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Components of Pathogenic *Leptospira* spp. with Potentials for Diagnosis of Human Leptospirosis

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SUMMARY Existing serological methods for diagnosis of leptospirosis are still unsatisfactorily due mainly to their low accuracy. In this study, serum samples of 18 clinically diagnosed-, IgM dipstick positive-, MAT positive-leptospirosis patients (group 1) were analyzed by IgG Western blotting against SDS-PAGE separated-whole cell homogenates of pathogenic and non-pathogenic *Leptospira* spp. belonging to 20 serovars of 15 serogroups. The samples of group 1 were collected from the patients at days 3 to 10 after the fever onset (fist samples). Second and third samples could be obtained from 4 patients. Sera of the 22 patients with other febrile illnesses (group 2) and 22 healthy counterparts (group 3) were used as patient- and normal- controls, respectively. Irrespective of the serovar or serogroup of the pathogenic *Leptospira* spp. used as antigen in the Western blotting, all of the 18 sera of patients with leptospirosis (group 1) gave characteristic diffuse antigen-antibody reactive bands located at ~35-38 and 22-26 kDa; and thus 100% diagnostic sensitivity of the Western blot assay. Some serum samples of the leptospirosis patients also reacted to components located at 80-100, ~70, 60, 54, and 48 kDa. More bands or the early recognized bands with increased intensity were observed when tested the second and third samples. The characteristic bands were not seen when homogenates of *L. biflexa*, serogroup Semaranga, serovar Patoc (saprophytic) and *L. biflexa*, serogroup Andamana, serovar Andamana (non-pathogenic but can infect host) were used in the assay. Sera of groups 2 and 3 did not react to the components at the seven locations implying 100% diagnostic specificity of the IgG Western blot assay. While awaiting validation with more patients' samples, the IgG Western Blot analysis aiming at the detection of the characteristic antigen-antibody reactive bands described in this study has high potential for early, rapid, simple and accurate diagnosis of human leptospirosis.

Leptospirosis is a worldwide infectious disease of humans and animals especially in the tropical and subtropical areas.[1-3] Symptoms and severity of human leptospirosis vary greatly from mild, flu-like illness to fatal (ictero) hemorrhagic form with severe involvement of vital organs, *e.g.* liver, lung and kidney.[4] During the early phase of illness, however, clinical manifestations of leptospirosis, *i.e.* fever, headache, myalgias, are not clearly distinguishable from other febrile illnesses.[5,6] Thus, laboratory methods are needed for confirmation of the clinical diagnosis. The presence of *Leptospira* spp. in the clinical specimens, *e.g.* blood, CSF and urine, by culture is the most definite means of diagnosis of leptospirosis but is not only insensitive but also slow and complicate. Several antibody detection assays have been

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developed as a serodiagnostic alternative of the disease.[7-9] Among them, the microscopic agglutination test (MAT) is the most accepted assay.[10] Nevertheless, MAT is laborious and requires living *Leptospira* spp. of various serovars and serogroups as the antigens. Other methods, *e.g*. assays for specific IgM are not satisfactorily sensitive during an early phase of infection.[11,12] at which time definite diagnosis is needed for treatment indication.

In this study, serum samples collected from clinically diagnosed leptospirosis patients, who were later in the course of the illness their serum samples were positive by IgM dipstick and MAT, were tested by Western blotting against SDS-PAGE separated-homogenates of both pathogenic and non-pathogenic *Leptospira* spp. belonging to 20 serovars of 15 serogroups. Serum samples of patients with other febrile illnesses and normal healthy individuals served as controls. Results of the assay indicate that the Western blot analysis for IgG antibody may be a serologically diagnostic alternative for human leptospirosis.

