

# LipL32, an Outer Membrane Protein of *Leptospira*, as an Antigen in a Dipstick Assay for Diagnosis of Leptospirosis

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**SUMMARY** Microscopic agglutination test (MAT), as well as other serological assays that aimed at detecting antibodies to *Leptospira*, supplements the leptospirosis diagnosis based on the clinical features. Nevertheless, false positive results have been occasionally reported when the crude antigen was used in those antibody-based tests due either to the presence of antibodies stimulated by other antigenically related pathogens in the patient's serum, or the antibodies in the serum may be stimulated by a previously unrecognized *Leptospira* infection, especially in the disease endemic areas. Thus, the more refined antigen should improve the serodiagnostic accuracy. Among *Leptospira* spp., LipL32, which is a pathogenic *Leptospira* outer membrane protein (OMP), expressed by the bacteria grown both *in vitro* and *in vivo*. In this study, recombinant LipL32 protein was tested by a dipstick method for its potential in serodiagnosis of leptospirosis. Preliminary results suggest that the recombinant LipL32 is a good diagnostic detection reagent for specific *Leptospira* IgG. Diagnostic sensitivity and specificity of the Lip32 dipstick assay, when compared to those of MAT, were 100% and 98.33%, respectively.

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Leptospirosis, a global re-emerging zoonotic infection caused by pathogenic *Leptospira* spp., is an important human and veterinary health problem, especially in the tropical and subtropical regions.<sup>1-3</sup> In Thailand, human cases of leptospirosis increased remarkably during 1995 to 2002. Currently, the disease prevention and control have received much attention and concern by the public health authority (Thailand Ministry of Public Health Reports 1995-2002).

Leptospirosis causes mild to severe clinical manifestations, which include fever, myalgia, headache, conjunctival suffusion, hepatomegaly, hemorrhage, jaundice and renal failure. Mortality rate may be high.<sup>4-5</sup> The clinical features at the early phase of illness are not specific and may be confused with

other febrile illness, such as dengue fever, malaria, melioidosis, typhoid, rickettsial infections, and others.<sup>6</sup> Thus laboratory tests are often required to confirm the clinical diagnosis and to help indicating the remedy. Positive *Leptospira* culture of specimen such as blood, urine and/or cerebrospinal fluid is the most specific diagnostic methods. However, it is limited by the poor sensitivity and the requirement of a long incubation time, i.e. several weeks, before the bacterial growth is seen. Thus, it is useful only as an

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epidemiological information and not for treatment indication. Alternative diagnostic methods are the antibody detection tests; among which the microscopic agglutination test (MAT) is the most common assay.<sup>7-8</sup> However, MAT has several drawbacks, especially the need to maintain several serogroups and serovars of live *Leptospira* spp. for antigen preparation<sup>8-10</sup>. Thus, many immunological tests, such as the macroscopic slide agglutination test (MSAT), indirect immunofluorescent test, indirect hemagglutination test, and ELISA<sup>11-21</sup> which do not need living *Leptospira* spp. have been developed as an alternative of the MAT. However, most tests still use antigens extracted from whole organisms, and thus problem of the false positive results due to cross reacting antibodies in patients with other diseases, such as syphilis, lyme disease, dengue hemorrhagic fever and hepatitis, have not been solved.<sup>22-25</sup> Recently, Saengjaruk *et al.*<sup>26</sup> used specific monoclonal antibody in an antigen detection assay for diagnosis of current leptospirosis.

In this study, the gene encoding LipL32, an outer membrane protein of ~32 kDa which is found only in pathogenic *Leptospira* spp., was cloned from *Leptospira interrogans* serovar bratislava genome. The recombinant protein was used as an antigen for detecting *Leptospira* spp. specific antibody by a dipstick assay. Our preliminary data suggested that the recombinant LipL32 protein is a suitable diagnostic antigen.

## MATERIALS AND METHODS

### Bacterial strains

Strains of *Leptospira* spp. used in this study were obtained from the Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. The bacteria were cultivated in a bovine serum albumin-Tween-80-supplemented Ellinghausen McCullough Johnson and Harris medium (EMJH) (Difco, USA).

### Serum samples

Serum samples were obtained from the patients admitted for treatment at King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The samples were divided into two groups; samples of the first

group were from 20 patients with clinically diagnosed leptospirosis whose other serum samples taken later in the course of the infection, were positive for antibody to *Leptospira* by MAT and indirect immunofluorescent assay (IFA). Samples of the second group were from 20 patients who were serologically positive for *Treponema pallidum* infection by FTA-ABS. Sera of 20 healthy medical students which had been kept in a freezer at the Department of Microbiology, Faculty of Medicine, Chulalongkorn University in Bangkok, Thailand, served as negative controls.

