Original Article

COMPARISON OF MICROSCOPIC AGGLUTINATION TEST AND INDIRECT ELISA IN THE DIAGNOSIS OF BOVINE LEPTOSPIROSIS

COMPARAÇÃO DA SOROAGLUTINAÇÃO MICROSCÓPICA E ELISA INDIRETO PARA O DIAGNÓSTICO DA LEPTOSPIROSE BOVINA

Mariana Assunção de SOUZA[1]; Jacqueline Ribeiro de CASTRO[1]; Tatiane Cristina Fernandes TAVARES[1]; Pollyanna Mafra SOARES[1]; Mariane Pacheco dos SANTOS[1]; Muriell Ribeiro GANDA[1]; Dayane Olímpia GOMES[2]; Anna Monteiro Correia LIMA-RIBEIRO[2]

1. Master in Veterinary Science, Faculty of Veterinary Medicine, Federal University of Uberlandia, Minas Grais, Brazil. mari_assuncao@yahoo.com.br ; 2. Resident in Veterinary Medicine, Federal University of Uberlandia, Minas Gerais, Brazil; 3. Professor Dr., Faculty of Veterinary Medicine, Federal University of Uberlandia, Minas Gerais, Brazil

ABSTRACT: The objective of this research was to compare the results obtained by the Microscopic Agglutination Test (MAT) and indirect ELISA using outer membrane proteins (OMP) of the serovar Hardjo as antigen (ELISA/OMP-Hardjo). Ninety-three samples of blood serum from 3 to 6-year-old cattle of both sexes with no history of vaccination for leptospirosis were used. To perform the MAT, live cultures of 14 *Leptospira* serovars representing 11 serogroups of *L. interrogans* were used as antigen. Outer membrane proteins (OMP) of the serovar Hardjo (ELISA-OMP/Hardjo) were used as the ELISA antigen. Of the 93 samples tested by MAT, 37 (40%) were positive. None of the samples testing positive in the MAT were negative in the ELISA. Sensitivity was 100% and specificity 64%. A comparison of the MAT and ELISA-OMP/Hardjo tests showed 78% agreement and the Kappa index was 0.58 ($p<0.0001$). The ELISA-OMP/Hardjo proved to be a sensitive test for the diagnosis of bovine leptospirosis, indicating its potential use as a screening test and for epidemiological studies. The titration results obtained by MAT and by optical density (OD) in the ELISA-OMP/Hardjo test showed a positive correlation, and as the antibody titers in the MAT increased, so did the OD in ELISA, demonstrating the correspondence between the tests evaluated here.

KEYWORDS: Immunodiagnosis. *Leptospira* spp. Outer Membrane Protein.

INTRODUCTION

Leptospirosis is a zoonosis caused by pathogenic bacteria belonging to the genus Leptospira, which currently comprises 19 species and over 300 serovars. The serovar classification of Leptospira is based on the expression of surface-exposed epitopes in a mosaic of lipopolysaccharide (LPS) antigens, while the specificity of epitopes depends on their sugar composition and orientation (ADLER; MOCTEZUMA, 2010).

The disease is distributed worldwide, with higher frequency in tropical and subtropical regions, where environmental conditions favor the transmission and maintenance of the epidemiological chain (BLANCO et al., 2009). In bovines, this infection has serious economic implications, with losses resulting mainly from reproductive disorders such as infertility, miscarriage, birth of weak calves and temporary decrease in milk production (CERVANTES et al., 2002).

The diagnosis can be made by cultivating and isolating the agent, but this technique has some limitations. Due to the slow growth of *Leptospira*, a culture may take up to two months to allow for a definitive diagnosis (SURUJBALLI; MALLORY, 2004). Therefore, serological tests based on the detection of *Leptospira* antibodies are usually performed (WHO, 2008).

The reference test for the diagnosis of bovine and human leptospirosis is the Microscopic Agglutination Test (MAT), a complex assay using live *Leptospira* strains. The test has low sensitivity, especially in the initial phase of the disease (WHO, 2008; MCBRIDE et al., 2005). The ELISA (enzyme-linked immunosorbent assay) has been the most widely used serological test. Different antigenic moieties have been used (PRIYA et al., 2003; BOMFIM et al., 2005; EL JALLI, 2008), in order to obtain a specific antigen of higher sensitivity.

