method towards its use as a screening or confirmation system (Silva *et al.*, 2006).

Table 4 shows the concordance between the two variants. The concordance is a measurable parameter; it is the percentage of coincident results between two tests performed on the same sample (NRGA 586, 1982; Silva *et al.*, 2006). The *kappa* index is one of the parameters used when it is required an evaluation of two systems against the same reference sample panel (Saegerman *et al*., 2004). In this study, a *kappa* index of 0.95 was obtained, resulting in a very good concordance between both variants.

Other authors have also used the concordance as a statistical method for the analysis of their results, and it was the case of Díaz *et al*. (2015), when they compared LFIA-PA system with the Rose Bengal reference test, which is one of the most used tests in Cuba and worldwide (NRAG 586, 1982), to carry out Brucellosis screening with 206 samples of bovine serum. The value of *k* they obtained was 0.95, meaning a very good concordance. Silva *et al*. (2006) also obtained similar concordance results (98%) with 1178 bovine samples for a *k* of 0.98 when they used an ELISA assay based on the inhibition principle, designed for brucellosis serological diagnosis in different animal species.

Although brucellosis is a well-managed disease, it continues posing a threat to the public and veterinary health in both developed and developing countries (Beesley, 1989; Nielsen *et al*., 2007; Aranís *et al*., 2008). Reaching an effective diagnosis is one of the premises to achieve good management of the disease (Beesley, 1989; Al Dahouk *et al*., 2013; Moreno, 2014). Some authors (Saegerman *et al*., 2004; Silva *et al*., 2006; Aranís *et al*., 2008; Genç *et al*., 2012; Montasser *et al*., 2012; Nicola *et al*., 2014; Díaz *et al*., 2015) suggest to use diagnostic strategies with highly sensitive tests and less specificity for screening, and then use a more specific test to confirm positive animals.

Conclusions

The developed LFIA-PG system was capable of detecting antibodies against *Brucella* spp. Its diagnostic performance showed high values in the analyzed parameters compared with the LFIA-PA system. Performance results together with the ease and speed in the execution of the test make possible its use as a screening method for diagnosis of brucellosis, as well as for this disease surveillance and management in cattle. We recommend following up this study carrying out field evaluations in bovine populations with different epizootiological conditions, as well as in other livestock species of interest.

Conflict of interest

The Authors declare that there is no conflict of interest.

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