

## A low molecular weight lipopolysaccharide antigen preparation reactive to acute leptospirosis heterologous sera

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**Abstract.** There is a need for identification of new infection markers against common *Leptospira* isolates in Malaysia. To achieve this goal, seven-day-old cultures of *Leptospira interrogans* serogroup Icterohemorrhagiae (L44) and *Leptospira interrogans* serogroup Javanica (L55) were used for antigen preparation by sequential extraction method using 40mM Tris, 8M Urea and 2M thiourea. Immunoblot analysis of the antigens were performed using serum samples from 46 local patients with confirmed acute leptospirosis, 28 patients with other infections and 14 healthy controls. The patients serum samples used in this study contained heterologous antibody against a number of different *leptospira* serovars. A strong IgM reactivity to a broad diffuse band of 10-15 kDa was observed. Combining results using L44 and L55 antigens showed sensitivity of 80.4% and specificity of 95.2% for detection of leptospirosis. Proteinase K and periodate treatment indicated that the band is likely to be lipopolysaccharide (LPS) in nature. This study showed that the 10-15 kDa antigen could potentially be useful for serodiagnosis of acute leptospirosis in Malaysia.

### INTRODUCTION

Leptospirosis is the most widespread zoonotic infection in the world (CDC, 1998). Its causative agent is highly motile spirochete bacterium. There are more than 250 different serovars identified worldwide, and 37 of them have been isolated in Malaysia (El Jalii & Bahaman, 2004). The disease occurs in both humans and animals, and the prevalence is much higher in tropical countries particularly during the hot and rainy seasons. It is an important public health problem, especially when heavy rainfall results in flooding in areas with poor housing and sewage infrastructure (Doungchawee *et al.*, 2008). Initial symptoms are not specific, often with

flu-like symptoms; however in human the infection may progress rapidly to serious complications with multi-systemic damage involving kidney, liver, lung and/or brain.

Malaysia is considered endemic for leptospirosis with prevalence of leptospiral antibody in 12% of normal population (Tan, 1970). A number of outbreaks have been reported from time to time (Koay *et al.*, 2004). During these outbreaks, confusion between the wide range of clinical presentations associated with leptospirosis and other hemorrhagic fever common to this region (e.g. dengue) caused delay in the early diagnosis required for the timely administration of antibiotic therapy (Sanders *et al.*, 1999; Levett *et al.*, 2000).

Most cases of leptospirosis are diagnosed by serology. This is because culture of leptospires is slow and expensive. Microscopic agglutination test (MAT) remains the most widely used reference technique (Ahmad *et al.*, 2005). It is highly specific but its low sensitivity (30-60%) makes it unsuitable for rapid diagnosis of leptospirosis (Cumberland *et al.*, 1999). Other serological tests such as indirect hemagglutination (Levett & Whittington, 1998) microcapsule agglutination (Arimitsu *et al.*, 1998) latex agglutination (Arimitsu *et al.*, 1998) and ELISA (Winslow *et al.*, 1997) do not offer satisfactory level of sensitivity and specificity for the early-phase diagnosis of leptospirosis. Although assays detecting IgM based on crude antigen (Winslow *et al.*, 1997) appear to be more sensitive for serodiagnosis, they may be subjected to variation in specificity (Guerreiro *et al.*, 2001). Research on leptospiral laboratory diagnosis are mostly directed to the outer membrane region of the cell, since it is clear that analysis of the cell surface is important in developing new sero-diagnostic tools. Immunoblotting technique using outer membrane antigenic components offers an attractive and suitable alternative. The main components of the outer membrane are lipopolysaccharide (LPS) and lipoproteins. LPS is the portion which is extremely antigenic and determines serotype specificity. It is an early target antigen for host antibodies that are agglutinating, opsonic and protective (Adler & Faine, 1978).

Thus in the effort towards developing a sensitive and specific test for acute leptospirosis, this study was conducted to identify potential infection markers in the outer membrane of pathogenic *Leptospira*, and to perform some characterization and evaluation of the marker.

## MATERIALS AND METHODS

### **Leptospiral strains and media**

Two pathogenic *leptospira* serovars that are commonly found in Malaysia i.e.

*Leptospira interrogans* serovar Icterohemorrhagiae (L44) and *L. interrogans* serovar Javanica (L55) were obtained in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium from the Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor, Malaysia. Bulk cultures were cultivated in liquid EMJH at 30°C in an incubator shaker for seven days. Cells ( $1-2 \times 10^8$  organisms/ml) were then harvested and washed once with phosphate buffered saline (PBS), 0.15M, pH 7.2, and stored in a -80°C freezer.

### **Serum samples**

A total of 46 retrospective serum samples from leptospirosis cases with acute symptoms were used. These samples originated from government hospitals in 12 of 14 states in Malaysia. The samples were positive by microscopic agglutination test (MAT), which is considered as the reference test. It is taken as positive when a single serum sample shows a titre of  $\geq 400$  (Faine *et al.*, 1999). In addition all samples were also positive by two commercial tests namely Lepto Tek Dri-Dot™ (BioMerieux, France) with overall sensitivity and specificity of 91% and IgM lateral flow test (Omega Diagnostics, United Kingdom) with 100% sensitivity and specificity, as reported by the respective manufacturers. This approach was taken to ensure that true positive and negative sera samples were used to evaluate the Western blot results in this study.

