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MICROBIOLOGY

Fluorescent polarization assay, two versions of indirect and competitive enzyme- linked immunoassay for brucellosis diagnosis in vaccinated buffaloes (*Bubalus bubalis*)

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ABSTRACT: *The fluorescence polarization assay (FPA), two variants (V) of the indirect enzyme-linked immunosorbent assay (I-ELISA) and the competitive enzyme-linked immunosorbent assay (C-ELISA) were evaluated in buffaloes to detect antibodies against Brucella spp. The V1 of I-ELISA identifies them through the monoclonal (M23) anti-bovine IgG (I-ELISAM23) and the V2 through the ProteinA / G (I-ELISA-A/G). Serum samples of 862 buffaloes (Bubalus bubalis) from the Northeast of Argentina (NEA) were analyzed using the complement fixation test (CFT) as the reference. Receiving Operator Characteristic (ROC) analysis defined for the area under the curve (AUC) determined the cutoff points, sensitivity (Se) and specificity (Sp) for each test. CFT identified 107 positive and 755 negative sera. The best AUC (0.986), Concordance with CFT (96.3%) and kappa value (0.843) was obtained by I-ELISA A/G test. This assay showed the highest Se (95.33%) and C-ELISA the highest Sp (97%). FPA failed to measure the antibodies in 23 (2.65%) serum samples due to unsuccessful reading. I-ELISA M23 proved to be ineffective to diagnose brucellosis in bubaline sera. The four serological tests showed cutoff points lower than those standardized for bovines. As conclusion, I-ELISA A/G, C-ELISA and FPA with its limitations would be effective techniques for the diagnosis of brucellosis in buffaloes in the NEA, requiring an appropriate cut-off point to guarantee their maximum performance in this species.*
Key words: *brucellosis, buffaloes, serological diagnosis.*

Teste de polarização fluorescente, duas versões de imunoensaio enzimático indireto e imunoensaio competitivo para diagnóstico de brucelose em búfalos vacinados (*Bubalus bubalis*)

RESUMO: *O ensaio de polarização de fluorescência (FPA), duas variantes (V) do ensaio imunoenzimático indireto (I-ELISA) e o ensaio imunoenzimático competitivo (C-ELISA), foram avaliados em búfalos para detectar anticorpos contra Brucella spp. O V1 do I-ELISA os identifica através do IgG monoclonal (M23) anti-bovino (I-ELISAM23) e o V2 através da Proteína A / G (I-ELISA-A / G). Amostras de soro de 862 búfalos (Bubalus bubalis) do Nordeste da Argentina (NEA) foram analisadas usando o teste de fixação do complemento (CFT) como referência. A análise Receiving Operator Characteristic (ROC) definida pela área sob a curva (AUC) determinou os pontos de corte, sensibilidade (Se) e especificidade (Sp) de cada teste. A CFT identificou 107 soros positivos e 755 soros negativos. Os melhores valores de AUC (0.986), concordância com CFT (96.3%) e kappa (0.843) foram obtidos pelo teste I-ELISA A / G. Este ensaio mostrou a maior Se (95.33%) e C-ELISA a maior Sp (97%). O FPA falhou em medir os anticorpos em 23 (2,65%) amostras de soro devido à falha na leitura. O I-ELISA M23 provou ser ineficaz para o diagnóstico de brucelose em soros bubalinos. Os quatro testes sorológicos mostraram pontos de corte inferiores aos padronizados para bovinos. Em conclusão, I-ELISA A / G, C-ELISA e FPA com suas limitações seriam técnicas eficazes para o diagnóstico de brucelose em búfalos no NEA, exigindo um ponto de corte adequado para garantir seu desempenho máximo nesta espécie.*
Palavras-chave: *brucelose, búfalos, diagnóstico sorológico.*

INTRODUCTION

The buffalo population in Argentina, according to official data (SIGSA - SENASA, 2019), reaches 123,680 cattle heads. Buffaloes are bred in nineteen of the twenty-three provinces, with 80% of the stock being concentrated in the northeast region of Argentina (NEA), where herds of bovines and buffaloes coexist in farms. Although, they are endowed with great rusticity, they are affected by the same diseases as cattle, as occur with brucellosis (OLIVEIRA, 2011).

