

Evaluation of LipL32 ELISA for Detection of Bovine Leptospirosis in West Java

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ABSTRAK

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Uji diagnosa Leptospirosis yang saat ini digunakan, yaitu micro Agglutination Test (MAT) dan isolasi, merupakan uji yang mahal, sulit dan membutuhkan keahlian khusus. Studi ini bertujuan mengembangkan ELISA berbasis protein rekombinan LipL32 sebagai uji yang mudah dan murah untuk mendeteksi Leptospirosis. DNA pengkode LipL32 dimurnikan dari bakteri *Leptospira Pomona*, dimasukkan ke dalam plasmid pRSET-C, kemudian diekspresikan sebagai protein poly-histidine-tagged pada sel *E.coli* BL21. Protein LipL32 berhasil dimurnikan dari supernatan sel lisis dengan Ni-NTA column sebanyak 1mg/l kultur. LipL32 tersebut digunakan sebagai antigen untuk koting dengan konsentrasi final 5µg/ml. Akurasi dari ELISA dievaluasi berdasarkan analisa ROC, dengan membandingkan hasil ELISA dan MAT dari 517 serum sapi. Pada studi ini diketahui bahwa area dibawah kurva (area under curve) sebesar 0.853, sehingga ELISA LipL32 dikategorikan sebagai uji dengan akurasi sedang (moderately accurate), dan menunjukkan bahwa ELISA mampu membedakan serum positive dan negative Leptospirosis. Selain itu, hasil yang diperoleh juga menunjukkan bahwa ELISA LipL32 mampu mendeteksi serum positive MAT spesifik terhadap serovar Hardjo, Grippytyphosa, Tarrasovi, Rachmati and Bataviae. Optimum ambang batas (cut off) untuk ELISA yang ditentukan berdasarkan kurva ROC adalah 0.504, dan diperoleh sensitivitas dan spesifisitas untuk ELISA sebesar 86.0% dan 69.5% berturut-turut. Secara keseluruhan dapat disimpulkan bahwa ELISA LipL32 pada penelitian ini dapat digunakan sebagai uji cepat untuk identifikasi antibody anti-Leptospira pada sapi.

Kata Kunci: ELISA, LipL32, Leptospirosis, Bovine

ABSTRACT

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The current diagnosis of leptospirosis, micro Agglutination Test (MAT) and isolation, is expensive, impractical and technically demanding. This study was aimed at developing an ELISA based on recombinant LipL32 as a practical, inexpensive test for Leptospirosis. The DNA encoding LipL32 was isolated from *Leptospira pomona*, inserted into pRSET-C plasmid then expressed in *E.coli* BL21 as a poly-histidine-tagged protein. The amount of LipL32 protein, which was purified from the supernatant of lysed cells by a Ni-NTA column, was 1mg/l culture. This purified LipL32 was used as the coating antigen at 5µg/ml. The accuracy of ELISA was evaluated based on ROC analysis, by comparing the ELISA and MAT results of 517 bovine sera. Result in this study showed that the area under curve (AUC) was 0.853, which categorised the LipL32 ELISA as a "moderately accurate" test and indicates that the ELISA was able to differentiate positive and negative Leptospirosis serum. The result also showed ELISA LipL32 could detect serum positive MAT to Hardjo, Grippytyphosa, Tarrasovi, Rachmati and Bataviae. The optimal cut off for OD ELISA determined based on ROC curve was 0.504, and it showed sensitivity and specificity of ELISA LipL32 relative to MAT were 86.0% and 69.5%, respectively. Overall, the result in this study showed that ELISA LipL32 can be used as a rapid test for identification of anti-Leptospira antibodies in bovine.

Key Words: ELISA, LipL32, Leptospirosis, Bovine

INTRODUCTION

Leptospirosis is one of the most important diseases both in human and animals. In Indonesia, the outbreak of this disease has been reported in human since the early 1900's and still occurring (Rahmawati 2013). The serological prevalence in animal, especially bovine, is high (Susanti et al. 2008). Although being endemic in Southeast Asia especially Indonesia, the disease is often

neglected or under-reported (Costa et al. 2015, Rajapakse et al. 2015). Human as accidental host can get infected from infected animal and contaminated environment. Common maintenance hosts for *Leptospira* are rodents, bovine and pig. Previous study in ICRIVS using MAT as serology test has reported high seropositivity of Leptospirosis in bovine and other mammals (Kusmiyati et al. 2005). Further, numbers of

cases was also occurred among livestock farm and abattoir workers (Fang et al. 2015).