### Microscopic agglutination test (MAT)

MAT was used as a reference method for identifying sera of patients with leptospirosis. For screening, aliquots of individual sera diluted 1:100 were incubated with live *Leptospira* spp., i.e., *L. interrogans* serovars autumnalis, ballum, bataviae, bratislava, canicola, celledoni, cynopteri, djasiman, gripotyphosa, hebdomadis, icterohaemorrhagiae, javanica, saigon, panama, pomona, pyrogenes, sejroe, and *L. biflexa* serovar patoc strain Patoc I) at 25°C for 2 hours. Aliquot of 10 µl from each mixture was examined for bacterial agglutination using a dark-field microscope. Serum which was positive for MAT at the dilution 1:100, was then diluted serially (started from 1:100) and retested against the respective serovar(s) to determine the agglutination titer. Serum samples with the titer 1:100 or higher was considered positive by MAT.

### Indirect immunofluorescent assay (IFA)

Beside tested by MAT, serum samples were also tested by IFA for specific IgG and IgM antibodies to *Leptospira*. *L. interrogans* serovar bratislava was used for preparing IFA antigen slides. Each serum sample was diluted serially, starting from 1:100. Each diluted aliquots was incubated with the *Leptospira* bacteria fixed on the slide, then washed. The antibodies bound to the fixed bacteria were revealed using either anti-human IgG- or IgM-conjugated with FITC (The Binding Site, Birmingham, UK). The IFA titer was the highest antibody dilution that ~50% of the leptospire on the slide were fluoresced. Antibody titer 1:100 or higher was considered positive for IFA.

### Amplification of *lipL32* DNA sequence by PCR

Genomic DNA extracted from *Leptospira* of various serovars, i.e. bratislava, autumnalis, ballum, bataviae, canicola, celledoni, cynopteri, djasiman, grippotyphosa, hebdomadis, icterohaemorrhagiae, javanica, louisiana, panama, pomona, pyrogenes, sejroe and non-pathogenic *L. biflexa* serovar patoc ) were amplified using primers specific for the *lipL32* gene: 5'-TTACCGCTCGAGGTGCTTTCGGTGGT-CTGC-3' and 5'-TGTTAACCCGGGTTACTTAGT-CGCGTCAGA-3'.<sup>31</sup> The PCR was carried out using the following conditions: denaturation at 94°C for 3 minutes, denaturation at 94°C for 1 minute, primer annealing at 59°C for 1 minute, and DNA extension at 72°C for 1 minute. The reaction was repeated for 20 cycles. Amplification of 16S RNA gene using primers: 5'-GGTAAAGATTTATTGCTCGG-3', and 5'-AAA TAA GCA GCA ATG AGA TG-3'<sup>33</sup> served as an internal control. PCR product was subjected to 1% agarose electrophoresis and the DNA amplicon was visualized under UV illumination. The PCR amplicon sizes for the *lip32* and 16R-RNA genes were 782 bp and 285 bp, respectively.

### Cloning and expression of LipL32 protein

Cloning of the *lip32* DNA into the plasmid, i.e. pRSETc (kindly provided by Professor Ming-Jeng Pan, Graduate Institute of Veterinary Medicine, National Taiwan University) was done according to method previously described.<sup>31</sup> Briefly, the *lipL32* DNA was purified from the agarose gel using Nucleospin (Macherey-Nagel, Germany) according to the manufacturer's instruction. The purified PCR product and the plasmid pRSETc were cut with appropriate restriction enzymes and ligated to the appropriately cut plasmid. The *lip32*-recombinant pRSETc plasmid was introduced into *E. coli* BL21 (DE3) (provided by Assistant Professor Wimon Chancham, Faculty of Science, Ramkhamhaeng University, Thailand). The recombinant *E. coli* clones were selected and confirmed for the presence of *lipL32* gene by PCR amplification and DNA sequencing (ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 and the Perkin Elmer 9600 automated nucleic acid sequencer).

The selected transformed *E. coli* clone was grown and Lip32-protein expression was induced by

IPTG (Bio-Basic, Canada). The appropriate IPTG-induction time for maximum protein expression was 2 hours. The Lip32 protein (with His-tag) was purified from the *E. coli* periplasmic proteins using the Ni-NTA spin column (Qiagen, USA) according to the manufacturer's instruction. Purity and reactivity of the purified protein were tested by immunoblotting against SDS-PAGE-separated *Leptospira* antigen.

### Antiserum to Lip32

The antiserum to Lip32 was a kind gift from Professor Ming-Jeng Pan.

