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The detection and characterization of pathogenic *Leptospira* and the use of OMPs as potential antigens and immunogens

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Abstract. The detection of leptospires in patient blood in the first week of the disease using PCR provides an early diagnostic tool. PCR using two sets of primers (G1/G2 and B64-I/B64-II) tested with samples seeded with 23 leptospiral strains from pathogenic and non-pathogenic strains was able to amplify leptospiral DNA from pathogenic strains only. Of the 39 antibody negative samples collected from patients suspected for leptospirosis, only 1 sample (2.6%) was PCR positive. Using LSSP-PCR, the G2 primers allowed the characterization of *Leptopira* species to 10 different genetic signatures which may have epidemiological value in determining species involved in outbreaks. Leptospiral outer membrane proteins from three strains were purified and reacted against patients sera and gave rise to different profiles for pathogenic and non-pathogenic strains. Lymphocytes of mice injected with OMPs proliferated and released IFN-^{3} when stimulated *in vitro* using *Leptospira* OMP as antigens. This suggests that an immune response could be established using leptospiral OMPs as a putative vaccine. OMPs were also used in a Dot-ELISA to detect antibodies against *Leptospira* pathogens in humans.

INTRODUCTION

Leptopirosis, caused by the spirochete *Leptospira*, is a major public health concern and has now been identified as one of the emerging infectious diseases worldwide. This has been exemplified by outbreaks in Nicaragua, Brazil, India, Southeast Asia, the United States, and more recently afflicting participants from several countries in the EcoChallenge Sabah 2000 competition in Malaysia (CDC, 2000). The spectrum of human disease caused by leptospires is wide, ranging from subclinical infection to a severe syndrome of multiorgan infection which includes intravascular coagulation followed by acute respiratory failure resulting in high mortality.

Diagnosis of leptospirosis mainly relies on the detection of *Leptospira* antibodies in patient's serum such as the microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA) (Dikken & Kmety, 1978). However, in humans the antileptospiral antibodies become detectable only after 7 days from the onset of illness. In addition to serological methods, culturing leptospires from clinical samples can be used as a diagnostic tool. However leptospires are fastidious organisms and cannot be easily cultured, and those that are successfully cultured will yield results after at least two weeks. Other methods of detection which include immunofluorescence, thin layer immunoassay and DNA hybridization are not widely used due to their lack of sensitivity (Le Febvre, 1987; Appassakji *et al*., 1995).

Polymerase chain reaction (PCR) has been proven to be a rapid, sensitive, and specific method for the detection of a wide range of microorganisms including those that are slow-growing and fastidious (Einstein, 1990). In addition, PCR has successfully been applied directly to clinical samples such as serum, urine, aqueous humor (Merien *et al*., 1992; Bal *et al*., 1994),

and has provided supporting evidence to the involvement of leptospires in ocular lesions and aseptic meningitis (Romero *et al.*, 1998).

Pathogenic *Leptospira* are capable of surviving in the host after urinary shedding for a long period of time. The current available leptospiral vaccines, either live attenuated whole cell vaccines or vaccines using LPS components, seem to provide short-term immunity, thus are not very effective at preventing disease. Leptospiral OMPs are an important alternative and have already been shown to elicit protective immunity in laboratory animals (Sonrier *et al.*, 2000). Only a few leptospiral OMPs have been well characterized including OMP L1 (Haake *et al.*, 1993; 2000).

This report describes the use of a PCR assay and LSSP-PCR for the specific detection and characterization of pathogenic strains of *Leptospira*. An attempt was also made to identify and characterize leptospiral OMPs from three serovars (two pathogenic and one non-pathogenic) that could serve as diagnostic reagents and a putative vaccine.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and clinical samples

Twenty-one reference strains of *Leptospira* were obtained from the WHO Collaborating Centre for Research and Reference on Leptospirosis, Queensland, Australia, and the Institut Pasteur, Paris (Table 1). All strains were maintained in Fletcher's media (Difco, USA), and grown in liquid Ellinghausen McCullough Johnson Harris (EMJH) media supplemented with 10% serum of hemolysed rabbit blood.

Blood, urine, and sera were obtained from patients suspected for leptospirosis at the intensive care unit (ICU), University of Malaya Medical Center (UMMC), Kuala Lumpur, and the Hospital Kuala Lumpur (HKL) between 2000 and 2002. Serum samples that were obtained from patients who were serologically negative for leptospirosis and pooled sera from dengue positive patients were used as negative controls.

Isolation and amplification of leptospiral DNA from cultures and clinical samples

An overnight culture of *Leptospira* was harvested by centrifugation at 13,000 x g for 10 min at 4°C. The pellet was resuspended in 800 µl sterile ddH_2O. Then, 200 µl of 0.1 M EDTA (pH 8.0) and 0.5% formaldehyde solution was added and the mixture was centrifuged at 13,000 x g for 10 min at 4°C. The pellet was washed with 1 ml 1 mM EDTA (pH 8.0), and again with 1 ml of sterile dH_2O leaving 100 µl of liquid to resuspend the pellet. The DNA was released by incubation at 100°C for 10 min, and was used directly in PCR, or stored at -20°C until further use.