The reason for the under-reported cases of leptospirosis is the difficulty in recognizing clinical signs for Leptospirosis, which is not specific and can vary from mild flu-like to severe illness. The standard diagnosis for leptospirosis, MAT and isolations, were impractical and technically demanding. Consequently, only limited laboratories are able to perform these two tests to confirm Leptospirosis infection. The drawbacks of MAT include the continuous maintenance culture of life *Leptospira*, time consuming, the difficulty in maintaining free-contaminating culture, requirement of high technical skill to perform the assay and difficulty for interpretation of the MAT results (Musso & La Scola 2013). Despite the fact that MAT has been recommended as a golden standard test, some studies proved the deficiency of MAT as a golden standard test because its sensitivity is too low (Limmathurotsakul et al. 2012). The isolation of *Leptospira* can be used as a definitive diagnostic method, which demonstrating the presence of *Leptospira* in clinical sample. However, this method is time consuming, technical demanding and not sensitive compared to other available method. Therefore, many diagnostic methods have been developed as alternative tests to overcome these problems, including immunological and molecular tools (Rajapakse et al. 2015).

LipL32 is the major outer membrane protein of Leptospire, which is highly conserved among pathogenic *Leptospira* species (Haake et al. 2000). Previous studies indicated that LipL32 protein as a potential candidate for rapid and accurate diagnosis of Leptospirosis (Bomfim et al. 2005). Furthermore, ELISA using recombinant LipL32 has been commercially available for detection of canine leptospirosis. However the suitability of such test for bovine leptospirosis is yet to be confirmed.

This study was aimed to develop indirect ELISA using recombinant protein LipL32 for detection of anti-*Leptospira* antibodies in bovine, and evaluate the performance by comparing this ELISA LipL32 to the MAT results.

MATERIALS AND METHODS

Preparation of recombinant LipL32 protein

Recombinant LipL32 protein was produced according to previous study (Sumarningsih et al. 2016) with some modification on expression method. Briefly, a single colony of *E. coli* BL21 cells containing plasmid- pRSET-C-LipL32 was propagated in 10 ml LB broth containing ampicillin (50 µg/ml) and chloramphenicol (35 µg/ml). After overnight incubation at 37°C, this culture was added into 250 ml fresh LB

broth containing 2% glucose, ampicillin (50 µg/ml) and chloramphenicol (35 µg/ml). Incubation was continued at 37°C until the density (OD600) reached 0.4-0.6, then the culture was induced by addition of 1 mM *isopropyl-beta-D-thiogalactopyranoside* (IPTG). After another incubation at 37°C for 4 hours, the culture was centrifuged at 7000 xG for 30 minutes. The supernatant was discarded and the pellets was collected and kept at -20°C until further processing. The cells were disrupted by sonication for 3 minutes at maximum amplitude. The LipL32 was purified from the disrupted cells using a native Ni-NTA purification kit (Fast Start, Qiagen). The purity of recombinant LipL32 protein was analyzed with SDS PAGE and the protein concentration was measured by Bradford assay using bovine serum albumin as standard.

Sequence Analysis

The plasmid of pRSET-C-LipL32 was purified from *E. coli* BL21 cells and sent to 1st base (http://www.base-asia.com/dna_sequencing/) for sequencing as described in previous study (Sumarningsih et al. 2016). Amino acid sequences of LipL32 from *Leptospira* serovar Pomona, Hardjo and Grippotyphosa were obtained from GeneBank with accession number AY609326, AY442332 and AY609327, respectively. All these LipL32 sequences were analyzed using bioinformatics software Geneious (version 7.1.2) and aligned against sequences P1 (AAKAKPVQKLDDDDGDDTYKEERHNK) and P2 (LTRIKIPNPPKSFDDLKN-IDTKKL) that has been identified as important epitopes for LipL32 (Lottersberger et al. 2009).

Production of positive and negative control sera for LipL32 ELISA

Ten calves from a nearby farm were bled and tested for leptospirosis using MAT. After the testing, one calf which was negative for leptospirosis was purchased. About 20 ml bloods were drawn, serum was prepared, aliquoted, kept at -20 and used as negative control serum. The calf was immunized with 1 mg of recombinant LipL32 and montanide ISA 70 M VG (SEPPIC) as adjuvant. The second immunization was carried out with 6x10⁸ formol-inactivated *Leptospira* hardjo and 2mg Quil A in 3.2ml PBS. Four weeks after second immunization, the serum was collected and tested with MAT and ELISA using recombinant LipL32 as coating antigen. After confirming that the serum was positive in both tests, 20 ml of blood was drawn, serum was prepared, aliquoted and used as a positive control serum.

