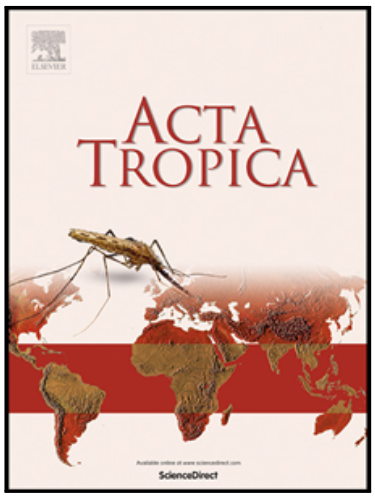

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Comparison of ELISA using recombinant LipL32 and sonicated antigen of leptospira for detecting bovine leptospirosis

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Abstract

Leptospirosis is one of the most widely distributed zoonosis in the world. Bovine leptospirosis is a serious problem in bovine production, causing reproductive losses. The aim of this work was to compare recombinant LipL32 with sonicated antigen for detecting anti-Leptospira IgG antibodies in bovine serum using ELISA. The Microscopic Agglutination Test (MAT) is used as the gold standard. Sonicated antigen from cultures of *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar copenhageni (strain M20) was used for the eELISA and rLipL32 for the rELISA. The performance of these assays was evaluated using serum samples from 166 bovines, 69 MAT positive and 97 MAT negative. At the optimal cut-off point recommended by the receiver operating characteristic (ROC) curve analysis, the sensitivity and specificity values were 98.6% and 97.9%, respectively, for eELISA, and 85.5% and 86.6% respectively, for rELISA. The value for the area under the ROC curve was 0.998 (0.994-1.0) (CI 95%) for eELISA and 0.929 (0.891-0.968) (CI 95%) for rELISA. The ROC curves for rLipL32 and sonicated antigen showed statistically significant differences (z = -3.826; p = 0.000). A three-way comparison showed statistically significant differences in the sensitivity and specificity of rELISA and eELISA. Our results showed that eELISA was more specific and sensitive than rELISA. The difference in performance (eELISA-rELISA) was 13.4% (4.03-23.28) (CI 95%) for sensitivity and 11.34 % (4.07-19.56) (CI 95%) for specificity. Our results show that the eELISA has a better diagnostic performance than rELISA for the detection of anti-Leptospira IgG antibodies in bovine serum.

Keywords

Bovine, ELISA, Leptospirosis, rLipL32, sonicated antigen

1. Introduction

Leptospirosis, a zoonosis widespread throughout the world, is caused by pathogenic bacteria of the genus *Leptospira* (Adler and Faine, 1983). Bovine leptospirosis

is a serious problem in bovine production, causing abortions, reduced milk yield, mortality in calves and decreased daily weight gain (Monte et al., 2015; Rocha et al., 2017). In Argentina, bovines are hosts of several Lepstospira serovar strains, mainly Pomona, Canícola, Wolffi and Grippotyphosa, and maintenance hosts of serovar Hardjo (Draghi et al., 2011, 2006).

The reference serological diagnosis of the infection has relied on the microscopic agglutination test (MAT) (OIE 2019); however, this technique is complex, time-consuming, and requires specific equipment, highly trained staff and the live cultures of several reference strains of Leptospira for use as antigens (De Souza et al., 2014).

Early detection of animals with symptoms compatible with leptospirosis can help to implement control measures of the disease (Soo et al., 2020). ELISA is ideal for demonstrating the absence of infection in the herd, contributing to leptospirosis eradication policies and determining the immunological status in animals or populations after vaccination (OIE 2019).