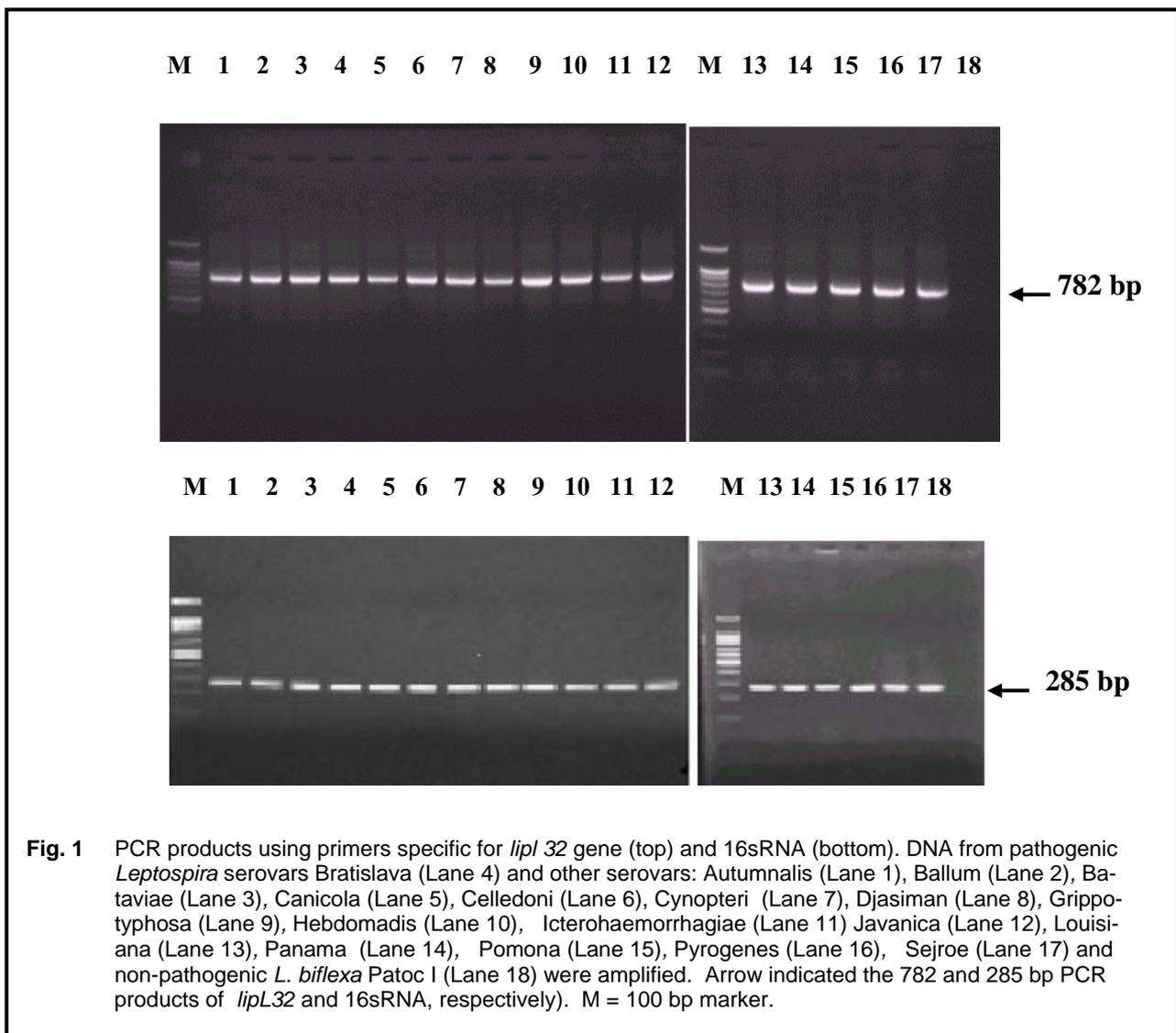
### Dipstick assay

Checkerboard titration for selecting the optimal conditions was done by varying the amounts of *Leptospira* antigen, various dilutions of the serum sample and the secondary antibody, i.e. anti-human IgG- or IgM- conjugated with alkaline phosphatase Recombinant LipL32 at appropriate concentration was dotted onto a nitrocellulose membrane (NC) strip attached to plastic. The strips were air-dried and blocked with 5% skim milk before incubating with appropriately diluted serum sample. Antigen-antibody reaction was visualized by incubating the strip with anti-human IgG- or IgM-alkaline phosphatase conjugate (Dako, Denmark). Color development was performed using NBT/BCIP substrate solution (Bio-Basic, Canada). Positive result was indicated by the appearance of a purplish-blue spot at the location where the antigen was dotted. Negative controls were spots on the same NC strip where only anti-human IgG- or anti-human IgM (20 ng) was dotted (no antigen or serum was applied).

## RESULTS

### Result on amplification of *lipL32* DNA from genomic DNA of various *Leptospira* spp.

As shown in Fig. 1, *lipL32* gene sequence could be amplified from genomic DNA of all tested pathogenic *Leptospira* serovars (lanes 1-17, Fig. 1 top), whereas the amplicon was not seen when genomic DNA of the non-pathogenic *Leptospira* was used as the template (lanes 18, Fig. 1 top). The 285 bp PCR products from 16sRNA were detected in all strains tested (lanes 1-18, Fig. 1 bottom).