These acute phase serum samples were collected  $\leq 10$  days from onset of symptoms. The clinical features were as follows: fever (100%), chills and rigors (32/46, 70%), cough (25/46, 55%), abdominal pain (8/46, 17%), joint pain/arthritis (5/46, 11%), jaundice (6/46, 13%), calf tenderness (3/46, 6.5%) and thrombocytopenia (4/46; 8.7%). Culture was unable to be performed on these samples, thus confirmation of the identity of the leptospiral serovars could not be made. However, based on the MAT test results, the presumptive identification of the serovars were as follows: Icterohaemorrhagiae, Autumnalis, Pyro-

genes, Bataviae, Grippotyphosa, Canicola, Australis, Pomona, Javanica, Sejroe, Djasiman, Tarassovi, Hepdomadis.

Other than the above samples, 28 serum samples from patients with other febrile diseases which may be misdiagnosed as leptospirosis were used, namely dengue (n=13), malaria (n=5), typhoid (n=3), toxoplasmosis (n=3) and amoebic liver abscess (n=4). These samples tested negative by MAT, Lepto Tek Dri-Dot and the IgM lateral flow test. In addition, 14 serum samples from healthy blood donors were used after confirming that they were negative by the above mentioned tests.

Prior to being used for specific IgM detection, all serum samples (n= 88) were pre-absorbed with rheumatoid factor (RF) absorbent (Virion-Serion, Germany) according to the manufacturer's instructions. This served to remove of IgM rheumatoid factors which preferably bind to IgG immune-complexes, and thus may cause non-specific reactivity. All serum samples were collected according to the guidelines of USM Human Research Ethics Committee.

#### **Antigen preparation**

Leptospiral antigen was prepared using sequential protein extraction technique, as described by Molloy *et al.* (1998); with some modifications. Seven-day *leptospira* culture was harvested and washed three times with phosphate-buffered saline (PBS, pH 7.2). The pellet was resuspended with 40mM Tris (pH 7.8) at the ratio of 1:2 and vortexed vigorously for 5 min. The bacterial suspension was then lysed by three cycles of freeze-thawing using liquid nitrogen and 37°C water bath. After centrifugation, the supernatant was stored at -20°C and the pellet was added to the sequential extraction buffer at a ratio of 1 mg pellet to 1 µl buffer. The buffer comprised 8M urea, 2M thiourea, 4% CHAPS, 50 mM DTT, 1mM protease inhibitor cocktail (Roche Diagnostic, Germany). The mixture was then vortexed and centrifuged at 10 000g for 10 min. The supernatant was collected and buffer exchanged to 10 mM Tris using

a spin column with 3 kDa MW cut off (Vivaspin, USA). The protein content was then determined by Lowry method using reagent compatible and detergent compatible kit (BioRad, USA), then stored at -80°C until use.

#### **SDS-PAGE and Immunoblotting**

SDS-PAGE was performed using Mini-PROTEAN 3 apparatus (Bio-Rad, Hercules, CA, USA) and standard protocol (Laemmli, 1970). Briefly the antigen was solubilised in final sample buffer then incubated in water bath (37°C) for 10 minutes and centrifuged. Approximately 20 µg protein was loaded onto a 12% resolving gel and electrophoresed at 100 volts. The gel was stained by Coomassie brilliant blue (BioRad, USA) and/or silver stain. For immunoblotting, the gel was transferred onto a nitrocellulose paper (NCP) with 0.45µm pore size (GE Osmonic, USA) using a semi-dry transblot (BioRad, USA). The NCP strips were blocked by Super Block® (Pierce, USA) and incubated overnight with diluted serum (1:50) at 4°C. After a washing step, the strips were incubated with monoclonal anti-human IgM and anti-human IgG conjugated with HRP (Zymed, USA) and the reactivity was detected by using chemiluminescence substrate (Roche Diagnosrtic, Germany) and developed on an X-ray film (Kodak, USA).

#### **Proteinase K and Periodate treatment**

The antigen preparation was treated with 10mg proteinase K and incubated at 37°C for 1 h and then stored at -20°C. Proteinase K-treated antigen and untreated control were then subjected to SDS-PAGE, staining (Coomassie and silver stain) and immunoblot analysis. In the latter, five pooled leptospirosis serum samples were used as the primary antibody and monoclonal anti-human IgM-HRP (Zymed, USA) as the secondary antibody.

A second antigen preparation was subjected to SDS-PAGE, followed by treatment using a lipopolysaccharide gel staining kit (Molecular Probes, USA). This kit specifically stains carbohydrate components of antigen and is considered to

be much more sensitive for LPS detection than periodate silver staining. The principle of the test is based on the carbohydrate component being oxidized by a periodate solution, which then reacts with Pro-Q® Emerald 300 dye. This created a bright green fluorescent signal which was visualized using a 300 nm UV trans-illuminator.

#### **Total lipopolysaccharide (LPS) Extraction**

A rapid commercial test kit (BOCA Scientific, Inc, USA) was used to extract total LPS from the bacterial culture. Briefly, bacterial cells were harvested and cell lysis buffer was added to the pellet, mixed and incubated for 5 min. Chloroform was then added to the cell lysate and centrifuged. Purification buffer was added to the supernatant and incubated at room temperature for 15min. LPS antigen (at optimized loading volume) were then separated by SDS-PAGE and tested for their reactivities with pooled leptospiral serum sample by IgM immunoblot analysis as described earlier.

#### **LPS elution**

Elution of the LPS-like component from gel slices was performed using Electro-Eluter Model 422 (Bio Rad, USA). Preparation of stock buffers and reagents were performed according to the manufacturer's recommendation (Instruction Manual, Electro-Eluter Model 422, Bio Rad, USA).