MATERIALS AND METHODS

Leptospira cultures and antigen preparation

Pathogenic and non-pathogenic *Leptospira* spp. belonged to 20 serovars of 15 serogroups were used in this study (Table 1). The bacteria were cultured in liquid Ellinghausen-McCullough Johnson-Harris (EMJH) medium. Bacterial cells from log phase cultures were harvested individually by centrifuging the cultures at 8,000 x *g*, 4°C for 30 minutes. The bacterial pellets were washed with fresh EMJH basal medium and finally the bacteria were suspended in small volumes of sterile distilled water. Whole cell homogenates were prepared by disrupting bacterial cells using sonicator (Ultrasonic Processor SONIC & MATERIALS, Inc., Newtown, CT, USA)

Table 1 List of *Leptospira* spp. which their whole cell homogenates were used as antigens in this study

No.	Species	Serogroups	Serovars (strains)
1	*L. biflexa*	Andaman	Andamana (CH11)
2	*L. interrogans*	Australis	Bangkok
3	*L. interrogans*	Australis	Bratislarva
4	*L. interrogans*	Autumnalis	Autumnalis (Akiyami A)
5	*L. borgptersenii*	Bullum	Bullum
6	*L. interrogans*	Bataviae	Bataviae
7	*L. interrogans*	Canicola	Canicola
8	*L. kirschneri*	Cynopteri	Cynopteri (3522C)
9	*L. interrogans*	Autumnalis	Djasiman
10	*L. borgptersenii*	Sejroe	Hardjo
11	*L. interrogans*	Hebdomadis	Hebdomadis
12	*L. interrogans*	Icterohaemorrhagiae	Copenhageni
13	*L. interrogans*	Icterohaemorrhagiae	Icterohaemorrhagiae
14	*L. borgptersenii*	Javanica	Javanica
15	*L. interrogans*	Pomona	Pomona
16	*L. interrogans*	Pyrogenes	Pyrogenes
17	*L. interrogans*	Louisiana	Saigon
18	*L. borgptersenii*	Sejroe	Sejroe
19	*L. interrogans*	Sejroe	Wolffi
20	*L. biflexa*	Semaranga	Patoc

at 20 kHz, 4°C, 5 minutes, 3 to 4 times. Dry weights of individual homogenates were determined and adjusted to the desired concentrations when used.

Subjects and serum samples

Serum samples were collected from three groups of subjects. The first group included 18 patients admitted to Khon-Kaen Provincial Hospital (about 390 km northeast of Bangkok, Thailand) during June to November 2000. They were clinically diagnosed leptospirosis according to WHO criteria.[10] The first serum samples were collected from them on the first day of their hospital arrival which were the third (1 patient), fourth (8 patients), fifth (4 patients), sixth (1 patient), seventh (2 patients), eighth (1 patient) and tenth days (1 patient) of fever onset (Table 2). Of these 18 samples, 14 were positive and 4 (patients no. 3, 6, 8 and 15) were negative for leptospirosis by IgM dipstick assay (Organon, Belgium). However, serum samples collected 5-6 days later from the 4 patients were positive by the IgM distick assay (Table 2). Different serum samples of these 18 patients collected later in the course of the illness were positive for leptospirosis by MAT. Follow-up serum samples could be obtained from patients no. 5, 7, 11 and 14 (Table 2). The second group included 22 patients with other febrile illnesses admitted for treatment also at Khon-Kaen Provincial Hospital. They were three patients with parasitologically confirmed *Plasmodium falciparum* infection, two patients who were serologically positive for melioidosis, three patients who were positive for IgM antibody to Dengue virus, one patient with hepatitis B, and 13 patients with fever of unknown causes. Subjects of the third group were 22 normal inhabitants of Khon-Kaen province. Written informed consent was obtained from each subject or the legal representative before serum sample collection.

SDS-PAGE and Western blot analysis

SDS-PAGE of whole cell homogenates of *Leptospira* spp. belonging to 15 serogroups and 20

Table 2 Information on serum samples of subjects of group 1

Patient no.	Days after fever onset when serum sample(s) was (were) collected from each patient			Days after fever onset when serum sample was positive by IgM dipstick for the first time
	First sample	Second sample	Third sample	
1	3	-	-	3
2	4	-	-	4
3	4*	-	-	10
4	4	-	-	4
5	4	5	6	4
6	4*	-	-	10
7	4	6	10	-
8	4*	-	-	9
9	4	-	-	4
10	5	-	-	5
11	5	7	18	5
12	5	-	-	5
13	5			5
14	6	8	19	6
15	7*	-	-	13
16	7	-	-	7
17	8	-	-	8
18	10	-	-	10