Specific multiplex PCR was carried out using primers G1 (5'-CTGAATCGCTGTA TAAAAGT) and G2 (5'-GGAAAACAAAT GGTCGGAAG), and primers B64-I (5'-CTGAATTCTCCATCTCAACTC) and B64-II (5'-GCAGAAATCAGATGGACGAT) as described previously (Gravenkamp *et al.*, 1993). For specificity analysis, 100 ng of DNA [each] from *Borrelia burgdorferi*, *Treponema reiteri*, *Mycobacterium* spp., *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Salmonella* spp., *Neisseria gonnorhoea*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Escherichia coli* and human were used and subjected to PCR amplification as described above. PCR amplification products were detected and identified by visualization of the bands of the expected size on 2% agarose gels.

Identification of *Leptospira* by Low Stringency Single Specific primer PCR (LSSP-PCR)

LSSP-PCR is a two-step procedure. Firstly, *Leptospira* DNA was amplified using G1 and G2 primers (20 pmol each), using the conditions as described earlier. After electrophoresis, the resulting band was excised, transferred to a sterile Eppendorf tube and heated at 95°C for 5 min to melt the agarose. Approximately 2 µl of the melted gel was used directly as template for the second PCR reaction and added into a 20 µl reaction mixture (10 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 200 uM [each] deoxynucleoside triphosphate, 48 pmol primer [either G1 or

Table 1. *Leptospira* strains used in the study

Serogroup	Serovar	Strain	Source[a]
Leptospira interrogans :			
Autumnalis	*autumnalis*	Akayami A	1,2
Icterohaemorrhagia	*icterohaemorrhagiea*	RGA	1
	copenhageni	Wijnberg	2
Hebdomadis	*hebdomadis*	Hebdomadis	2
Pomona	*pomona*	Pomona	2
Pyrogenes	*pyrogenes*	Salinem	2
Bataviae	*bataviae*	Van Tienen	2
Australis	*australis*	Ballico	2
Canicola	*canicola*	Hond Utrcht IV	2
Leptospira santarosai :			
Shermani	*Shermani*	1342 K	1
Leptospira borgpetersenii :			
Javanica	*javanica*	Veldrat Bat 46	1
Sejroe	*sejroe*	M 84	1,2
	hardjobovis	Sponselee	2
Ballum	*castellonis*	Castellon 3	2
Leptospira noguchii :			
Panama	*Panama*	CZ 214 K	1,2
Leptospira weillii :			
Celledoni	*Celledoni*	Celledoni	1
Leptospira inadai :			
None	*Lyme*	#10	1
Leptospira meyeri :			
Ranarum	*Ranarum*	ICF	1
Leptospira geomospecies 5 :			
Semaranga	*sao Paulo*	Sao Paulo	1
Leptospira kirschneri :			
Cynopteri	*cynopteri*	3522 C	1,2
Grippotyphosa	*grippotyphosa*	Moskva V	2
Leptospira illini	*Illini*	3055	1
Leptospira biflexa :			
Semaranga	*Patoc*	Patoc I	2

[a] : (1) WHO/FAO/OIE Collaborating Center for Reference and Research on Leptospirosis, Queensland, Australia
: (2) *Leptospira* CNR, Institut Pasteur, Paris, France

G2] and 1.6 U *Taq* DNA polymerase [PROMEGA]). After an initial denaturation step at 94°C for 6 min, the PCR was as follows: 1 min at 94°C followed by 30°C for 1 min, for a total of 35 cycles. The resulting PCR products were diluted with equal amount of formamide loading buffer prior to electrophoresis on a 10% polyacrylamide gel followed by silver staining as described by Sanguinetti *et al.* (1994).

Preparation of leptospiral OMPs, Western blotting, and immunodetection. Leptospiral OMPs were prepared according to the method of Haake *et al.* (1998) and solubilised in 2X sample buffer (60 mM Tris-

HCl (pH 6.8), 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.1% bromophenol blue). Proteins were separated on a 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel at 50 mA and stained with Coomasie brilliant blue or transferred to nitrocellulose membranes for immunoblot analysis. The membranes were blocked with Tris-buffered saline-0.1% Tween-20 (TTBS) containing 2% BSA, incubated with Leptospirosis-positive human sera (1:500 dilution) for 1 hour, and probed with goat anti-human antibody conjugated to alkaline phosphatase. Visualisation of the antigen-antibody binding was carried out using NBT/BCIP substrate (Amersham).

Determination of immunogenic response of BALB/C mice to leptospiral OMPs, lymphocyte separation and proliferation

Purified OMPs (10 µg) were injected subcutaneously into 6 to 8 week-old BALB/C mice. Lymphocytes were separated from mice spleens, using the method of Morgan & Darling (1994), and were harvested 5 days post-inoculation which were then used in a MTT cell proliferation assay and ELISPOT detection of interferon gamma (IFN-^{3}) (Weichert *et al.*, 1991; Reece *et al.*, 2002). Lymphocytes from inoculated BALB/C mice were incubated with purified leptospiral OMPs using a standard MTT assay (according to manufacturers instructions) to detect the specific lymphocytic response to the leptospiral OMPs. Mouse IFN-^{3} was measured using the ELISPOT kit (Mabtech AB, Sweden).

Leptospirosis diagnosis using Dot-ELISA

A simple Dot-ELISA was performed using leptospiral OMPs spotted on a nitrocellulose membrane and reacted against pooled human sera. Briefly, 330 mg/ml OMP solution was serially diluted (two-fold), manually blotted onto nitrocellulose strips and air dried. The strips were incubated in blocking buffer (5% skimmed milk in PBS) for 10 minutes , washed thrice in 0.01 M PBS (pH 7.4) before incubation with diluted human sera (1:500) for 20 min. The strips were washed and incubated with biotinylated goat anti-human IgG or IgM (1:8000 dilution) for 20 min, washed and incubated with alkaline phosphatase-conjugated strepavidin for another 20 min. The strips were washed three times with 0.01 M PBS, once with 0.15 M Tris-HCl before being incubated with the developing solution (BCIP/NBT substrate solution) (Sigma, Germany). After 5 to 10 min, the strips were washed with distilled water and air dried.