Approximately equal amounts (based on the protein content) of leptospiral antigen from L44 and L55 serovars were pooled and separated according to SDS-PAGE procedure described earlier. In order to locate the 10 -15 kDa region on the gel, pre-stained protein molecular weight marker (Kaleidoscope™, Bio Rad, USA) was used and the band of interest was cut and chopped in small pieces for elution procedure, using membranes with molecular weight cut off (MWCO) of 3500 Daltons. Elution was performed at 8-10mA/glass tube with constant current for about 3-5 hours; and the liquid level slightly above the membrane was collected. Buffer

exchange (to 10mM Tris buffer) and concentration of the eluted LPS was simultaneously performed using a spin column with 2000 Daltons MW CO (Vivapsin, USA). The carbohydrate (LPS) content of the eluted antigen fraction (hereafter named as 'EAF') was determined using a rapid commercial test kit (Glycoprotein Carbohydrate Estimation Kit, Pierce, USA).

#### **Dot enzyme immunoassay (EIA)**

Pieces of rectangular nitrocellulose membrane, 9 x 9mm (0.45um; Micro-filtration Sys., Ca, USA) were dotted with one microlitre of EAF, each containing decreasing carbohydrate content i.e. neat (2µg), 1:2 dilution (1 µg), and 1:4dilution (0.5 µg). After the optimized LPS content of the antigen to be dotted was determined, the dot EIA was used to test the same panel of serum samples as that used for the Western blots. One microlitre of EAF was spotted onto the center of the membrane, air dried and blocked for one hour. After blocking, membranes were washed and transferred to 24 well flat bottom polystyrene culture plates. The membranes were then incubated with patients serum diluted at 1:50 in TBS for 3 hours at room temperature. The membranes were washed with TBS-T after aspirating off the sera in the wells. The washing step was repeated two more times each for 5 minutes. For the HRP-conjugated IgM antibody (Zymed, USA) a dilution of 1:3000 was prepared in TBS and added to all the wells containing the membranes for 1 hour incubation at room temperature. Upon completion of incubation, the conjugate solution was aspirated off and the membranes were washed three times in TBS as described above. The antigen and antibody reactions were detected by chemiluminescent substrate (Roche Diagnostic, Germany) and developed on x-ray films (Kodak, USA). The positive and negative results were determined by visual comparison of the dot intensity for each test.

#### **Statistical analysis**

The sensitivity obtained using antigen

prepared from *Leptospira* L44 serovar; and the sensitivity after including the positive results using antigen prepared from *Leptospira* L55 serovar, was compared using McNemar's test. A p value of <0.05 was considered statistically significant.

## RESULTS

### SDS-PAGE and Immunoblotting

The protein content of the antigen preparation was determined to be 2600-3400ug/ml. Coomassie stain of L44 and L55 antigen profiles revealed a complex pattern of protein bands with molecular weights ranging from less than 15 kDa to more than 150 kDa. Many proteins were shared however they were some band differences between two isolates. Silver staining of SDS-PAGE (Fig. 1a) detected a diffuse double band at 10-15 kDa region in both L44 and L55 antigen preparations, which was absent in Coomassie stained profiles (Fig.1b).

Further analysis of the antigen preparation was carried out by Western blot assays using serum samples from acute leptospirosis cases containing antibodies towards a variety of different serovars, and pooled sera from five blood donors (Fig. 2). A broad, diffused antigenic band of 10-15kDa was reactive with almost all leptospiral positive serum samples but not reactive with normal sera. The sensitivity of seroreactivity with L44 antigens was found to be 71.7% (33/46) when tested with serum of patients with acute leptospirosis; and the specificity was 95.2% (40/42) when tested with sera of normals and other infections (Table 1). The 13 false-negative sera which did not show reactivity with the L44 antigen preparation were further tested with L55 antigen preparation, and four samples tested positive, thus resulting in an increase of the sensitivity to 80.4% (37/46) (Table 2). This increase in sensitivity was found to be statistically significant ( $p < 0.05$ ). Figure 3 shows reactivities of 10-15 kDa band prepared from L55 antigen, using serum samples of patients which contain antibodies against

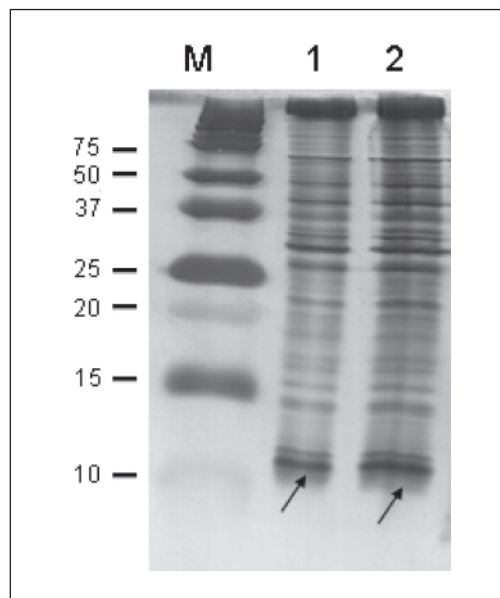


Figure 1a. Silver stained profiles of L44 (lane 1) and L55 (lane 2); (M) molecular weight markers. The arrow shows diffuse double band between 10-15 kDa