*Patient whose first serum sample was negative by IgM dipstick
-, not available.

serovars (Table 1) was carried out in a PROTEAN-III cell apparatus (Bio-Rad) according to the method of Laemmli.[13] One hundred and fifty micrograms of the *Leptospira* homogenate was used per one slab gel. SDS-PAGE separated-components were electroblotted from the gel onto a nitrocellulose membrane (NC). After the unoccupied sites on the blotted NC were blocked with a blocking solution (3% BSA in PBS, pH 7.4) for one hour, the membrane was then vertically stripped. Individual strips were reacted with serum samples (diluted 1:200). The antigen-antibody reactive bands were revealed by using the alkaline phosphatase labeled-goat anti-human IgG (Promega, Wisconsin) at dilution 1:7,500 and a substrate solution (BCIP/NBT [KPL, USA]). Relative molecular mass of individual *Leptospira* component reacted to the serum was worked out by comparing with the locations of Broad Range pre-stained standard proteins (BioRad, CA, USA) which were concurrently run in the same gel.

RESULTS

It was found that all (100%) of the first serum samples of the 18 patients with leptospirosis (group 1) reacted as diffuse antigen-antibody reactive bands to components located at ~35-38 and ~22-26 kDa of the SDS-PAGE separated-homogenates of pathogenic *Leptospira* spp. used as antigen in the Western blot analysis, irrespective of the bacterial serogroup and/or serovar. The sera also gave reactive bands to other five components of the pathogenic *Leptospira* spp., *i.e.*, diffuse band at 80-100 kDa (12 patients, 66.7%), thick single band at 70 kDa (9 patients, 50%), single band at 60 kDa (9 patients, 50%), single band at 54 kDa (14 patients, 77.8%), single band at 48 kDa (4 patients, 22%) (Fig. 1A) Table 3 summarizes the reactivity of all sera of group 1 to SDS-PAGE separated- homogenate of pathogenic *Leptospira* spp. None of the serum samples of groups 2 and 3 reacted to the *Leptospira* spp. at these locations (data not shown).

When the homogenates of non-pathogenic *L. biflexa, i.e.,* serogroup Semaranga, serovar Patoc and serogroup Andamana, serovar Andamana, were used in the Western blotting, none (0%) of the patients' sera of group 1 revealed characteristic diffuse posi-

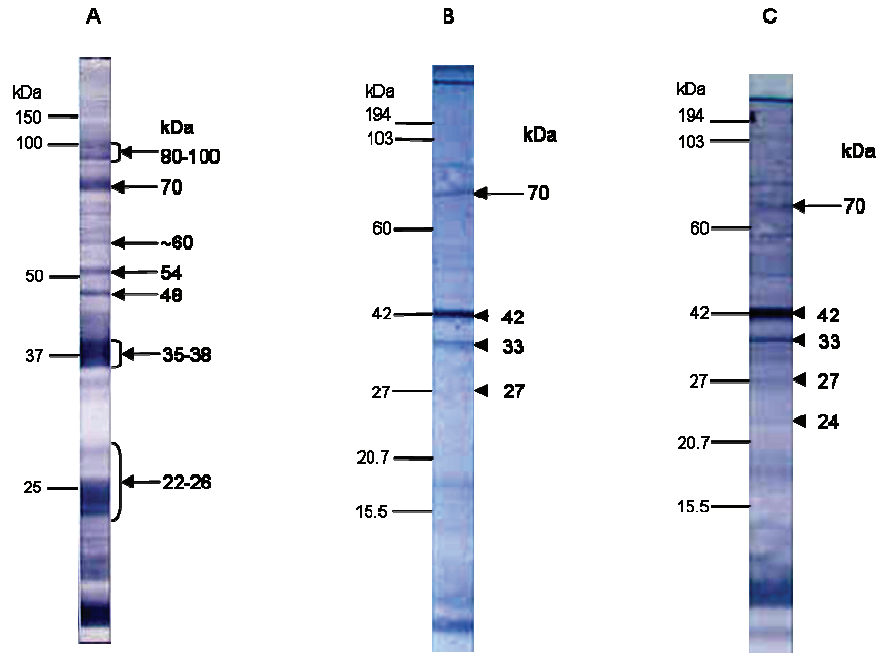

Fig. 1 Western blot-patterns of the first serum sample of a representative patient of group 1 against SDS-PAGE separated-homogenate of *Leptospira interrogans*, serogroup Pyrogenes, serovar Pyrogenes (A), *Leptospira biflexa,* serogroup Semaranga, serovar Patoc (B), and *Leptospira biflexa,* serogroup Andaman, serovar Andamana (C). Numbers at the left and right of each strip indicate molecular masses in kDa.

