UDC 619:616.98-078:57.083.33:579.834.115:636.2:636.4:636.7

THE VALIDATION OF ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DIAGNOSIS LEPTOSPIROSIS AMONG DOGS, PIGS AND CATTLE

Pyskun A. V.[1], Spiridonov V. G.[2], Ukhovskyi V. V.[1], Rybalchenko D. Y.[2], Homenko Y. V.[2]
[1] Institute of Veterinary Medicine of National Academy of Agrarian Sciences of Ukraine, Kyiv, Ukraine, e-mail: uhovskiy@ukr.net
[2] Ukrainian Laboratory of Quality and Safety of Agricultural Products, Kyiv, Ukraine

Summary. This study was aimed to develop a modification of ELISA for the diagnosis of leptospirosis in animals that would correspond to the following requirements:

1) ability to detect antibodies to all serogroups of *Leptospira* that are recommended for the diagnosis of this zoonoses in Ukraine (high sensitivity and specificity);

2) minimization of the risk of laboratory-acquired infection at the preparation of reagents for ELISA and its conducting.

The article presents the results of validation of ELISA for the diagnosis of leptospirosis among dogs, pigs, and cattle. During the validation, several technological stages were conducted. The first stage was carried out by selection and determination of the optimal concentration and titers of the main components of ELISA (antigen, enzyme conjugate, enzyme substrate, and blocking substance), and the second stage — by statistical analysis of the results after testing ELISA on the reference panel of sera blood samples.

Established that the sensitivity of the developed ELISA test is less than its specificity (89.8% against 96.7%), but the general efficiency of this method is high and equals 93.6%.

Keywords: leptospirosis, microscopic agglutination test, enzyme-linked immunosorbent assay, validation, titer

Introduction. Leptospirosis is the most wide spread zoonosis worldwide, which is present in all continents except Antarctica and evidence for the carriage of Leptospira has been found in virtually all mammalian species examined (Adler and la Peña Moctezuma, 2010).

The wide spectrum of symptoms confuses the clinical diagnosis and makes it undependable. The laboratory diagnosis of leptospirosis, a prerequisite for treatment, is usually achieved either by isolation of the causative organisms or by serological evidence indicating recent infection (Sharma et al., 2007). The microscopic agglutination test (MAT) is the reference test for diagnosis and detects antibodies at serovar levels (Levett, 2001). MAT has many advantages, but there are significant deficiencies. The maintenance of stock cultures and use of live organisms creates a risk of laboratory-acquired infection (Wautkins and Zochowski, 1990). Therefore, several methods have been developed for use in diagnosis of leptospirosis as an alternative to MAT, of which IgM ELISA is the most promising and detects genus-specific antibodies (Kurstak, 1985; Sharma et al., 2007; Bolin, 2008).

ELISA was also developed in the Laboratory of Leptospirosis of Farm Animals of the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences (IVM NAAS) for identifying the immunoglobulins G in the blood sera samples of cattle. Inactivated serovar wijnberg (serogroup Icterohaemorrhagiae) was used as the antigen. However, it has not gained widespread among specialists of veterinary medicine in Ukraine and needed, like MAT, working with pathogenic Leptospira culture (Ivanska et al., 2003).

The aim of this work is to develop a modification of ELISA for the diagnosis of leptospirosis in animals that would correspond to the following requirements:

1) ability to detect antibodies to all serogroups of Leptospira that are recommended for the diagnosis of this zoonoses in Ukraine (high sensitivity and specificity);

2) minimization of the risk of laboratory-acquired infection at the preparation of reagents for ELISA and its conducting.

Materials and methods. During the validation, we have conducted several technological stages. The first stage was carried out by selection and determination of the optimal concentration and titers of the main components of ELISA (antigen, enzyme conjugate, enzyme substrate, and blocking substance), and the second stage — by statistical analysis of the results after testing ELISA on the reference panel of sera blood samples.

To perform this research, ELISA was carried out with field samples of blood sera from healthy and sick with leptospirosis dogs, pigs and cattle, and seven samples of reference sera (OIE), that were obtained from the Royal Tropical Institute, Amsterdam.

The research had been carried out during the 2013–2015 in the Laboratory of leptospirosis of farm animals

of the IVM NAAS and in the Ukrainian Laboratory of Quality and Safety of Agricultural Products (ULQSP APC).