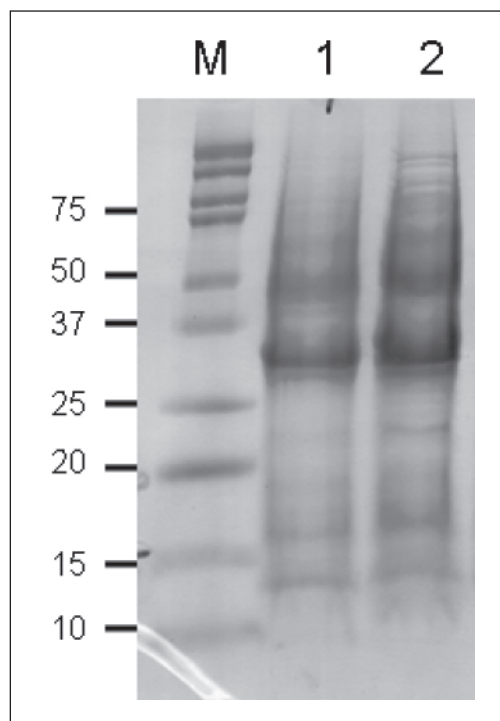


Figure 1b. Coomassie blue stained profiles of L44 (lane 1) and L55 (lane 2). (M) molecular weight markers. There is no diffuse double band between 10-15 kDa



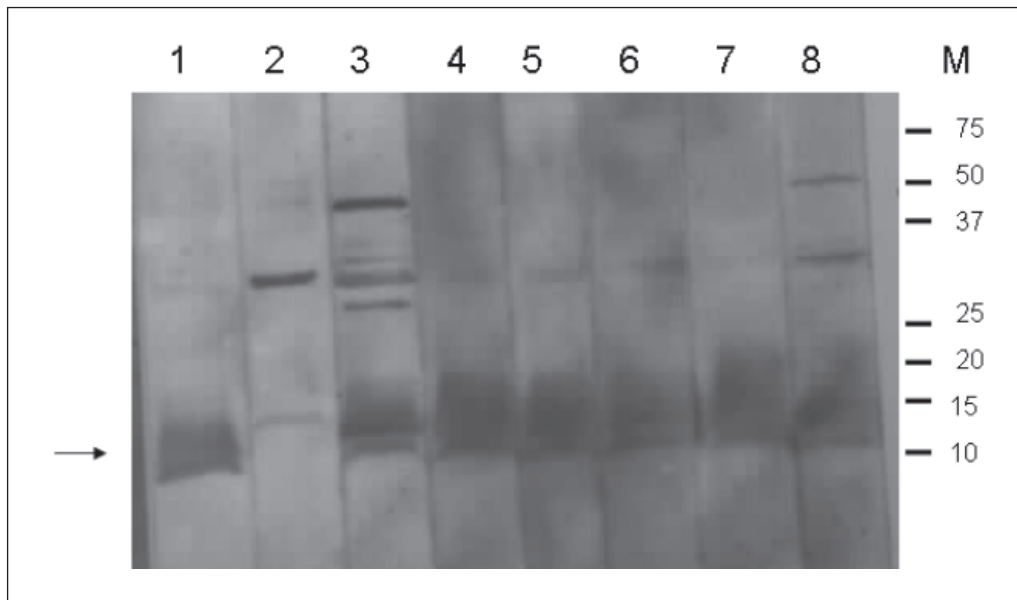


Figure 2. A representative IgM Western blot showing the reactivities of sequentially extracted antigen preparation (L44) with the patients' sera containing leptospiral antibodies from various *leptospira* serovars (presumptive) and pooled normal sera: Djasiman (lane 1); pooled normal sera (lane 2); Canicola (lane 3); Javanica (lane 4); Bataviae (lane 5); Australis (lane 6); Autumnalis (lane 7); Icterohaemorrhagiae (lane 8); (M) molecular weight markers. Arrow shows the intense bands of 10-15 kDa on all strips incubated with sera from leptospirosis patients

Table 1. Overall IgM reactivities of leptospirosis and control sera against 10-15 kDa band from L44 isolate by sequential extraction technique using Tris, urea, and thiourea

Samples	*MAT		Western blot		Total
	Pos	Neg	Pos	Neg	
Leptospira patients	46	0	33	13	46
Other infections	0	28	1	27	28
Healthy people	0	14	1	13	14

Sensitivity of Western blot : 33/46 = 71.7 %  
 Specificity of Western blot : 40/42 = 95.2%  
 Positive Predictive Value : 33/35 = 94.2%  
 Negative Predictive Value : 40/53 = 75.4%

\* These serum samples were tested by two commercial tests namely Lepto Tek Dri-Dot™ (BioMerieux, France) and the IgM lateral flow test (Omega Diagnostics, United Kingdom). Out of 33 samples which were positive by Western blot, the patients had the following clinical symptoms: fever (n=33, 100%), chills and rigors (n=32, 97%), cough (n=20; 60%), abdominal pain (n=5; 15%), joint pain/arthralgia (n=3, 9%), jaundice (n=4, 12%), calf tenderness (n=3, 9%) and thrombocytopenia (n=3.9%)

Table 2. Overall IgM reactivities of leptospirosis and control sera against 10-15 kDa band from L44 +L55 isolates by sequential extraction technique using Tris, urea and thiourea

Samples	*MAT		Western blot		Total
	Pos	Neg	Pos	Neg	
Leptospira patients	46	0	37	9	46
Other infections	0	28	1	27	28
Healthy people	0	14	1	13	14

Sensitivity of Western blot : 37/46 = 80.4 %  
 Specificity of Western blot : 40/42 = 95.2%  
 Positive Predictive Value : 37/39 = 94.8%  
 Negative Predictive Value : 40/49 = 81.6%