Brucellosis is a zoonotic infectious disease caused by bacteria of the genus *Brucella* that produces abortions in domestic and wild animals and a serious disease in humans (PAPPAS et al., 2006). There are several reports that indicate the presence of brucellosis in buffaloes around the world (PARADISO et al., 2018; MEGID et al., 2005). In Argentina, biovar 1 of *B. abortus* is the one that habitually infects buffaloes

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and bovines (SAMARTINO, 2002; LUCERO et al., 2008) and recently the presence of biovar 5 of *B. abortus* has also been described in buffaloes (MARTÍNEZ et al., 2014).

MARTINEZ et al. (2018) reported 10.94 % of buffaloes serologically positive to brucellosis in the NEA region. In Argentina, the strategy for the control of brucellosis in buffaloes is the same as that regulated for bovines and both species are under a National Plan for the Control and Eradication of brucellosis. This plan establishes the vaccination with *B. abortus* S19 of female bovines and female buffaloes between 3 and 8 months old and culling of all the positive animals detected by the official serological tests, performed from 18 months old in females or 6 months old in males (not vaccinated). The lipopolysaccharide (LPS) of smooth *B. abortus* elicit a long-lasting antibody response in both vaccinated and infected animals. Most of test based on the whole bacteria or LPS are unable to distinguish the origin of the infection and may lead to misdiagnosis. The administration of the vaccine to young animals, between 3 and 8 months of age, generally results in insufficient antibody levels to cause misdiagnosis by the time animals reach sexual maturity and are tested for brucellosis; however, some animals do have residual antibodies from vaccination (NIELSEN & YU, 2010). The tests officially recognized in Argentina for the diagnosis of bovine brucellosis are the buffered plate agglutination test (BPA) and the indirect enzyme-linked immunoassay (I-ELISA) both as screening tests; the tube agglutination test (SAT), 2-mercaptoethanol test (2ME), competitive enzyme-linked immunoassay (C-ELISA) and fluorescent polarization assay (FPA) as confirmatory tests, and the complement fixation (CFT) test as the reference test (SENASA, 2006).

In Argentina, BPA followed by FPA is the most frequent combination of tests used for the serological diagnosis of brucellosis in buffaloes, with the same criteria for interpreting results as that used for cattle. FPA has been evaluated in buffaloes in countries other than Argentina, by comparing their results with other serological or molecular techniques (MONTAGNARO et al., 2008).

ELISA tests have been also evaluated in buffaloes, demonstrating different performance than in bovine (ARIF et al., 2018). When I-ELISA is based on conjugates that react with bovine immunoglobulin, its performance for the diagnosis in buffaloes presents certain difficulties (PAULIN et al., 2012). Protein A from *Staphylococcus aureus* and protein G from group G *Streptococcus* spp are molecules able to nonspecifically bind the Fc region of the immunoglobulins from different animal species and have been used for the development of multispecies diagnostic tests (KUMAR & CHAND, 2011). An indirect ELISA based on these recombinant proteins conjugated with peroxidase (I - ELISA A / G) has been reported for brucellosis diagnosis (NIELSEN et al., 2004).

Since serological tests must be validated for each animal species before their implementation in a new region (OIE, 2016), the performance of FPA, the two variants of I-ELISA and C-ELISA for brucellosis diagnosis were evaluated in buffaloes from the NEA using CFT as “Gold standard”.

MATERIALS AND METHODS

Animals

Serum samples from 862 buffaloes between 2 and 15 years old, belonging to 11 herds from Corrientes (n=561) and 3 herds from Formosa (n=301), provinces in the NEA, were evaluated. The majority of buffalo females had received the *B. abortus* S 19 vaccine according to the sanitary regulations, between 3 and 8 months of age.

Serum samples

Blood samples were taken from the jugular vein or middle coccygeal, with disposable syringes and needles. Samples were centrifuged at 1200 x*g* for 10 min and the serum obtained was stored at -20 ºC until processing.

Convenience samples were taken from each farm as follows: a hundred percent of the animals were analyzed when the total was < 50 animals, 20% when there were between 50 and 500 animals, and at least 100 samples when the size of the herd exceeded 500 animals.

The negative population included buffaloes from herds where all animals were negative to brucellosis by CFT, and the positive population included positive buffaloes to CFT from herds with at least one animal with titers of 41 UIFC or more according to the cut-off recommended for vaccinated population (ALTON et al., 1975).