Results and discussion. Recombinant protein LipL 32 was used as the antigen for ELISA. It is a modified analog one of the major outer membrane lipoprotein of Leptospira (Tokuda, 2009). It is experimentally proved, that this protein is a part of the outer membrane only in pathogenic Leptospira species and is a secure to use for laboratory staff (Tokuda, 2009; Murray et al., 2009). All these qualities contribute to its widespread in the formulation of leptospirosis diagnosticums in different countries (Bomfim, Ko, and Koury, 2005; Sharma et al., 2007).

To determine the optimal dose of sensitizing antigen, we conducted its sorption within 0.5–4 µl volume in carbonate-bicarbonate buffer (CBB), pH 9.6. At the same time we have tested substances that eliminate the nonspecific interaction of antibodies with antigen-edible gelatin and skimmed milk powder (respectively, 0.5% and 5% solutions). This combination allows prevention false reactions and reduces the time for titration of the components.

The results of antigen titration with different blocking substances are shown on Fig. 1 and Fig. 2.

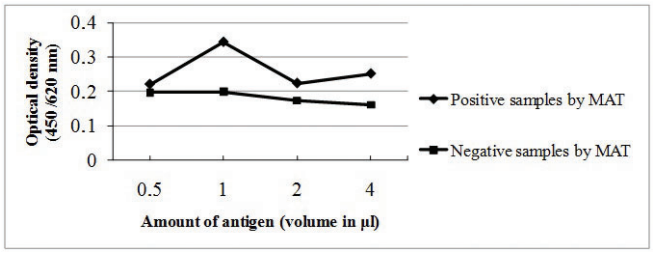

Figure 1. The sorption curve of antigen on polystyrene microplates with the use of 5% solution of skimmed milk powder

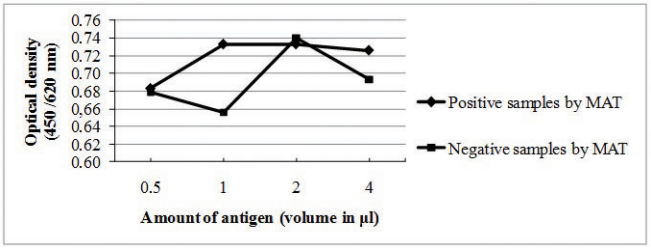

Figure 2. The sorption curve of antigen on polystyrene microplates with the use of 0.5% solution of gelatin

As the analysis of titration had shown, the optimum amount of LipL 32 in both cases was 1 µl volume. With such amount of antigen the high correlation coefficients were observed between the indicators of optical density in positive and negative blood sera samples by MAT, representing, respectively, 1.73 and 1.12.

However, in all investigated wells of microplate the high background reactions were registered when dilution of blood sera samples by phosphate buffer with 0.5% gelatin solution. Specifically, when the amount of antigen was 2 µl, the value of optical density of the negative control was higher than of the positive (respectively, 0.74 o. d. and 0.733 o. d.). At the same time, when a 5% skim milk solution was used, the indicators of the optical density of negative controls were much smaller, and the correlation coefficients of positive and negative blood sera samples were higher (Figs 1–2).

As a result of the previous research on the comparison of enzyme conjugates directed to different classes of immunoglobulins, it was found that the conjugate based on recombinant protein LipL 32 with horseradish peroxidase as the enzyme label (directed to Ig M and Ig G) is much more effective in the diagnosis of leptospirosis than the conjugate based on recombinant protein G of Streptococcus spp. and protein A of Staphylococcus aureus (directed on Ig G) (Pyskun, 2015). The titration of enzyme conjugate, based on recombinant protein LipL 32, and determination of the optimal dilution of blood sera samples for statement of ELISA with it, were conducted (Tabs 1–2).

The range of the titration for determination of the optimal blood sera dilution in ELISA was between 1/2.5–1/40 (2.5–40 µl volumes of blood sera) (Tab. 2). It was selected to provide the convenience for the further conducting an ELISA, for the reason that the process of the blood sera collecting (by doser) in volume less than 2 µl, and contributing to its homogenous dissolution in 98 µl of solution for the sample dilution is a very laborious process. At the same time, there is a high probability of background reactions in negative controls, *in case if* it is necessary to add blood sera in volume more than 40 µl in wells of microplate.

The results of titration are shown on Fig. 3 and Fig. 4.