Given MAT drawbacks, sensitive techniques such as ELISA have been developed as alternative methods to detect leptospiral antibodies using a single serum dilution (Bomfim et al., 2005; Bourhy et al., 2013; De Souza et al., 2014; Dey et al., 2004; Hartleben et al., 2012; Lottersberger et al., 2002; Musso and La Scola, 2013; Penna et al., 2017; Zhylkibayev et al., 2018). ELISA and other immunochemical tests have used whole-cell and recombinant leptospiral antigens as an approach to the screening of leptospiral infection (Bomfim et al., 2005; Bourhy et al., 2013; Hartleben et al., 2012; Lottersberger et al., 2002). The ELISA for detecting bovine leptospirosis is not currently standardized and shows substantial differences in the coating antigen (bacteria vs. recombinant protein) (Chen et al., 2018).

Recombinant surface proteins or lipoproteins have been used as antigens in ELISA to detect Leptospira-specific antibodies. In general, the antigens used for ELISA may not cover the full diversity of circulating strains, resulting in a limited sensitivity (Chen et al., 2018). In this work, we performed a comparative analysis of two different ELISA to detect Leptospira-specific antibodies: one that uses recombinant LipL32 as antigen and the other that uses sonicated antigen of *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar copenhageni strain M20.

2. Materials and methods

2.1 Samples of bovine serum

Serum samples were obtained from the laboratory of the National Institute of Agriculture and Livestock Technology (INTA), Veterinary Research Center, Institute of Veterinary Pathobiology, Buenos Aires, Argentina. They were collected from the Argentine Pampas region between 2017 and 2019. A total of 166 bovine serum samples were used; 69 MAT-positive sera for leptospirosis and 97 MAT-negative sera for leptospirosis**.** For this comparative study, high MAT titres were selected (MAT ≥1 / 1600) to ensure that the antibodies were associated with the disease and not with the vaccine(OIE, 2014). Vaccination status is unknown of these bovines.

2.2 Microscopic agglutination test (MAT)

MAT was performed using as antigen live cultures of Leptospira grown at 30 °C under aerobic conditions in liquid medium EMJH (Difco). The used strains were *Leptospira interrogans* serogroup Pomona serovar Pomona strain Pomona, *L.i.* Canicola, Canicola Hond Utrecht IV; *L.i.* Sejroe Wolffi 3705; *L.i.* Sejroe Hardjo Hardjoprajitno; *L. i.* Icterohaemorrhagiae Copenhageni M 20; *L.i.* Tarassovi Tarassovi Perepelicin; *L. borgpetersenii* Ballum Castellonis Castellon 3 and *L. kirschneri* Grippotyphosa

Grippotyphosa Moskva V. Sera were screened at 1:100 dilution and positive sera were titrated to the end point using standard methods (OIE, 2019). Nikon® dark field microscope Eclipse E200 with a 10X objective was used. The end point titer was determined as the highest serum dilution showing agglutination of at least 50% of the bacterial cells (OIE, 2019).

2.3 Antigen for eELISA (sonicated L. i. serogroup Icterohaemorrhagiae serovar Copenhageni strain M 20)

Antigen was prepared according to Surujballi et al. (2004) (Surujballi and Mallory, 2004), with modifications. Briefly, 5-ml of pre-culture of *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain M 20 in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco, BD, Sparks, Maryland, USA) was used to inoculate 500 ml of EMJH. This culture was incubated at 30 ºC with constant shaking (50 RPM) for one week until an absorbance value at 420 nm (A_{420}) of ≥ 0.5 was reached. The culture was harvested by centrifugation (20.000 x g at 4 °C for 30 min) and washed twice by centrifugation in phosphate buffered saline (PBS). The cells were resuspended in PBS and then killed by heating at 56 °C overnight and at 70 ºC for 2 h. Aliquots (1 ml) of the mixture were then cooled on ice at 0 ºC and sonicated with a 375 W cell disruptor for 15 seconds every 45 seconds, twice (Heat Systems-Ultrasonics, Farmingdale, New York, USA). The resulting material was stored at -70 °C and used as antigen in the ELISA. This preparation was used directly as antigen for the eELISA. The antigen preparation was analyzed by SDS-PAGE (12 %) under reducing conditions (Laemmli, 1970) and quantified by the Bradford method (Bradford, 1976). Immunological reactivity was confirmed by immunoblotting (Kurien and Hal Scofield, 2015). For this, they were used five bovine sera MAT- positives (titre ≥ 1/1600 and

reactives to *L. i.* serogroups Sejroe, Pomona, Icterohamorrhagiae and Canicola) and five bovine sera MAT-negatives (titre <1/100). The sera were dilutor to 1/100.