Optimization of ELISA LipL32

ELISA optimization was performed to determine the optimal concentration of recombinant LipL32 protein as coating antigen for ELISA. Briefly, recombinant LipL32 protein was serially diluted by twofold dilution; start at 12.5 µg/ml to 0.195 µg/ml in carbonate-bicarbonate buffer pH 9.6 and 100 µl of this solution was added into microtitre plate. Negative and positive serum control were included as standard for optimization of ELISA LipL32 in this study. Both serum were used with dilution of 1/100 and 1/200 in PBS pH 7.4. HRP-goat anti bovine IgG (Jackson Immuno Research) as secondary antibody was used with dilution 1/3000 and 1/6000 in PBS pH 7.4. After adding the substrate solution and ABTS, the absorbance at wavelength 420 nm was measured with Spectrophotometer microtitre plate reader.

Sample collection

Blood samples were collected from 517 bovine (dairy and beef cattle) of various ages in West Java in 2016. The blood samples were handled and processed carefully to prevent hemolysis. The serum was then carefully prepared and stored at -20°C until used.

Micro Agglutination Test (MAT)

MAT for bovine serum was performed as described in previous study (Sumarningsih et al. 2016). Briefly, panels of antigens were added into serum samples that had been diluted in PBS, pH 7.4 at 1 : 50, 1 : 100, 1 : 400, 1 : 1600. The panels of antigens consisted of 14 *Leptospira* serovars including: *L. icterohaemorrhagiae*, *L. javanica*, *L. celledoni*, *L. canicola*, *L. ballum*, *L. pyrogens*, *L. cynopteri*, *L. rachmati*, *L. australis*, *L. pomona*, *L. grippityphosa*, *L. hardjo*, *L. bataviae*, and *L. tarrasovi*. After incubation for 2 hours at 30°C, the mixture was examined under a dark-field microscope. The MAT titer was determined as the highest serum dilution that agglutinated at least 50% the life leptospire.

ELISA LipL32 for field bovine sera

ELISA LipL32 for field sera was performed using microtitre plates (NUNC Maxisorb). Briefly, the plate were coated with diluted recombinant LipL32 protein (5 µg/ml) in carbonate-bicarbonate buffer and incubated overnight at 4°C. This coating solution was removed and plate was washed twice with PBS-Tween 0.05%.

The well was blocked by adding 150 µl of blocking buffer (5% normal goat serum in PBS) and incubated for 2 hours at room temperature. After washing the plate twice, 100 µl of diluted bovine serum (1/100) was added into each well and incubated for 1.5 hours at room temperature. The solution was discarded and the plate was washed four times. Goat anti-bovine IgG-HRP was added at 1/6000 dilution in PBS (100 µl/well) and incubated for 1.5 hours at room temperature. The solution was discarded and plate was washed four times. Substrate and chromogenic (ABTS) solution) was added, 100 µl/well. After incubation at room temperature for 10 minutes, the absorbance (OD ELISA) was read using Spectrophotometer microtitre plate reader at wavelength 420 nm.

Statistical analysis

Data were analyzed using descriptive statistics. The accuracy of LipL32 ELISA as a serological test was analyzed using a ROC-curve based on MAT as the golden standard. A MAT titre of 1/100 was used as the cut-off for negative and positive for leptospirosis. The Area under curve (AUC) is used as the discriminating power of LipL32 ELISA for positive and negative leptospirosis. The cut-off of the ELISA was determined as the OD at which the test gave the highest sensitivity and specificity. All statistical analyses were carried out using a commercial statistical package.

RESULTS AND DISCUSSION

Quantity and quality of recombinant LipL32 protein

The concentration of recombinant LipL32 measured by Bradford assay was 1mg/l culture. The quantity of LipL32 protein produced in present study was as low as 1/8 as that in previous study (Sumarningsih et al. 2016). As in previous study, the purity of recombinant protein LipL32 produced in present study was high (see Figure 1).

Recombinant protein LipL32 in this study was successfully purified using a native buffer system. Similar to the previous study which using pRSET-B to express recombinant protein LipL32 as a solubilized protein and revealed that recombinant protein LipL32 was located in the cytoplasm of *E. coli* (Chalayon et al. 2011). This is in contrast to *Leptospira* since in this bacteria LipL32 is located in the outer membrane as a dominant lipoprotein (Cullen et al. 2005)