RESULTS

PCR sensitivity and specificity

To assess the specificity of the primers, DNA isolated from the 21 *Leptospira* reference strains representing 11 *Leptospira* pathogenic and saprophytic serogroups were subjected to multiplex PCR using primer sets G1/G2 and B64-I/B64-II. The resulting PCR products are shown in Figures 1A and 1B. PCR using the G1/G2 primer pair gave rise to the expected band size of 285 bp and belongs to 8 serogroups. However amplification using the B64-I/B64-II primer pair generated a 360 bp fragment from DNA belonging to two of the *Leptospira kirschneri* serogroup instead of the expected 563 bp fragment obtained by Gravekemp *et al.* (1993). The expected 563 fragment was obtained only when mixed DNA from *Leptospira kirschneri* and *Leptosira interrogans* were used as a template for the PCR. The size of the 360 bp fragment was not modified whether the B64-I/B64-II primers were used alone or together with G1/G2 primers. Both primer sets did not amplify DNA isolated from non-pathogenic strains namely, *Leptospira biflexa* strain Patoc I, and *Leptospira illini* strain 3055.

To determine the sensitivity of the PCR and the inhibitory effect of any substances present in the clinical samples (urine and serum) in both the DNA extraction and PCR processes, the urine and serum samples were spiked with *Leptospira* cells and DNA was extracted using the methods described earlier. PCR products corresponding to the expected sizes of 285 and 360 bp were obtained in all spiked samples (Figure 1).

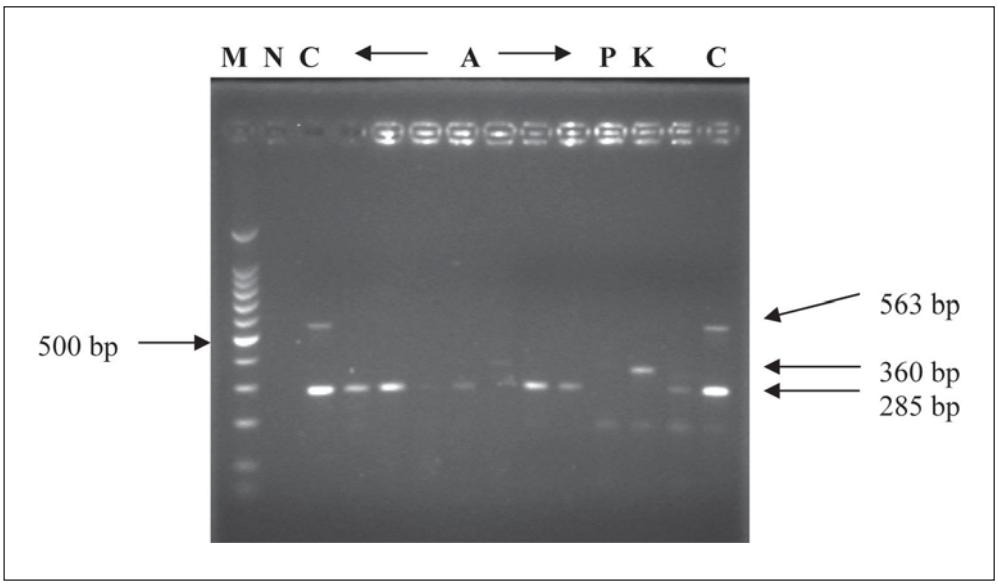

Figure 1. PCR amplification of Leptospiral DNA using primer sets G1/G2 and B64-I/B64-II. Expected PCR product size of 285 bp obtained using both primer sets from strains belonging to the *Leptospira interrogans* serogroups (Lanes indicated by the letter A). DNA from *Leptospira kirschneri* strain 3522 C amplified producing a 360 bp fragment (Lane K). When mixed DNAs from *L. interrogans* strain RGA and *L. kirschneri* strain 3522 C were amplified, fragment bands of 285 and 563 bp were noted (Lane C). DNA from *Leptopsira biflexa* strain Patoc I (Lane P).

Sequence polymorphism between PCR products generated from different strains of *Leptospira*

A 285 bp fragment was specifically amplified from most of the *Leptospira* serovars used in this study. The amplicons were further characterized in LSSP-PCR using primers G1 or G2 and yielded distinct profiles comprising multiple bands ranging from <100 bp to >1500 bp. It was noted that genetic signatures obtained from *Leptospira* species with G2 were more informative than those obtained with G1. The latter yielded very similar profiles for all serovars analysed which mainly consisted of the first PCR fragment (Figure 2). The main constitutive fragments obtained with G2 are listed in Table 2. The sizes of the main fragments which represent the PCR product from the first amplification from each *Leptospira* strain using both primers show different electrophoretic mobility in the polyacrylamide gel, ranging from approximately 255 bp to 310 bp. These fragments were almost similar in size within a serovar in most of the strains but showed distinct profiles between different leptospiral serovars.