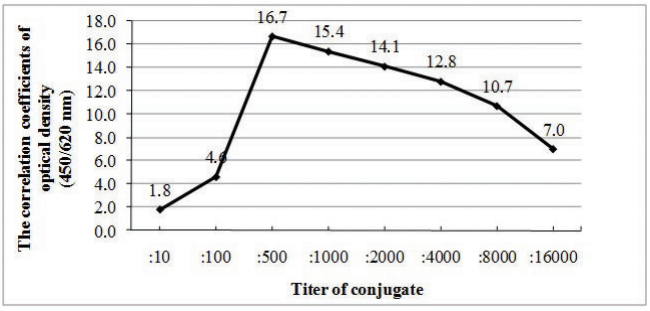

Figure 3. Dependence of the correlation coefficients of optical density between positive and negative samples on the titers of conjugate

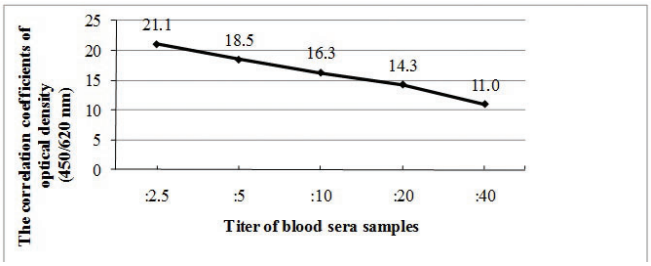

Figure 4. Dependence of the correlation coefficients of optical density between positive and negative samples on the titers of blood sera

The results of conjugate titration had shown, the highest correlation coefficients of optical density between positive and negative samples of blood sera were registered in its titer 1/500 and dilution of sera 1/2.5 (40 µl volume). Coefficients were, respectively, 16.7 and 21.1 (Figs 3–4).

Beyond that, the specific reagent in the development of ELISA is an enzyme substrate (substrate-chromogen). Due to the enzymatic reaction of conjugate with the substrate by using chromogen, the reaction products become colored that enables visually or automatically evaluate the presence of antibodies in the test material.

Table 1 – The results of the titration enzyme conjugate based on recombinant protein LipL 32

The titer of blood sera (OIE) by MAT	Indicators of optical density at 450/620 nm in a different dilutions							
	1:10	1:100	1:500	1:1000	1:2000	1:4000	1:8000	1:16000
Grippotyphosa ++ 1:4000	3.981	3.429	2.580	2.430	1.952	0.833	0.561	0.211
Icterohaemorrhagiae ++ 1:8000	3.320	3.111	2.133	1.721	1.514	1.128	0.623	0.114
Sejroe ++ 1:32000	2.247	1.914	1.052	0.613	0.543	0.327	0.221	0.094
Tarassovi ++ 1:16000	2.671	2.113	1.370	0.945	0.613	0.333	0.228	0.107
negative sample	1.706	0.573	0.107	0.092	0.082	0.051	0.038	0.019

Table 2 – The results of titration blood sera samples for statement ELISA with conjugate based on recombinant protein LipL 32

The titer of blood sera (OIE) by MAT	Indicators of optical density at 450/620 nm in a different dilutions				
	1:2.5	1:5	1:10	1:20	1:40
Grippotyphosa ++ 1:4000	2.412	2.121	1.921	1.66	1.23
Icterohaemorrhagiae ++ 1:8000	2.745	2.192	1.85	1.441	0.733
Sejroe ++ 1:32000	1.721	1.268	0.922	0.712	0.512
Tarassovi ++ 1:16000	1.32	0.947	0.647	0.422	0.22
negative sample	0.097	0.088	0.082	0.074	0.061

The o-phenylenediamine (OFD) and tetrametilbenzidin (TMB) are the most sensitive solutions of substrate-chromogens that are used for conducting ELISA nowadays. So we compared the obtained results of ELISA with these compounds (Tab. 3).

As the comparison of the enzyme substrates showed, the indicators of the optical density in the positive samples of blood sera by MAT from all species and in reference to blood sera were significantly higher by using TMB solution than indicators by using OFD chromogen, respectively, 0.836 ± 0.089 o. d. against 0.601 ± 0.066 o. d. and 1.490 ± 0.14 o. d. against 1.069 ± 0.13 o. d. The difference in optical values in both cases was significant ($p < 0.05$). At the same time, the optical indicators of the negative blood sera samples by MAT were not significantly different in both cases (0.098 ± 0.002 o. d. against 0.085 ± 0.0017 o. d.).