Several highly immunogenic proteins have been identified recently in the outer membrane of leptospira (CULLEN et al., 2004). These structures are considered potential targets for the development of new diagnostic tests (CULLEN et al., 2005). The largest class of *Leptospira* membrane proteins is composed of the lipoproteins LipL32, LipL21, LipL36, LipL48, and LipL41 (CULLEN et al., 2004; CULLEN et al., 2005; MATSUNAGA et al., 2006).

Received: 26/09/12
Accepted: 20/02/14

Because this disease has economic consequences resulting from the reduced reproductive efficiency of cattle herds, correct diagnosis and epidemiological surveillance are essential for its effective control. The objective of this research was to compare the results obtained by MAT against those obtained by indirect ELISA, using outer membrane proteins of the serovar Hardjo as antigen (ELISA/OMP-Hardjo) for the diagnosis of bovine leptospirosis.

MATERIAL AND METHODS

Samples: 93 samples of blood serum from 3 to 6-year-old cattle of both sexes with no history of vaccination for leptospirosis were used. The samples were supplied by the Laboratory of Infectious Diseases of the School of Veterinary Medicine (FAMEV), Federal University of Uberlândia (UFU).

Microscopic Agglutination Test (MAT): The *L. interrogans* samples were cultivated in Ellinghausen-McCullough-Johnson-Harris liquid medium (EMJH – DIFCO), to which was added 10% sterile rabbit serum free of anti-*Leptospira* antibodies, and the mixture incubated for seven days at 30°C.

The MAT was performed using as antigen live cultures of 14 serovars of *Leptospira* (Australis, Autumnalis, Bataviae, Bratislava, Canicola, Copenhageni, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Pomona, Pyrogenes, Tarassovi and Wolffi), representing 11 different serogroups of *L. interrogans*.

A dilution of 1:100 was used to determine the seroreactive animals, considering samples reactive when they presented agglutination equal to or higher than 50% (FAINE et al., 1999). After screening, these samples were diluted and examined until their final titers were defined. In cases of co-agglutination, the serovar that showed the highest serological title was adopted as the criterion of positivity.

Production of Antigen (OMP/Hardjo): Outer membrane proteins (OMP) of the serovar Hardjo (ELISA-OMP/Hardjo) were used as the ELISA antigen. The extraction was performed with the detergent Triton X114 (HAAKE et al., 1991), and the extracted proteins were precipitated with acetone (CUNNINGHAM et al., 1988).

ELISA-OMP/Hardjo: The indirect ELISA test was performed based on described modifications (TOMICH et al., 2007). Polystyrene microtiter plates were sensitized for 14h with 100 µL/well of the antigen (OMP/Hardjo) at a concentration of 0.08 µg/µL in carbonate bicarbonate buffer (0.05 M, pH 9.6) at 4ºC in a moist chamber. The plates were then washed manually three times using an automated micropipette with (PBS 0.01M pH 7.4), 200 µL of PBS-M blocking solution (5% skim milk powder diluted in PBS) were added and the plates incubated for 1h at 37°C. After further washing, 100 µL of bovine serum was distributed in duplicate, diluted 1:50 in PBS-TM (PBSM with 0.05% (v/v) Tween) and incubated for 1h at 37ºC. The plates were washed again with PBS-T (PBS containing 0.05% (v/v) Tween) and 100 µL of conjugated anti-bovine IgG labeled with peroxidase diluted 1:5.000 in PBS-TM were added for 1 hour at 37ºC. After additional washing, the reaction was developed by adding 100 µL/well of OPD (orthophenylenediamine – Sigma) supplemented with H_2O_2. After 15 min at room temperature without exposure to light, the reaction was interrupted by adding 25 µL of 4N H_2O_2 solution. Reactivity was evaluated based on optical density (OD) readings determined at a wavelength of 492 nm in a Thermoplate TP-Reader spectrophotometer.

The cutoff point was determined by the sum of the mean ODs of the negative samples plus two standard deviations (MADRUGA et al., 2001).

