IDENTIFICATION OF CLINICAL ISOLATES OF *LEPTOSPIRA* SPP BY PULSED FIELD GEL-ELECTROPHORESIS AND MICROSCOPIC AGGLUTINATION TEST

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Abstract. Twenty-five clinical isolates of *Leptospira* spp were characterized by microscopic agglutination test (MAT) and pulsed field gel-electrophoresis (PFGE) in comparison with 23 reference *Leptospira* serovars and with the saprophytic *L. biflexa* serovar Patoc. PFGE DNA profiling was more specific and reliable than MAT.

INTRODUCTION

Leptospirosis caused by pathogenic *Leptospira interogans* is an infectious disease of local and wild animals. Humans can become infected by contacting with urine of carrier animals or contaminated environment (Faine, 1998). The disease is a major public health problem in Thailand, especially in the northeastern part of the country where the disease is endemic. The annual number of reported cases has been increasing in Thailand since 1996. Symptoms and signs of leptospirosis are frequently presented as cases with undifferentiated febrile illness. The majority of affected cases are people or farmers in rural areas, but the precise source of infection is usually uncertain (Tangkanakul and Kingnate, 1998).

The species of *Leptospira interrogans* includes 212 serovars arranged in 23 serogroups on the basis of antigenic classification (Kmety and Dikken, 1988). The conventional identification of leptospires is based on microscopic agglutination test (MAT) and cross agglutinin absorption test (CAAT) with serogroup and serovar specific hyperimmune serum (Dikken and Kmety, 1978). Serovar identification of leptospiral strains has become faster with the use of monoclonal antibodies (mAbs) (Terpstra *et al*, 1987). Serotypic identification is non-specific, tedious and extremely time consuming, as it requires the maintenance of reference strains and the preparation of their corresponding immune sera (Faine *et al*, 1999). Recently, genomic classification of pathogenic *Leptospira* spp has allowed identification of *L. interrogans, L. weilii, L. borgpetersenii, L. inadai, L. noguchii* and *L. santarosai* (Zuerner and Bollin, 1990). Genetic identification of leptospires can be done using DNA-DNA hybridization (Nielsen *et al*, 1989), restriction endonuclease analysis (REA) (Marshal *et al*, 1981; Ellis *et al*, 1991) and pulsed field gel-electrophoresis (PFGE) (Taylor *et al*, 1991). PFGE is a method that allows study of the molecular basis of various bacterial genomes (Levett, 2001). Study of leptospiral DNA using PFGE overcomes some limitations of other DNA procedures, offering the advantage of a simple, reliable and reproducible interpretation, which is in contrast to

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serotyping (Herrmann *et al*, 1992).

The purpose of this study was to determine by PFGE the serovar types of leptospires isolated from human blood culture and compare the results with those of MAT.

MATERIALS AND METHODS

Blood culture isolation

Blood samples were collected in Buri Rum and Surin provincial hospitals from patients with suspected leptospirosis during 2004. Blood specimens were cultured in semi-solid Ellinghausen and McCullough, modified by Johnson and Harris (EMJH) medium and incubated at 28-30ºC in the dark. Cultures were examined weekly for 8 weeks using a dark field microscope. Samples with leptospiral growth were considered positive. In addition, whole live leptospires were cultured in liquid neopeptone medium for antigen preparation for MAT testing.

Preparation of bacteria and specific antisera

Twenty-three reference strains of *L. interrogans* serovar Australis, Bangkok, Bratislava, Autumnalis, Rachmati, Ballum Bataviae, Canicola, Cellidoni Djasiman, Grippothyphosa, Hebdomadis, Icterohaemorrhagiae, Copenhageni, Javanica, Pomona, Pyrogenes, Hardjo, Sejroe, Saigon, Ranarum, Sarmin, and the saprophytic *L. biflexa* serovar Patoc were obtained from National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. Leptospires were periodically checked using specific reference antisera supplied from the US Center for Disease Control and Prevention (CDC), in Atlanta, Georgia (Table 1). In addition, twenty-five

Table 1
Serovar reference strains from pathogenic and non - pathogenic *Leptospira* spp.