\* These serum samples were tested by two commercial tests namely Lepto Tek Dri-Dot™ (BioMerieux, France) and the IgM lateral flow test (Omega Diagnostics, United Kingdom). Out of 37 samples positive by Western blot, the clinical symptoms of the patients were as follows: fever (n=37, 100%), chills and rigors (n=32, 86%), cough (n=24; 65%), abdominal pain (n=6, 16%), joint pain/arthralgia (n=5, 14%), jaundice (n=6, 16%), calf tenderness (n=3, 8%) and thrombocytopenia (n=4, 11%)

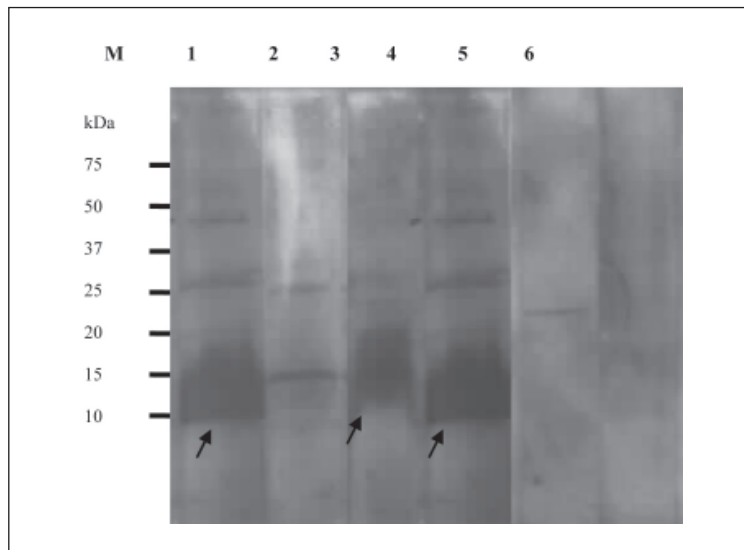


Figure 3. A representative IgM Western blot showing the reactivities of sequentially extracted antigen preparation (L55) with the patients' sera containing leptospiral antibodies from various serovars (presumptive) and pooled normal sera samples : Djasiman (lane 1); pooled normal sera (lane 2); Canicola (lane 3); Javanica (lane 4); Dengue (lane 5); amoebic liver abscess (lane 6); (M) molecular weight markers. The arrows show the intense bands of 10-15 kDa on strips incubated with sera from leptospirosis patients (lanes 1, 3, 4)

various serovars, and sera from two non-leptospiral infections.

The predominant antibody recognized by the reactive antigen was IgM; there was no reactivity to the same antigen when IgG antibody conjugate was used (Fig. 4).

#### **Proteinase K and Periodate treatment**

Proteinase K treated antigenic preparation showed that the 10-15 kDa band was resistant to the proteinase K, thus it was not protein in nature. The resistant band did not stain by Coomassie however silver staining showed the presence of a prominent band in 10-15kDa region (Fig. 5). Western blot analysis of proteinase K treated and untreated antigen showed that the 10-15 kDa reacted positively with pooled leptospirosis serum samples (Fig. 6).

SDS-PAGE separated antigens stained by the LPS staining kit demonstrated fluorescence band at the 10-15kDa region (Fig. 7). This indicated the presence of carbohydrate component in the antigenic band.

#### **Immunoreactivity to total LPS extraction**

Western blot analysis of total LPS extraction from the leptospiral culture did not show any seroreactivity against serum sample from pooled leptospirosis patients. In contrast, concurrent immunoblot of the antigen preparation used in this study showed good reactivity with pooled leptospira serum. (Fig. 8)

#### **Dot EIA**

The carbohydrate content of one ml of pooled and concentrated eluted LPS antigen (EAF) was determined to be 2000 µg/ml. Following optimizations, the minimum concentration of the EAF that showed clear positive results with leptospirosis samples and negative results with other serum samples was determined to be 1000 µg/ml (1 µg/µl), thus was selected for dot blot analysis using the panel of serum samples. Overall dot EIA results for patients and control sera indicated equivalent level of sensitivity (80.4%) and specificity (95.2 %)

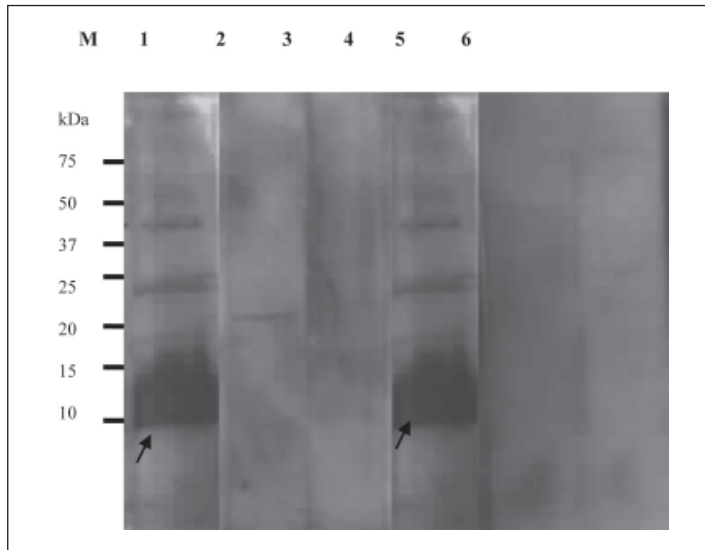


Figure 4. A representative Western blot showing reactivities of L44 sequentially extracted antigen preparation with patients and control sera probed with IgG and IgM conjugated antibodies. Lanes 1 and 4 are leptospirosis sera probed with IgM-HRP (arrows indicate area of intense reactivities at 10-15 kDa). Lanes 2 to 3 are the same leptospirosis sera used in lanes 1 and 4, but blotted with IgG-HRP (no reactivity seen at 10-15 kDa region). Lanes 5 and 6 are strips incubated with pooled normal serum and with dengue serum respectively and probed with IgG-HRP; (M) molecular weight marker

