Comparison of Leptospiral Serovars Identification by Serology and Cultivation in Northeastern Region, Thailand

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Blood from patients suspected of leptospirosis 148 specimens were cultured for leptospira. Twenty two specimens were positive (15%). The isolated leptospira were tested against the 24 serovars of standard antisera by Microscopic Agglutination Test (MAT). It was found that all 22 leptospira isolates reacted strongly against L. autumnalis, except 1 isolate that also reacted againts serovar djasiman. The patient's sera were collected from only 14 cases. When the sera of the 14 patients were tested with the 24 reference serovars by MAT, it was found that sera reacted the most against L. australis and in decreasing order against L. bratislava, L. autumnalis, L. rachmati, L. copenhageni, L. javanica. There had some cross reactions against several serovars in a single patient.

The present study showed inconsistency between culture results and serum assays. Since sera showed cross reactivities against several serovars, it was not possible to determine which serovar was etiologic. Therefore, the isolation of leptospira though time consuming is specific in the identification of the serovar.

Keywords: *Leptospirosis, Leptospire, Cultivation, Microscopic Agglutination Test*

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Leptospirosis, a disease caused by pathogenic Leptospira interrogans, is thought to be the most wide spread zoonotic disease in the world and often related to occupation of the patients. Various animals such as rodents, dogs, cattle and swine are natural sources of infection. Such animals shed the leptospires in their urine which contaminate water, soil and sewage. Humans get infection when come into contact with the contaminated environment and the organisms will penetrate through the broken skin or mucosa. Leptospirosis has a wide range of clinical manifestations ranging from inapparent or mild febrile illness to severe illness which involving multi-system of humans body[1,2]. Thailand is the endemic area of the disease especially in the northeast of the country[3] where most of the people are farmers. Since 1996, the reported cases increased markedly. In 2000, 14,285 leptospirosis cases were reported and 10,217 cases in 2001with 362 deaths and 171 deaths respectively[4]. Numerous serovars of leptospires were claimed as the cause of infection even in the same geographical area.

Early diagnosis is the most important. This depends on clinical features, occupation, history of contact with water or soil, and laboratory findings. Laboratory tests usually rely on serological test by demonstrating leptospira antibodies. There are various commercial serological tests available in the market. They are simple, rapid, sensitive and effective for leptospirosis screening[5,6]. Microscopic Agglutination Test (MAT) is the standard reference test for detection of leptospiral antibodies definite to serovars[3,7]. Isolation of leptospires from clinical specimens provides the most specific diagnosis but it is inconvenient due to complicated techniques, less sensitivity and time consuming[3,8]. Accuracy of serovars identification may also depend on determining animal reservoirs and

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occupational risks which can also play the important roles in disease prevention and control[9].

In this preliminary study, the authors identified leptospire serovars from clinical specimens by serological technique compared with cultivation groups in order to verify the specific serovars for further prevention and control of the disease.

Material and Method

Sample collection

First blood samples were collected from the suspected leptospirosis patients on the first day of admission to hospitals in the northeast of Thailand during 2002. Five ml of blood was taken. One, two, three drops of blood were placed in each tube of Elling hausen McCullough Johnson Harris media (EMJH)[10], and the remaining blood were centrifuged. The sera were kept for serological testing. Convalescent sera were collected as paired sera on 7-14 days after the first blood was taken.

Panel of Leptospires use for Microscopic Agglutination Test (MAT)

The MAT was performed by using a battery of leptospires, 24 serovars of which were known to exist in Thailand[11]. These serovars include *L. interrogans serovars australis, bangkok, bratislava, autumnalis, rachmati, bataviae, canicola, celledoni, cynopteri, djasiman, grippotyphosa, hebdomadis, copenhageni, icterohaemorrhagia, javanica, saigon, pomona, pyrogenes, ranarum, sarmini, sejroe, wolffi, tarassovi* and *L. biflexa* for non-pathogen. All reference antigens and antisera were obtained from the Center for Disease Control and Prevention (CDC) in Atlanta, Georgia.

Serovars identification by cultivation

Leptospires were isolated from blood specimens which were inoculated into EMJH medium. The culture media were incubated at 30°C for 4 days and then they were checked for bacterial contamination or leptospiral growth by dark field microscope every week for 8 weeks[11].

Leptospires were adjusted to McFarland 0.5 units or 10^8 cells/ml as antigen concentration. The reference antisera of 24 serovars of leptospires were used to agglutinate leptospires prepared from cultivation, and were examined by MAT. The serial two-fold dilution of each of the 24 serovars of reference antisera were performed in microtiter plates (1:50-1:51,200). Then 50 µl of leptospiral culture concentration was added into every well of diluted reference antisera. The agglutination was examined by dark field microscope. The serovars were considered positive with titers $\geq$ 1:100[12,13].