2.4 Recombinant antigen for rELISA (rLipL32)

rLipL32 antigen was provided by the Laboratory of Applied Immunology, Nucleus of Biotechnology, Center for Technological Development, Federal University of Pelotas, Brazil, and was prepared according to Hartleben et al.(Hartleben et al., 2012). The purified protein was dialyzed against phosphatebuffered saline (PBS) and glycine 0.1 %, pH 8.0, for16 h at 4 ºC. Fractions of the purified rLipL32 were analyzed by SDS-PAGE (12 %) under reducing conditions (Laemmli, 1970) and quantified by the Bradford method (Bradford, 1976). Purity was >95% as determined by gel electrophoresis. Immunological reactivity of rLipL32 was confirmed by immunoblotting (Kurien and Hal Scofield, 2015). For this, they were used five bovine sera MAT- positives (titre ≥ 1/1600 and reactives to *L. i.* serogroups Sejroe, Pomona, Icterohamorrhagiae and Canicola) and five bovine sera MAT-negatives (titre <1/100). The sera were dilutor to 1/100.

2.5 Development of eELISA and rELISA.

Flat-bottomed polystyrene plates (MaxiSorp of Nunc TM USA, Cat No. 6366) were coated overnight at 4 °C with 50 µl of the antigen preparation; *L. interrogans* Icterohaemorrhagiae Copenhageni *M 20* sonicated and rLipL32. Different amounts of antigen were tried to determine the optimum amount for coating. The amount 125 ng/well for eELISA and 50 ng/well for rELISA was chosen because a higher amount did not increase the ELISA signal more than the background signal. The plates were washed three times with PBS (pH 7.2) and then blocked with 200 µl of 2% skimmed milk solution in PBS (PBSM; pH 7.2) at 37 °C for 1 h. Plates were washed five times with PBS plus

0.05% Tween 20 of Promega® (PBST) and incubated at 37 °C with 50 µl of a 200-fold dilution of the serum in PBSM for 1 h. All reactions were performed independently in duplicate. A pool of five MAT-positive (sera 1-5) and five MAT-negative (sera 1-5) bovine sera were included in each plate as internal standards. Plates were washed five times with PBST and incubated at 37 °C for 1 h with 50 µl of 2500-fold dilution of horseradish peroxidase conjugated antibody anti-Bovine-IgG (whole IgG) (KPL). Plates were washed four times with PBST and once with PBS; 50 µl 3,3´, 5, 5´-tetramethylbenzidine peroxidase substrate (Thermofisher Scientific) were added per well. The plates were incubated in the dark at room temperature for 15 min. The reaction was stopped with 1N HCl and was measured using an ELISA plate reader (A_{450}) (Biotek, model Elx 808).

2.6 Data analysis

Bovine sera (69 positive and 97 negative) with diagnostic status defined by MAT were analyzed, with rLipL32 and sonicated antigen as antigens in two different ELISA. The results obtained with both ELISA were compared with the gold standard by applying Receiver Operator Characteristic Curves (ROC) (Cerda and Cifuentes, 2012; CLSI, 2014). Subsequently, the diagnostic performance values (sensitivity = Se and specificity = Sp) of both methods with their respective 95% confidence intervals (CI 95%) were estimated. The optimal cut-off point (CO) based on optical density at 450 nm (OD) reading were determined by means of the highest Index of Youden (J = Se + Sp-1) (CLSI, 2014; Youden, 1950). Finally, the diagnostic parameters obtained with both ELISA were compared following the CLSI EP 12 protocol (three-way method) (CLSI, 2014), which establishes that there are no differences between the diagnostic methods when the CI 95% of the difference between the diagnostic parameters contains the value zero. The analyses

were performed using the software Microsoft Excel. Sera were considered positive when OD results were greater than the CO.