SDS-PAGE analysis and immunodetection of *Leptospiral* whole cell antigens

The protein profiles of the two *L. interrogans* strains were similar and had few indistinct bands with molecular masses of more than 80 kDa. The non-pathogenic *L. biflexa* strain, which harboured five high molecular weight proteins (75, 83, 97, 111 and 133 kDa), had a more complex protein profile (Figure 3a). The protein complex representing the flagellar structure appeared to be different in all three serovars. In serovar autumnalis, there were two major bands at 36 and 32 kDa, accompanied by a minor band at 30 kDa, whereas in serovar icterohaemorrhagiae only the two major bands were visible (36 and 32 kDa). In serovar patoc, 3 bands at 32, 31 and 28 kDa were considered as the major bands forming the flagellar protein complex (Figure 3b).

In the immunoblot analysis of leptospiral antigens, patient's sera detected distinct leptospiral antigen bands from each serovar (Figure 3b). The two pathogenic serovars (Akiyami and RGA) had similar profiles with three common bands : two of the bands were

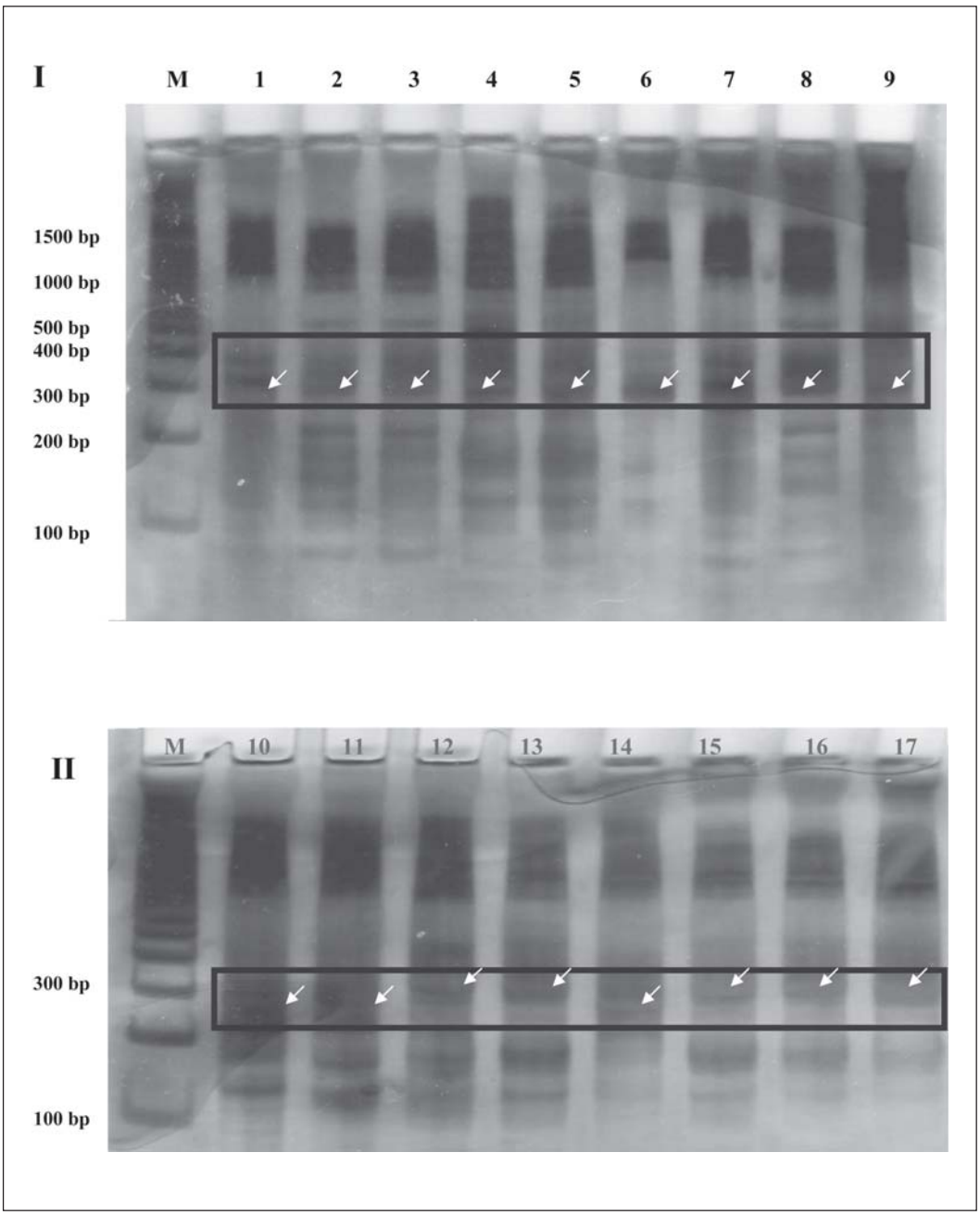

Figure 2. LLSP-PCR genetic signature obtained from different species of *Leptospira* with primer G2. LSSP-PCR reaction products were separated on a 10% (w/v) polyacrylamide gel and silver stained. Lanes: (M): Molecular size marker (100 bp); (1): *Leptospira weillii:* serovar *celledoni*, (2, 3, 4, 5, 8, 13, 15, 16, and 17): *Leptospira interrogans*: serovars *autumnalis*, *icterohaemorrhagiea*, *hebdomadis*, *pyrogenes*, *bataviae*, *copenhageni*, *australis*, *canicola* and *Pomona*, respectively (6): *Leptospira meyeri:* serovar *ranarum*, (7): *Leptospira santarosai:* serovar *shermani*, (9). Bands inside the box are PCR products from the first amplification with primers G1/G2 (bands position marked with arrows).