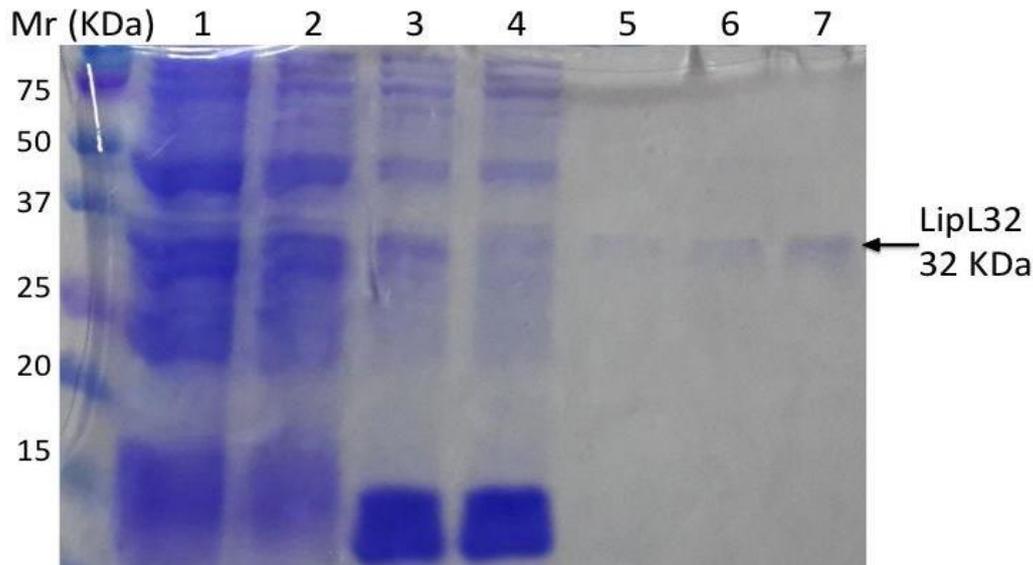


Figure 1. SDS PAGE of recombinant LipL32 protein at different stages of purification. Line 1: uninduced cell, Line 2: induced cell, Line 3: supernatant of lysed cell, Line 4: Flow through, Line 5: Wash buffer, Line 6: LipL32 elution fraction 1, Line 7: LipL32 elution fraction 2. (arrow ← : LipL32 protein 32kDa).

Optimization of ELISA LipL32

In preliminary experiment, the optimum concentration of recombinant protein LipL32 was determined by checkerboard titration (Figure 2). The optimum dilutions of secondary antibody and coating antigen were determined based on the OD differences between positive and negative serum for anti-*Leptospira*. Serum anti-*Leptospira* generated by immunised the bovine using inactivated *Leptospira* serovar Hardjo was included in this study for comparison.

The OD for positive serum control in ELISA LipL32 using conjugate diluted 1 in 3000 was higher than that when using conjugate 1 in 6000 (Figure 2a vs Figure 2b). However, the background ELISA with the 1 in 3000 diluted conjugate was high as indicated by the increased (above 0.5) OD for negative serum. To obtain a better specificity, a dilution of 1 in 6000 of secondary antibody was used in all assays. Based on the OD difference between positive and negative serum was shown in Figure 2.b, the optimum concentration of LipL32 protein for ELISA coating was determined to be 5 µg/ml.

Identification of bovine Leptospirosis using MAT

MAT was used in this study to determine the status of Leptospirosis. Bovine sera from the field were tested with MAT using panels of 14 *Leptospira* serovars. The result of MAT summarized in Table 1 showed that 164 of a total 571 serum samples were positive MAT and the titers ranged from 100 to 1600. This 28.7% seroprevalence was comparable with that of previous study performed in Indonesia and other country (Kusmiyati et al. 2005, Subharat et al. 2012; Alonso-Andicoberry et al. 2001).

According to previous study on seroprevalence of bovine Leptospirosis in Indonesia, bovine sera that were positive on MAT were dominated against serovar *Hardjo* (Kusmiyati et al. 2005). However, in this study as shown in table 2, the majority of MAT positive sera were against serovar *Grippotyphosa* (50%). Of 164 serum positive MAT, 82 to serovar *Grippotyphosa*, 71 to serovar *Hardjo*, 3 to serovar *Bataviae*, 3 to serovar *Rachmati*, 1 serum to *Tarrasovi* and 1 serum to *Icterohaemorrhagiae*. Two sera were positive for both serovars *Grippotyphosa* and *Bataviae*, and 1 serum was positive for *Grippotyphosa* and *Rachmati*.

