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Development of In-House ELISA using recombinant LipL32 for Detection of Human Leptospirosis in Indonesia

Pengembangan ELISA In-House untuk Deteksi Leptospirosis pada Manusia di Indonesia

Sumarningsih[1], Gita Sekarmila[1], Andi Mulyadi[1], Ahpas[1], Simson Tarigan[1]

[1]Indonesian Research Center for Veterinary Science, Jl. Raya Jakarta-Bogor Km.46 Cibinong, National Research and Innovation Agency, Bogor
**Email:* suma028@brin.go.id

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Abstrak

Keberhasilan pengobatan pasien leptospirosis sangat bergantung pada diagnosis dini yang dilakukan secara akurat melalui konfirmasi hasil laboratorium. MAT yang merupakan uji standar utama untuk mendeteksi leptospirosis pada manusia memiliki banyak keterbatasan dan hanya tersedia di laboratorium rujukan. Oleh karena itu, banyak penelitian menyarankan protein LipL32 sebagai kandidat yang baik untuk pengembangan alat deteksi leptospirosis karena bersifat lestari (*conserved)* dan hanya diproduksi pada spesies Leptospira patogen. Penelitian ini bertujuan untuk menyelidiki kinerja ELISA yang kami kembangkan di labororium menggunakan LipL32 rekombinan untuk mendeteksi kasus leptospirosis di Indonesia. Empat belas serum manusia digunakan dalam penelitian ini dan status infeksi ditentukan menggunakan MAT. Hasil penelitian menunjukkan sembilan dari sebelas serum positif MAT berhasil dikenali dengan ELISA LipL32. Ikatan antibodi pada LipL32 juga dikonfirmasi dengan immunoblot. Terdapat satu dari tiga serum negatif MAT yang memiliki OD tinggi di atas 0,5 pada pemeriksaan ELISA, namun menunjukkan reaksi negatif pada hasil imunoblot. Secara keseluruhan, penelitian ini menunjukkan bahwa protein rekombinan LipL32 dapat mengenali antibodi dari manusia yang terinfeksi leptospirosis dan dapat digunakan sebagai antigen universal untuk mendeteksi infeksi oleh serovar leptospira patogen.

Kata kunci : ELISA; human; leptospirosis; LipL32.

Abstract

Early laboratory confirmation is important for the accurate diagnosis and treatment of patient infected by leptospirosis. Microscopic agglutination test (MAT) as the gold standard for detection of human leptospirosis has many limitation and only available in reference laboratories. Therefore, many studies suggested LipL32 protein as a good candidate for development of leptospirosis detection kit because it is highly conserved and produced only in pathogenic Leptospira species. In this study, we aim to investigate the performance of our in-house ELISA using recombinant LipL32 to detect leptospirosis in Indonesia. Fourteen human sera were used in this study and the infection status were determine using MAT. The result showed that nine of eleven MAT positive sera were successfully recognized by LipL32 ELISA. The antibody binding to LipL32 was also confirm by immunoblot. There was one of three MAT negative sera has high OD above 0.5 in ELISA, but it showed negative reaction in immunoblot result. Overall, this study demonstrated that recombinant LipL32 protein can recognized antibody from human leptospirosis and can be used as a universal antigen to detect infection by any serovars of pathogenic leptospira.

Keywords : ELISA; human; leptospirosis; LipL32.

Introduction

Leptospirosis is an important zoonotic disease that can be transmitted between animals and humans by direct or indirect contact. The clinical signs may vary from a subclinical infection to a severe illness that can cause mortality—(Levett, 2001). Estimated annual morbidity of leptospirosis in human is 1.03 million cases with 58,900 deaths for each year (Costa *et al.,* 2015). Furthermore, high number of leptospirosis cases (1.408 cases with 139 deaths) has been reported during 2022 in Indonesia (Nurhidayat, 2023). However, this number can be higher since Leptospirosis commonly underdiagnosed because of the non-specific symptoms and signs (Gasem *et al.,* 2020).

The current diagnosis of leptospirosis, Microscopic agglutination test (MAT) and isolation, are expensive, impractical, technically demanding and only can be performed in three laboratories in Indonesia (Gasem *et al.,* 2020; Goris *et al.,* 2013; Musso and La Scola, 2013). To date, there are more than 200 serovars within the species L. interrogans (Levett, 2001). There is possibility that the causative Leptospira serovar present in the field not included in the panel used in MAT and giving the false negative for MAT result. Furthermore, previous study reported that MAT has many limitation and was imperfect as gold standard for evaluation of the new diagnostic test (Limmathurotsakul *et al.,* 2012).

LipL32 protein is found in outer membrane of leptospira, highly conserved and only produced in pathogenic Leptospira species (Bomfim *et al.,* 2005; Haake *et al.,* 2000). Recently, many diagnostic tests use recombinant protein as an antigen because it is safe and more specific compare to native protein from pathogenic bacteria. Therefore, many study suggested recombinant LipL32 as a good antigen candidate for diagnostic kit because it can detect infection to any serovars of pathogenic Leptospira.