Control sera

A strong positive serum (C ++), a weak positive serum (C +) and a negative serum (C -) standardized against the OIE International Standard Serum anti-*B. abortus*, were included in duplicate in the evaluated serological tests (OIE, 2016).

Serological tests

None of the serological tests available in Argentina for the diagnosis of brucellosis in bovine have been standardized for their use in buffaloes. All tests were carried out following the official recommendations for cattle defined by The Animal Health National Service (SENASA, 2019).

a. Fluorescent polarization test (FPA)

The basis for the FPA is that the rate of rotation of a molecule in solution is inversely proportional to its size. A small molecule will rotate rapidly while larger molecules rotate more slowly. For brucellosis, FPA is based on the rotational difference between the soluble antigen (LPS) fluorochrome-labeled and the same molecule attached to the antibody when a plane of polarized light at an appropriate wavelength excites it (NIELSEN & GALL, 2001).

FPA is very accurate and the sensitivity/ specificity can be manipulated by altering the cut-off value between positive and negative reactions to provide a highly sensitive screening test as well as a highly specific confirmatory test. The FPA can distinguish vaccine-induced antibodies in most animals vaccinated between 3 and 8 month of age (NIELSEN & YU, 2010). A commercial kit (Biotandil®) was used according to the manufacturer specification. Readings were carried out in a polarization analyzer Fluorescent Sentry 200® (Diachemix, USA™). Results were expressed in millipolarization units (UmP) (NIELSEN & GALL, 2001).

b. Enzyme-linked immunosorbent assay (ELISA)

Two variants (V) of indirect ELISA (I-ELISA) and competitive ELISA (C-ELISA) were performed using the smooth lipopolysaccharide (sLPS) of *B. abortus* S1119-3 as antigen passively attached to polystyrene plates of 96 wells (NUNC 69620).

I-ELISA

I-ELISA was carried out according to OIE Manual of Diagnostic Standards and Tests (OIE, 2016). The test rely on the detection of the immune complex constituted by the sLPS and the specific antibodies present in the test serum, using a 'marker' molecule.

I-ELISA has a very high sensitivity, but because it is unable to distinguish antibodies generated by *B. abortus* S19 from those induced by wild-type strains, the specificity may be slightly lower. I-ELISA is a recommended screening assay for the diagnosis of brucellosis, particularly in individual animal tests of serum or milk (NIELSEN & YU, 2010).

In the V-1 (I-ELISA M23), the antigen-antibody reaction was revealed using a monoclonal antibody M23 (mAb M23) specific for bovine immunoglobulin G1 (IgG1), conjugated with horseradish peroxidase (HRPO). The mAb M23 has shown cross-reaction with IgG of other species as sheep and goats (HENNING & NIELSEN, 1992). In the V-2 (I-ELISA A/G), the antigen - antibody reaction was revealed using a recombinant Protein A/G (Pierce Biotechnology, Rockford, IL, USA) which has the ability to establish a non-specific binding with IgG from different animal species (NIELSEN et al., 2004), conjugated with HRPO (NIELSEN et al., 2004). Hydrogen peroxide (H_2O_2) and [2, 2'–azino-bis (3-ethylbenzene-thiazoline-6-sulfonic acid)] (ABTS) was used as chromogenic substrate and the reading was made at 405 nm. Results were expressed in percentage of positivity (PP) calculated by (OD_{405} of the test sample)/ (OD_{405} of C++) × 100, where OD_{405} is the optical density at 405 nm.

C-ELISA

This test was carried out according to OIE Manual of Diagnostic Standards and Tests (OIE, 2016). C-ELISA was based on the monoclonal antibody (mAb) M84 specific for the O-polysaccharide epitope of *B. abortus* S1119-3. The anti-*Brucella* antibodies present in the serum compete with the M84 to attach to the specific epitope. ELISA overcomes some of the problems arising from residual *B. abortus* S19 vaccine induced-antibodies. The mAb M84 has slightly higher affinity for the antigen than most of the vaccine induced- antibodies, but lower affinity than most antibodies arising from infection. The specificity of C-ELISA is very high and it is recommended as confirmatory assay (NIELSEN & YU, 2010).

A goat anti-mouse IgG1 conjugated to HRPO (Jackson Immunoresearch) was used as detector system. H_2O_2 ABTS was used as chromogenic substrate and the reading was made at 405 nm. Results were expressed in percentage of inhibition (PI) calculated by [(1 − $OD_{405}$of test sample)/ (OD_{405} of conjugate control)] × 100, where OD_{405} is the optical density at 405 nm.