Statistical Analysis: The efficiency of the ELISA-OMP/Hardjo test was determined by calculating the relative values of sensitivity and specificity (THRUSFIELD, 2004), using the MAT results as reference. The degree of concordance was measured by the Kappa index (FERREIRA; ÁVILA, 2001). To evaluate the correlation of antibody titers obtained by MAT and ELISA optical density values, Pearson's correlation coefficient test was applied, using the statistical software BioEstat version 5.0.

RESULTS

Of the 93 samples tested by MAT, 37 (40%) were positive, with titers equal to or higher than 100, while 56 (60%) were negative. Table 1 presents the MAT data for the different serovars evaluated and the frequency of titers from positive reactions. Only eight of the 14 researched serovars were detected.

Table 1. Frequency distribution of positive results in the Microscopic Agglutination Test for different serovars of *Leptospira interrogans* in samples of bovine serum; Uberlândia MG, 2011.

Serovars	Results and serological titers					Total	(%)
	100	200	400	800	1600		
Canicola	1	1	-	-	-	2	5,4
Grippotyphosa	2	1	2	1	-	6	16,2
Hardjo	1	8	3	4	5	21	56,7
Hebdomadis	1	-	1	-	1	3	8,2
Icterohaemorrhagiae	2	-	-	-	-	2	5,4
Wolffi	1	-	-	-	-	1	2,7
Pomona	1	-	-	-	-	1	2,7
Tarassovi	1	-	-	-	-	1	2,7
Total	10	10	6	5	6	**37**	**100**

The negative serum samples showed a mean OD of 0.147 with a standard deviation of 0.048. The cutoff point of the ELISA-OMP/Hardjo test was 0.244. Of the 93 samples evaluated by ELISA-OMP/Hardjo, 57 (62%) tested positive and 36 (38%) negative. No sample that tested positive in the MAT was negative in the ELISA.

The relative sensitivity of the ELISA was 100% and specificity 64% (Table 2). The comparison of MAT and ELISA-OMP/Hardjo tests showed 78% agreement and the Kappa index 0.58 ($p<0.0001$). The agreement found in the Kappa analysis is good and suggests that the differences between the results obtained in the tests did not occur randomly.

The mean values of OD obtained in the ELISA-OMP/Hardjo test were 0.329, 0.504, 0.707, 0.851 and 1.215, corresponding to the titrations of 100, 200, 400, 800 and 1600 in the MAT.

Table 2. Comparison of the results of bovine serum samples tested by the MAT and the ELISA-OMP/Hardjo test for the serological diagnosis of bovine leptospirosis.

ELISA/OMP	MAT		
	Positive	Negative	Total
Positive	37	20	57
Negative	0	36	36
Total	37	56	93

Sensitivity = 100% = 64% Specificity = 78% concordance Kappa = 0.58

The analysis of the Pearson coefficient indicated a strong positive correlation between antibody titers obtained with MAT and the OD of ELISA ($r = 0.925$ and $p< 0.0001$), i.e., with increasing MAT titration, the OD values in ELISA-OMP/Hardjo also increased (Figure 1).

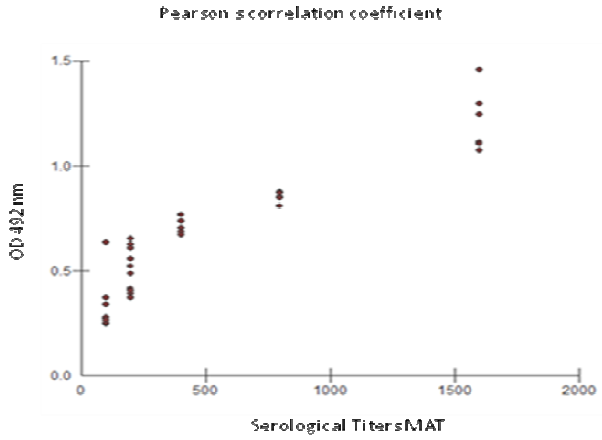

Figure 1. Graphic representation of optical density values obtained in the ELISA-OMP/Hardjo and antibody titers of the Microscopic Agglutination Test by Pearson's correlation coefficient.