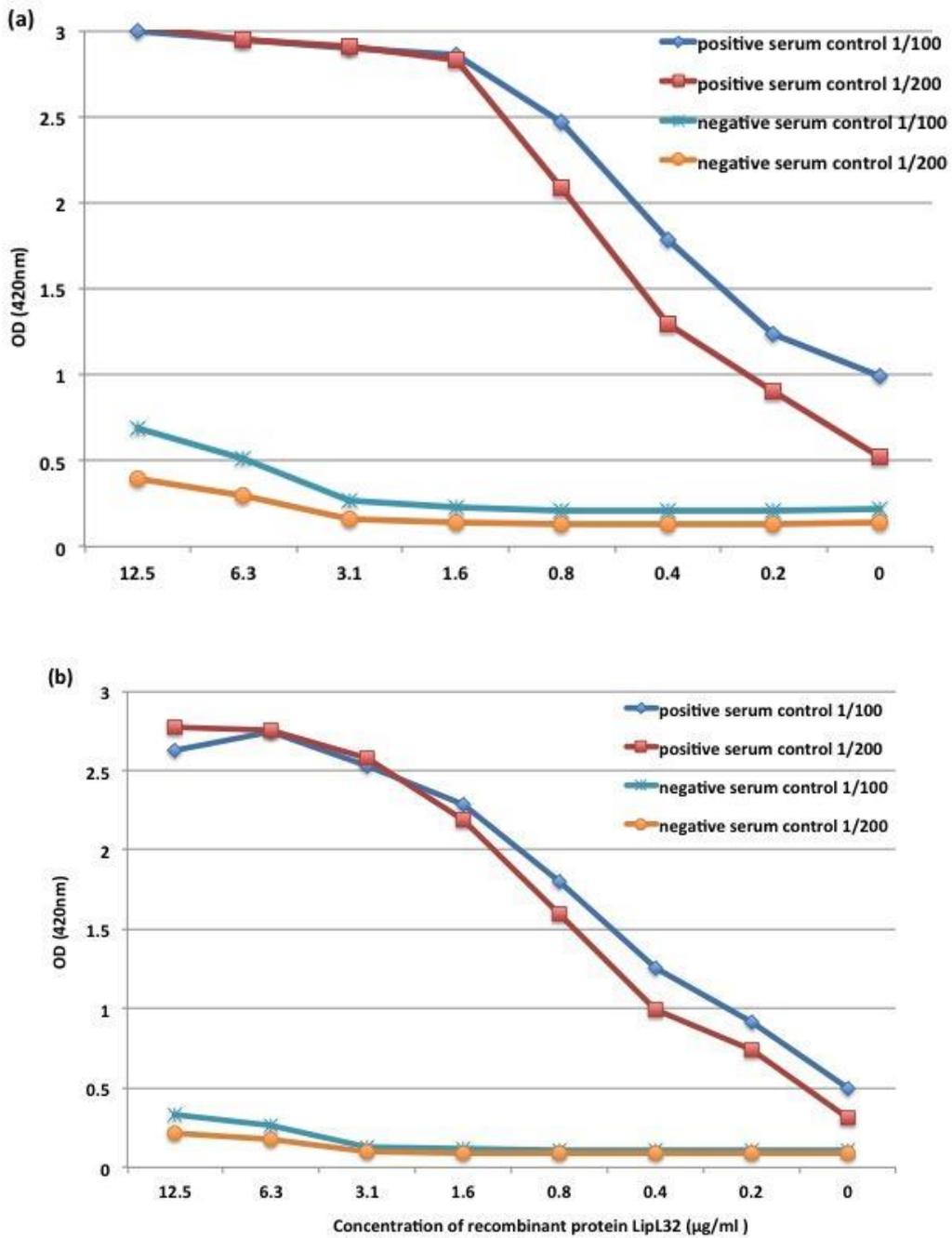


Figure 2. Optimisation of ELISA LipL32; Titration of recombinant protein LipL32 for ELISA coating: (a) ELISA using Goat-anti-Bovine-IgG-HRP diluted in 1/3000, and (b) ELISA using Goat-anti-Bovine-IgG-HRP diluted in 1/6000. Twofold dilution of LipL32 start at 12.5 µg/ml to 0.195 µg/ml in carbonate-bicarbonate buffer pH 9.6.

Table 1. MAT result of field bovine sera

MAT results	Number of samples	Percentage (%)
Positive	164	28.7
Negative	407	71.3
Total	571	100

Table 2. Serospecific of bovine sera positive in MAT

Serovar	Number of sample	Percentage (%)
Grippotyphosa	82	50.00
Hardjo	71	43.29
Bataviae	3	1.83
Rachmati	3	1.83
Tarrasovi	1	0.61
Icterohaemorrhagiae	1	0.61
Grippotyphosa and Rachmati	1	0.61
Grippotyphosa and Bataviae	2	1.22
Total	164	100

Performance of ELISA LipL32 compared to MAT

The LipL32 ELISA developed in this study was able to differentiate between bovine serum that were positive and negative Leptospirosis. As shown in Figure 3, 95% confidence intervals of the MAT-negative serum OD was well-separated with that of MAT-positive serum, even with the lowest titer. The mean OD of MAT-negative serum was 0.451. The mean OD of the positive sera with titer of 100, 400 and 1600 were 0.742, 0.881 and 0.986, respectively. Although, the OD was increased by the increasing MAT titer, the differences were not significant.

