2012

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CIENCIA RURAL, SANTA MARIA, v. 42, n. 9, pp. 1621-1626, SEP, 2012
http://www.producao.usp.br/handle/BDPI/35436

Ciência Rural, Santa Maria, v.42, n.9, p.1621-1626, set, 2012
ISSN 0103-8478

Fluorescence polarization assay, competitive enzyme-linked immunosorbent assay (ELISA-C) and indirect ELISA for the diagnosis of brucellosis in buffaloes (*Bubalus bubalis)*

Teste de polarização fluorescente, teste imunoenzimático competitivo (ELISA-C) e ELISA indireto para o sorodiagnóstico da brucelose em búfalos (*Bubalus bubalis)*

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ABSTRACT

*The objective of the present study was to compare the performance of three serological tests for diagnosis of **Brucella abortus** infections in buffaloes (**Bubalus bubalis**). Serum samples collected from 696 adult females were submitted to the competitive enzyme-linked immunosorbent assay (ELISA-C), (I-ELISA), fluorescence polarization test (FPA), 2-mercaptoethanol test (2-ME) and complement fixation test (CFT). The **gold standard** was the combination of CFT and 2-ME, considering as positive the reactors in both CFT and 2-ME, and as negative those non-reactors. ROC analyses were done for C-ELISA, I-ELISA and FPA and the Kappa agreement index were also calculated. The best combinations of relative sensitivity (SEr) and relative specificity (SPr) and Kappa were given by C-ELISA (96.9%, 99.1%, and 0.932, respectively) and FPA (92.2%, 97.6 and 0.836, respectively). The C-ELISA and FPA were the most promising confirmatory tests for the serological diagnosis of brucellosis in buffaloes, and for these tests, cut-off values for buffaloes may be the same as those used for bovines.*

Key words: *serological tests, serodiagnosis, buffaloes, brucellosis.*

RESUMO

*O presente estudo objetivou comparar o desempenho de três testes para o sorodiagnóstico da **Brucella abortus** em búfalos (**Bubalus bubalis**). Soros de 696 fêmeas bubalinas adultas foram submetidos aos testes: teste imunoenzimático indireto (ELISA-I), teste imunoenzimático competitivo (ELISA-C), teste de polarização fluorescente (TPF), 2-mercaptoetanol (2-ME) e teste de fixação do complemento (FC). Foi empregada, como **gold standard**, a combinação de dois testes, FC e 2-ME. A curva ROC foi construída para os três testes: ELISA-I, ELISA-C e PF e, com base nos resultados desta análise, foi calculado o índice de concordância Kappa para cada teste. As melhores combinações de sensibilidade (Sr) e especificidade (Er) e os melhores resultados de Kappa foram alcançados pelo ELISA-C (96,9%, 99,1% e 0,932, respectivamente), seguido pelo PF (92,2%, 97,6%, e 0,836, respectivamente). Concluiu-se que os resultados dos estudos com os testes ELISA-C e PF em bovinos podem ser inferidos para búfalos com razoável segurança e que ambos demonstraram ser testes confirmatórios promissores para a espécie estudada.*

Palavras-chave: *teste sorológico, sorodiagnóstico, búfalo, brucelose.*

INTRODUCTION

Brucellosis is a very important disease for bovines and buffaloes due to the reproductive problems it causes (NASIR et al., 2004) and also the risk for public health. It is a barrier to the international trade of animals and animal products (OIE, 2009).

Bovine brucellosis has become the target of many control programs in several countries since the beginning of the 20th century. These control programs are based on the certification of brucellosis-free herds by a routine of serological tests and vaccination. The reactors are culled until two of more negative results are obtained for all animals. Therefore,

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Received 04.18.11 Approved 04.06.12 Returned by the author 07.10.12
CR-5172

the intrinsic characteristics of the tests are very important, because false-positive results lead to the sacrifice of healthy animals, and false-negatives may keep the infection sources inside the herd. Intense scientific efforts have been done toward the improvement of the methods used in the diagnosis of brucellosis. Because the economic interest on buffaloes is limited, the number of studies involving the species is very low.

The choices of tests used in brucellosis control programs consider performance, cost and simplicity. Performance is mainly based on the detection of IgG1 antibodies, the most prevalent immunoglobulin in naturally infected animals. The indirect and competitive ELISA (I-ELISA and C-ELISA), the complement fixation test (CFT) and the fluorescence polarization (FPA) have the lowest detection threshold for this class of antibodies (NIELSEN & DUNCAN, 1990).

