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

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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.


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 surveillance. 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. tarassovi. 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-dried 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.
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. tarassovi, 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 tarassovi, 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 immublot 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.

**Conclusion**

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.

<table>
<thead>
<tr>
<th>Human Sera</th>
<th>MAT</th>
<th>OD LipL32 ELISA</th>
<th>LipL32 Immunoblot</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Serum 1:100</td>
<td>Serum 1:200</td>
</tr>
<tr>
<td>h1</td>
<td>ict 1/100; aus 1/50</td>
<td>0.442</td>
<td>0.243</td>
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<tr>
<td>h2</td>
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<tr>
<td>h3</td>
<td>har 1/400; bat 1/100</td>
<td>0.869</td>
<td>0.547</td>
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<tr>
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<td>ict 1/100; jav 1/100</td>
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<td>1.019</td>
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<tr>
<td>h5</td>
<td>bat 1/400</td>
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<tr>
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<tr>
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<td>0.163</td>
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<tr>
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<tr>
<td>h12</td>
<td>ict 1/100; rach 1/100</td>
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References