Serogroup	Serovar	Reference strain
Australis	Australis	Ballico
	Bangkok	BD 92
	Bratislava	Jez-bratislava
Autumnalis	Autumnalis	Akiyami A
	Rachmati	Rachmat
Ballum	Ballum	Mus 127
Bataviae	Bataviae	Swart
Canicola	Canicola	Hond Utrecht IV
Cellidoni	Cellidoni	Cellidoni
Djasiman	Djasiman	Djasiman
Grippotyphosa	Grippotyphosa	Moskva V
Hebdomadis	Hebdomadis	Hebdomadis
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
	Copenhageni	M 20
Javanica	Javanica	Veldrat Bataviae
Pomona	Pomona	Pomona
Pyrogenes	Pyrogenes	Salinem
Sejroe	Hardjo	Hardjoprojitno
	Sejroe	M 84
Louisiana	Saigon	LSU 1945
Ranarum	Ranarum	ICF (Iowo City frog)
Sarmin	Sarmin	Sarmin
Biflexa	Patoc	Patoc I

clinical isolates from human blood were identified by MAT and cross-absorbed before being characterized by PFGE. The organisms were grown at 30ºC in neopeptone medium or EMJH medium and were used when the log phase of growth was reached.

Identification of leptospires

Leptospires were agglutinated with 23 serovars of reference antisera by MAT and compared with PFGE.

Preparation of leptospiral DNA plugs

Leptospires in liquid medium, at a density of 10^8 cells/ml, were sedimented at 10,000*g* for 15 minutes at 4ºC, then washed and resuspended in cell suspension buffer. The cell suspension was then heated at 50ºC in 1% agarose. The cell suspension was then transfered to plug molds using sterile transfer pipettes. The agarose was allowed to solidfy at 4ºC for 10-15 minutes. The solid agarose plugs were placed in a 50 ml conical centrifuge tube containing lysozyme solution (25 mg/ml) and incubated for 2 hours at 37ºC. The lysozyme solution was removed and the plugs were rinsed with sterile water. Proteinase K solution (600 U/ml) was added and the plugs were incubated overnight at 50ºC. The plugs were washed four times with wash buffer (Tris-EDTA buffer: 10.0 mM Tris, 1.0 M EDTA, pH 7.5), by incubating for 1 hour at room temperature using 1 ml of wash buffer for each plug. The plugs were stored at 4ºC in wash buffer. The plugs were stable for 3 months.

Restriction enzyme digestion of DNA plugs

Each plug was placed in sterile 1.5 ml microcentrifuge tube and incubated for 1 hour in 1 ml of 0.1x wash buffer. The 0.1x wash buffer was removed by aspiration and 0.5 ml of 1x restriction enzyme buffer was added and the mixture was incubated for 1 hour with gentle agitation at room temperature. The buffer was aspirated off and 0.3 ml of fresh 1x enzyme buffer was added. Then 40-60 U of *Not I* restriction enzyme (Toyobo, Japan) were added and the mixture was incubated 2-3 hours at 37ºC. The enzyme solution was removed and the plug was incubated with 1 ml of 1 x wash buffer for approximately by 30 minutes with gentle agitation. The wash buffer was removed and the plug was equilibrated in 0.5x TBE buffer (Tris-Borate EDTA; 0.9M Tris base, 0.9M boric acid, 0.02M EDTA pH 8.0).

PFGE

Pulsed field gel-electrophoresis was preformed in a clamped homogenous field (CHEF) electrophoresis apparatus (CHEF-DR III, Bio-rad Laboratories, Richmond, California) at 14ºC with buffer recirculation. The initial switch and final switch time was 5.5 seconds and 63.5 seconds respectively, for 22 hours. DNA from Lambda Ladder (Promega Corp, Madison, Wisconsin) and *Saccharomyces cerevisiae* chromosome (Bio-rad) were used as standard markers. Electrophoresis was conducted at 120V in 0.5x Tris-borate-EDTA buffer. Gels were stained with ethidium bromide (0.5 µg/ml) and visualized under ultraviolet light.