The official tests used in the control and eradication program for bovine brucellosis in Brazil are buffered acidified plate antigen test (AAT) for screening, and for confirmation of positive results a combination of Wright test with 2-mercaptoethanol test (2-ME), and CFT or FPA has being used (BRASIL, 2010).

The 2-ME is efficient for cattle and buffaloes (PINTO et al., 2005; PAULIN, 2006). However, it is longstanding, requires a large volume of reagents and lab glassware, and uses a toxic reagent. Besides, 2-ME has to be carried out together with the tube seroagglutination test (SAL) because it does not detect IgM. Another disadvantage is related to the prozone phenomenon, which may produce false-negative results (PAULIN et al., 2009).

The CFT shows the best correlation with ***Brucella abortus*** isolation in naturally or experimentally infected animals (NIELSEN, 1995). NARDI JÚNIOR (2009) reported that vaccine antibodies interfere less with CFT than with agglutination tests. However, the test is cumbersome and requires specialized labor and strict quality control of the reagents. Besides, in rare situations, when sera show excess or predominance of IgG2 antibodies, reading may be similar to that of the prozone phenomenon, leading to false-negative results. This fact occurs because IgG2 does not fixate the complement but reacts with the antigen, preventing IgG1 from binding to it (CHAPPEL, 1989). In these rare cases, another test of high sensitivity and specificity, such as FPA or immunoenzymatic tests, would have an important role in the final analysis of the sample.

Differently from CFT, immunoenzymatic tests have high sensitivity and specificity, do not show the prozone phenomenon and can be automated. Disadvantages are related to the initial investment in the equipment and the impossibility, as in the other tests, of differentiating vaccinated from infected animals. Furthermore, if the process is not automated, it will take longer than AAT (MATHIAS, 2010).

The FPA is based on rotational differences between the soluble antigen and the complex antigen-antibody. It is quick and easy to be used, reagents do not have to be rinsed, and the equipment is portable (NIELSEN et al., 2001). The test was validated for the diagnosis of brucellosis in cattle, goats, pigs and wild animals (PAULIN, 2006). It requires a smaller volume of serum than conventional tests and is not affected by hemolysis (SAMARTINO et al., 1999). Moreover, NIELSEN et al. (1996) reported that FPA differentiates S19 vaccinated from unvaccinated animals. The downside is the investment in equipment and kits.

Herd certification process is based on the indirect diagnosis; therefore, due to the lack of research regarding the efficacy of diagnostic tests for detecting brucellosis in buffaloes, the objective of the present research was to compare the performance of I-ELISA, C-ELISA and FPA in the diagnosis of brucellosis in this species.

MATERIAL AND METHODS

Serum samples were collected from 696 Murrah female buffaloes in eight farms from Vale do Ribeira region, state of São Paulo, Brazil. All farms had history of reproductive failures compatible with the brucellosis and a history of irregular vaccination with the strain 19. All samples were tested with the AAT. Samples were analyzed by five different serological tests for brucellosis diagnosis, carried out simultaneously: 2-ME, CFT, I-ELISA, C-ELISA and FPA.

Antigen used in 2-ME and CFT was produced at *Instituto Biológico* according to the protocol by CEPANZO (1969). Antigen used in I-ELISA developed by the Institute for Animal Science and Health (IASH, 2000) to detect antibodies IgG against ***B. abortus*** in bovines. Antigen used in C-ELISA was LPS obtained from ***B. abortus*** 1119-3 produced at the *Instituto Nacional de Tecnología Agropecuaria* (INTA), according to the protocol by NIELSEN et al. (1998). Antigen used in FPA was developed by Diachemix Corporation and was based on the lipopolysaccharide LPS containing the O chain of ***B. abortus*** 1119-3 conjugated with fluorescein isothiocyanate.

2-ME was carried out according to ALTON et al. (1988) and the cut-off was set as for unvaccinated animals with the strain 19, according to the Brazilian

regulations (BRASIL, 2004) because, at the time of sampling, the S19 vaccination coverage of cattle and buffaloes in Brazil was very low (PAULIN & FERREIRA NETO, 2003). Dilutions 1:400 and 1:800 were added to 2-ME in order to prevent the prozone phenomenon.

CFT in microplate, and titration of hemolysin, complement and antigen were carried out as described by OIE (2010). Serum titers were obtained by determining the reciprocal of the greatest dilution in which 25% of the complement was fixated, and results were converted in international units (UI) using the standardized technique from the Central Veterinary Laboratory and based on international standard serum acquired from the same laboratory. Positive sera showed titers equal or greater than 20UI (MAFF, 1991).