Previous study reported that ELISA using recombinant protein LipL32 had excellent performance for detection of bovine with Leptospirosis (Bomfim *et al.,* 2005). We also developed in-house ELISA using recombinant LipL32 and evaluated the accuracy using bovine sera from west java that infected leptospirosis. This LipL32 ELISA showed the relative sensitivity and specificity were 86.0% and 69.5%, respectively was compared to MAT (Sumarningsih *et al.,* 2017). LipL32 ELISA has been considered as an alternative for MAT and can be used as a screening test Leptospirosis sSurveillance. The study aim to investigate the performance of in-house LipL32 ELISA for detection of human Leptospirosis.

Materials and Methods

Human sera

Fourteen samples of human sera were obtained from diagnostic unit of Indonesian Research Center of Veterinary Science in Bogor since 2014 to 2020. These sera were previously tested by MAT using panel of 15 different serovars including L. icterohaemorrhagiae, L. javanica, L. celledoni, L. canicola, L. ballum, L. pyrogens, L. cynopteri, L. rachmati, L. australis, L. pomona, L. grippotyphosa, L. hardjo, L. bataviae, and L. tarrasovi. The MAT was performed using procedure as described in our previous study (Sumarningsih *et al.,* 2017).

LipL32 enzyme linked immunosorbent assay (ELISA)

LipL32 protein was produced from *Escherichia coli* and purified using Ni-NTA column as described in previous study (Sumarningsih *et al.,* 2017). The size and purity of recombinant LipL32 protein was analysed by SDS-PAGE. Eluted LipL32 protein were collected from all fractions and the concentration was measured using spectrophotometer (Ultraspec 1100 pro) at wavelength 280nm. Some buffers were prepared in this ELISA including Phosphate-buffered saline-Tween 0.05% (PBST) for washing buffer, 5% Casein in PBS for blocking buffer and 4% normal goat serum in PBS for diluent. The ELISA plate (NUNC Maxisorb) was coated with 100µl LipL32 protein (5µg/ml) in carbonate-bicarbonate buffer (pH 9.6), and incubated for 16 hours at 4°C. After washing, 150µl blocking buffer was added into each well and incubated for 2 hours at 25°C. ELISA plate was then washed

and 100µl of diluted human sera (1:100 and 1:200) was added and incubated for 1.5 hours at 25°C. After washing four times, HRP Goat anti-human immunoglobulin G (IgG) diluted 1:3000 was then added and incubated for 1.5 hours at 25°C. After washing four times, ABTS substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) was added into each well and incubated for 15 minutes at 25°C. The optical density (OD) was then read using ELISA microplate reader at wavelength 405nm.

LipL32 Immunoblot

Recombinant LipL32 was blotted in two different spot (2.5µl and 1.5µl) in a nitrocellulose membrane strip. The membrane was air-dryed and blocked with 5% casein in PBS solution for 2 hours. Human sera were diluted at 1:200 in diluent and incubated with membrane for 1.5 hours at 25°C. The membrane was then washed four times with PBST and incubated in HRP Goat anti human IgG diluted 1:3000 in diluent for 1.5 hours at 25°C. The membrane was then washed four times and antigen-antibody binding was probed by adding chromogenic DAB (3, 3 –diaminobenzidine) substrate.

Result and Discussion

Leptospirosis is an important infectious disease and mostly difficult to diagnose because the infection cause no symptoms or mild symptoms that similar to other disease. Current gold standard for serological test is MAT. However, previous study have reported high number of misdiagnosed Leptospirosis case in patient at the hospital sites (Gasem *et al.,* 2020). It is important to develop a rapid diagnostic test using universal antigen for Leptospirosis detection.

In this study, we used recombinant LipL32 produced in *E.coli* with high purity percentage (above 90% (figure 1) as antigen in our in-house ELISA and the performance was evaluated to detect human leptospirosis (Figure 2). A total of fourteen human sera were used in this study, eleven sera were MAT positive and three were MAT negative. The highest OD among these MAT negative sera (dilution 1 in 200), which was 0.49, used as the cut off value for LipL32 ELISA. ELISA result with the OD above 0.5 will be categorized as a positive reaction, while the OD less than 0.5 as a negative reaction.

Figure 1. SDS-Page (12.5%) of purified recombinant LipL32 protein. LipL32 protein was eluted in fraction 1 (E1), fraction 2 (E2), fraction 3 (E3), fraction 4 (E4) and fraction 5 (E5). 10 µl of each fraction was analyzed to confirm the size and purity of LipL32 protein. The blue arrow showed the LipL32 protein with correct size 32kDa and high purity (>90%).

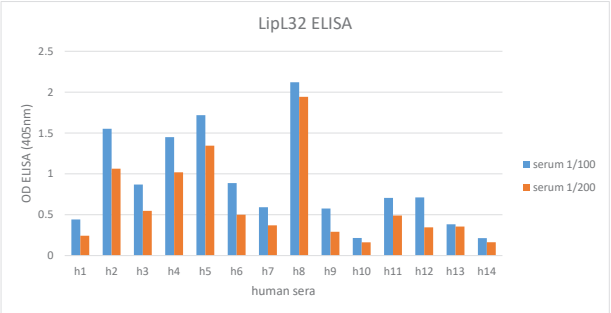

Figure 2. LipL32 ELISA using fourteen human sera (h1 to h14). Sera were diluted 1:100 and 1:200 in diluent. The antibody binding was detected by adding ABTS substrate and read at wavelength 405nm. Eleven sera were MAT positive and three sera were MAT negative (h11, h13 and h14). The OD 0.5 was used as cut off value.

