



Evaluation of the recombinant LipL32 in enzyme-linked immunosorbent assay for the serodiagnosis of bovine leptospirosis

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Abstract

The recombinant leptospiral protein LipL32 was evaluated for use in the diagnosis of bovine leptospirosis by enzyme-linked immunosorbent assay (rLipL32 IgG ELISA). The microscopic agglutination test (MAT) of 150 serum samples from cattle suspected of leptospirosis showed that 125 (83.3%) samples had positive reciprocal agglutination titres, which ranged from 100 to 1600. The highest titres were observed for the serovars Hardjoprajitno and Bratislava. In the rLipL32 IgG ELISA, 83.3% of the samples were positive. The sensitivity of IgG ELISA for 125 bovine sera, which had MAT titres of greater than or equal to 100, was 100%. ELISA showed a specificity of 100% with 58 bovine sera, which were negative at a 1:50 dilution in the MAT for *Leptospira interrogans* serovars. When analytical specificity of the IgG ELISA was evaluated using 60 bovine serum samples from animals showing serum antibodies to other pathogens that cause abortion in cattle, such as *Babesia* sp., *Anaplasma* sp. and *Brucella* sp. and no cross-reaction was observed. The recombinant LipL32 IgG ELISA can be an alternative to the MAT for diagnosis of leptospiral infection in cattle.

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1. Introduction

Leptospirosis is a worldwide zoonotic infection. In cattle, this disease causes agalactia, abortion, infertility, stillbirths, birth of weak calves, retention of fetal

membranes and can culminate in the death of the infected animal (Ellis, 1984; Thiermann, 1984b). Cattle can be infected by several pathogenic *Leptospira* serovars, although they are the maintenance hosts of the serovar Hardjo. Thus, cattle represent an important reservoir for the transmission of the infection to humans since they excrete live leptospire in their urine into the environment for prolonged periods (Ellis, 1984, 1994).

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The most commonly used serological test for diagnosis of leptospirosis is the microscopic agglutination test (MAT). However, this test has several disadvantages: it does not differentiate titres of infection from vaccine titres and fails to identify most chronic shedders (Thiermann, 1984a). In addition, it is laborious and is potentially hazardous to laboratory staff (Ellis, 1984). The diagnosis of bovine leptospirosis using ELISA has been directed at the detection of the serovar-specific antibodies. The antigens used for ELISA have been prepared using a variety of techniques, such as a hot phenol–water extraction (Thiermann, 1983a; Thiermann and Garret, 1983), sonicated whole leptospires (Adler et al., 1982; Cousins et al., 1985; Fairbrother, 1985; Bercovich et al., 1990; Surujballi et al., 1997b; Ramadass et al., 1999; Surujballi and Mallory, 2001, 2004), mechanical disruption (Trueba et al., 1990), detergent extraction (Cho et al., 1989; Goddard et al., 1991), acetic acid extraction (Surujballi et al., 1997a), ethanol extraction (Fairbrother, 1985) or dodecyl sulphate extraction (Cho et al., 1989; Goddard et al., 1991; Yan et al., 1999). Variations in the techniques used for preparation of antigens for the ELISA have contributed to the poor reproducibility of the results (Levett and Whittington, 1998). Development of a single specific antigenic reagent suitable for serological detection of infections with all serovars remains a great challenge. Recently, recombinant antigens have been produced using porin transmembrane protein (OmpL1), lipoproteins (LipL32, LipL36 and LipL41) and a heat-shock protein (Hsp58), all of which have been used in ELISA for serodiagnosis of human leptospirosis (Flannery et al., 2001). The LipL32 is a major leptospiral outer membrane protein whose expression is restricted to pathogenic *Leptospira* species (Haake et al., 2000). In this study, an IgG ELISA using the LipL32 recombinant protein as an antigen was evaluated for the diagnosis of bovine leptospirosis.

2. Materials and methods

2.1. *Leptospira* strains

Leptospira serovars were obtained from the reference laboratory of the Royal Tropical Institute

of Amsterdam, Holland. Leptospires were cultivated in semi-solid and liquid EMJH media (Ellinghausen and McCulloch, 1965).