Results

eELISA and rELISA were easily performed and neither well-to-well variation nor plate-to-plate variation was higher than 10%, confirming their repeatability. Regarding MAT results of positive sera, titres reached up to 6400, and predominant serogroups were Sejroe serovar Wolffi 3705 (47, 69.2 %) and Pomona (29, 41.7%). The results of the MAT for the 69 positive sera according to the serogroup and serovar are shown in **table 1**. Performance of eELISA and rELISA was evaluated with serum samples of 166 bovines, 69 MAT positive and 97 MAT negative.

Serogroup reported	No. of samples and % seropositivity (overall)
***Leptospira interrogans* serogroup Sejroe serovar Wolffi 3705**	**47 (69.2%)**
***L. i.* serogroup Pomona serovar Pomona Pomona**	**29 (41.7%)**
***L. i.* serogroup Sejroe serovar Hardjo Hardjoprajitmo**	**8 (12.1%)**
***L. i.* serogroup Icterohaemorrhagiae serovar Copenhageni M 20**	**2 (3.3%)**
***L.i.* serogroup Canicola serovar Canicola Hond Utrecht IV**	**1 (2.1%)**

Table 1. Results of the MAT for the 69 sera positive according to the serogroup.

The optimal cut-off point (CO) was 0.297 for eELISA and 0.203 for rELISA. ROC curve analysis, which evaluates sensitivity and specificity along a curve, produced fitted areas of 0.998 (0.994-1.0), ($p < 0.05$) for the detection of IgG against sonicated *L. i.* Icterohaemorrhagiae Copenhageni M 20, and 0.929 (0.891-0.968), ($p < 0.05$) for the detection of IgG against rLipL32 **(Figure 1 and table 2)**, with both of them being significantly different from 0.5 **(Figure 1 and table 2)**. The comparison of both areas showed statistically significant differences between them ($z = -3.826$; $p = 0.000$) **(Figure 1)**.

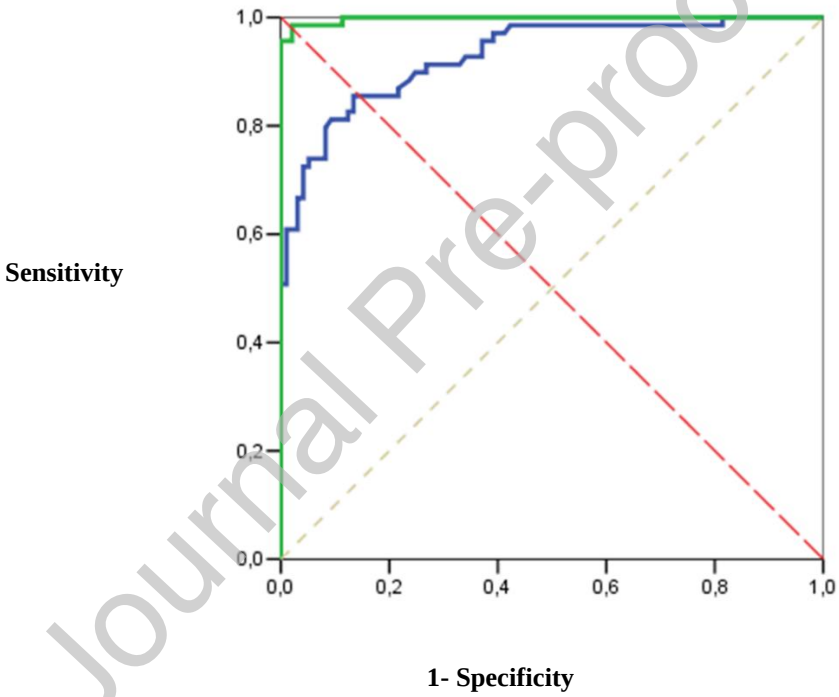

Figure 1. Receiver Operating Characteristics curves for eELISA (green curve) and rELISA (blue curve). AUC: 0.998 (0.994-1.0), ($p < 0.05$) for the detection of IgG against sonicated *L. i.* Icterohaemorrhagiae Copenhageni M 20 (eELISA) and 0.929 (0.891-0.968), ($p<0.05$) for the detection of IgG against rLipL32 (rELISA).
Note: The ROC curves for rLipL32 and sonicated antigen show statistically significant differences ($z = -3.826$; $p = 0.000$).