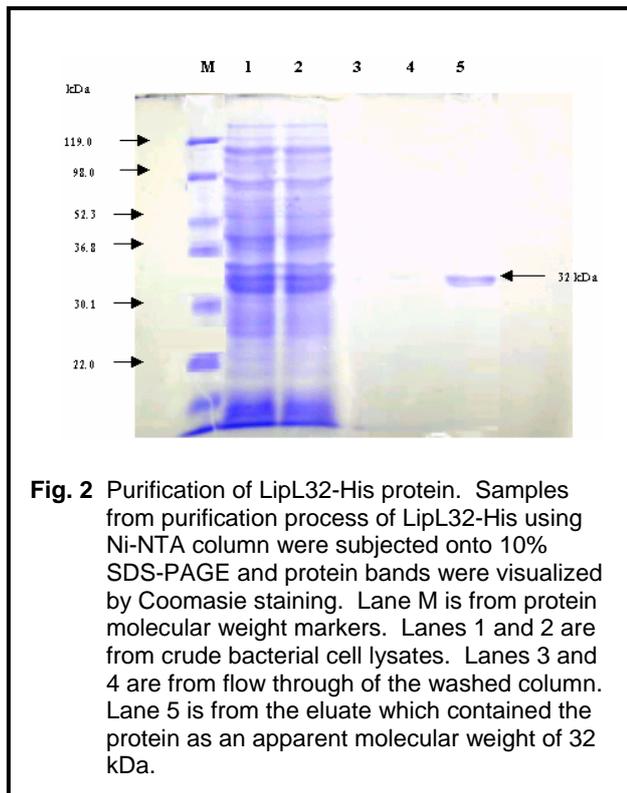
### Immunological reactivity of the Recombinant LipL32

The DNA amplicon of recombinant pRSETc carrying *lip32* from PCR was sequenced to ensure accuracy of the *lip32* DNA insert. The plasmid was shown to contain the *lip32* insert (with a sequence homology to that of the *lipL32* deposited in the Genbank (accession number AF111292) (data not shown). Lip32 was induced to be expressed from a selected transformed *E. coli* clone and the protein was purified. SDS-PAGE-separated recombinant Lip32 stained with Coomassie Brilliant Blue dye showed a stained band at ~32-kDa (Fig. 2). Immunoblotting of the SDS-PAGE separated-recombinant Lip32 with specific antibody to Lip32 or serum

of MAT/IFA positive leptospirosis patient revealed an antigen-antibody reactive band at ~32 kDa (lane 1, Fig. 3 top). However, there was no band in the immunoblot when the SDS-PAGE-separated recombinant Lip32 was probed with serum sample positive for antibody to *T. pallidum* or serum from a healthy individual (lanes 2 and 3, respectively, Fig. 3 bottom).

### Recombinant LipL32 as an antigen in a dipstick assay

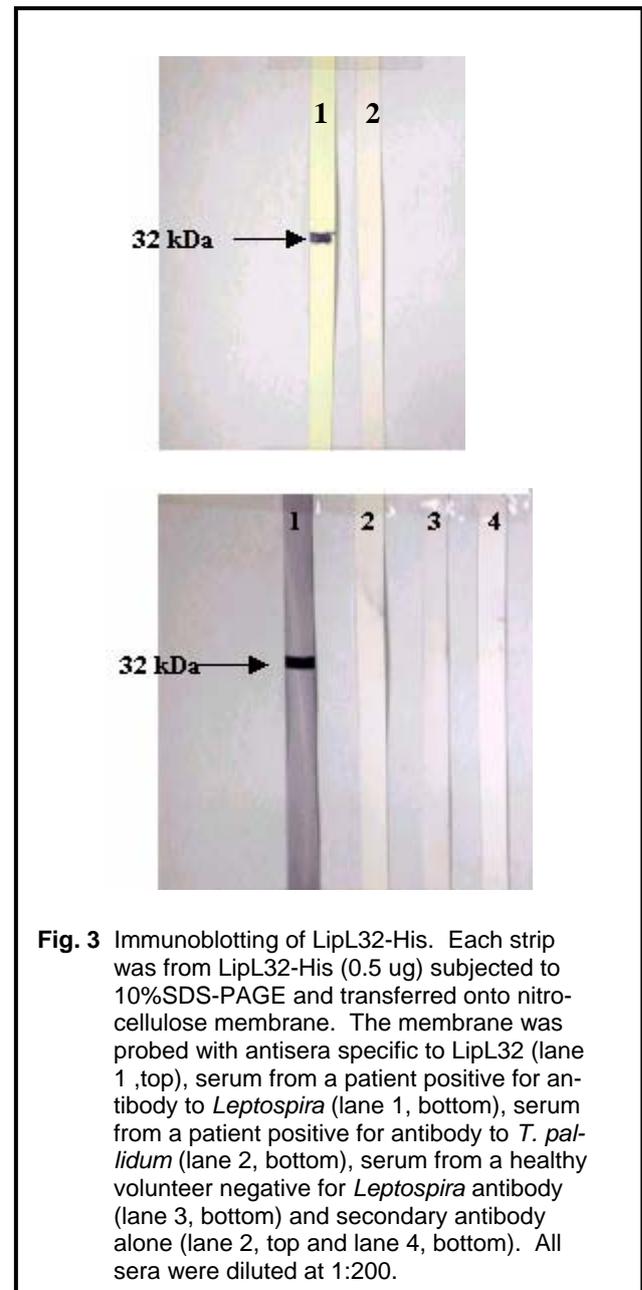
From titration, the optimum concentration of the recombinant Lip32 in the dipstick assay was 50 ng per dot; while the optimum dilutions of the leptospirosis patient's serum and the anti-human IgG-



alkaline phosphatase conjugate were 1:50 and 1:2,000, respectively. Dipstick assay using these conditions showed positive spots distinguishable from the non-specific background in the negative control spots. Serum samples of group 2, i.e. positive for antibody to *T. pallidum* did not reveal the positive spot when tested by the same condition using the Lip 32 as the antigen, implying high specificity of the dipstick assay.