Table 2. Estimated molecular size of DNA fragments obtained by LSSP-PCR with G2 primer

Strain	Serovar	LLSP-PCR bands	Profile
L. interrogans			
Akiyami A	*autumnalis*		
RGA	*icterohaemorrhagia*	550,295,210,120,70	1
Van Tienen	*bataviae*		
Hebdomadis	*hebdomadis*		
Salinem	*pyrogenes*	500,295,170,120,70	2
Wijnberg	*copenhageni*		
Ballico	*australis*		
Hond Utrecht IV	*canicola*	295,185,140	3
Pomona	*Pomona*		
L. borgpetersenii			
Castellon 3	*castellonis*		
Veldrat Bataviae 46	*javanica*	265, 210,180,140	4
L. santarosai			
1342 K	*shermani*	300,255,60	5
L. weilii			
Celledoni	*celledoni*	260,70	6
L. meyeri			
Iowa City Frog (ICF)	*ranarum*	270,140,60	7
L. noguchii			
CZ 214 K	*panama*	310,210,195,140	8
L. genomospecies 5			
Sao Paulo	*sao paulo*	450,295	9
L. inadai			
#10	*lyme*	280,210	10

32 kDa and 61 kDa, assumed to be LipL32 and the heat shock protein GroEL, respectively which are highly conserved across leptospiral species as well as a band at 45 kDa (Haake *et al*., 2000). The 32 kDa band was also detected in the non-pathogenic serovar Patoc I.

Determining the immunogenic response to leptospiral OMPs in BALB/c mice

To investigate the possibility that leptospiral OMPs produce an immune response in mice, lymphocytes from mice innoculated with leptospiral OMPs were collected and used in a lymphocyte proliferation assay, while the production of IFN-^{3} was measured using ELISPOT assay.

Absorbance values obtained using the MTT assay were almost similar in groups inoculated with leptospiral OMPs (Figure 4a). The average absorbance reading in mice inoculated with OMPs extracted from the pathogenic RGA strain were higher compared to those from both the groups inoculated with the non-pathogenic Patoc I strain and control mice (without antigen) respectively. However this difference is not significant ($p > 0.005$).

The ELISPOT assay measures the IFN-^{3} produced by individual lymphocytes in response to stimulation by an antigen. Figure 4b shows the mean ELISPOT counts and standard error values obtained from each group. ELISPOT counts from the three groups inoculated with OMPs were significantly higher than the control group, the highest being from the group inoculated with OMPs from the pathogenic RGA strain.

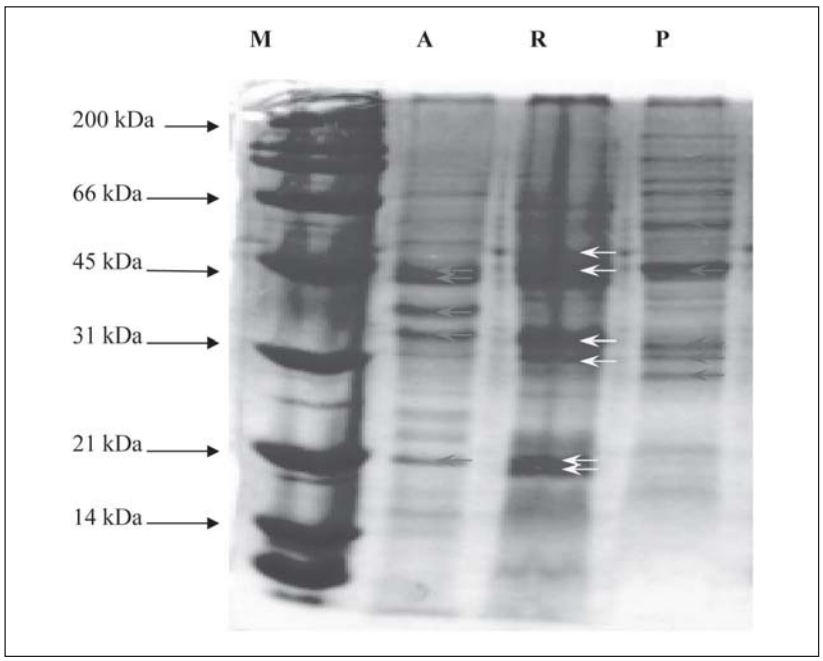

Figure 3a. Protein profiles of three Leptospira serovars in SDS-PAGE.
Whole cell lysate of three *Leptospira* strains (Akayami, RGA and Patoc I) resolved on a 12% polyacrylamide gel and stained with Coomasie blue. Arrows point to the positions of major proteins of each strain. Lanes : Lane M = broad range SDS-PAGE molecular weight standard; lane A = strain Akayami; lane R = strain RGA; lane P = strain Patoc I.