Serovars identification by serological test

The 24 reference serovars of Leptospires were grown in EMJH medium and incubated at 30°C for 7 days and then adjusted to 10^8 cells/ml. The serial dilutions of patient sera were mixed with reference antigens and examined for agglutination by dark field microscope. The end point titer was the highest dilution in which 50% of the leptospires agglutinates and unagglutination in negative control. Test was considered positive at the titer $\geq$ 1:400 in single serum or a much higher titer of $\geq$ 4 fold dilution in an acute phase serum than in the convalescent-phase serum[12]. The result of serum and all specimens were presented by descriptive frequency table.

Results

In 2002, 148 blood samples of leptospirosis suspected cases from the northeast of the country were examined by culture and serological testing. Twenty - two specimens (15%) were positive for leptospires by culture. All 22 leptospires were positive for serovar *autumnalis* by MAT except specimen no. 6 which was also positive for serovar *djasiman*. Among 22 culture confirmed patients, 8 cases had single serum while 6 cases had paired sera.

Fourteen of isolated leptospires were determined using MAT with reference antisera raised against 24 leptospiral serovars. All isolates demonstrated high titer ranging from 1:800 to 1:25,600 for only serovar *autumnalis* and titer for *djasiman* were in a range of 1:100 to 1:3,200 as shown in Table 1and 2.

For single serum, the specimens were tested with 24 leptospire reference antigens. All specimens agglutinated with serovar australis at titers ranging from 1:200 to 1:1,600. Other serovars: *bratislava, autumnalis, copenhageni* and *rachmati* were agglutinated with most of the specimens at titers 1:100 to 1:1,600. The serum and isolated leptospire from the same patient were used for homologous agglutination. Seven cases showed positive reaction at titers 1:100 to 1:800 and only one case gave negative result (Table 1).

For paired serum, all specimens gave high titers to serovar *australis* while 5 specimens were also positive for serovar bratislava (Table 2). Other serovars: *autumnalis, javanica* and *copenhageni* were positive for some sera samples. Homologous aggluti-

Table 1. Test results for patient with single serum

No.	Cultivation		Serological test					Homologous agglutination
	autumnalis	djasiman	australis	bratislava	autumnalis	copenhageni	rachmati	
1	1:6400	1:100	1:800	1:1600	1:1600	N	N	1:800
2	1:25600	1:800	1:1600	1:800	1:1600	N	1:400	1:800
3	1:6400	1:100	1:800	1:200	1:100	1:400	N	1:200
4	1:1600	1:400	1:200	N	1:100	N	N	N
5	1:25600	1:400	1:1600	N	N	1:1600	N	1:100
6	1:3200	1:3200	1:1600	1:800	N	1:200	1:100	1:800
7	1:12800	1:400	1:1600	1:1600	1:800	1:400	1:100	1:400
8	1:3200	1:200	1:1600	1:400	N	1:400	1:400	1:400

Table2. Test results for patient with paired serum

No.	Cultivation		Serological test					Homologous agglutination
	autumnalis	djasiman	australis	bratislava	autumnalis	javanica	copenhageni	
1.1	1:1600	1:800	1:1600	1:800	N	N	N	1:100
1.2	ND	ND	1:12800	1:1600	1:800	1:800	N	1:1600
2.1	1:800	1:400	1:200	N	N	N	N	N
2.2	ND	ND	1:100	N	N	N	N	N
3.1	1:6400	1:400	1:800	1:400	N	N	N	N
3.2	ND	ND	1:3200	1:12800	N	1:800	1:6400	1:12800
4.1	1:12800	1:400	1:1600	1:6400	1:1600	1:800	N	1:400
4.2	ND	ND	1:6400	1:6400	1:400	1:800	1:400	1:1600
5.1	1:25600	1:800	1:1600	1:800	1:200	N	1:100	N
5.2	ND	ND	1:3200	1:1600	N	1:800	N	1:1600
6.1	1:12800	1:400	N	N	N	N	N	N
6.2	ND	ND	1:1600	1:200	N	1:200	1:800	N

N: Negative ND: Not done

nation of patient sera and isolated leptospires in this group were positive in 4 cases.

Discussion

Serology is the routine diagnostic approach for leptospirosis. The MAT with a number of live antigens is considered to be the standard serological procedure to detect definite serovar of leptospiral antibodies with quantitative measurement[12]. However, crossed reaction between serovars are common[14] and several serovars are detected in the same serum[14]. Culture is the gold standard method which can detect causative organisms during the first week (leptospiremia) of the illness. Although the cultivation method has disadvantages such as time consuming, insensitive and difficult to process but it is specific for serovars of causative leptospires. The aim of this project was to study the relation between serology and cultivation of leptospires in clinical specimens from patients in endemic area of leptospirosis.