Complement fixation tests (CFT)

It was standardized and carried out at 50% of hemolysis according to the OIE Manual of Diagnostic Standards and Tests (OIE, 2016) and used as reference test. CFT is standardized against the WHO Second International Standard Anti-*Brucella abortus* Serum (ISAbS). The principle relies on two antigen-antibody complex that fix complement, one

specific (*B. abortus* cells- IgG1 specific isotypes), and the second (3% of sheep erythrocytes- hemolysin) as detector system. If the primary immune complex were formed would consume the C‘, which will not be available to react with the second complex (the hemolytic system) resulting in little or no lysis of erythrocytes. Alternatively, if the primary immune complex were not formed, the C‘ would cause lysis of all sensitized sheep erythrocytes. Therefore, the amount of hemoglobin in solution is a measure of the activity of the anti-*Brucella* antibody (ALTON et al., 1975; OIE, 2016). The high specificity of the test is based on the ability of IgG1 isotype to fix C’ efficiently. The test does not discriminate between antibodies induced by *B. abortus* S19 and those generated by the wild types of *B. abortus*. CFT has been and is a widely used as a confirmatory test in control/eradication programs (NIELSEN & YU, 2010; OIE, 2016). The titer obtained (50% hemolysis) for each serum was expressed in International Units of Complement-Fixing antibodies (ICFTU) / mL and values ≥ 41 ICFTU were considered positive (ALTON et al., 1975; SENASA, 2019). Three categories were established according to the antibody titers, low (41-83 ICFTU), medium (106 and 331 ICFTU) and high (425-1323 ICFTU) level of antibodies.

Statistics

For the skill to discriminate positive and negative samples to FPA, I-ELISA in its two versions and C-ELISA, the Receiver Operator Characteristic (ROC) curve analysis (METZ, 1978) was used. The Area under the curve (AUC), the cutoff point, the sensitivity (Se) and specificity (Sp) were estimated by MedCalc software (SCHOONJANS, 2005). Agreement among tests and *kappa* value were estimated using the WinEpiscope program.

RESULTS AND DISCUSSION

The *in vitro* isolation of *Brucella* spp is the recommended gold standard to determine the Se of serological tests for brucellosis diagnosis (GODFROID et al., 2010). However, the difficulty to isolate this intracellular bacterium from the majority of infected cattle (OIE, 2016) obligate to use CFT as the most suitable gold standard (PAULIN et al., 2012).

From 862 buffaloes analyzed, CFT identified 107 positives and 755 negatives, which were categorized as brucellosis infected or uninfected respectively. The distribution of the positive samples according to antibody titers (CFT) categorized by ranges (ICFTU) resulted in 29 samples with low (41 -83 ICFTU), 22 with medium (106 - 331 ICFTU) and 56 with high (425 – 1323 ICFTU) antibody levels.

Based on the ROC analyses the FPA, I-ELISA A/G and C-ELISA showed satisfactory performance for brucellosis diagnosis, while I-ELISA M23 was unable to properly discriminate between the positive and the negative populations. The AUC (A) and the scatter diagrams (B) for each test are shown in figure 1.

The efficiency of the evaluated tests to discriminate positive/negative results related to CFT were analyzed by comparison of the technical criteria displayed in table 1. Although, the performance of FPA was elevated (AUC=0.952), its effectiveness was affected due to reading failure, since it did not allow defining the positive or negative status of 23 serum samples (2.67%) that were left with an inconclusive result. Reading failure using FPA were previously described and could be related to the riboflavin presence in serum that, as it was demonstrated in milk samples, generates a nonspecific fluorescence that interferes with that emitted by the fluorescein in FPA (GALL et al., 2002). Although, the concentration in milk is higher, riboflavin is also reported in the blood and tissues and would originate from chromogenic compounds present in foods such as corn under specific environmental and culture handling conditions (SOLARES JUÁREZ, 2003). Since inconclusive results imply in other sampling or complementary analyses, as long as this is not resolved FPA would be less useful than the other techniques, especially when used in inhospitable areas of the NEA.