2.2. Serum samples from cattle

Blood samples were obtained from cattle suspected of leptospirosis ($n = 150$) at a dairy farm in the state of Minas Gerais, Brazil. Clinical suspicion was based on finding reproductive problems: repeat breeding after artificial insemination or natural breeding, spontaneous abortion, birth of weak calves or mummified foetuses and mastitis. These animals had not been vaccinated against leptospirosis and had no clinical signs of active disease. Bovine blood samples were collected by puncturing the jugular vein with a sterile needle under aseptic condition. The animals received humane care as outlined in the “Guide for the Care and Use of Laboratory Animals” of the National Research Council (1996). The blood samples were incubated at room temperature until coagulation was complete. Sera were collected, aliquoted and stored at -20°C . Control serum samples were obtained from healthy cattle ($n = 33$) at a different farm and from cattle with other diseases than leptospirosis ($n = 60$). These samples were comprised of sera from animals with positive serology for babesiosis ($n = 24$) and anaplasmosis ($n = 21$) as confirmed by an indirect immunofluorescence test. Sera positive for brucellosis ($n = 15$) were tested by the Card-Test and the 2-Mercaptoethanol Test. Control sera were tested for leptospiral antibodies with the MAT (1:50 dilution) to determine that animals were not positive for leptospirosis. These animals had not been vaccinated against leptospirosis and showed no clinical signs of the disease.

2.3. Microscopic agglutination test

The microscopic agglutination test (MAT) was carried out according to Faine (1982), using reference strains of 21 *Leptospira interrogans* serovars: Australis, Autumnalis, Ballum, Bataviae, Bratislava, Canicola, Grippotyphosa, Hardjobovis, Hardjoprajitno, Hebdomadis, Icterohaemorrhagiae, Javanica, Pyrogenes, Pomona, Tarassovi, Wolffii, Castellonis, Sejroe, Copenhageni, Djasiman and Shermani. Reciprocal agglutination titres of greater than or equal to 100 were considered positive reactions.

2.4. Enzyme-linked immunosorbent assay

Recombinant LipL32 (rLipL32) was purified as previously described (Haake et al., 2000) from *Escherichia coli* transformed with *L. interrogans* serovar Copenhageni LipL32 in the pRSET expression vector (Invitrogen). Flat-bottomed polystyrene microtitre plates (Corning) were coated with 50 μ l of the rLipL32 (5 ng/well) suspended in 0.05 mM sodium carbonate buffer (pH 9.6) (Flannery et al., 2001). Each plate was incubated under constant agitation for 2 h at room temperature. The plates were washed twice with distilled water and three times in wash solution (PBST: PBS (pH 7.4, 0.01 M) with 0.05% (v/v) Tween 20). Plates were incubated with a blocking solution of 1% (w/v) bovine albumin with a 99% grade of purity (Sigma) in PBST for 4 h at room temperature. After three washes with PBST, the plates were stored at -20° until used. Each sample of sera was diluted 40-fold in blocking solution and 100 μ l of this solution was added per well. The plates were incubated for 1 h at room temperature with agitation. After three washes with PBST, each well was incubated with 100 μ l of a 40,000-fold dilution of anti-bovine γ -chain rabbit antibodies conjugated to horseradish peroxidase (Sigma) previously diluted in PBST for 1 h at room temperature with agitation. Subsequently, the plates were washed three times with PBST and twice with PBS. Orthophenylenediamine (Sigma, 20 mg) was dissolved in 50 ml of 0.03% (v/v) hydrogen peroxide, 25 mM citric acid, 50 mM Na_2HPO_4 (pH 5.0). Fifty microlitres of this solution was added per well and the plate was incubated for 20 min in the dark at room temperature. The colorimetric reaction was stopped by adding 25 μ l of 2 M H_2SO_4 per well, and the optical density (OD) of each assay was measured at 492 nm

using an ELISA reader (TITERTEK MULTISKAN (MCC/340-MKII).

Initial assays were performed to determine the ideal serum dilution using 28 samples of bovine serum that were positive in the MAT (a reciprocal titre of ≥ 100). Sera were diluted in serial two-fold dilutions from 1:10 to 1:1280. The best performance was obtained with sera samples diluted at 1:40. Individual serum samples were tested in duplicate on three different plates, and the mean of the measurements was calculated for analysis. The serum that showed the highest value of absorbance at this dilution was chosen as a positive control. The cut-off point for the IgG ELISA was defined as OD equal to 0.159 among serum samples ($n = 25$) from those animals, which were negative in the MAT.