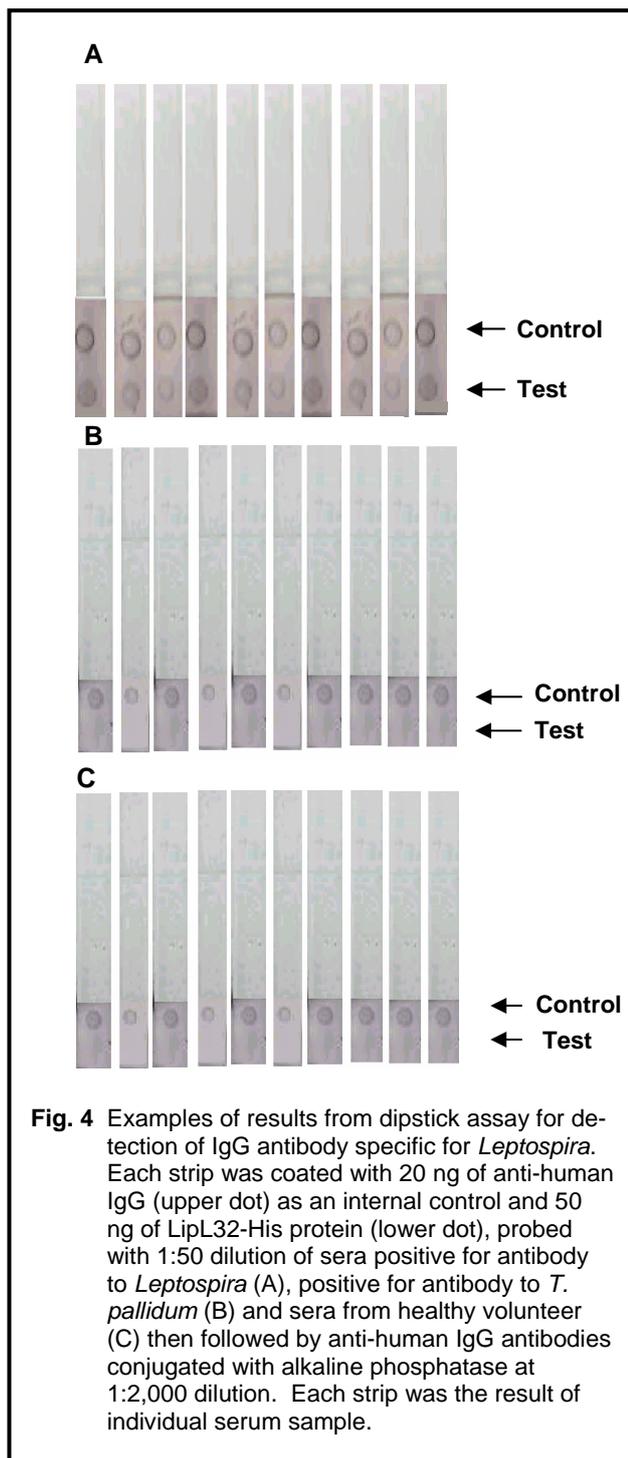
Anti-human IgM-alkaline phosphatase conjugate was also subjected to the checkerboard titration to determine the optimum concentrations of recombinant Lip32 and the optimum dilutions of patients' sera and the second antibody for the dipstick assay. However, no visible positive spots were revealed using serum samples of both groups 1 and 2. When the anti-human IgM-alkaline phosphatase at higher concentration (e.g. 1:500) was used, weakly positive spots were shown with serum samples of the group 1 (diluted 1:25, 1:50 and 1:100). Besides, positive reactions were also observed when sera of group 2 were tested under these conditions, implying the low specificity of the assay.

The optimum concentration of the recombinant Lip32 (50 ng per dot), and the optimum dilu-



tions of the patients' sera (1:50) and the anti-human IgG-alkaline phosphatase conjugate (1:2,000) were used for testing sera of all three groups.

Figs. 4A, B and C show the representative of IgG dipstick positive results tested on serum samples of patients with leptospirosis (group 1). Table 1 shows results of sera of all groups tested by the IgG-dipstick assay. All sera of group 1 (leptospirosis patients whose sera were MAT/IFA positive) were positive by the dipstick assay. One of 20 samples of



group 2 (patients whose sera were FTA/ABS positive) was also positive by the dipstick assay, while no sample of group 3 (healthy controls) gave positive results. Thus, the IgG dipstick assay has 100% sensitivity compared with MAT and IFA and 98.33% specificity.

Seventeen of 20 samples of group 1 were negative and 3 gave weakly positive by IgM dipstick assay. None of the sera of groups 2 and 3 was positive by the IgM dipstick assay.