The cut-off point for FPA (≥ 87 UmP) was lower than that standardized for cattle, where suspect status is given to individuals with values ≥ 94 Ump and ≤ 104 Ump and positive ≥ 105 UmP (SENASA, 2019). The distribution of the Ump values for FPA did not show an adequate polarization between positive and negative results, and there was a grouping of the negative values immediately below the cutoff point, which could affect the definition of the infection status of buffaloes. Despite the limitation described, the Se (85.39%) and the Sp (95.07%) of this technique indicated that could be used as confirmatory test, in the same way as recommended by the Argentine health legislation. Analogous result, were reported by MONTAGNARO et al. (2008) in Italy when they used FPA in buffalo and compared it with CFT, ROC analysis showed similar values of AUC (0.957), Se (92.6%) and Sp (91.2%) than those reported in the present research, although the cutoff point was higher (117 UmP). Conversely, PAULIN et al. (2012), using FPA vs. CFT and 2ME in buffaloes from Brazil,

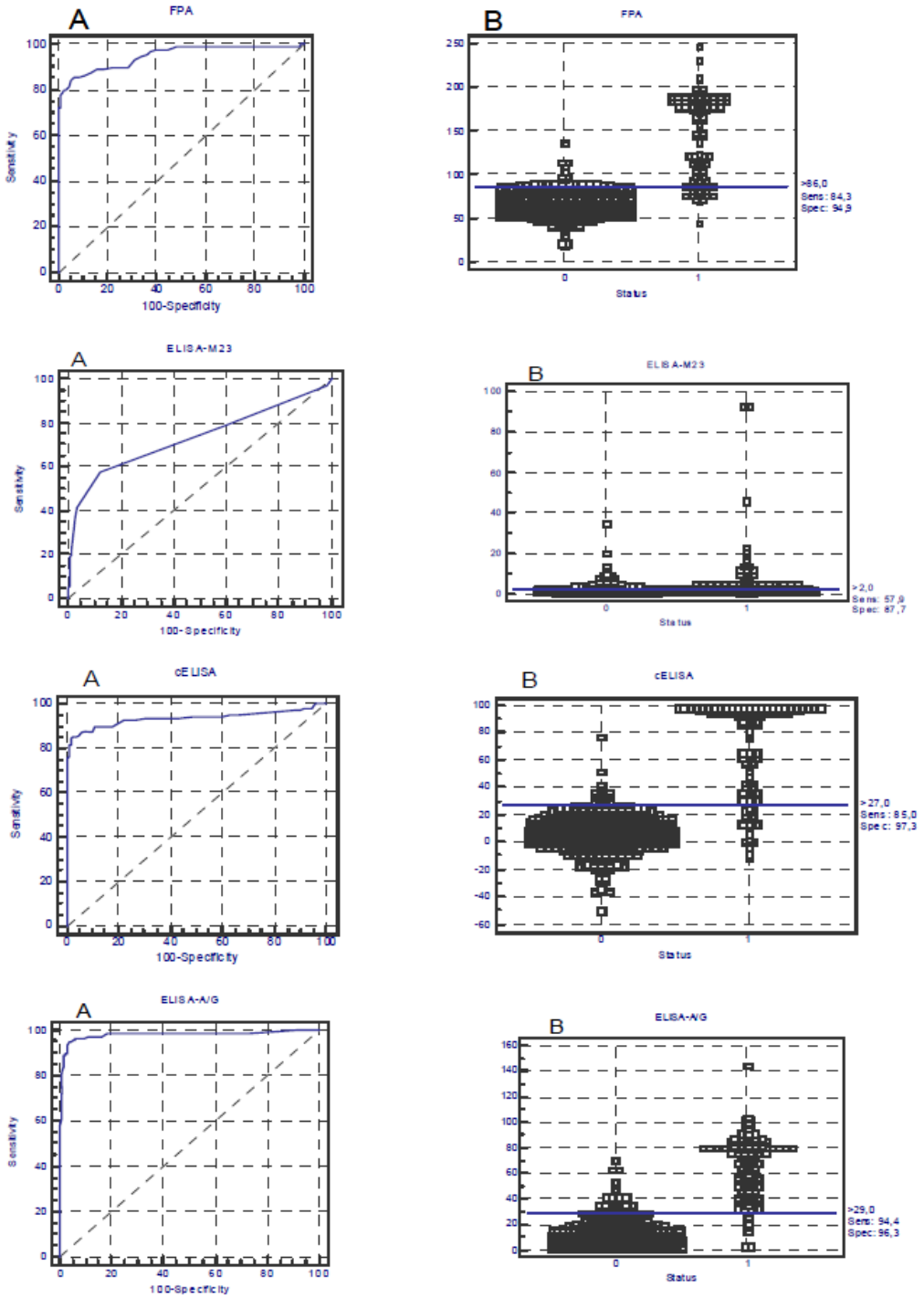

Figure 1 - Receiver operator characteristic (ROC) analysis. A. Area under the curve (AUC) and cut-off point determination to optimize the relative sensitivity and specificity of different serological tests related to complement fixation test (CFT) for the diagnosis of brucellosis in vaccinated buffaloes. B. Distribution of positive and negative results obtained by fluorescence polarization assay (FPA), two variants of the indirect enzyme-linked immunosorbent assay (I-ELISA M23, I-ELISA A/G) and the competitive enzyme-linked immunosorbent assay (C-ELISA).

Table 1 - Performance of fluorescence polarization assay (FPA), two variants of the indirect enzyme-linked immunosorbent assay (I-ELISA M23, I-ELISA A/G) and the competitive enzyme-linked immunosorbent assay (C-ELISA) for the diagnosis of brucellosis in 862 vaccinated buffalo sera, 107 positives and 755 negatives by complement fixation test (CFT) used as reference test.

Test (units)	FPA (Ump)	I –ELISA M23 (PP)	I –ELISA A/G (PP)	C –ELISA (PI)
Serum sample (n)	839*	862	862	862
AUC*	0.952 (0.935-0.965)	0.736 (0.706-0.766)	0.986 (0.975-0.993)	0.942 (0.924-0.957)
Cut-off	≥ 87	≥ 3	≥ 30	≥ 28
Positives mean (±SD)	132.97 (±43.9)	6.75 (±11.39)	64.55 (±22.74)	72.39 (±27.8)
Negatives mean (±SD)	68.94 (±10.39)	1.51 (±0.55)	7.80 (±6.17)	5.27 (±9.62)