Variables	**AUC**	**Error** [a]	**Sig.**	**Asymptotic at 95 %**	
Contrast			**Asymptotic** [b]	Lower limit	Upper limit
Result					
***L. i.* sonicated**	0.998	0.002	0.000	0.994	1.000

rLipl32	0.929	0.020	0.000	0.891	0.968

Table 2. Curve fitted area for the IgG ELISA results of each antigen.
AUC: Area under curve.
a: non parametric assumption
b: Null hypothesis: true area: 0.5

The sensitivity of eELISA in the identification of MAT positive bovines was 98.6% (it identified 68 of the 69 positive bovines for MAT), whereas the specificity was 97.9% (95 of the 97 negative sera were correctly identified). The sensitivity of rELISA in the identification of MAT positive bovines was 85.5% (it identified 59 of the 69 positive bovines for MAT), and the specificity was 86.6% (84 of the 97 negative sera were correctly identified). The diagnostic performance for rLipL32 (CO = 0.203; Se = 85.5%; Sp = 86.6%; J = 0.72) and for sonicated *L. i.* Icterohaemorrhagiae Copenhageni M 20 (CO = 0.297; Se = 98.6%; Sp = 97.9%; J = 0.96) is shown in **table 3**. **Table 3** shows the effect of varying the cut-off value on the diagnostic sensitivity and diagnostic specificity of each assay (eELISA and rELISA) as determined by receiver operating characteristic (ROC) curve analysis. A three-way comparison showed statistically significant differences in the Se and Sp of rELISA and eELISA **(Tables 4 and 5).** The eELISA is 13% more sensitive and 11.3% more specific than rELISA **(Table 5)**.

CO eELISA	P	N	WP	WN	Se	Sp	J
0,2930	68	94	3	1	98,6	96,9	0,955
0,2970	68	95	2	1	98,6	97,9	0,965
0,2995	67	95	2	2	97,1	97,9	0,95

CO rELISA	P	N	WP	WN	Se	Sp	J
0,2010	59	83	14	10	85,50	85,6	0,71

0,2030	59	84	13	10	85,50	86,6	0,72
0,2040	59	84	13	10	85,50	86,6	0,72
0,2050	58	84	13	11	84,10	86,6	0,71

Table 3. Effect of varying the cut-off value (selected examples shown) on the diagnostic sensitivity and diagnostic specificity of each enzyme linked immunosorbent assay (eELISA and rELISA) as determined by receiver operating characteristic (ROC) curve analysis. CO: cut-off point. P: positives; N: negatives; WP: wrong positives; WN: wrong negatives; Se: sensitivity; Sp: specificity; J: Youden Index.

Method		**Total samples**	**Diagnostic accuracy criteria MAT**	**Diagnostic accuracy criteria MAT**
eELISA	**rELISA**	n	POSITIVE	NEGATIVE
Positive	Positive	59	58	1
Positive	Negative	11	10	1
Negative	Positive	13	1	12
Negative	Negative	83	0	83
Total	**Total**	166	69	97

Table 4. Three-way comparison. MAT, eELISA and rELISA.

Performance	**Difference %** (eELISA –rELISA)	**CI 95%**
Se	13.04	(4.03-23.28)
Sp	11.34	(4.07-19.56)

Table 5. Difference in performance of eELISA and rELISA. Se: sensitivity; Sp: specificity. CI: Confidence Interval; eELISA: *L. i.* Icterohaemorrhagiae Copenhageni M 20 sonicated; rELISA: rLipL32.
Note. The differences between Se and Sp of the methods are statistically significant.