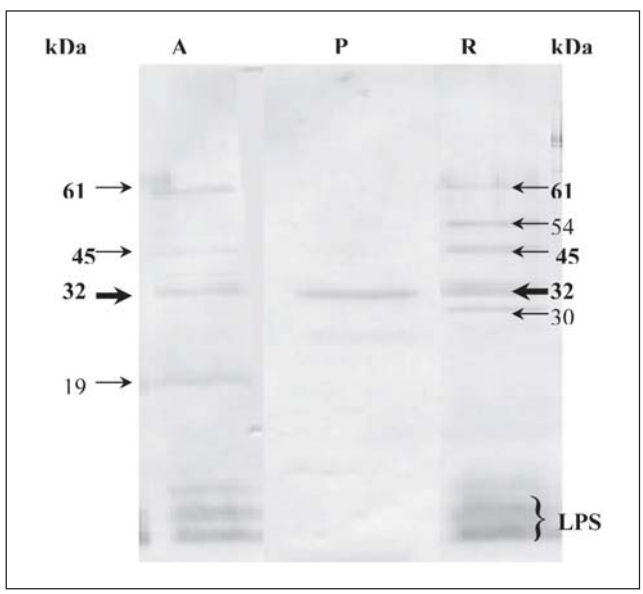

Figure 3b. Immunoblot analysis of leptospiral OMPs.
Immunoblot of leptospiral detergent-enriched proteins probed with pooled sera from patients laboratory-confirmed with leptospirosis. Three common antigens (arrows at 61, 45 and 32 kDa) were shared in the pathogenic strains Akayami and RGA. The 32 kDa antigen (thick arrow)was also detected in the non-pathogenic strain. (Lane A = Strain Akayami A, lane R= Strain RGA, lane P = Strain Patoc I, and LPS = Lipopolysacharides).

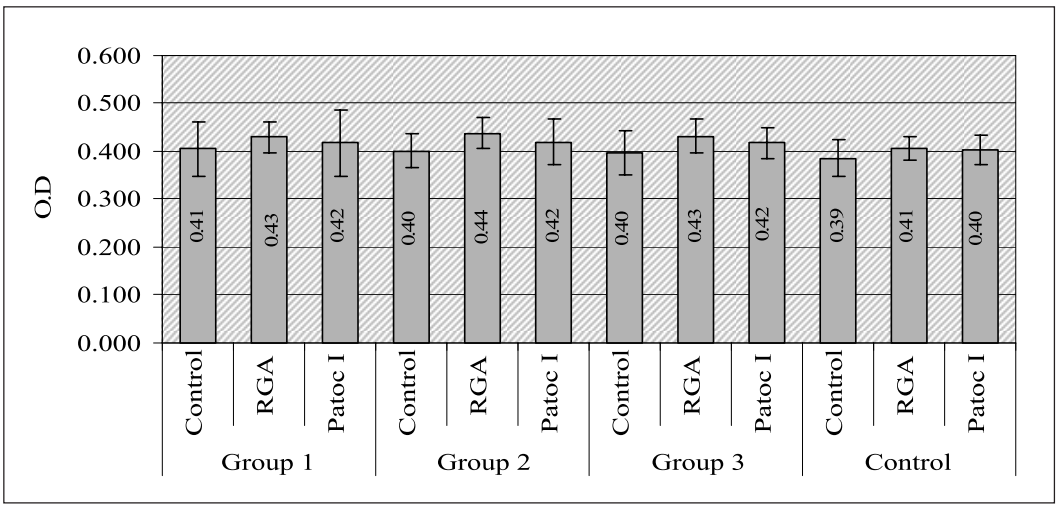

Figure 4a. Lymphocyte proliferation response to leptospiral OMPs.
Group 1: Mice injected with OMPs extracted from strain Akayami A.
Group 2: Mice injected with OMPs extracted from strain RGA.
Group 3: Mice injected with OMPs extracted from strain Patoc I.
Group 4: Control group, mice were mock injected.
Stimulant antigens:
RGA: OMPs extracted from strain RGA.
Patoc I: OMPs extracted from strain Patoc I.
Control: No antigen added

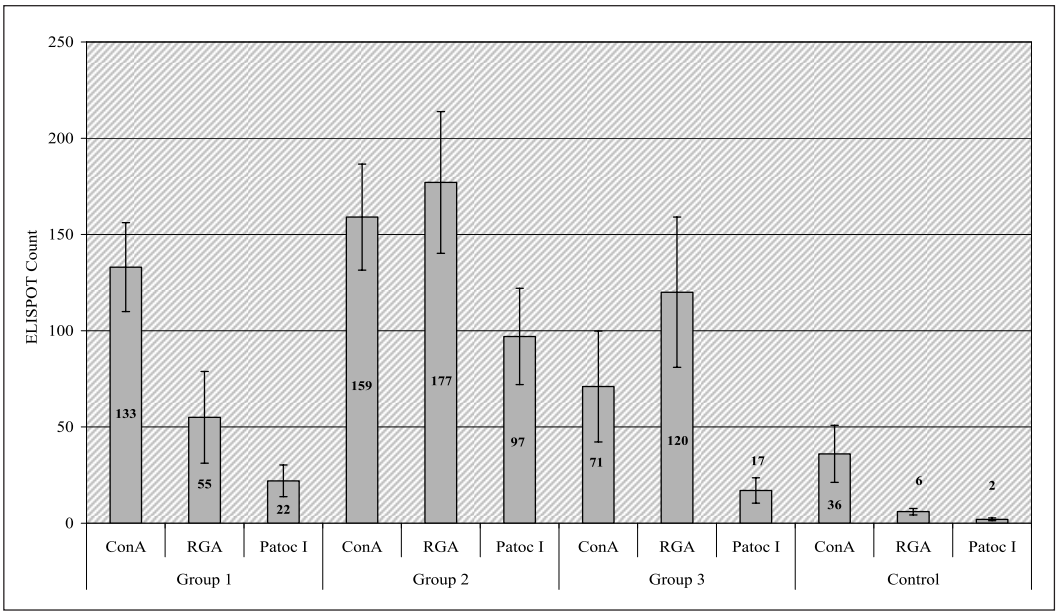

Figure 4b. Measurement of Interferon-γ released from lymphocytes.
Group 1: Mice injected with OMPs extracted from strain Akayami A.
Group 2: Mice injected with OMPs extracted from strain RGA.
Group 3: Mice injected with OMPs extracted from strain Patoc I.
Group 4: Control group, mice were mock injected.
Stimulant antigens:
Con A: 5µg/ml Concanavalin A.
RGA: OMPs extracted from strain RGA.
Patoc I: OMPs extracted from strain Patoc I.