Thus, at the end of the first stage in the development of ELISA, we made the selection of antigen, enzyme conjugate, blocking reagent and chromogenic substrate, and established their optimum concentration.

The last technological stage was carried out by a statistical analysis of the results after testing of ELISA on the panel of blood sera samples.

Table 3 – The results of comparison the sensitivity of enzyme substrates OFD and TMB

The positive and negative by MAT blood sera samples	The number of samples	The mean values of optical density, o. d.	
		TMB	OFD
The positive blood sera samples from dogs	5	0.330	0.195
The negative blood sera samples from dogs	3	0.101	0.094
The positive blood sera samples from pigs	19	0.591	0.491
The negative blood sera samples from pigs	6	0.096	0.083
The positive blood sera samples from cattle	12	1.436	0.943
The negative blood sera samples from cattle	2	0.099	0.082
Reference blood sera (OIE)	7	1.490	1.069

To perform this phase of research, we used the blood sera panel that consisted of 128 positive samples (including 7 reference sera OIE) and 152 negative samples by MAT. The results of research are shown in Table 4.

As shown in Table 4, the indicators of the optical density in blood sera samples from dogs, pigs and cattle were increased respectively to antibodies titers by MAT. At the same time, the difference between them was highly significant in most cases ($p < 0.001$).

The limits and the average values of the optical density within each group of the blood sera by MAT were similar to each other in all of the listed species. Based on the obtained results, we have decided to calculate the statistical indicators of ELISA simultaneously by values of optical density of blood sera samples from all three species and the reference sera (OIE).

For further research, it was necessary to find with accuracy the determination of the limits of optical density indicators that interpreted as true or false. For this purpose, we used the method «cut-off», on the basis of which Table 5 was formed.

Table 4 – The results of testing ELISA on the panel of blood sera samples

The species of animal	The titers of antibodies by MAT	The number of samples	The values of optical density, o. d.	
			Lim	M ± m
Dogs	negative samples	48	0.079–0.125	0.101 ± 0.00076
	Monoreaction in titer ++1:50 – ++1:100	17	0.116–0.401	0.194 ± 0.012***
	Mixed reactions in titer ++1:50 – ++1:100	12	0.124–0.428	0.266 ± 0.015***
	Monoreaction in titer ++1:500 – ++1:2500	4	0.361–1.116	0.672 ± 0.08***
	Mixed reactions in titer ++1:100 – ++1:2500	4	0.763–3.175	1.762 ± 0.325*
Pigs	negative samples	53	0.075–0.129	0.102 ± 0.0008
	Monoreaction in titer ++1:50 – ++1:100	16	0.122–0.326	0.182 ± 0.0094***
	Mixed reactions in titer ++1:50 – ++1:100	9	0.136–0.425	0.246 ± 0.018**
	Monoreaction in titer ++1:500 – ++1:2500	11	0.117–1.1	0.508 ± 0.035***
	Mixed reactions in titer ++1:100 – ++1:2500	8	0.681–3.138	1.831 ± 0.192***
Cattle	negative samples	51	0.071–0.124	0.102 ± 0.0023
	Monoreaction in titer ++1:50 – ++1:100	14	0.119–0.392	0.212 ± 0.013***
	Mixed reactions in titer ++1:50 – ++1:100	13	0.122–0.505	0.321 ± 0.017***
	Monoreaction in titer ++1:500 – ++1:2500	6	0.119–0.945	0.483 ± 0.05**
	Mixed reactions in titer ++1:100 – ++1:2500	7	0.823–3.345	1.647 ± 0.239***
blood sera (OIE)	Monoreaction in titer ++1:4000 – ++1:32000	7	0.698–2.583	1.52 ± 0.139

Footnote: * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$

Table 5 – The results of comparison developed ELISA with MAT

The positive and negative blood sera samples by ELISA	The positive and negative blood sera samples by MAT	
	MAT+	MAT–
ELISA+	115	5
ELISA–	13	147

Analyzing the results, the indicators of diagnostic sensitivity (D-SN), specificity (D-SP) and overall efficiency (Ef) were calculated by the following formulas.