DISCUSSION

Due to the complexity and drawbacks of MAT, the search for new ELISA-type diagnostic tests is constant (PALANIAPPAN et al., 2004). Proteins in the outer membrane of Leptospira are preserved among the different serovars (FAINE et al., 1999; LEVETT, 2001). Due to the similarity of the OMP of the serovar Hardjo with the membrane proteins of *L. interrogans* (LAFETÁ et al., 2008), as well as the prevalence of this serovar in bovines (TOMICH et al., 2007), these proteins were chosen for use as antigen in this assay. Although only the OMP of the serovar Hardjo was used as antigen in the ELISA test, it successfully identified the *Leptospira* reactive samples, regardless of the presumptive infecting serogroup.

Tomich et al. (2007), evaluated indirect ELISA for the diagnosis of bovine leptospirosis, using LipL32 as antigen. When they compared the results to those obtained with the MAT, they found that of 282 samples analyzed, 143 were positive in MAT and 161 in ELISA rLipL32. The sensitivity observed was 99.30% and the specificity was 86.33%.

These results demonstrate the high sensitivity of ELISA tests to recognize a greater number of reactive animals than the MAT. In the present study, the sensitivity observed was 100% and specificity was 64%. ELISA assays have proved to be highly sensitive, reproducible, and with a high degree of reliability, in addition to detecting antibodies earlier than the MAT (AHMAD et al., 2005).

Sakhaee et al. (2010), analyzed 355 samples of bovine serum from five dairy farms, using the MAT and ELISA techniques, and obtained 55 positive samples in MAT and 77 in ELISA. The authors also reported that 2.25% of the samples were reactive in MAT but negative by ELISA, and 8.45% were negative in MAT and positive in ELISA. In the present study, MAT showed 37 positive while ELISA-OMP/Hardjo showed 57 positive, and all the samples testing positive in MAT also tested positive in ELISA.

El Jalli (2008), performed an ELISA test using the antigen lipopolysaccharide (LPS) extracted from a *Leptospira* serovar Hardjo culture by heat shock, and found 100% sensitivity. Of the 170 samples of bovine serum examined, 47 tested positive in the MAT and 96 in the ELISA. Priya et al. (2003), used LPS from *L. biflexa* serovar Patoc in the ELISA test reported sensitivity of only 48%. The authors attributed this low sensitivity to the fact that the LPS was serovar-specific and suggested that the use of LPS from different serovars might enhance the sensitivity of the test.

Unlike the present study, Surujballi; Mallory (2004), developed ELISA and used as antigen a mixture of whole dead and sonicated cells from six *Leptospira* serovars, obtaining sensitivity of 93.5% and specificity of 94.7%. According to the authors, the use of a mixture of whole cells from several serovars allows for the detection not only of antibodies that cross-react against proteins but also antibodies to the immunodominant components specific to each individual serovar (LPS).

Bomfim et al. (2005), evaluated indirect ELISA in the diagnosis of bovine leptospirosis, using LipL32 as antigen, and compared the results to the MAT test, but found no difference in the results of the two tests. The authors also found that titrations of 1:100, 1:200, 1:400, 1:800 and 1:1600 in MAT yielded mean OD of 0.244, 0.242, 0.267, 0.249 and 0.365, respectively, in ELISA. In the present study, the MAT titration results showed a strong positive correlation with the mean values of OD in the ELISA-OMP/Hardjo test.

Because it is a technique with a high level of sensitivity and reproducibility, which can be automated, the ELISA is the method of choice for testing large numbers of samples (TIZARD, 2002). The requirement of a small volume of reagents and its biological safety are the main advantages of this test.

CONCLUSIONS

The ELISA-OMP/Hardjo test proved sensitive for the diagnosis of bovine leptospirosis, indicating its promising potential as a screening test and for epidemiological studies, since it recognized a larger number of reactive animals than the Microscopic Agglutination Test.

The results obtained by titration in the MAT and by the OD in the ELISA-OMP/Hardjo test showed a positive correlation, and the antibody titers in MAT and the OD in ELISA increased proportionally, demonstrating the analogousness of the tests.

ACKNOWLEDGMENT

The authors received financial support for the research, authorship, and/or publication of this article from the Brazilian research funding agency CNPq (National Council for Scientific and Technological Development).