Table 3 Antigenic components of pathogenic *Lepospira* spp. bound by antibodies in the 18 leptospirosis patients' first sera and the second and third sera of four patients (no. 5, 7, 11 and 14) revealed by Western blot analysis

Patient no.	Days of fever onset when first sample was collected	Antigenic components (indicated in kDa) reacted with antibodies in the																				
		1st serum sample							2nd serum sample							3rd serum sample						
		80-100	70	~60	54	48	35-38	22-26	80-100	70	~60	54	48	35-38	22-26	80-100	70	~60	54	48	35-38	22-26
1	3	–	+	+	+	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2	4	–	+	+	+	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3	4*	+	+	+	+	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
4	4	+	+	+	+	+	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5	4	–	–	–	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	4*	+	–	–	+	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
7	4	+	–	–	–	+	+	+	+	+	–	–	+	+	+	+	+	+	–	+	+	+
8	4*	+	+	+	+	+	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
9	4	–	–	+	+	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10	5	+	–	+	+	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
11	5	–	–	+	–	–	+	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+
12	5	–	–	–	+	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
13	5	+	+	+	+	+	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
14	6	+	+	–	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	7*	+	–	–	–	+	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
16	7	+	–	–	–	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
17	8	+	+	–	–	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
18	10	+	+	–	+	–	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
% Positivity		66.66	50.00	50.00	77.77	22.22	100	100	100	100	75	50	100	100	100	100	100	100	50	100	100	100

*Patient whose first serum sample was negative by IgM dipstick
nd, not done.

tive bands at the ~35-38 and/or 22-26 kDa locations of the non-pathogenic bacteria, although some of the sera gave relatively faint, single band(s) at locations 42, 33, 27 and 24 kDa (Fig. 1B and 1C). Nine (50%) and 12 (67%) samples of leptospirosis patients gave faint bands to the 70 kDa component, respectively (Fig. 1B and 1C). No bands at the locations of 80-100, 60, 54, and 48 kDa were seen when the homogenates of the two non-pathogenic *Leptospira* spp. were used in the assay. Serum samples of groups 2 and 3 did not react to any component in homogenates of the non-pathogenic *L. biflexa* (data not shown).

Western blot patterns of the second and the third serum samples of four patients, *i.e.,* no. 5, 7, 11 and 14 showed an increase in number and intensity the antigen-antibody reactive bands to most, if not all, components located at the seven locations (80-100, 70, 60, 54, 48, 35-38, and 22-26 kDa of the pathogenic *Leptospira* spp. (Fig. 2).

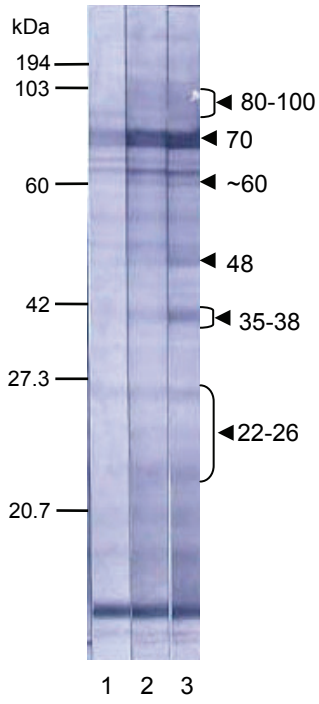

Fig. 2 Western blot-patterns of the serially collected sera of a representative patient of group 1 (patient no. 11) against the SDS-PAGE separated- homogenate of *Leptospira interrogans*, serogroup Icterohaemorrhagiae, serovar Icterrohaemorrhagiae. The serum samples were collected on days 5, 7, and 18 after fever onset (lanes 1, 2, and 3, respectively). Molecular masses are indicated by numbers at the left; Individual *Leptospira* components (80-100, 70, ~60, 48, 35-38 and 22-26 kDa) reacted to antibodies in the patient's samples are indicated by the numbers and the arrow heads on the right.