Sensitivity (%)	85.39 (76.32-91.99)	58.88 (48.95-68.30)	95.33 (89.43-98.47)	85.98 (77.93-91.44)
Specificity (%)	95.07 (93.26-96.50)	87.81 (85.27-90.06)	96.42 (94.84-97.63)	97.48 (96.09-98.48)
Concordance with CFT (%)	94.04	84.2	96.3	96.1
Kappa value	0.719 (0.652-0.786)	0.392 (0.326-0.457)	0.843 (0.777-0.909)	0.821 (0.755-0.888)

* 23 samples could not be assessed because of reading failure.
AUC: area under the curve; UmP: millipolarization units; PP: % Positivity; PI: % Inhibition.

obtained a Se of 92.2% and an SP of 97.6% with a cut-off value > 104 UmP.

With regard to I-ELISA, there were marked differences when the variants were evaluated. The V-1 based on mAb M23-peroxidase shows less discriminative capacity (AUC=0.736) than the V-2 based on Protein A / G-peroxidase (AUC = 0.986). The A / G chimeric protein showed high affinity for binding with the bubaline IgG, as was described for other domestic and wild mammals (NIELSEN et al., 2004). I-ELISA A/G showed an adequate distribution between positive and negative results, with negative values grouped around 15 PP, while the cut-off point was ≥30 PP (Figure 1B). This test showed the highest Se (95.33 %) over all the evaluated techniques, a desirable characteristic for a screening test, in addition to the high Sp (96.42%).

Since C-ELISA reached the second best performance (AUC= 0.942) and the best Sp (97.48%) than the other tests and showed an adequate Se and scattering between positive and negative results, it is eligible as confirmatory test. Similarly to the other tests evaluated, the cut-off point for C-ELISA in buffaloes (≥ 28 PI) was also lower than that in bovines (≥ 40 PI) (SENASA, 2019). ARIF et al. (2018) studied brucellosis in buffaloes in Pakistan through the Bayesian analysis and showed that C-ELISA (Se= 77%, Sp= 99.4%) had better performance than an I-ELISA (Se= 51%, Sp= 98.8%). In a study of brucellosis in buffaloes in Brazil, PAULIN et al. (2012) reported the best performance of diagnosis using C-ELISA, with 96.9% Se and 99.1% Sp and a lower efficiency using I-ELISA with 64.1%Se and 71.1%Sp. The inclusion of the mAb specific for one epitope on the "O" chain of the LPS of *Brucella* spp, and the capability to detect antibodies in multiple species, without depending of an antibody specific of specie, provide to C-ELISA a high performance for the diagnosis of brucellosis (PAULIN et al., 2012).

