Leptospirosis, a disease caused by pathogenic Leptospira interrogans, is thought to be the most widespread 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 2001 with 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...
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 Ellinghausen 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*, *javania*, *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 leptospiiral 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 \(\mu\)l of leptospiiral 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\(^11\). 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 leptospiiral 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 leptospirosis 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*, *javania* and *copenhageni* were positive for some sera samples. Homologous aggluti-
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

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<th>Table 1. Test results for patient with paired serum</th>
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N: Negative  ND: Not done
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

มยุรา กุสุมาร, น깍วรรณ บุญสาธร, มณฑาชิน เบียงกลาง, อุมาพร สินา, ปัญจ สารรัตน์ปัญญาเลิศ, พิมพ์ใจ นัยโกวิท

นำตัวอย่างเลือดของผู้ป่วยที่สงสัยว่าเป็นโรคเลปโตสไปโรซิสจำนวน 148 รายมาเพาะเชื้อพบว่ามีเชื้อเลปโตสไปราขึ้น 22 รายคิดเป็นร้อยละ 15 เมื่อนำเชื้อที่ได้มาทดสอบกับแอนติซีรัมมาตรฐาน 24 ซีโรวาร์ด้วยวิธีไมโครสโครปิคแอคกรูติเนชั่น (Microscopic Agglutination Test, MAT) พบว่าเชื้อทุกด้วยทำปฏิกิริยากับซีเวิร์ร์ autumnalis และมี 1 รายที่พบซีเวิร์ร์ djasiman รวมด้วย ในจำนวนผู้ป่วยที่ตรวจพบเชื้อ 22 รายนี้มี 14 รายที่เก็บซีรัมได้เมื่อนำซีรัมของผู้ป่วยทั้ง 14 รายมาทำปฏิกิริยาด้วยวิธี MAT กับเชื้ออ้างอิง 24 ซีโรวาร์พบว่าเกิดปฏิกิริยาที่มากที่สุดของซีโรวาร์ autumnalis, bratislava, copenhageni, rachmati, และ javanica โดยพบว่าเกิดปฏิกิริยาข้ามกับหลายซีโรวาร์ในผู้ป่วยคนเดียวกัน

ผลการทดสอบนี้แสดงให้เห็นความไม่สอดคล้องกันระหว่างผลการเพาะเชื้อและผลการทดสอบซีรัมของผู้ป่วยและเนื่องจากการทดสอบซีรัมในปฏิกิริยาข้ามเกี่ยวกับหลายซีโรวาร์ทำให้ไม่สามารถบอกได้ซึ่งผู้ป่วยซึ่งโรคได้เป็นเพราะโรคที่แท้จริง ดังนั้นการเพาะแยกเชื้อและใช้วิธีทดสอบพบเชื้อจะมีความจำเป็นในการพิสูจน์ชี้วิวภาระต่อไป