DISCUSSION

A rapid, simple and accurate method is needed for diagnosis of human leptospirosis. Direct demonstration of *Leptospira* in patient's clinical samples such as blood, urine, CSF performed by bacterial culture takes too long time and is insensitive.[14-16] Serum antibody detection, thus, serves as an indirect alternative means of leptospirosis diagnosis. MAT is one of the commonly used antibody detection assays.[17] It is specific provided that a four-fold or greater rise in the MAT titer could be demonstrated using paired serum samples taken at an interval of about 10 days or a titer higher than that of the cut-off value was found.[10] MAT is insensitive during the early phase of infection due to the nature of the test, an agglulination assay, which requires some amount of antibodies to the *Leptospira* surface components before the bacterial clumps could be visualized under the dark-field microscope. Besides, MAT requires a large battery of living *Leptospra* spp. of various serogroups and serovars which are laborious and costly. Other simpler antibody detection methods have been developed, such as indirect immunoflorescent assay, IgM ELISA, IgM dipstick.[6,17-19] Sensitivity of these assays, however, is still limited by the *Leptospira* spp. used for preparing the antigens. False negative results may occur if the infecting *Leptospira* spp. does not match the *Leptospira* spp. used as antigen in the assays. Alternatively, false positive results may be obtained by antibodies in serum of patient which have been stimulated by previously unrecognized *Leptospira* infection or exposure to antigenically related organisms especially in the leptospirosis endemic areas.[20]

In this study, we analyzed the diagnostic potential of homogenates prepared from 15 serogroups and 20 serovars of both pathogenic and non-pathogenic *Leptospira* spp. by IgG-immunoblotting. Results indicated that any serogroup and any serovar of the pathogenic *Leptospira* spp. but not the non-

pathogenic *L. biflexa* could be used in the assay. Sera of all clinically diagnosed, MAT-positive, IFA-positive leptospirosis patients reacted to components of the pathogenic *Leptospira* spp. located at 35-38 and 22-26 kDa with characteristic diffuse bands and thus 100% diagnostic sensitivity. These characteristic antigen-antibody reactive bands were revealed by the patients' sera collected as early as three days after the fever onset (earlier samples were not available). Few other components of the pathogenic *Leptospira* spp. that some of the first serum samples of the patients reacted were ~80-100, 70, 60, 54 and 48 kDa (Fig. 1A). More reactive band(s) and/or increased intensity of the early seen antigen-antibody reactive band(s) were observed when the followed-up samples were tested; indicating the higher magnitude of immune response to the *in vivo* expressed *Leptospira* antigenic component(s) during the later phase of infection and thus implying current *Leptospira* infection. The IgG-Western blotting was negative for the seven antigen-antibody reactive bands when serum samples of the patients with other febrile illnesses (group 2) and healthy controls (group 3) were tested; indicating 100% diagnostic specificity of the assay.

Although some of the sera of patients with leptospirosis reacted to components located at ~70, 42, 33 and 22-26 kDa of the non-pathogenic, *L. biflexa* serogroup Semaranga, serovar Patoc (saprophytic) (Fig. 1B) and serogroup Andamana, serovar Andamana (can infect host but cannot cause disease) (Fig. 1C), but the reactive bands were either faint and/or did not show diffuse characteristic. Thus, we recommend using homogenate of pathogenic *Leptospira* spp. as the antigen in the assay. The IgG-immunoblotting is simple. It can be used in testing several samples at the same time without much increase in the test time. It is rapid because the antigen blotted strips can be pre-prepared and kept ready in the laboratory for a long time (we kept the antigen blotted strips for longer than 18 months and still could be used in the Western blotting) without changing the reactivity of the separated *Leptospira* components (data not shown). The immunoblotting does not need live *Leptospira* spp. and thus is not hazardous. Thus, the IgG-immunoblotting has high diagnostic potential for diagnosis of current human leptospirosis. The method awaits further validation with larger sample size.

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