The authors found that almost all of the isolations from clinical specimens agglutinated at high titers for serovar autumnalis and cross reacted with serovar *djasiman*. Only one specimen gave high titer to both serovars. The results showed that all isolated leptospires from patients in acute phase were specific to serovar *autumnalis* except one case had co-infection with serovar *djasiman*. Further surveillance study in rodent animal should be performed in order to know the specific reservoir of both serovars.

For serological test, high titers were demonstrated for serovars *australis* followed by *bratislava* and cross reacted with several other serovars. These were due to the fact that antibodies of *australis* and *bratislava* were rather stable which could be found in high titers for long period and both serovars were in

the same serogroup[7]. Since the northeast of Thailand is the endemic area of leptospirosis where people have been exposed to leptospires infection , these people possess non specific immunity to leptospires and thus reacted to several serovars[7]. No antibody responses were detected for sera no. 5, 6, 8, 2.1, 3.1, and 6.1 which supporting the previous study elsewhere that there was no correlation between serological finding and identity of the isolation[15].

Homologous agglutination was tested using 14 isolations agglutinated with serum from the same patient, positive results were found in 11 cases (78.5%). Four out of 11 cases had no antibody to serovar *autumnalis* but yielded high titer to other serovars especially serovar *australis*, these may cause the agglutination to the isolated organisms[7].

The remaining 3 cases, 1 case (No. 4) had MAT titer of 1:100, 2 cases (No. 2.1,6.1) had no antibody to serovar *autumnalis* or others. These data suggested that they got primary infection.

According to the present study, most of the results of cultured identification did not match the serological findings. The MAT serological results in endemic area could demonstrate the prevalence of leptospire serovars. However, for confirmation of serovars, genetic characterization using polymerase chain reaction or pulse field gel electrophoresis or restriction endonuclease analysis may be the alternative methods for identification of leptospires but these techniques require special equipment[16,17].

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การศึกษาเปรียบเทียบซีโรวาร์ของเชื้อเลปโตสไปราจากการทดสอบน้ำเหลืองและการเพาะเชื้อในผู้ป่วยในภาคตะวันออกเฉียงเหนือของประเทศไทย

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นำตัวอย่างเลือดของผู้ป่วยที่สงสัยว่าเป็นโรคเลปโตสไปโรซิส จำนวน 148 ราย มาเพาะเชื้อ พบว่ามีเชื้อเลปโตสไปรา ขึ้น 22 ราย คิดเป็นร้อยละ 15 เมื่อนำเชื้อที่ได้มาทดสอบกับแอนติซีรัมมาตรฐาน 24 ซีโรวาร์ด้วยวิธีไมโครสโครปิค แอคกรูติเนชั่น (Microscopic Agglutination Test, MAT) พบว่าเชื้อทุกตัวทำปฏิกิริยาสูงสุดต่อซีโรวาร์ autumnalis และมี 1 รายที่พบซีโรวาร์ djasiman ร่วมด้วย ในจำนวนผู้ป่วยที่ตรวจพบเชื้อ 22 รายนี้ มี 14 รายที่เก็บซีรัมได้ เมื่อนำซีรัมของผู้ป่วยทั้ง 14 ราย มาทำปฏิกิริยาด้วยวิธี MAT กับเชื้ออ้างอิง 24 ซีโรวาร์ พบว่าเกิดปฏิกิริยากับเชื้อ australis มากที่สุด รองลงมาคือ bratislava, autumnalis, copenhageni, rachmati, และ javanica ตามลำดับ โดยพบว่าเกิดปฏิกิริยาข้ามเกี่ยวกับหลายซีโรวาร์ในผู้ป่วยคนเดียวกัน

ผลการทดสอบนี้ แสดงให้เห็นความไม่สอดคล้องกันระหว่างผลการเพาะเชื้อและผลการทดสอบซีรัมของผู้ป่วยและเนื่องจากการทดสอบซีรัมให้ปฏิกิริยาข้ามเกี่ยวหลายซีโรวาร์ ทำให้ไม่สามารถบอกได้ชัดเจนว่าซีโรวาร์ใดเป็นสาเหตุก่อโรคที่แท้จริง ดังนั้นการเพาะแยกเชื้อแม้จะใช้เวลาทดสอบนานแต่จะมีความจำเพาะในการพิสูจน์ซีโรวาร์ได้ดีกว่า