RESUMO: O objetivo desta pesquisa foi comparar os resultados obtidos pela Soroaglutinação Microscópica (SAM) e pelo ELISA indireto empregando proteínas de membrana externa (PME) do sorovar Hardjo como antígeno (ELISA/PME-Hardjo). Utilizou-se 93 amostras de soro sanguíneo de bovinos de ambos os sexos com idade entre três a seis anos e sem histórico de vacinação para leptospirose. Para a realização da SAM utilizou-se como antígeno culturas vivas de 14 sorovares de *Leptospira*, representando 11 sorogrupos de *L. interrogans*. Empregou-se como antígeno do ELISA, proteínas de membrana externa (PME) do sorovar Hardjo (ELISA-PME/Hardjo). Das 93 amostras testadas na SAM, 37 (40%) foram positivas. Já o ELISA-PME/Hardjo identificou 57 (62%) positivas e 36 (38%) negativas. Nenhuma amostra positiva na SAM foi negativa no ELISA. A sensibilidade foi de 100% e especificidade 64%. A comparação dos testes de SAM e ELISA-PME/Hardjo demonstrou concordância de 78% e índice *Kappa* de 0,58 ($p<0,0001$). O ELISA-PME/Hardjo revelou-se como um teste sensível para o diagnóstico da leptospirose bovina, indicando seu uso potencial como exame de triagem e para estudos epidemiológicos. A correlação dos resultados obtidos pela titulação na SAM e DO no ELISA-PME/Hardjo foi positiva, sendo que a medida que os títulos de anticorpos na SAM aumentaram, a DO no ELISA também aumentou, demonstrando haver correspondência entre os exames avaliados.

PALAVRAS-CHAVE: Imunodiagnóstico. *Leptospira* spp. Proteína de Membrana Externa

REFERENCES

ADLER, B.; MOCTEZUMA, A. P. *Leptospira* and leptospirosis. **Veterinary Microbiology,** Amsterdam, v. 140, n. 3/4, p. 287-296, 2010.

AHMAD, S. N.; SHAH, S.; AHMAD, F. M. H. Laboratory diagnosis of Leptospirosis. **Journal of Postgraduate Medicine**, Mumbai, v. 3, p. 195-200, 2005.

BLANCO, R. M.; TAKEI, K.; ROMERO, E. C. Leptospiral glycolipoprotein as a candidate antigen for serodiagnosis of human leptospirosis. **Letters in Applied Microbiology**, Oxford, v. 49, p. 267-273, 2009.

BOMFIM, M. R. Q.; KO, A.; KOURY, M. C. Evaluation of the recombinant LipL32 in enzyme-linked immunosorbent assay for the serodiagnosis of bovine leptospirosis. **Veterinary Microbiology**, Amsterdam, v. 109, p. 89-94, 2005.

CERVANTES, L. P. M.; PUEBLA, M. A. C.; ROSAS, D. G.; SERRANÍA, N. R.; BARRANCA, J. I. T. Estudio serológico de leptospirosis bovina en México. **Revista Cubana de Medicina Tropical**, Ciudad de La Habana, v. 54, p. 24-27, 2002.

CULLEN, P. A.; HAAKE, D. A.; ADLER, B. Outer membrane proteins of pathogenic spirochetes. **FEMS Microbiology Letters**, Oxford, v. 28 p. 291-318, 2004.

CULLEN, P. A.; XU, X.; MATSUNAGA, J.; SANCHEZ,Y.; KO, A. I.; HAAKE, D. A.; ADLER,B. Surfaceome of *Leptospira* spp. **Infection and Immunity,** Washington. v. 73, n. 8, p. 4853-4863, 2005.

CUNNINGHAM, T. M.; WALKER, E. M.; MILLER, J. N.; LOVETT, M. A. Selective release of *Treponema pallidum* outer membrane and associated polypeptides with triton x-114, **Journal of Bacteriology,** Washington. v. 170, n. 12, p. 5789-5796, 1988.

EL JALLI, I. M. Comparison between ELISA and the Microscopic Agglutination Test for the Diagnosis of Bovine Leptospirosis. **Revue d'élevage et de medicine vétérinaire des pays tropicaux.** Montpellier Cedex 5, v. 61, n. 2, p. 73-75, 2008.