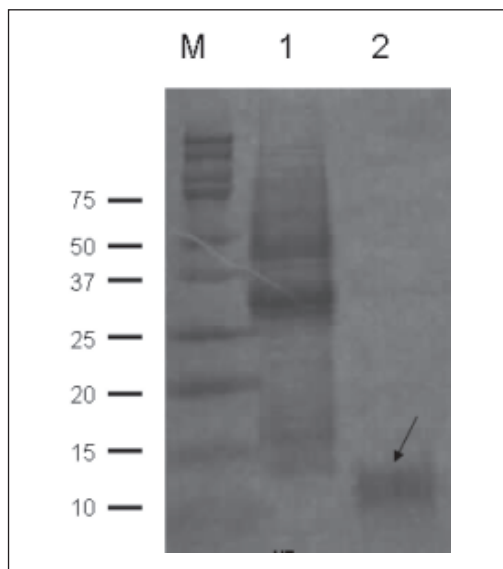


Figure 5. SDS-PAGE of Coomassie stained leptospiral antigen (lane 1); silver stained of proteinase -K treated leptospiral antigen (lane 2); (M) molecular weight markers. The arrow shows that the intense 10-15 kDa band is a proteinase-K resistant antigen which is not in Coomassie stained antigen

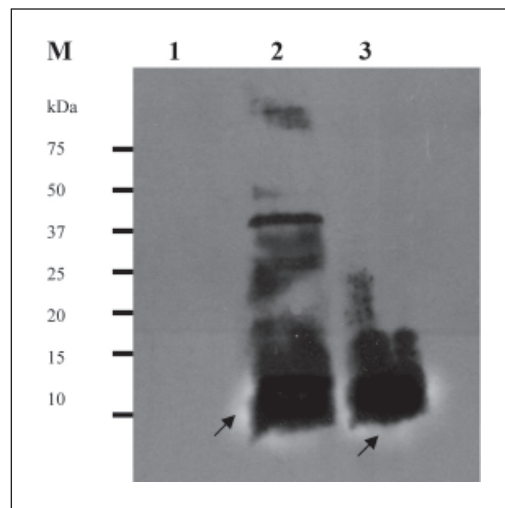


Figure 6. Immunoblot of proteinase K-treated antigen incubated with pooled normal sera (lane 1); untreated antigen incubated with pooled leptospira sera (lane 2); proteinase K treated antigen incubated with pooled leptospira sera (lane 3); (M) molecular weight markers. The arrows show the intense 10-15 kDa reactive bands irrespective of whether proteinase K-treated or untreated antigen was used



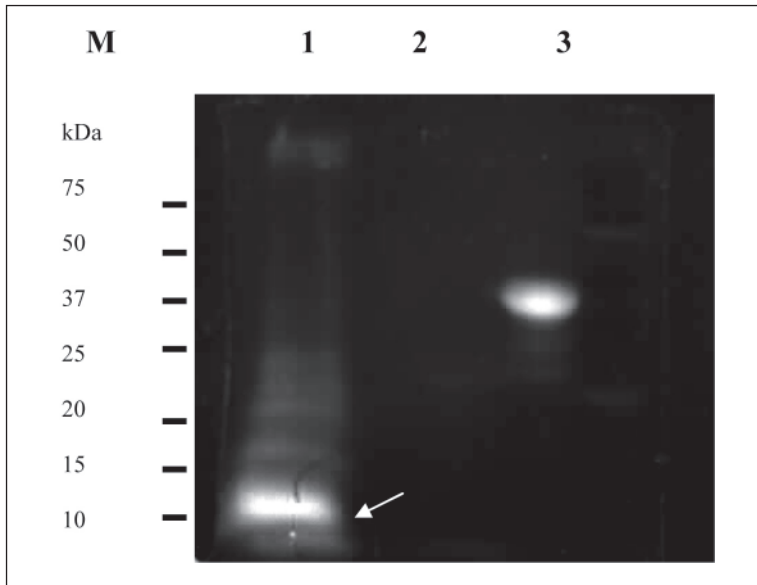


Figure 7. SDS-PAGE analysis of L44 antigen stained by LPS gel staining kit. Fluorescence seen (arrow) at 10-15 kDa region (lane 1); *BmR1* recombinant protein expressed by *E.coli* as LPS negative control (lane 2); LPS positive control (HRP) provided in the kit (lane 3); (M) molecular weight markers