RESULTS

Cultivation of suspected leptospirosis specimens from human blood showed 25 positive cases. Using MAT, twenty-four of the isolated leptospires were positive for serovar Autumnalis, which demonstrated titer ranging from 1:1,600 to 1:25,600 except for isolate strain No.16 which was positive for serovar Mini at titer of 1:800 only (Table 2). Twenty-one strains of isolated leptospires were positive for both serovar Autumnalis and Djasiman. Some serovars of leptospires could be agglutinated by more than two reference antisera but at low titers. Mixed serovars of leptospires were shown as being Djasiman, Grippotyphosa, Muencheni, Icterohaemorrhagiae and Zanoni with titers ranging take 1:100 to 1:400.

PFGE DNA band patterns of the 23 reference serovars of leptospires are shown in Fig 1A and 1B. PFGE banding patterns were

Table 2
Serovars of leptospires found from human's blood 25 cases by MAT.

Serovar isolate No.	*L.* Autumnalis	*L.* Djasiman	*L.* Mini
1	1:1,600	-	-
2	1:12,800	1:100	-
3	1:6,400	1:400	-
4	1:400	1:400	-
5	1:3,200	1:100	-
6	1:12,800	-	-
7	1:6,400	1:200	-
8	1:1,600	1:100	-
9	1:6,400	1:100	-
10	1:6,400	1:100	-
11	1:6,400	1:100	-
12	1:1,600	1:800	-
13	1:25,600	1:800	-
14	1:6,400	1:100	-
15	1:1,600	1:400	-
16	-	-	1:800
17	1:25,600	1:400	-
18	1:3,200	1:3,200	-
19	1:3,200	-	-
20	1:12,800	1:400	-
21	1:12,800	1:1,600	-
22	1:25,600	1:800	-
23	1:12,800	1:400	-
24	1:3,200	1:800	-
25	1:6,400	1:400	-

quite specific for each serovar and could be used to classify the serovars of leptospires. The clinical strains of human blood showed bands in lane N and lane M of Fig 1A and 1B, respectively. Twenty-two clinical strains had similar bands but three clinical strains (isolates 4, 6 and 16) had differential patterns (Fig 1C and 1D). PFGE of clinical strain (isolate 4, Fig 1C, lane E) had MAT titer of serovar Autumnalis and Djasiman of 1:400 and 1:400, respectively. PFGE of clinical strain, isolate 6 (Fig C, lane G) was positive for leptospire serovar Autumnalis with MAT titer 1:12,800. The clinical strain (isolate 16 (Fig D lane F) did not show agglutination to reference hyperimmune leptospires. The reference serovar Autumnalis was used in comparison with clinical strains (lane A of Fig C and D).

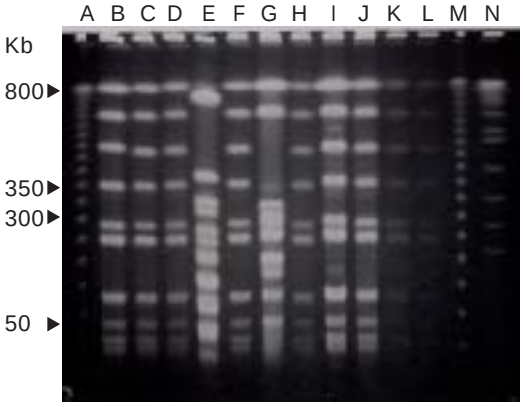
DISCUSSION

Leptospire is an important pathogen as it causes a life threatening disease. To assess the epidemiology of the disease, leptospires need to be detected and properly identified. Classification based on MAT and CAAT phenotyping requires the maintenance of a comprehensive collection of reference strains and their corresponding rabbit immune sera (Dikken and Kmety, 1978). The time required for identification of an unknown strain takes at least 2 months, mainly because of the time needed for the preparation of immune serum in rabbit. To overcome this problem, a number of genetic tests have been developed, including randomly amplified polymorphic DNA fingerprinting (RAPD) (Gerristsen *et al*, 1995), restriction endonuclease analysis (REA) (Ellis *et al*, 1991) and PFGE (Prashanth and Badrinath, 2005). RAPD analysis is very sensitive to changes in reaction conditions and presence of contamination (Williams *et al*, 1990). REA patterns are difficult and time-consuming to obtain. PFGE technique can be used to separate large DNA molecules or fragments up to approximately 10 Mbp in size and does not require a collection of microorganism (Vollrath and Davis, 1987). PFGE has proven useful in characterizing and verifying genomes of leptospira serovars and has served as a tool for culture identification (Herrmann *et al*, 1991, 1992).