2.5. Statistical analysis

Statistic analysis was done using the χ^2 tests with the SAS Statistics Programme, Version 5. Sensitivity was defined as the capacity of IgG ELISA to identify positive sera samples which were also positive on MAT, while specificity was defined as the capacity of IgG ELISA to identify negative sera samples which were also negative on MAT.

3. Results

3.1. Microscopic agglutination test

Out of 150 bovine serum samples submitted to the MAT, 125 were positive (83.3%) with titres that varied from 100 to 1600. Among these, 28 showed a titre of 100 (22.4%), 31 a titre of 200 (24.8%), 33 a titre of 400

Table 1
Results of the MAT and rLipL32 IgG ELISA for bovine leptospirosis diagnosis

MAT negative titres (<100)	MAT positive		IgG ELISA positive		IgG ELISA negative	
	No. of samples (%)	Titres (≥ 100) ^b	Mean OD \pm S.D. ^a	No. of samples (%)	Mean OD \pm S.D. ^a	No. of samples (%)
25 (16.6%)	28 (22.4%)	100	≤ 0.244 (0.053)	16 (57.1%)	≤ 0.105 (0.018)	10 (40.0%)
	31 (24.8%)	200	≤ 0.242 (0.069)	19 (61.2%)		
	33 (26.4%)	400	≤ 0.267 (0.094)	20 (60.6%)		
	19 (15.2%)	800	≤ 0.249 (0.060)	10 (52.6%)		
	14 (11.2%)	1.600	≤ 0.356 (0.182)	8 (57.1%)		

^a Means of the means of the ODs of three different days of experiments and standard deviation (S.D.).

^b Maximum reciprocal MAT titres.

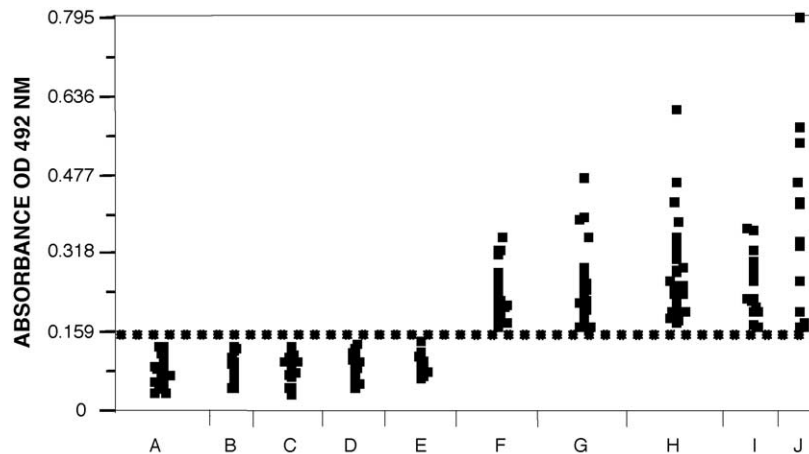


Fig. 1. IgG antibody response to the recombinant leptospiral protein (LipL32) 5 ng/well in the ELISA in cattle suspected of leptospirosis and control groups. Serum samples from healthy animals (A); other diseases such as brucellosis (B); babesiosis (C); anaplasmosis (D) and serum samples from 25 animals with MAT < 100 (E); 28 with MAT = 100 (F); 31 with MAT = 200 (G); 33 with MAT = 400 (H); 19 with MAT = 800 (I); and 14 with MAT = 1600 (J). The defined cut-off value of 0.159 is shown as a dashed line.

(26.4%), 19 a titre of 800 (15.2%) and 14 a titre of 1600 (11.2%) (Table 1). Out of 21 *Leptospira* serovars used in the MAT, six showed no reaction with any of the tested sera. The antibodies most frequently found in the MAT were for serovars: Hardjoprajitno (33.6%), Tarassovi (8.8%), Grippytyphosa (6.4%) and Wolffi (4%). The highest agglutination titres observed were for serovars Hardjoprajitno and Bratislava.