Diagnostic sensitivity:

$$D-SN=\frac{115}{(115+3)}\times 100=89.8\%$$

Diagnostic specificity:

$$D-SP=\frac{147}{(147+5)}\times 100=96.7\%$$

Overall efficiency:

$$Ef=\frac{(115+147)}{(115+5+147+13)}\times 100=93.6\%$$

Also, the additional efficiency indicators were calculated: the predictive values of positive (95.8%) and negative (91.9%) tests, the Youden's index (0.865), and the likelihood coefficients of positive (27.2) and negative (0.105) results.

Conclusions. 1. The selection of the main components for conducting ELISA for leptospirosis and determination of the optimal concentration were done.

2. Established that the sensitivity of the developed ELISA is less than its specificity (89.8% against 96.7%), but overall efficiency of this method is high and equal 93.6%.

3. The predictive values of positive and negative tests are high and equal, respectively, 95.8% and 91.9%.

References

Adler, B. and la Peña Moctezuma, A. de (2010) 'Leptospira and Leptospirosis', *Veterinary Microbiology*, 140(3–4), pp. 287–296. doi: 10.1016/j.vetmic.2009.03.012.

Bolin, C. A. (2008) Chapter 2.1.9. Leptospirosis. In: Manual of diagnostic tests and vaccines for terrestrial animals. 6th ed. Vol. 2. Available at: http://web.oie.int/eng/normes/MMANUAL/2008/pdf/2.01.09_LEPTO.pdf.

Bomfim, M. R. Q., Ko, A. and Koury, M. C. (2005) 'Evaluation of the recombinant LipL32 in enzyme-linked immunosorbent assay for the serodiagnosis of Bovine Leptospirosis', *Veterinary Microbiology*, 109(1–2), pp. 89–94. doi: 10.1016/j.vetmic.2005.05.002.

Ivanska, N. V., Kucheriavenko, O. O., Kucheriavenko, O. O., Rezunenko, Y. V. and Hanova, L. O. (2003) *Practical guide to work with the ELISA test system for the detection of antibodies against Leptospira 'ELISA-Leptospirosis-cattle' [Praktychnyi posibnyk po roboti z imunofermentnoiu test-systemoiu dlia vyiavlennia antytil proty leptospir 'IFA-leptospiroz-VRKh']*. Edited by M. Ya. Spivak. Kyiv: Diaprof-Med. Available at: http://diaproph.com.ua/pdf/metodichky/ua/10_ifa_leptospiroz_kpc.pdf. [in Ukrainian].

Kurstak, E. (1985) 'Progress in enzyme immunoassays: Production of reagents, experimental design, and interpretation', *Bulletin of the World Health Organization*, 63(4), pp. 793–811. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2536367/pdf/bullwho00087-0162.pdf.

Levett, P. N. (2001) 'Leptospirosis', *Clinical Microbiology Reviews*, 14(2), pp. 296–326. doi: 10.1128/cmr.14.2.296-326.2001.

Murray, G. L., Srikram, A., Hoke, D. E., Wunder, E. A., Henry, R., Lo, M., Zhang, K., Sermswan, R. W., Ko, A. I. and Adler, B. (2008) 'Major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans*', *Infection and Immunity*, 77(3), pp. 952–958. doi: 10.1128/iai.01370-08.

Pyskun, A. V. (2015) 'Selection of optimal conjugate for the enzyme-linked immunosorbent assay against Leptospirosis' [Pidbir optymalnoho koniuhatu dlia postanovky imunofermentnoho analizu na leptospiroz], *Veterinary Biotechnology [Veterynarna Biotekhnolohiia]*, 26, pp. 156–163. Available at: http://vetbiotech.kiev.ua/volumes/JRN26/23.pdf. [in Ukrainian].

Sharma, R., Tuteja, U., Khushiramani, R., Shukla, J. and Batra, H. V. (2007) 'Application of rapid dot-ELISA for antibody detection of Leptospirosis', *Journal of Medical Microbiology*, 56(6), pp. 873–874. doi: 10.1099/jmm.0.46926-0.

Tokuda, H. (2009) 'Biogenesis of outer membranes in Gram-negative bacteria', *Bioscience, Biotechnology and Biochemistry*, 73(3), pp. 465–473. doi: 10.1271/bbb.80778.

Wautkins, S. A. and Zochowski, W. J. (1990) 'Leptospira', in Wreghitt, T. G. and Morgan-Capner, P. (eds.) ELISA *in the clinical microbiology laboratory*. London: Public Health Laboratory Service, pp. 225–237. ISBN: 9780901144249.