FAINE, S.; ADLER, B.; BOLIN, C.; PEROLAT, P. 2nd ed . Methods. *In*: Leptospira and Leptospirosis. **Medical Science Melbourne**, Austrália,1999. p. 169-184

FERREIRA, A. W.; ÁVILA, S. L. M. **Sorologia: importância e parâmetros. Diagnóstico Laboratorial das principais doenças infecciosas e auto-imunes**. 2ª Edição. Rio de Janeiro: Guanabara Koogan, p.1-8. 2001.

HAAKE, D. A.; WALKER, E.; BLANCO, D. R.; BOLIN, C. A.; MILLER, J. N. LOVETT, M. A. Changes in the surface of Leptospira interrogans serovar grippotyphosa during “in vitro” cultivation. **Infection and Immunity,** Washington , v. 59, n. 3, p. 1131-1140, 1991.

LAFETÁ, B. N.; SANTOS, S.; SILVA, V. L.; CARVALHO, M. A. R.; DINIZ, C. G.; SILVA, N. Determinação do perfil proteico da membrana externa da Leptospira interrogans sorovariedade Hardjoprajitno. **Arquivo Brasileiro Medicina Veterinária e Zootecnia,** Belo Horizonte **,** v. 60, n. 6, p. 1301-1306, 2008.

LEVETT, P. N. Leptospirosis. **Clinical Microbiology Reviews,** Washington **,** v. 14,n. 2, p. 296-326, 2001.

MADRUGA, C. R.; ARAÚJO, F. R.; SOARES, C. O. **Imunodiagnóstico em Medicina Veterinária**. Campo Grande: Embrapa Gado de Corte, 2001 360 p.

MATSUNAGA, J.; WERNEID, K.; ZUERNER, R. L.; FRANK, A.; HAAKE, D. A. LipL46 is a novel surface-exposed lipoprotein expressed during leptospiral dissemination in the mammalian host. **Microbiology**.Spencers Wood , v. 152, p. 3777-3786, 2006.

MCBRIDE, A. J. A; ATHANAZIO, D. A.; REIS, M. G.; KO, A. I. Leptospirosis, **Current Opinion of Infectious Diseases,** London, v. 18, n. 5, p. 376-386, 2005.

PALANIAPPAN, R. U.; CHANG, Y. F.; HASSAN, F. Expression of Leptospiral immunoglobulin-like protein by *Leptospira interrogans* and evaluation of its diagnostic potential in a kinetic ELISA. **Journal of Medical Microbiology,** Spencers Wood, v. 53, n. 10, p. 975-984, 2004.

PRIYA, C. G.; BHAVANI, K.; RATHINAM, S. R.; MUTHUKKARUPPAN, V. R. Identification and evaluation of LPS antigen for serodiagnosis of uveitis associated with leptospirosis. **Journal of Medical Microbiology,** Spencers Wood, v. 52, n. 8, p. 667-673, 2003.

SAKHAEE, E.; ABDOLLAHPOUR, G.; BOLOURCHI, M.; TABRIZI, S. S. Comparison between microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA) for detection of leptospiral antibodies in cattle. **Comparative Clinical Pathology,** London, v. 19, n. 1, p. 5-9, 2010.

SURUJBALLI, O.; MALLORY, M. An indirect enzyme-linked immunosorbent assay for the detection of bovine anti-bodies to multiple *Leptospira* serovars, **Canadian Journal of Veterinary Research,** Ottawa, v. 68, n. 1, p. 1-6, 2004.

THRUSFIELD, M. **Epidemiologia Veterinária**, 2. ed. São Paulo: Roca, 2004. 556 p.

TIZARD, I. R. **Imunologia Veterinária**, 6. ed. São Paulo: Roca, 2002. 532 p.

TOMICH, R. G. P.; BOMFIM, M. R. Q.; KOURY, M. C.; PELLEGRIN, A. O.; PELEGRIN, L. A.; KO, A. I.; BARBOSA-STANCIOLI, E. F. Leptospirosis serosurvey in bovines from brazilian pantanal using IgG ELISA with recombinant protein LipL32 and microscopic agglutination test. **Brazilian Journal of Microbiology,** São Paulo, v. 38, n. 4, p. 674-680, 2007.

WORLD ORGANIZATION FOR ANIMAL HEALTH (OIE), **Diagnostic techniques.** *In*: Leptospirosis, OIE, Paris, France, p. 252-255, 2008.