3.2. Determination of cut-off values

The cut-off value for interpretation of the IgG ELISA was determined by the mean OD obtained with the 25 negative sera on MAT from the herd studied. They were submitted to ELISA at a dilution of 1:40, and each reaction was repeated on different days. The average OD was 0.105 and the value for the standard deviation was 0.018. The final average plus three times the standard deviation determined that the cut-off point for the reactions was OD 0.159. All 150 serum samples were tested in duplicate on three different plates. In Table 1 were shown the means of the means of the ODs from three different days of experiments, as well as the number and percentage of serum samples, which were within these means. Fig. 1 shows the results obtained with the IgG ELISA when considering a positive reaction an OD value greater or equal to 0.159. The average of the ODs of all serum

samples tested in IgG ELISA were distributed according to results shown in the MAT.

3.3. ELISA performance

The sensitivity of the IgG ELISA was calculated using the χ^2 tests and the MAT as a reference standard. The 125 bovine sera that had reciprocal MAT titres of higher than or equal to 100 for 15 serovars were used to estimate the sensitivity of the ELISA relative to that of the MAT. The sensitivity of this IgG ELISA with the rLipL32 antigen relative to the MAT was 100%. The analysed results were considered statistically significant ($p < 0.05$).

Sera that were negative to different serovars in the MAT were used to determine the specificity of the IgG ELISA. The analytical specificity was 100% for 60 sera from cattle positive for other diseases, such as babesiosis, anaplasmosis and brucellosis and the specificity was 100% for 33 sera from healthy cattle.

4. Discussion

The method most commonly used for the diagnosis of leptospiral infection in animals and humans is the detection of specific serum antibody using the microscopic agglutination test (MAT). Culture isola-

tion shows better sensitivity to detect leptospires in cattle, but it is difficult to perform and is often unsuccessful (Thiermann, 1983b). ELISA has been used previously to assay antibodies against leptospires in cattle using a variety of techniques to prepare antigens. This paper describes the use of an indirect IgG ELISA, using the recombinant LipL32 antigen for the detection of bovine anti-leptospire antibodies. ELISA with the recombinant LipL32 antigen developed in this study had a sensitivity of 100% relative to the MAT using bovine sera that showed MAT titres ≥ 100 when different serovars were tested. The sensitivity found in this study is higher than that reported by Flannery et al. (2001) who evaluated the same recombinant antigen for the diagnosis of human leptospirosis. Recently, the recombinant protein LipL32 IgG ELISA has been used for serodiagnosis of canine leptospirosis (Dey et al., 2004). The sensitivity and specificity obtained was of 97% relative to MAT. In this study, the ELISA had an specificity of 100% relative to the MAT when 58 MAT-negative sera were used and analytical specificity was 100% when sera from animals with other common bovine diseases, such as babesiosis, anaplasmosis and brucellosis were tested. Together, these findings suggest that rLipL32 antigen is a specific, sensitive and practical test for the detection of antibodies against bovine leptospiral infection.

In the present work, the highest MAT titres were observed against serovars Hardjoprajitno, and Bratislava. In seroepidemiological studies carried out by Vasconcellos et al. (1997) in the state of Minas Gerais, the highest titres were observed for serovars: Hardjo, Wolffi, Pomona and Grippotyphosa.

In this study, the use of IgG ELISA was reported for the first time with the recombinant LipL32 antigen for the detection of leptospiral antibodies in serum samples from cattle suspected of leptospirosis. The advantages of using the recombinant LipL32 antigen in the diagnosis of human and animal leptospirosis include: high sensitivity and specificity, reproducibility of 100% when performed under the conditions described, faster and easier to perform, particularly for testing large numbers of samples, safe since it eliminates the preparation of whole cell antigenic extracts of leptospires (which eliminates all the extensive quality control required to monitor large

volumes of cultures), the stability of antigens and an objective interpretation of results.

The recombinant leptospiral protein LipL32 was prepared from *L. interrogans* serovar Copenhageni. Serum from cattle used in the present study demonstrated antibodies for different serovars in the MAT and showed in the IgG ELISA 100% of sensitivity and specificity. The conserved nature and high level of expression of LipL32 among pathogenic *Leptospira* spp. (Haake et al., 2004) suggest that rLipL32 ELISA may exhibit similar performance regardless of the locally predominant serovar. Thus, use of the recombinant LipL32 antigen in ELISA has the potential to become a useful tool for serodiagnosis of bovine leptospiral infection.

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