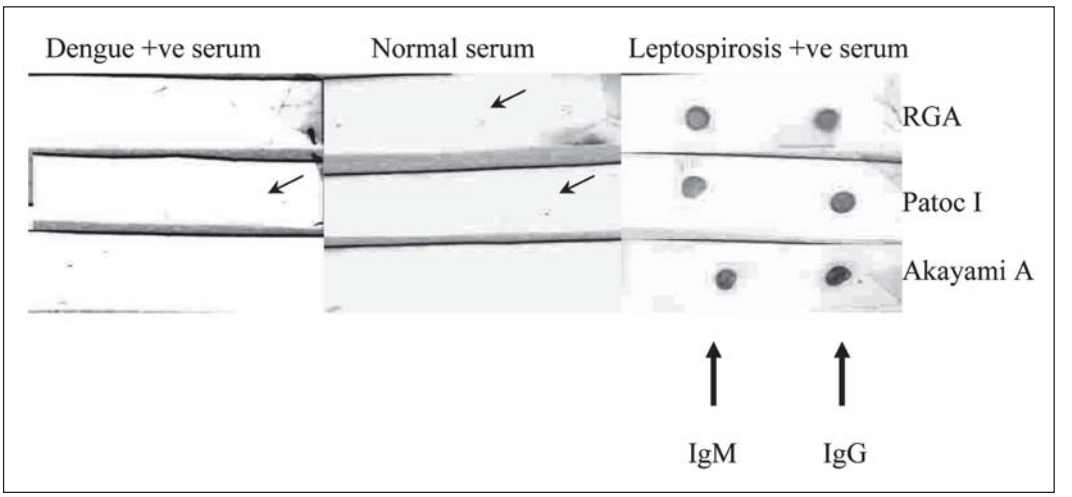

Figure 5. Dot-ELISA results with control sera.
Control sera from healthy humans and from dengue positive patients tested in a dot-ELISA using 1µg of OMPs per spot. Fine arrows indicate no reactivity between OMPs and sera. Bold arrows indicate reactivity between OMPs and IgM & IgG respectively.

Leptospirosis diagnosis using Dot-ELISA
Dot-ELISA was performed using concentrated OMPs extracted from each strain and probed with leptospirosis positive human sera (Figure 5). It was noted that the limit of detection of human IgM by leptospiral OMPs was 2 ng as opposed to 2 to 8 ng for IgG. This is because IgM is a pentamer with 10 binding sites, thus could be picked by lower levels of OMP. Control sera which consisted of normal human serum and sera from dengue infected patients reacted against OMP at concentrations as high as 1 mg and was undetectable which showed the specificity of this assay.

DISCUSSION

Leptospirosis is rapidly emerging as a major public health concern. The clinical presentation of leptospirosis is biphasic. During leptospiraemia, *Leptospira* organisms can be found in the blood, cerebral spinal fluid (CSF), aqueous humor, and most tissues, but less commonly from urine. In this study, no leptospires were recovered from serum or urine samples. The failure to recover leptospires from the PCR positive sample however is probably due to the presence of non-viable leptospires.

The difficulty in culturing *Leptospira* spp. is attributed to the general fastidiousness of the organism. Sejvar *et al.* (2003) investigated an outbreak of leptospirosis in athletes who participated in the EcoChallenge – Sabah 2000 multisport endurance race, held in Malaysian Borneo in the year 2000. Out of the 36 serum samples from athletes who met the case definition of leptospirosis, 26 were tested positive with ELISA but only one was culture confirmed (Sejvar *et al.*, 2003).

The difficulties in isolating *Leptospira* spp. from clinical samples and body fluids have been reported in several studies (Harkin *et al.*, 2003). PCR, bacteriological culture, and serological testing were compared as diagnostic tools and PCR has proven to be the most sensitive, specific and rapid method for the detection of *Leptospira* spp. (Harkin *et al.*, 2003).

Detection of leptospires at an early stage of infection increases the chance of successful treatment thereby decreasing the severity of infection and reducing the threat to public health and safety (Faine, 1982). Although serological detection of leptospiral antibodies has been the preferred diagnostic

method, one major drawback of these techniques is that in many cases, particularly in the initial stage of infection, lack of antibodies in patients sera has resulted in inability to confirm diagnosis at this stage. In this study, PCR amplification of DNA obtained from 23 *Leptospira* reference strains representing 11 leptospiral pathogenic and saprophytic serogroups, was evaluated. PCR using the B64-I/B64-II primers generated a 360 bp fragment from *Leptospira kirschneri* strains as opposed to the 560 bp fragment obtained by Gravekamp *et al.* (1993). These findings concurred with those of Parma *et al.* (1997). The PCR assay was able to detect leptospiral DNA in all of the spiked blood and urine samples.

Clinical samples collected from 39 patients admitted to ICU in UMMC and HKL were screened by the PCR assay and we were able to detect one positive case which was negative by MAT. Unfortunately the patient died the same day of sample collection. MAT detected three other positive cases that were negative with PCR. In these patients, antibiotics had been administered four days before sample collection, which may have destroyed any remaining leptospires in the patients' body fluids, therefore explaining the failure to detect any leptospiral DNA. However, as previous studies have shown that leptospires can be detected in the urine of patients suspected of leptospirosis within the first seven days from the onset of infection, PCR analysis of urine can be extremely useful for early diagnosis of leptospirosis in comparison to serological assays (Bal *et al.*, 1994).