The best concordance with CFT was observed for I-ELISA A/G (k=0.843) and C-ELISA (k= 0.821), while I-ELISA M23 showed the lower. The reduced efficacy of this test could be attributed to a low affinity between the buffalo IgG and the mAb M23. Although, this mAb had been developed against an epitope of the bovine IgG, it also recognizes the IgG in small ruminants (GALL et al., 2003). However, the identification of variants in the genomic sequences that encode the Ig in buffaloes could explain differences in the spatial conformation of the antibodies (KUMAR & CHAND, 2011) that would affect the binding with the mAb. The concordance of the four serological tests and CFT was greater as the CFT antibody titers (ICFTU) increased. FPA, I-ELISA A/G and C-ELISA reached 100% agreement when CFT antibody titers were between 425 – 1323 ICFTU (Table 2).

The persistence of residual antibodies after vaccination with *B. abortus* S19 could occur in inhospitable areas such as in the NEA, since the vaccination in cattle not always is possible to do before 8 months old. Only the administration of the vaccine to young animals, usually between 3 - 8 months of age, make possible to discriminate vaccinated from those naturally infected heifers, when

Table 2 - Concordance (%) between the positive results obtained by fluorescence polarization assay (FPA), two variants of the indirect enzyme-linked immunosorbent assay (I-ELISA M23, I-ELISA A/G) and the competitive enzyme-linked immunosorbent assay (C-ELISA) according to the antibody titers determined by Complement fixation test (CFT) in 107 positive serum samples from vaccinated buffaloes, except for FPA (n=89).

----------CFT----------	----------Concordance (%)----------			
n	FPA	I-ELISA M23	I-ELISA A/G	C-ELISA
29	59.3	37.9	82.8	55.2
22	88.9	31.8	100	90.9
56	100	80.4	100	100

ICFTU: International Units of Complement-Fixing antibodies.

they are analyzed at 18 months of age (NIELSEN &YU, 2010; SENASA, 2019). In a previous report, the extinction of the post vaccinal antibodies was detected earlier by C-ELISA and FPA than by I-ELISA and CFT; although, most of the positive animals showed low levels of complement-fixing antibodies (ICFTU) (AGUIRRE et al., 2002). This could explain the low concordance of FPA (59.3%) and C-ELISA (55.2%) related to CFT observed in this research in the smallest range of titles (48-83 UICFT) in the buffaloes. These results suggested that low titers of complement -fixing antibodies could be residual from vaccination, therefore C-ELISA and FPA could specifically discriminate between vaccinated and infected buffaloes. Seventeen weeks after vaccination with *B. abortus* S19, more than 90% of the calves were negative to C-ELISA, FPA and CFT and 75% to I-ELISA, what allowed to conclude that residual antibodies from vaccination were detected not only by I-ELISA and CFT but also by C-ELISA and FPA, though in a different proportion of bovines (AGUIRRE et al., 2002). The differences in the cut-off points obtained for each technique among different studies carried out in other regions of the world for the diagnosis of brucellosis, and between the cut-off points obtained for buffaloes and cattle in the NEA, support the OIE recommendation of validate serological techniques in the target species when tests are being introduced into a region.

CONCLUSION

I-ELISA A / G, C-ELISA and FPA with its limitations could be useful for the diagnosis of brucellosis in buffaloes in the NEA. The use of the appropriate cut-off points would guarantee the best performance of the tests in the context of the brucellosis control and eradication programs. I-ELISA M23 would not be suitable for the diagnosis of brucellosis in buffaloes.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

We authors of the article declared, for all due purposes, the project that gave rise to the present data of the same has not been submitted for evaluation to the Ethics Committee of the University /Research Institute "Universidad Nacional del Nordeste", but we are aware of the content of the Brazilian resolutions of the National Council for Control of Animal Experimentation - CONCEA "http://www.mct.gov.br/index.php/content/view/310553.html" if it involves animals.

Thus, the authors assume full responsibility for the presented data and are available for possible questions, should they be required by the competent authorities.

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