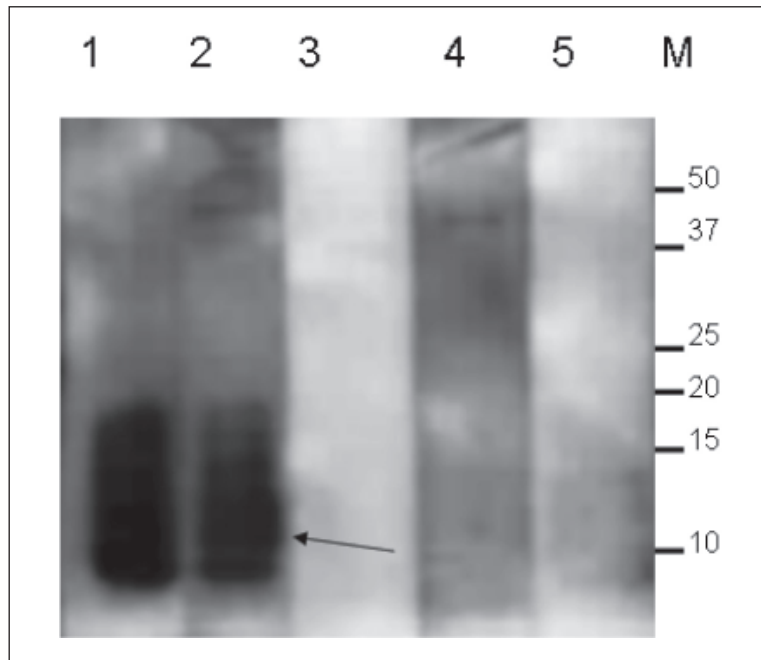


Figure 8. Comparison of total LPS and sequentially extracted LPS antigen preparation probed with pooled leptospirosis sera. Sequentially extracted LPS antigen of L44 (lane 1); sequentially extracted LPS antigen of L55 (lane 2); total LPS extraction of L55 antigen (lane 3); total LPS extraction of L44 antigen (lane 4); and LPS negative antigen of *BmR1* (lane 5); (M) molecular weight markers

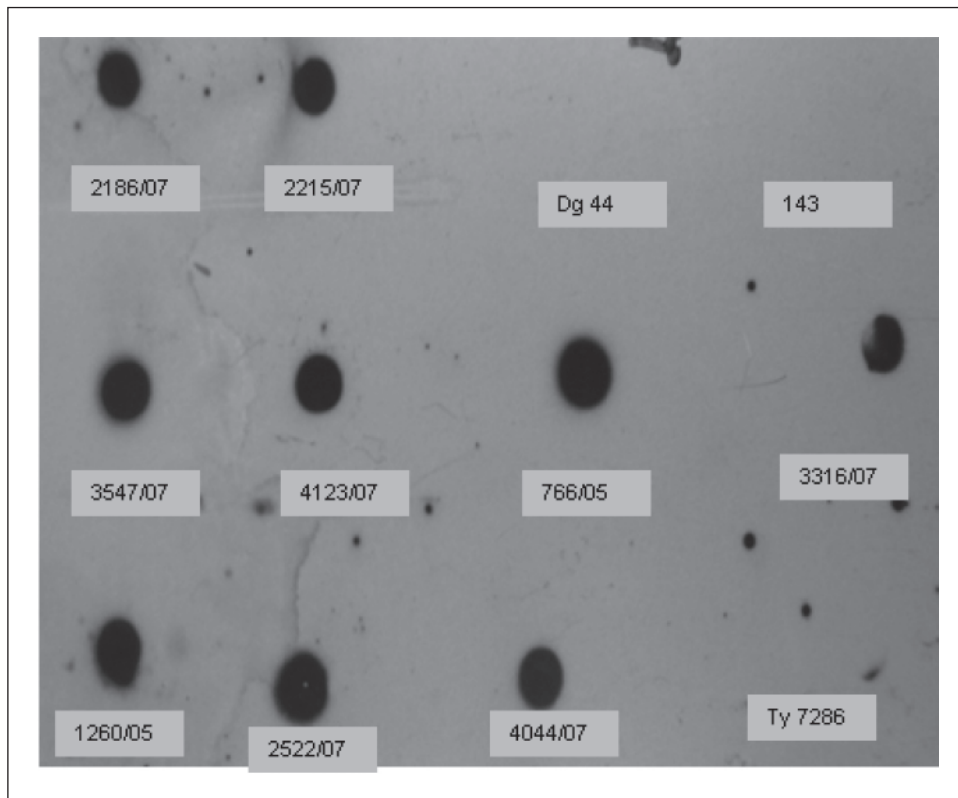


Figure 9. A representative IgM dot-blot using eluted 10-15 kDa antigen preparation. Reactive dots are shown on those incubated with leptospirosis serum samples (2186/07, 2215/07, 3547/07, 766/05, 4123/07, 3316/07, 1260/05, 2522/07, 4044/07) and not reactive with dots incubated with serum samples from patients with dengue (Dg44), and typhoid (TY7286), and pooled normal sera (143)

when compared with Western blot assay. A representative result of the dot EIA is shown in Figure 9.

#### DISCUSSION

Patients with leptospirosis often present with mild and non-specific clinical symptoms such as fever, headache, nausea and muscle pains; all of which are easily confused with symptoms of other febrile illnesses such as influenza, dengue, typhoid and malaria (Koay *et al.*, 2004). Misdiagnosed and mistreated patients are at risk for life-threatening multi-organ complications such as renal failure, liver damage, massive pulmonary hemorrhage, brain damage, cardiac arrhythmia, and circulatory collapse (Levett, 2001).

Therefore sensitive and specific diagnostic tests are needed to detect the disease at the early stage for prompt and correct treatment to prevent patients from developing severe complications. Thus detection of specific antibody against *Leptospira* at the acute stage of the infection is crucial.

The microscopic agglutination test (MAT) is considered as the standard reference test for serodiagnosis of leptospirosis, but was reported to be only 30-60% sensitive (Cumberland *et al.*, 1999). This is not adequately sensitive to detect early stage of the disease which is important for initiation of early and effective chemotherapy. In addition it is a complex, time consuming procedure and poses biological hazard since it requires maintenance of a panel of live cultures.