Indirect Enzyme-Linked Immunosorbent Assay (I-ELISA) was carried out in Ceditest™ kit for ***B. abortus*** using bovine serum conjugate developed by the Institute for Animal Science and Health (IASH, 2000). Results were expressed as percentage of positivity - PP (WRIGHT et al., 1993).

C-ELISA was carried out as described by GALL et al. (1998). Results were also expressed as PP.

FPA was performed following the protocol developed by NIELSEN et al. (2001). Results were expressed in millipolarization units (mP). Cutoff values adopted by SAMARTINO et al. (1999) were used, considering the fact that animals could have been vaccinated with strain B19. Therefore, non-reactors were considered those animals showing values below to 94mP; results from 95mP to 104mP were considered to be inconclusive; and reactors were considered those sera showing values above 104mP, which is the criterion used for bovines.

According to MARTIN et al. (1987) and MATHIAS et al. (2010), the gold standard was made up of the combination of CFT and 2-ME. Animals classified as positive in both CFT (titer =20IU) and 2-ME (not vaccinated criteria) were considered to be positive gold standards and negative in both, CFT (titer <20IU) and 2-ME (not vaccinated criteria), were considered to be negative gold standards. Sera with inconclusive results for the 2-ME and with not agreeable results between CFT and 2-ME were excluded from the analysis. Thus, from the initial 696 tested sera, 650 were selected for ROC (receiver operating characteristic) analysis.

ROC analysis were done for C-ELISA, I-ELISA and FPA based on the gold standards, and generated cut-off values that optimized the results for SEr and SPr of each test. Cut-off values were also used in the calculation of Kappa agreement index between each test and the gold standards. All calculations were carried out in MedCalc free software (MEDCALC, 2011).

RESULTS AND DISCUSSION

In buffaloes, important studies were carried out by MATHIAS et al. (1998) and MOLNÁR et al. (2002) and MONTAGNARO et al. (2007). MATHIAS et al. (1998) evaluated serological tests using 465 serum samples collected from female buffaloes of unknown vaccination history. The positive animals to AAT and C-ELISA were considered to be the positive gold standards. The relative sensitivity (SEr) of C-ELISA was 100% and Kappa index, 0.97. MOLNÁR et al. (2002) analyzed serum samples from 440 adult female buffaloes and vaccination history was also unknown. The C-ELISA was considered the gold standard. SEr of I-ELISA, with an anti-bovine conjugate made up of monoclonal antibodies, was 98.6 and Kappa index, 0.93. SEr of C-ELISA2, a commercial C-ELISA, was 97.1 and Kappa index, 0.91. MONTAGNARO et al. (2007) evaluated the FPA test using 912 serum samples collected from female buffaloes. The CFT was considered the gold standard. SEr and relative specificity (SPr) for FPA were 92.6% and 88.9 percent and Kappa index, 0.715.

The results of ROC analysis for C-ELISA, I-ELISA and FPA are shown in table 1. The Kappa index are in the table 2. The gold standards were exclusively based on indirect diagnosis (2-ME and CFT), and did not incorporate the health status of the herds or direct methods of diagnosis. Because 2-ME has a range of results considered to be inconclusive – which were not used in the analysis - negative and positive gold standard groups were built with great stringency. However, the results allowed the comparison of I-ELISA, C-ELISA and FPA tests in relation to the gold standards.

The cut-off value suggested for the C-ELISA (41PP, Table 1) was almost the same (40PP) adopted for bovines by GALL et al. (1998) and the cut-off value suggested for the FPA (92, Table 1) was exactly the same adopted for bovines by NIELSEN et al. (1996).

The Institute for Animal Science and Health (IASH, 2000) recommended the percentage of positivity =45% to classify bovines as positive for brucellosis by the I-ELISA. Our results showed that if the same criterion is adopted for buffaloes, the SEr will be 4.7%. Cut-off value suggested in the ROC analysis was 7, leading to SEr and SPr values of 64.1% and 71.1%, respectively (Table 1).

Table 1 - Key data of the ROC analysis for competitive enzyme-linked immunosorbent assay (ELISA-C) indirect ELISA (I-ELISA) and fluorescence polarization test (FPA) used for diagnosis of brucellosis in buffaloes. Titers expressed as percentage of positivity (PP).