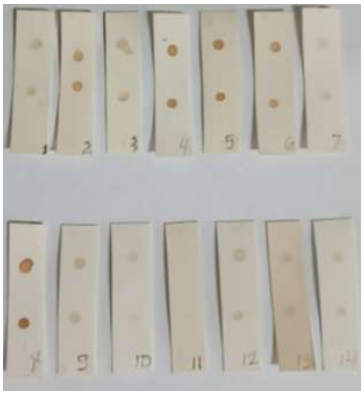

Figure 3. Immunoblot of recombinant LipL32 protein using fourteen human sera (h1 to h14). The nitrocellulose membrane was spotted with LipL32 protein in two different volume, 2.5µl (upper) and 1.5µl (lower). The Ab-Ag binding was detected using HRP Goat-anti human IgG.

The antibody binding to LipL32 in ELISA was also confirmed by immunoblot and then compared to MAT (Table 1). Figure 2 showed nine of MAT positive sera (h2, h3, h4, h5, h6, h7, h8, h9 and h12) were reacted to LipL32 and the ELISA OD was high above 0.5. Similar findings were obtained in immunoblot, but sera number h7 and h12 failed to reacted with LipL32 (figure 3). This result demonstrated that recombinant LipL32 protein were successfully binding to IgG from human that infected by different serovar of Leptospira (L. icterohaemorrhagiae, L. javanica, L. bataviae, L. hardjo, L. tarrasovi, L. celledoni and L. rachmati). Previous study also reported that LipL32 is a conserved protein among the pathogenic Leptospira species, immunoblot result showed serum anti-LipL32 was able to reacted with native LipL32 protein from *L. interrogans pomona, L. interrogans bratislava, L. kirschneri grippotyphosa, L. kirschneri mozdok, L. borgpetersenii hardjo, L. borgpetersenii tarrasovi, L. inadai lyme, L. noguchii fort bragg, L. santarosai canalzonae, L. santarosai bakeri and L. weilii celledoni* (Haake *et al.,* 2000). Therefore, it supported that recombinant LipL32 is a good candidate for universal antigen in rapid in-house diagnostic kit.

Results showed that two of MAT positive sera (h1 and h10) have negative reaction in ELISA and immunoblot. This can be explained by antibody response in human leptospirosis where IgM will present earlier than IgG (Picardeau, 2013). MAT detect both IgG and IgM antibody, but the ELISA and immnublot we used in this study only detect IgG. Therefore, it can give a false negative result to MAT positive sera in the case of acute infection because the IgG was not present yet in patient serum.

Result from immunoblot showed that all three MAT negative sera (h11, h13 and h14) cannot bind to LipL32. This negative reaction was also found in ELISA except for serum h11. This false positive result from h11 serum is a non-specific reaction commonly found in indirect ELISA which mostly caused by hydrophobic binding of immunoglobulin in serum sample to the solid surfaces (Waritani *et al.,* 2017).

We cannot perform statistical analysis data to determine the accuracy of LipL32 ELISA because of very limited sample. However, this study showed that LipL32 ELISA successfully recognized nine of eleven MAT positive sera. This result supporting further research for in-house development of diagnostic test for Leptospirosis detection in Indonesia.

Conclussion

This study demonstrated that our in-house ELISA using recombinant LipL32 protein was able to detect leptospirosis infection in human. It also supported that LipL32 is a good antigen candidate for for development of diagnostic kit. However, further study on ELISA optimization and validation is required using more serum sample from human leptospirosis.

Tabel 1. Comparison of MAT, LipL32 ELISA and LipL32 Immunoblot using sample from human sera.

Human Sera	MAT	OD LipL32 ELISA		LipL32 Immunoblot Serum 1:200
		Serum 1:100	Serum 1:200	
h1	ict 1/100; aus 1/50	0.442	0.243	negative
h2	ict 1/400; jav 1/400; bat 1/100	1.552	1.063	positive
h3	har 1/400; bat 1/100	0.869	0.547	positive
h4	ict 1/100; jav 1/100	1.449	1.019	positive
h5	bat 1/400	1.718	1.345	positive
h6	ict 1/400; bat 1/400; tar 1/100	0.887	0.500	positive
h7	ict 1/100; bat 1/100; tar 1/100	0.592	0.370	negative
h8	bat 1/100; tar 1/400	2.121	1.943	positive
h9	cel 1/100; bat 1/100	0.576	0.291	positive
h10	ict 1/100; can 1/50; tar 1/100	0.215	0.163	negative
h11	negative	0.706	0.490	negative
h12	ict 1/100; rach 1/100	0.712	0.345	negative
h13	negative	0.383	0.356	negative
h14	negative	0.213	0.164	negative

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