The leptospiral serovars that we isolated are particularly interesting as almost all strains were agglutinated at high titer for serovar Autumnalis and cross-reacted with serovar Djasiman. Only two cases reacted specifically to serovar Autumnalis alone. Previous surveillance studies in kidney tissues from rodent

A. PFGE of reference strains of *Leptospira* spp; lanes A, Lambda DNA markers; B, serovar Autumnalis; C, Australis; D, Bataviae; E, Canicola; F, Grippothyphosa; G, Icterohaemorrhagiae; H, Javanica; I, Pomona; J, Pyrogenes; K, Bratislava; L, Copenhageni; M, Bangkok; N, clinical strain and lane O, *S. cerevisiae* markers.

B. PFGE of reference strains of *Leptospira* spp; lanes A, Lambda DNA markers; B, serovars Sejroe, C, Hardjo; D, Hebdomadis; E, Djasiman; F, Ballum; G, Saigon; H, Cellidoni; I, Rachmati; J, Sarmin; K Patocl; L, Ranarum; M, clinical strain and N, *S. cerevisiae* markers.



C. PFGE of clinical strains ; lanes A and M, Lambda DNA markers; B, isolate 1, C, isolate 2 ; D, isolate 3; E, isolate 4; F, isolate 5; G, isolate 6; H, isolate 7; I, isolate 8; J, isolate 9; K, isolate 10; L, isolate 11; lane N, *S. cerevisiae* markers.

D. PFGE of clinical strains; lanes A, reference serovar Autumnalis ; B, isolate 12; C, isolate 13; D, isolate 14; E, isolate 15; F, isolate 16; G, isolate 17; H, isolate 18; I, isolate 19; J, isolate 20; K, isolate 21; L, isolate 22; M, isolate 23; N, isolate 24; O, isolate 25.

Fig 1–Pulse field gel-electrophoresis patterns of clinical isolates and reference strains of *Leptospira* spp. Experiental conditions are described in Materials and Methods.

animals collected from Buri Rum Province reported that serovar Canicola (93%) and Autumnalis (77%) are the predominant serovars detected in urban and rural animals, respectively (Doungchawee *et al*, 2005). Previous studies showed that *Leptospira* serovar Bratislava, Sejroe and Pyrogenes are the most seroprevalant in northeast Thailand (Chaifoo *et al*, 1998; Arjkienn 2000; Boonyod *et al*, 2001; Tangkanakul *et al*, 2002).

In this study, PFGE patterns from clinical isolates showed differences from reference

strains, but almost all clinical strains had similar band patterns. However, the *Not* I restriction patterns of 22 out of 25 clinical isolates were similar to serovar Autumnalis pattern, but the human isolates patterns have some addition molecular weight bands of 46 bp, 60 bp, 350 bp and 540 bp, and also they lack of molecular weight bands 165 bp and 140 bp. Five of the 22 clinical isolates had an extra band (160 kb) (Fig 1D, lanes E, H, I, L, O) suggesting that a genetic rearrangement had occured in these clinical isolates (Zuerner *et al*, 1988). Isolate no 4 (Fig 1C, lane G) and isolate no 6 (Fig 1 lane G) was related to serovar Cellidoni and Pyrogenes, repectively. Isolate no 6 (Fig 1D lane F) was related to serovar Pomona. Our study differed from that previously reported by Herrman *et al* (1991) who found no corelation between different serovars by serotyping and their PFEG digestion fingerprinting. The explanation may be that the restriction site distributions are related to differences in DNA methylation. Discrepancy between the patterns observed in previous study and our study could be due to pulse and run times, which caused DNA migration to form different patterns and changes in band intensity. Other methods, such as gene sequencing, should also be used to help identify clinical isolates of *Leptospira*.

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