Commercially available kits for the diagnosis of leptospirosis also have limited sensitivity of less than 72% particularly for diagnosis of early stage infection (McBride *et al.*, 2007).

Specific anti-leptospiral IgM antibodies appear as early as three days post-infection (Faine *et al.*, 1999), thus IgM antibody is the relevant response for early diagnosis of acute leptospiral infection and the detection of such antibody can greatly contribute to rapid diagnosis of leptospirosis (Faine *et al.*, 1999). Therefore this study was focused on identifying leptospiral antigens that react specifically with anti-human IgM antibodies.

Two *Leptospira* serovars which are prevalent in Malaysia were used for antigen preparation. Sequential extraction method using Tris, urea, and thiourea were employed for the leptospiral antigen preparation. This is a highly reproducible method capable of separating soluble (hydrophilic) cytoplasmic proteins from poorly soluble (hydrophobic) membrane-associated proteins. The membrane-associated proteins were targeted since they are mainly responsible for early immunologic response in patients. SDS-PAGE followed by immunoblotting were performed to investigate the sero-reactivities of the hydrophobic components of the leptospiral antigen preparation against acute patient sera whose blood were taken within 10 days of exhibiting the acute symptoms. The most prominent reactivity was observed at 10-15kDa region which appeared as a broad diffused band on X-ray films. This reactivity was observed in IgM blots and not in IgG blots, thus indicating that the band is a potential acute infection marker. The sensitivity for detection of acute leptospira cases using antigen from L44 was 71.7% (33/46). When the false negative samples were tested with L55 antigen, the sensitivity significantly ( $p < 0.05$ ) increased to 80.4% (37/46). The specificities for both antigens were 95.2% (40/42) when tested with sera from other febrile diseases and healthy control samples. Thus the 10 -15 kDa

antigen is potentially very useful as an acute infection marker for leptospirosis.

Diffuse banding pattern with less than 30 kDa molecular mass is usually characteristic of lipopolysaccharide (LPS) components of the outer membrane layer (Doungchawee *et al.*, 2008). The silver-stained SDS-PAGE of LPS profile in the antigen preparation used in this study revealed a simple doublet banding pattern, in contrast to the ladder-like pattern of other enterobacterial LPS. Further characterization studies performed on this antigen by proteinase K treatment and subsequent immunoblotting assay indicated that the antigen was resistant to the enzyme treatment, however the seroreactivity to pooled acute leptospirosis serum samples was intact, thus confirming that the reactive band was not protein in nature. When polysaccharide staining was performed, it gave a strong positive reaction, demonstrating that the antigen contained carbohydrate moiety. Therefore it can be concluded that the antigen most probably originated from the LPS layer of the *leptospira* outer membrane.

To further characterize the antigen, total LPS extraction of *leptospira* culture was performed, and subjected to SDS-PAGE and Western blot analysis. It was interesting to note that unlike our expectation, the antigen prepared from the total LPS extract did not react with the leptospirosis serum samples. The difference in immunogenicity could thus be attributed to the different method used in antigen preparation which may have led to a difference in the antigenic epitopes available for binding to the anti-leptospiral antibodies.

The serum samples tested in this study were from acute leptospirosis patients collected from various states in Malaysia (12 out of 14 states) who were infected with a variety of *leptospira* serovars. The results showed that the 10-15 kDa LPS band from *Leptospira* L44 and L55 demonstrated a high degree of reactivity towards this group of serum samples. This indicated that the antigen has multivalent capacity to detect heterologous leptospiral antibodies.

It was clear the LPS antigen preparation used in this study was capable of cross-reacting with a broad range of *leptospira* serovars, unlike previous reports that showed serovar specificity of leptospiral LPS antigen (Guerreiro *et al.*, 2001; Dounghawee *et al.*, 2007).

Using proteinase K-treated leptospiral lysate antigen prepared from *L. interrogans* serovar Copenhageni, Ribeiro *et al.* (1992) showed immunoblot reactivity of a diffused 14.8-22k Da band with sera from acutely infected patients in both IgM and IgG blots. In a subsequent report Ribeiro *et al.*, (1995) developed an IgM dot-blot assay using the proteinase K-resistant antigen from *L. interrogans* serovar Icterohaemorrhagiae which showed 92.1% sensitivity and specificity of 97.5%. Similarly, a 14-18 kDa derived from a mixture of ten serovars of leptospira was reported to be able to detect a large percentage (82.4%) of sera from leptospirosis patients during the first 3 days after the onset of symptoms (Dounghawee *et al.*, 2008). In a related study by our group, SDS-PAGE was performed using leptospiral antigen extracted with Triton X-114 and the seroreactivity was investigated against acute patients sera in IgM immunoblots. The most prominent reactive antigen was a diffused band at 15-20kDa region (results not shown). This antigen may be similar to that reported by the above two investigators. In contrast, the LPS infection marker reported in this study is clearly of a lower molecular weight, thus it seemed to be different from the LPS antigens reported previously. To the best of our knowledge, there are no other reports that showed leptospiral antigenic bands of lower than 14 kDa. The dot-blot EIA using the eluted antigen showed the same sensitivity and specificity as the Western blot assay, thus providing an approach for development of a rapid dot EIA using this antigen.

In conclusion, this study has identified a low molecular weight LPS antigen as a potential infection marker for immunodetection of acute leptospirosis. Currently candidates for protein infection markers are

being studied to complement this assay in order to develop an assay with greater than 90% sensitivity.

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