## DISCUSSION

In Thailand, most reported leptospirosis cases were from rural areas, therefore, diagnostic tests should not only be specific but also rapid for timely administration of antibiotics or other treatments.<sup>3,32,34</sup> Many diagnostic tests using a variety of immunological principles have been established in order to supplement the MAT assay. Most tests utilized whole-cell extracts as the antigen.<sup>21, 35-37</sup> False positive reactions have been reported when samples from patients with other diseases such as hepatitis, syphilis, infections by dengue virus, cytomegalovirus, mycoplasma and Epstein-Barr virus were tested<sup>22-25, 38-43</sup>.

Because a more specific test for laboratory diagnosis of leptospirosis is required, purified antigens have been the next step in the diagnostic assays. Gene and protein expression in bacteria change in response to the environment in order to adapt and interact with their specific environment. For this reason, the candidate antigen for antibody detection should be the one that is expressed during infection and is able to induce an immune response. Outer membrane proteins were selected since their surface exposure makes them accessible to immune recognition and attack. Outer membrane proteins of *Leptospira* have been characterized by both biochemical and molecular biology methods. These include OmpL1, LipL32, LipL36, LipL41 and LipL48 and other less well-characterized outer membrane proteins such as proteins at molecular masses of 16, 21, 21.5, 22, 31, 36, 44, 48, 90 and 116 kDa.<sup>44-49</sup>

LipL32 is an outer membrane protein which is highly conserved among pathogenic leptospires and was found to be expressed in the proximal tubules of infected animals. In addition, it is a prominent immunogen during leptospirosis. From immunoblotting experiments, acute and convalescent phase sera of human leptospirosis showed great reactivity against LipL32 protein.<sup>31</sup> Expression of this protein in laboratory cultures has also been demonstrated. For those reasons, LipL32 was chosen to be the center of this study.

**Table 1** Comparison of results from MAT, IFA and dipstick assays for the detection of antibody to *Leptospira*

Serum samples	N	MAT	IFA		Dipstick (His-LipL32 Ag)	
			IgG	IgM	IgG	IgM
A	20	P	P	P	P	W = 3, N = 17
B	20	N	N	N	N = 19, P = 1	N
C	20	N	N	N	N	N

A = sera positive for antibody to *Leptospira*; B = sera positive for antibody to *T. pallidum*; C = sera negative for antibody to *Leptospira*; N = negative, P = positive, W = weakly positive

Although, LipL32 gene has been cloned and protein expression has been done from *Leptospira* serovar Kirschneri and Shermani,<sup>31</sup> the serovar most commonly reported in Thailand, *L. interrogans* serovar Bratislava has not been investigated. This study successfully cloned and expressed LipL32 *in vitro* as demonstrated earlier. The cloned gene has a sequence homology to *lipL32* from other serovars, which confirms that *lipL32* is conserved. As mentioned earlier, there are more than 200 serovars of leptospires identified throughout the world, only a few of which have been demonstrated to possess the *lipL32* gene. In addition to the serovar Bratislava, in this study, the *lipL32* gene was also demonstrated in 16 other pathogenic serovars reported in Thailand.

Since we considered that a rapid test is needed for leptospirosis diagnosis, establishing a dipstick assay using purified His-LipL32 was initiated. We attempted to detect antibodies specific to *Leptospira* with both IgM and IgG subtypes. However, only specific IgG antibody could be detected. According to our preliminary data, the sensitivity and specificity for IgG detection were quite high (100 and 98.33%, respectively) when compared with MAT. IFA was also done to demonstrate the subtypes of antibodies. Serum samples positive for antibody to *T. pallidum* were included for non-specificity testing since bacteria of the Genus *Treponema* are also spirochetes with demonstrated cross-reactivity when sera from syphilis patients were tested with leptospiral antigens.<sup>44,49</sup> In our study, only one out of 20 samples gave a false positive result. Moreover, all sera from healthy volunteers gave quite clear negative results. Although the results from IgG detection were promising for further study, specific IgM antibody was weakly detected in only 3 out of 20 samples proven positive for antibody to *Leptospira*. This

may be due to the fact that certain antigens can only induce certain subtypes of immunoglobulin. It has been shown that most IgM antibodies were specific to lipopolysaccharide (LPS) whereas most IgG antibodies were specific to protein fractions. Chapman *et al.*<sup>50</sup> demonstrated that when leptospiral antigens were treated with proteinase K, the pattern of IgM antibody recognition was unchanged, whereas most bands obtained from IgG antibody were lost when compared with non-treated antigens. However, larger and more diverse groups of samples and different types of antigens are needed to be examined in order to clearly explain the observed results.

#### ACKNOWLEDGEMENTS

The authors thank the Rachadapiseksompoch Fund, Faculty of Medicine, Chulalongkorn University and the Inter-Department of Medical Microbiology, Faculty of Graduate School, Chulalongkorn University for supporting this work. We are grateful to Miss Apiradee Theamboonlers and the staff at the Hepatitis Research Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University for their DNA sequencing facilities and their valuable advice.