Titer(PP)	Relative sensitivity (SEr)	CI95% (SEr)	Relative specificity (SPr)	CI95% (SPr)	SEr.SPr
C-ELISA					
> 38	96.9	(89.1 - 99.5)	97.8	(96.1 - 98.8)	9476.82
> 39	96.9	(89.1 - 99.5)	98.5	(97.1 - 99.4)	9544.65
> 40	96.9	(89.1 - 99.5)	98.9	(97.6 - 99.6)	9583.41
> 41	96.9	(89.1 - 99.5)	99.1	(97.9 - 99.7)	9602.79
> 45	93.7	(84.7 - 98.2)	99.3	(98.1 - 99.8)	9304.41
> 46	92.2	(82.7 - 97.4)	99.3	(98.1 - 99.8)	9155.46
> 53	90.6	(80.7 - 96.5)	99.4	(98.4 - 99.9)	9005.64
I-ELISA					
> 4	93.7	(84.7 - 98.2)	30.4	(26.5 - 34.4)	2848.48
> 5	85.9	(75.0 - 93.3)	47.6	(43.3 - 51.9)	4088.84
> 6	68.7	(55.9 - 79.8)	62	(57.8 - 66.1)	4259.4
> 7	64.1	(51.1 - 75.7)	71.1	(67.1 - 74.9)	4557.51
> 8	53.1	(40.2 - 65.7)	79.6	(76.0 - 82.9)	4226.76
> 9	48.4	(35.8 - 61.3)	84.6	(81.3 - 87.6)	4094.64
> 10	40.6	(28.5 - 53.6)	88.9	(85.9 - 91.4)	3609.34
> 44	4.7	(1.0 - 13.1)	99.4	(98.4 - 99.9)	467.18
FPA					
> 89	92.2	(82.7 - 97.4)	95.6	(93.5 - 97.1)	8814.32
> 90	92.2	(82.7 - 97.4)	96.3	(94.3 - 97.7)	8878.86
> 91	92.2	(82.7 - 97.4)	97.2	(95.5 - 98.4)	8961.84
> 92	92.2	(82.7 - 97.4)	97.6	(95.9 - 98.7)	8998.72
> 93	90.6	(80.7 - 96.5)	98.1	(96.6 - 99.1)	8887.86
> 94	90.6	(80.7 - 96.5)	98.5	(97.1 - 99.4)	8924.1
> 95	87.5	(76.8 - 94.4)	98.7	(97.3 - 99.5)	8636.25

The best results of SEr, SPr and Kappa were observed for the C-ELISA (Tables 1 and 2). This excellent result can be explained because the conjugate used was directed against "O" chain, a specific part of the bacterial wall, and that's why, when applying the test, there is no difference in results between the sera of different species. MATHIAS et al. (1998) and MOLNÁR et al. (2002) reported similar values of SEr (100% for both) and SPr (95.5% and 99.3%, respectively) for this species, even though these authors have adopted different strategies to make up the gold standards.

The low performance observed for I-ELISA (Tables 1 and 2) can be related to the conjugate used, which was made up of anti-bovine polyclonal antibodies. The use of anti-buffalo IgG_1 monoclonal antibody could increase this performance. MOLNÁR et al. (2002), studying the diagnosis of buffalo brucellosis, reported a better performance for the I-ELISA when the conjugate used was a monoclonal anti-bovine IgG instead anti-bovine total IgG. GUARINO et al. (2001) reported great results with the anti-buffalo conjugate.

The FPA also presented a good performance, showing that it is also adequate for buffaloes (Tables 1 and 2). Similar values of SEr and SPr were also reported for bovines by DAJER et al. (1999), SAMARTINO et al. (1999) and MONTAGNARO et al. (2007).

Any comparison between SEr and SPr values obtained in this study and those reported by other authors should be drawn with caution, because of the differences in relation to the composition of the gold standards and cut-off values. Even variations among the techniques should be taken into account. In spite of that, the best combinations of SEr and SPr were observed by C-ELISA (96.9% and 99.1%, respectively) and FPA (92.2% and 97.6%, respectively).

CONCLUSION

The C-ELISA and FPA were the most promising confirmatory tests for the serological

Table 2 - Kappa index of enzyme-linked immunosorbent assay (ELISA-C) indirect ELISA (I-ELISA) and fluorescence polarization test (FPA) according to the ***gold standard*** based on the combination of complement fixation test and 2-mercaptoethanol test for diagnosis of brucellosis in buffaloes.

Test	Kappa	Interpretation according to LANDIS; KOCH (1977)
C-ELISA	0.932	almost perfect agreement
FPA	0.836	almost perfect agreement
I-ELISA	0.135	slight agreement

diagnosis of brucellosis in buffaloes, and for these tests, cut-off values for buffaloes may be the same as those used for bovines. However, due to the ease and fast of execution of FPA, this test may be more suitable to Brazil's PNCEBT than C-ELISA.

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