Discussion and Conclusions

Bovine leptospirosis is underdiagnosed in most cases, causing severe reproductive and productive anomalies that lead to significant economic losses to the bovine industry. *Leptospira interrogans* serogroups Sejroe and Pomona were recorded as the dominant serogroups in bovines; these results are consistent with those previously obtained for Argentina (Draghi et al., 2011, 2006).

MAT is considered the reference method for serological detection of leptospirosis. However, this test requires the maintenance of a large panel of live pathogenic cultures. The use of live leptospira also poses a risk of laboratory-acquired infection to the laboratory technicians (Chen et al., 2018). MAT is a complex and time-consuming method. The results are observed under a dark field microscope for agglutination, and interpretation is not precise. A well-trained technician can only perform MAT testing on about 30-40 samples in a single day. By contrast, ELISA is much easier to perform, can test a large number of samples at the same time, is less subjective, and yields more accurate and precise results (Bourhy et al., 2013).

To overcome the limitations of MAT, several recombinant protein-based serological tests have been developed using outer membrane proteins from pathogenic species (Bomfim et al., 2005; Deneke et al., 2014; Dey et al., 2004; Karthik et al., 2013; La-ard et al., 2011; Padilha et al., 2019; Sankar et al., 2010; Zhylkibayev et al., 2018) and dead bacteria (Bourhy et al., 2013; Penna et al., 2017; Surujballi and Mallory, 2004). The LipL32 protein from pathogenic *Leptospira* spp. is the major target of the humoral immune response and has been used in immunochemical approaches to detect antibodies in human (Matsunaga et al., 2003) and animal sera (Hartleben et al., 2012).

In this study, we compared two ELISA (rELISA and eELISA) using recombinant LipL32 and sonicated antigen of leptospira for detecting bovine leptospirosis. The ELISA with sonicated *L. i.* Icterohaemorrhagiae Copenhageni M 20 showed 98.6% sensitivity with a specificity of 97.9%, whereas the ELISA with rLipL32 showed 85.5% sensitivity with a specificity of 86.6% **(Table 3).** A three-way comparison showed statistically significant differences in the Se and Sp of rELISA and eELISA. Our results showed that eELISA was more specific and sensitive than rELISA. The results for rLipL32 are consistent with those obtained by other authors when used this recombinant

protein in an ELISA (Bomfim et al., 2005; Sankar et al., 2010). Our findings agree with those provided by Chen et al 2018 (Chen et al., 2018); they showed that no single recombinant antigen can detect antibodies in all samples from different serogroup infections, even with the use of highly conserved proteins (such as LipL32) in ELISA.

Overall, our results indicate that eELISA has a better diagnostic performance than rELISA for the detection of anti-Leptospira IgG antibodies in bovine serum. However, the value for the area under the ROC curve was 0.998 (0.994-1.0) (CI 95 %) for eELISA and 0.929 (0.891-0.968) (CI 95 %) for rELISA, indicating a high level of accuracy for both ELISA. Both ELISA could be used as an alternative immunoserological screening method to know the epidemiological situation in bovine herds and can be an alternative to MAT for the diagnosis of leptospira infection in bovines, in regions where MAT cannot be performed. When the signs of leptospirosis appear in a herd, the infection has been some months ago, so the presence of IgG will be common. There we find the importance of IgG detection. Nevertheless, to establish vaccination programs in the herd, it is important to identify the serogroups of *Leptospira* spp. present in the herd by MAT or PCR (Martínez et al., 2018). This is the first comparison of ELISA using recombinant LipL32 and sonicated antigen from *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni M 20 for the detection of bovine leptospirosis. The study of antigens for their application in the diagnosis of leptospirosis is of utmost importance. This assay may be an important tool for the diagnosis of bovine leptospirosis.

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