#### REFERENCES

- Farr RW. Leptospirosis. Clin Infect Dis 1995; 21: 1-6.
- Waitkins S. Leptospirosis? Br Med J (Clin Res Ed) 1985; 290: 468.
- Vinetz JM, Glass GE, Flexner CE, Mueller P, Kaslow DC. Sporadic urban leptospirosis. Ann Intern Med 1996; 125: 794-8.
- Sitprijia V, Moolaoor P, Suwangool S, Chroonruangrit S. Leptospirosis: clinical manifestations and pathogenesis. Proceeding of the International Symposium on Leptospirosis, Seoul, 1985; pp. 88-101.
- Yunibandha J. First report of Weil's disease in Thailand. J Med Assoc Thailand 1943; 26: 83-136.

6. Guidelines for the control of leptospirosis. WHO Offset Publ 1982; pp. 1-171.
7. Palmer MF. Laboratory diagnosis of leptospirosis. *Med Lab Sci* 1988; 45: 174-8.
8. Turner LH. Leptospirosis II. Serology. *Trans R Soc Trop Med Hyg* 1968; 62: 880-99.
9. Hussaini SN. A comparative study on the use of whey and serum for microscopic agglutination test for the detection of *Leptospira* antibodies. *Br Vet J* 1976; 132: 621-6.
10. Myers DM. Effect of culture medium on the agglutination of leptospire by microscopic agglutination test. *Rev Agent Microbiol* 1976; 8: 14-20.
11. Galton MM, DK Powers, AM Hall, RG Cornell. A rapid microscopic slide screening test for the serodiagnosis of leptospirosis. *Am J Vet Res* 1958; 19: 505-12.
12. Torten M, Shenberg E, van der Hoeden J. The use of immunofluorescence in the diagnosis of human leptospirosis by a genus-specific antigen. *J Infect Dis* 1966; 116: 537-43.
13. Appassakij H, Silpapojakul K, Wansit R, Woodtayakorn J. Evaluation of the immunofluorescent antibody test for the diagnosis of human leptospirosis. *Am J Trop Med Hyg* 1995; 52: 340-3.
14. Imamura S, Matsui H, Ashizawa Y. Indirect hemagglutination test for detection of leptospiral antibodies. *Jpn J Exp Med* 1974; 44: 191-7.
15. Sakamoto N, Yanagawa R, Ono E, Kida H, Mori M, Arimitsu Y, *et al.* Detection of antibodies to *Leptospira* genus-specific antigen in human and animal sera by indirect hemagglutination test with a partially purified genus-specific protein antigen. *Zentralbl Bakteriell Mikrobiol Hyg [A]* 1985; 259: 548-56.
16. Sulzer CR, Glosser JW, Rogers F, Jones WL, Frix M. Evaluation of an indirect hemagglutination test for the diagnosis of human leptospirosis. *J Clin Microbiol* 1975; 2: 218-21.
17. Terpstra WJ, Ligthart GS, Schoone GJ. Serodiagnosis of human leptospirosis by enzyme-linked immunosorbent-assay (ELISA). *Zentralbl Bakteriell A* 1980; 247: 400-5.
18. Pappas MG, Ballou WR, Gray MR, Takafuji ET, Miller RN, Hockmeyer WT. Rapid serodiagnosis of leptospirosis using the IgM-specific dot-ELISA: comparison with the microscopic agglutination test. *Am J Trop Med Hyg* 1985; 34: 346-54.
19. Adler B, Murphy AM, Locarnini SA, Faine S. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. *J Clin Microbiol* 1980; 11: 452-7.
20. Mailloux M, Mazzonelli JG, Dufresne Y. Application of an immuno-enzyme technique to titration of antibodies in leptospirosis: ELISA (enzyme-linked immunosorbent assay). *Zentralbl Bakteriell Mikrobiol Hyg [A]* 1984; 257: 511-3.
21. Terpstra WJ, Ligthart GS, Schoone GJ. ELISA for the detection of specific IgM and IgG in human leptospirosis. *J Gen Microbiol* 1985; 131(Pt 2): 377-85.
22. Turner LH. Leptospirosis I. *Trans R Soc Trop Med Hyg* 1967; 61: 842-55.
23. Smits HL, Ananyina YV, Chereschsky A, Dancel L, Lai AFRF, Chee HD, *et al.* International multicenter evaluation of the clinical utility of a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human serum specimens. *J Clin Microbiol* 1999; 37: 2904-9.
24. Smits HL, Hartskeerl RA, Terpstra WJ. International multicentre evaluation of a dipstick assay for human leptospirosis. *Trop Med Int Health* 2000; 5: 124-8.
25. Yersin C, Bovet P, Smits HL, Perolat P. Field evaluation of a one-step dipstick assay for the diagnosis of human leptospirosis in the Seychelles. *Trop Med Int Health* 1999; 4: 38-45.
26. Saengjaruk P, Chaicumpa W, Watt G, *et al.* Diagnosis of human leptospirosis by monoclonal antibody-based antigen detection in urine. *J Clin Microbiol* 2002; 40: 480-9.
27. Goodsens HAT, AE van der Bogaard, MKE Nohlmans. Evaluation of fifteen commercially available serologic tests for diagnosis of lyme borreliosis. *Eur J Clin Microbiol Infect Dis* 1999; 18: 551-60.
28. Rodriguez I, Alvarez EL, Fernandez C, Miranda A. Comparison of a recombinant-antigen enzyme immunoassay with *Treponema pallidum* hemagglutination test for serological confirmation of syphilis. *Mem Inst Oswaldo Cruz* 2002; 97: 347-9.
29. Schmidt BL, M Edjlalipour, A Luger. Comparative evaluation of nine different enzyme linked immunoabsorbent assays for determination of antibodies against *Treponema pallidum* in patients with primary syphilis. *J Clin Microbiol* 2000; 38: 1279-82.
30. Picardeau M, Brenot A, Saint Girons I. First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa* flaB results in non-motile mutants deficient in endoflagella. *Mol Microbiol* 2001; 40: 189-99.
31. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, *et al.* The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect Immun* 2000; 68: 2276-85.
32. Heisey GB, Nimmanitya S, Karnchanachetane C, Tingpalapong M, Samransamruajkit S, Hansukjariya P, *et al.* Epidemiology and characterization of leptospirosis at an urban and provincial site in Thailand. *Southeast Asian J Trop Med Public Health* 1988; 19: 317-22.
33. Patarakul K, Lertpocasombat K. Detection of pathogenic leptospiral DNA in Urine by Polymerase chain reaction. *J Med Assoc Thai* 2005; 87 (Suppl) S134-139.
34. Phraisuwan P, Whitney EA, Tharmaphornpilas P, Guharat S, Thongkamsamut S, Aresagig S, *et al.* Leptospirosis: skin wounds and control strategies, Thailand, 1999. *Emerg Infect Dis* 2002; 8: 1455-9.
35. Khisamov GZ, Morozova NK. Fatty acids as resource of carbon for *Leptospira*. *J Hyg Epidemiol Microbiol Immunol* 1988; 32: 87-93.
36. Matsuo K, Isogai E, Araki Y. Occurrence of [-> 3)- $\beta$ -D-Manp-(1 -> 4)- $\beta$ -D-Manp-(1 -> )n units in the antigenic