The ELISA based on recombinant protein is relatively easier and safer to perform compared to MAT. Bomfim et al. (2005) evaluated LipL32 ELISA for detection of bovine Leptospirosis and reported that the agreement was perfect with MAT. The LipL32 ELISA was reported to have 100% of sensitivity and specificity. LipL32 ELISA was also available commercially from IDEXX, but it is intended to be used for Leptospirosis detection in canine and it need to be imported with high cost for application in Indonesia. This problem motivated us to develop another LipL32

ELISA suitable for detection of bovine Leptospirosis in Indonesia.

The Receiver Operating Characteristic (ROC) curve was used to measure the performance of LipL32 ELISA in this study by comparing the ELISA's OD and MAT results. Results (Figure 4) showed that the area under curve for the ROC curve, which indicates the discriminating power of the test, was 0.853. A test with $0.7 < \text{AUC} < 0.9$ is categorized as a moderately accurate (Greiner et al. 2000).

ROC curve was also used to determine the cut off, sensitivity and specificity of LipL32 ELISA. Figure 4 and Table 4 showed the optimum cut off OD for ELISA LipL32 in this study was 0.504. Based on this cut off values, the sensitivity and specificity were 86.0% and 69.5%, respectively.

Carcass

Table 4 presented the distribution of OD ELISA LipL32 based on MAT titer. The OD for MAT negative serum was found mostly less than 0.5, and the OD for bovine serum positive to MAT varied and mostly

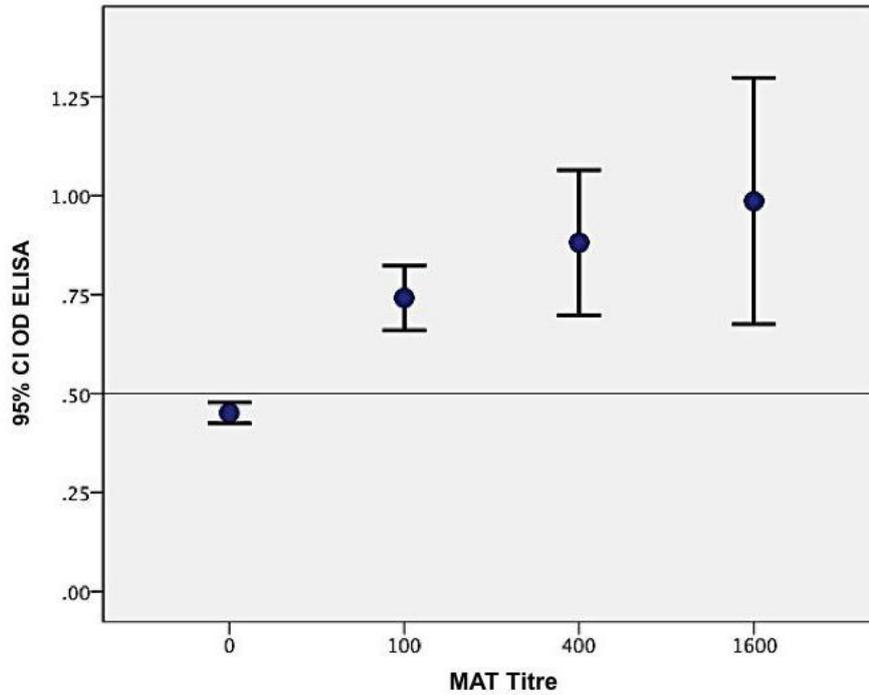


Figure 3. ELISA LipL32 evaluation (with 95% Confidence Intervals). Graph was generated by plotting the OD values based on the peak titre of MAT (100, 400 and 1600), and peak titre 0 was indicated for MAT negative serum.