LSSP-PCR using primer G2 produced genetic signatures composed of multiple fragments between 100 and 1500 bp which are the characteristics of LSSP-PCR (Pena *et al.*, 1994; Barreto *et al.*, 1996). In this study, 10 different genetic signatures were obtained with the primer G2 in the analysis of serovars obtained from different species of *Leptospira*. LSSP-PCR profiles generated from serovars of the same species were similar. Similar findings have been reported in previous studies, therefore justifying the proposal of a taxonomical classification of the genus *Leptospira*, which is based on intraspecific genetic homogeneity and G+C contents of DNA (Yasuda *et al.*, 1987; Bao *et al.*, 1997; Marluce *et al.*, 2003).

The protein antigens of two pathogenic strains of *L. interrogans* (*L. icterohaemorrhagiae* and *L. autumnalis*, the most common leptospiral pathogens in human leptospirosis in South-East Asia) and one saprophytic strain of *L. biflexa*, were identified and compared. Protein profiles of the three reference strains revealed only minor differences between the two pathogenic strains. The protein profile of *L. biflexa* was more complex and differed significantly from the *L. interrogans* strains (Figure 3a). Immunoblot analysis of the antigens from the three serovars identified three common proteins (32, 45 and 61 kDa) among the two pathogenic strains of *L. interrogans* (Akayami and RGA). The 32 kDa protein, which was identified as the LipL32 protein was also detected in the non-pathogenic *L. biflexa* strain (Patoc I), thereby indicating the probable importance of this protein as an immunodominant antigen which is recognized by the humoral response during a natural infection. This conclusion is consistent with the findings of Chapman *et al.* (1991) which identified the 32 kDa protein as a major immunoreactive antigen in leptospirosis, and confirmed to be LipL32 using 2D electrophoresis (Chapman *et al.*, 1991). The 61 kDa protein which could be the 62 kDa heat shock protein GroEL identified by Guerreiro *et al.* (2001). In addition to their potential use in serodiagnosis, leptospiral proteins expressed during mammalian infection may also have immunoprotective potential. This has been shown in the study of Sonrier *et al.* (2000) which reported that innoculation of whole leptospiral proteins conferred protection against heterologous as well as homologous leptospiral serovars in experimental animal models. These findings indicate that the three common leptospiral proteins recognized during natural infection may serve as potential candidates for both serodiagnosis as well as vaccine development, thereby helping to curb the current threat of leptospirosis to public health. Cellular immune response to *Leptospira* OMPs

extracted from the three serovars used in this study (*L. icterohaemorrhagiae*, *L. autumnalis*, and *L. biflexa)* was investigated using the lymphocyte proliferation assay and ELISPOT assay. The results showed that the lymphocytes from mice inoculated with OMP antigen produced IFN-^{3} in response to stimulation *in vitro* by different OMP antigens, thus indicating the induction of a cell-mediated response by leptospiral OMPs. However, the response was found to be heterologous as well as homologous since the lymphocytes were activated by both OMP antigens from strain RGA as well as Patoc I. Further studies in a susceptible animal model with live virulent leptospires should be carried out to investigate the immunoprotective properties of extracted leptospiral OMPs and their probable use as a potential vaccine candidate for leptospirosis.

The diagnosis of leptospirosis is difficult due to among other factors, the complexity of the disease. To date, the definitive serological test is the MAT, which requires significant expertise as well as maintenance of a panel of live cultures. Moreover, the diagnosis of acute leptospirosis by MAT often requires paired serum samples, thus delaying the diagnosis which could considerably affect the patients' chances of survival. In this study, OMP-enriched Triton X-114 fractions from pathogenic and non-pathogenic strains of *Leptospira* were used in a Dot-ELISA for detection of antibodies against *Leptospira* in human serum. Serum from leptospirosis MAT-confirmed patients reacted with the OMPs of the three serovars used. Control serum from healthy persons or from dengue-infected patients did not react with the OMP spots thereby indicating the specificity of this assay. However, further studies with a larger panel of leptospirosis positive and negative control sera will have to be carried out to validate the sensitivity and specificity of this assay. The preliminary data from this assay are in accordance with previous findings by Sekhar *et al.* (2000) and Levett *et al.* (2001).

PCR is a potentially valuable diagnostic tool in leptospirosis. Patients presenting with clinical symptoms suggestive of leptospirosis will have an opportunity for early diagnosis, thereby enhancing patient management through timely commencement of appropriate medical treatment. This, in turn, will result in reducing the overall threat of leptospirosis to public health.

However, there is still a need to develop rapid serodiagnostic strategies especially now that leptospirosis has been recognized as an emerging cause of epidemics worldwide. Pathogenic *Leptospira* species possess a number of protein antigens that are expressed during infection and identification of these proteins has potentially important implications particularly in the development of new serodiagnostic and immunoprotective strategies. This study has shown that the OMPs of 32, 45 and 61 kDa are expressed during infection and elicit a humoral immune response, thus revealing immunoprotective potential and possible use in vaccine development of the proteins. Furthermore, the application of OMP-enriched fractions in Dot-ELISA for the detection of IgM antibodies against *Leptospira* in human serum revealed a serodiagnostic property that could lead to the development of a rapid, specific and sensitive assay for the early diagnosis of leptospirosis.

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