- polysaccharides from *Leptospira biflexa* serovar patoc strain Patoc 1. Carbohydr Res 2000; 328: 517-24.
37. Matsuo K, Isogai E, Araki Y. Utilization of exocellular mannan from *Rhodotorula glutinis* as an immunoreactive antigen in diagnosis of leptospirosis. J Clin Microbiol 2000; 38: 3750-4.
  38. Yang CW, Wu MS, Pan MJ, Hsieh WJ, Vandewalle A, Huang CC. The *Leptospira* outer membrane protein LipL32 induces tubulointerstitial nephritis-mediated gene expression in mouse proximal tubule cells. J Am Soc Nephrol 2002; 13: 2037-45.
  39. Sagdeeva LG. One of the causes of nonspecific false positive reactions in syphilis. Vestn Dermatol Venerol 1970; 44: 59-64.
  40. Bajani MD, Ashford DA, Bragg SL, Woods CW, Aye T, Spiegel RA, et al. Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. J Clin Microbiol 2003; 41: 803-9.
  41. Gussenhoven GC, van der Hoorn MA, Goris MG, Terpstra WJ, Hartskeerl RA, Mol BW, et al. LEPTO dipstick, a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human sera. J Clin Microbiol 1997; 35: 92-7.
  42. Levett PN. Leptospirosis: re-emerging or re-discovered disease? J Med Microbiol 1999; 48: 417-8.
  43. Sanders EJ, Rigau-Perez JG, Smits HL, Deseda CC, Vornadam VA, Aye T, et al. Increase of leptospirosis in dengue-negative patients after a hurricane in Puerto Rico in 1996 [correction of 1966]. Am J Trop Med Hyg 1999; 61: 399-404.
  44. Haake DA, Walker EM, Blanco DR, Bolin CA, Miller MN, Lovett MA. Changes in the surface of *Leptospira interrogans* serovar grippityphosa during *in vitro* cultivation. Infect Immun 1991; 59: 1131-40.
  45. Haake DA, Champion CI, Martinich C, Shang ES, Blanco DR, Miller JN, et al. Molecular cloning and sequence analysis of the gene encoding OmpL1, a transmembrane outer membrane protein of pathogenic *Leptospira* spp. J Bacteriol 1993; 175: 4225-34.
  46. Shang ES, Exner MM, Summers TA, Martinich C, Champion CI, Hancock RE, et al. The rare outer membrane protein, OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. Infect Immun 1995; 63: 3174-81.
  47. Shang ES, Summers TA, Haake DA. Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect Immun 1996; 64: 2322-30.
  48. Haake DA, Matsunaga J. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. Infect Immun 2002; 70: 4936-45.
  49. Barnett JK, Barnett D, Bolin CA, Summers TA, Wagar EA, Cheville NF, et al. Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. Infect Immun 1999; 67: 853-61.
  50. Chapman AJ, Everard CO, Faine S, Adler B. Antigens recognized by the human immune response to severe leptospirosis in Barbados. Epidemiol Infect 1991; 107: 143-55.