Table 3. Analysis of OD_ELISA based on the peak titre of MAT

MAT result (peak titre)	N	Mean	Std. Dev	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
Negative MAT	407	.451	.206	.0102	.431	.471	.115	1.823
Positive MAT : 100	94	.742	.301	.031	.680	.803	.343	2.045
400	49	.881	.479	.068	.743	1.019	.367	2.669
1600	21	.986	.501	.109	.758	1.215	.506	2.089
Total	571	.555	.320	.013	.529	.581	.115	2.669

ranged between 0.5-1.0. Some serum with MAT titer 100 and 400 had low OD (less than 0.5). The OD ELISA for all bovine serum with MAT titer 1600 was above 0.5. The sensitivity of bovine serum in ELISA LipL32 was higher as the titer of MAT increased. Similar finding has been reported by previous study that evaluated the ELISA LipL32 for leptospirosis canine (SNAP Lepto), which is available commercially from IDEXX. The study showed the agreement percentage between MAT and ELISA LipL32 (SNAP Lepto) for serum with MAT titer 100, 200, 400, 800 and 1600 was 62.5%, 55%, 72.4%, 69.8% and 73.5%, respectively (Curtis et al., 2015). The sensitivity of ELISA LipL32 developed in this study is apparently higher than that

study. However, the specificity of our ELISA is lower. These results indicate that LipL32 ELISA is potential to be used as a rapid test for identification of leptospirosis especially as a screening test for anti-Leptospira antibodies in bovine. For a screening approach, the sensitivity of ELISA LipL32 can be improved by lowering the cut off OD. It would decrease the specificity, but it can be overcome by follow-up confirmatory test (Table 5).

In this study, LipL32 ELISA was able detected sera that were positive against various serovars (hardjo, grippotyphosa, tarrasovi, rachmati, bataviae, Tarrasovi and Icterohaemorrhagiae). This is in agreement with fact that LipL32 is highly conserved among serovars of

pathogenic *Leptospira*. The ELISA OD was presented in table 6, for sera that were MAT-positive against serovar *Grippotyphosa*, *Hardjo*, *Bataviae*, *Rachmati*, *Tarrasovi*, and *Icterohaemorrhagiae*. One serum which was MAT-positive against both serovar *Grippotyphosa* and *Rachmati* has 0.92 OD, and the other two sera were MAT-positive against both serovar *Grippotyphosa* and *Bataviae* with the mean OD of 0.61. This findings was

also supported previous study that reported mice sera anti recombinant LipL32 was successfully recognised and reacted to 32kDa band (protein LipL32) from whole cell lysate of *Leptospira* from 17 different serovar (Chalayon et al. 2011). The fact that LipL32 ELISA can detect antibody to different serovars provides further support the usefulness of LipL32 ELISA as a screening test for Leptospirosis

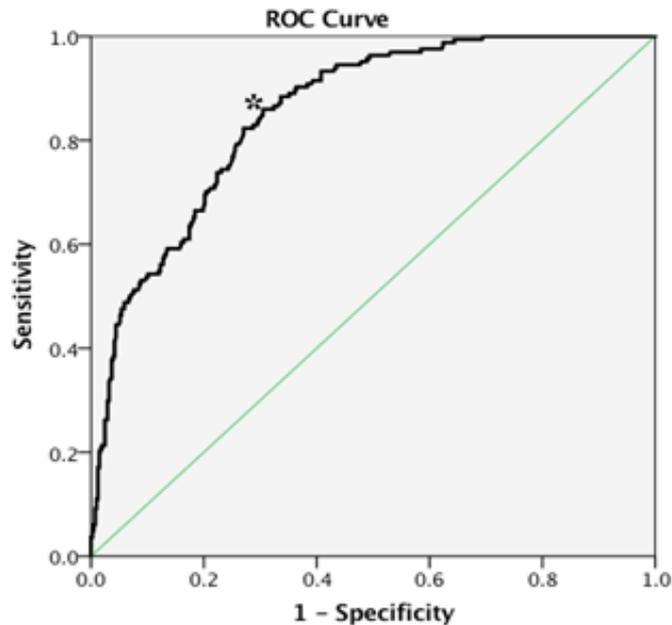


Figure 4. Receiver Operating Characteristic (ROC) Curve of ELISA LipL32. Analysis of ELISA LipL32 using bovine sera in which 164 were MAT positive and 407 were MAT negative. Optimal cut off point is indicated (*) for OD 0.504 with 86% sensitivity and 69.5% specificity, and area under curve of 0.853 (95% CI).

Table 4. Distribution of OD ELISA LipL32 based on MAT titre of bovine serum

MAT result	N	Frequency of serum sample (f) based on OD ELISA				
		(0.1 – 0.5)	(0.5 – 1.0)	(1.0 – 1.5)	(1.5 – 2.0)	(> 2.0)
Negative MAT	407	278	119	7	3	0
Positive MAT						
100	94	15	60	17	1	1
400	49	8	29	5	6	1
1600	21	0	13	4	3	1

Table 5. Comparison between the percentage of sensitivity-specificity and cut off OD ELISA LipL32 based on ROC curve analysis

Cut Off for OD ELISA	% Sensitivity (95% CI)	% Specificity (95% CI)
0.400	97.0	42.8
0.451	93.3	57.7
0.477	90.2	63.1
0.504*	86.0	69.5
0.532	75.0	75.7
0.544	72.6	77.6

* Optimal Cut off for OD ELISA LipL32 with 86% and 69.5% of sensitivity and specificity, respectively.

Table 6. Analysis of OD_ELISA based on the Leptospira serovar specific

MAT result (serovar specific)	N	Mean	Std. Dev	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
-ve MAT	407	.451	.206	.010	.430	.471	.115	1.823
+ve MAT: Grippotyphosa	82	.883	.475	.052	.779	.988	.367	2.669
Hardjo	71	.712	.254	.030	.652	.773	.343	1.503
Bataviae	3	1.345	.621	.358	-.196	2.887	.860	2.045
Rachmati	3	.881	.189	.109	.409	1.351	.769	1.100
Tarrasovi	1	.911912	.912
Icterohaemorrhagiae	1	.830830	.830
(Grippo and Rach)	1	.917918	.918
(Grippo and Bat)	2	.614	.331	.234	-2.356	3.583	.380	.847
Total	571	.555	.320	.013	.529	.581	.115	2.669

Table 7. Amino acid sequence of important epitope (P1 and P2) from LipL32 protein

Name	Amino acid sequence of LipL32	Source
<u>P1 (151-177)</u>	<u>AAKAK PVQKL DDDDD GDDTY KEERH NK</u>	<u>Lotterberger et al. 2009</u>
rLipL32_4f2	AAKAK PVQKL DDDDD GDDTY KEERH NK	Present study
L. Pomona	AAKAK PVQKL DDDDD GDDTY KEERH NK	Genebank AY609326
L. Hardjo	AAKAK PVQKL DDDDD GDDTY KEERH NK	Genebank AY442332
L. Grippotyphosa	AAKAK PVQKL DDDDD GDDTY KEERH NK	Genebank AY609327
<u>P2 (181-204)</u>	<u>LTRIK IPNPP KSFDD LKNID TKKL</u>	<u>Lotterberger et al. 2009</u>
rLipL32_4f2	LTRIK IPNPP KSFDD LKNID TKKL	Present study
L. Pomona	LTRIK IPNPP KSFDD LKNID TKKL	Genebank AY609326
L. Hardjo	LTRIK IPNPP KSFDD LKNID TKKL	Genebank AY442332
L. Grippotyphosa	LTRIK IPNPP KSFDD LKNID TKKL	Genebank AY609327



Figure 5. Sequence alignments for the important epitope of LipL32 protein located in amino acids number 151 to 177 (P1) and 181-204 (P2). LipL32 protein in this study (rLipL32_4f2) was aligned to P1, P2 and other LipL32 sequences obtained from genbank.

Previous studies performed using simultaneous and parallel solid phase peptide synthesis on derivatives cellulose membranes has been identified two important epitopes of LipL32, P1 and P2 which located in two different regions 151–177 and 181–204, respectively. Both epitopes were highly conserved between pathogenic *Leptospira* (Lottersberger et al. 2009). Sequences analysis showed 100% homologous for both peptides (P1 and P2) with recombinant LipL32 protein produced in this study (rLipL32_4f2) and LipL32 protein from *Leptospira* serovar Pomona, Hardjo, and Grippotyphosa obtained from gene bank with accession number AY609326, AY442332 and AY609327, respectively (Table 7) .

MAT is a gold standard for Leptospirosis and used in this study to determine the true positive and true negative status of Leptospirosis (OIE 2013). However, previous studies found that MAT has many limitation and was imperfect as gold standard for evaluation of the new diagnostic test (Limmathurotsakul et al. 2012). Beside serology assay, PCR targeting LipL32 gene has been developed in order to increase the sensitivity of Leptospirosis detection. The PCR had higher sensitivity and spesificity compared to MAT (De Abreu Fonseca et al. 2006; Ooteman et al. 2006). Thus, bovine serum that positive in ELISA LipL32 can be confirmed by PCR test for eliminating false positive.

CONCLUSION

The result showed ELISA LipL32 in this study can be used as rapid test for identification of leptospirosis and for screening anti-*Leptospira* antibodies in bovine. This ELISA LipL32 was able to detect serum MAT-positive to hardjo, grippotyphosa, tarrasovi, rachmati,

and bataviae. The optimal cut off for OD ELISA determined based on ROC curve was 0.504, and its sensitivity and specificity of ELISA LipL32 were 86.5% and 69.5%, respectively.

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