Leptospirosis in Takeo Province, Kingdom of Cambodia, 2003

Heng Seng MD*,**, Touch Sok MD, MPH**,
Waraluk Tangkanakul MD, MPH***, Wimol Petkanchanapong PhD****,
Uraiwan Kositanont PhD*****, Hem Sareth******,
Bunleng Hor MD*******, Chuleeporn Jiraphongsa MD, PhD*

** International Field Epidemiology Training Program, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand*
*** Communicable Disease Control Department of Ministry of Health, Cambodia*
**** Bureau of General Communicable Diseases, Department of Disease Control, Ministry of Public Health, Thailand*
***** National Institute of Health, Department of Medical Science, Ministry of Public Health, Thailand*
****** Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*
******* Takeo provincial hospital, Cambodia*
******** National HIV/AIDS, Dermatology and STD, Cambodia*

Background: *In Cambodia, epidemiology and disease burden of leptospirosis were not addressed as they do not have an existing surveillance system and have limitations on their laboratory diagnosis.*
Objective: *Define the existence of leptospirosis and determine the antibodies to serovars of leptospires in Cambodia.*
Material and Method: *One hundred and twenty-one suspected cases of leptospirosis were enrolled in this cross-sectional study, between September 8 and November 30, 2003 from Takeo Provincial Hospital in Doun Keo District, Cambodia.*
Results: *Common clinical manifestations were fever (96%), headache (92%), and myalgia (87%). Common risk behaviors were throwing garbage on the ground (84%), pulling out sprouts (77%), fertilizing (49%), and plowing (47%). Microscopic agglutination test result confirmed four cases and polymerase chain reaction test result confirmed seven cases. Two cases each showed antibodies to serovars Javanica and Australis. An estimated annual incidence of leptospirosis in Takeo province was 7.65 per 100,000 populations. Further studies to define epidemiology and burden of disease are needed.*
Conclusion: *Increasing awareness and knowledge on leptospirosis among people are necessary to decrease the impact of leptospirosis in Cambodia.*

Keywords: *Leptospirosis, Leptospirae, Cambodia*

J Med Assoc Thai 2007; 90 (3): 546-51
Full text. e-Journal: *http://www.medassocthai.org/journal*

Cambodia is located in the southwest of Indochina and shares the border with Thailand (North), Laos PDR (Northeast), and Vietnam (the East and South). The country of 181,035 square kilometers has a large fresh water lake (Tonle Sap) that connects to the Mekong River. Population in the year 2003 was 13,287,053. Thirty-five percent was in the 10-19 age group and 47.8% was in the working age group (20-49 years). It is divided into 24 provinces or 75 operational districts. There are 68 referral hospitals and 942 health centers. The main occupation is agriculture.

Leptospirosis is a zoonotic and endemics disease in tropical and sub-tropical areas. The early symptoms of mild leptospirosis cases are similar to those of influenza. Even in severe cases, the disease

Correspondence to : Tangkanakul W, Bureau of General Communicable Diseases, Department of Disease Control, Ministry of Public Health, Tiwanond road, Nonthaburi 11000, Thailand. Phone: 0-25903-180-4, Fax: 0-2591-8436. E-mail: waraluk @health.go.th

can be mistaken for malaria, hepatitis, dengue or rat-borne hanta virus infection, hemorrhagic fevers, or typhus. From early headache, fever, and muscular pain, leptospirosis can lead to kidney and liver failure, meningitis, bleeding through the lungs, and death[1]. The case-fatality was as high as 4%[2]. However, only 39.5% of infected persons have clinical manifes tations[3]. The burden of leptospirosis should have been much larger than those figures suggest as many relatively mild cases are going unreported. The main reason for this underestimation is the extremely low awareness of having leptospirosis among people and health personnel.

An epidemic of leptospirosis happened in Thailand. The lowland northeastern provinces adjacent to Cambodia were especially hard hit, and at least 80% of cases were farmers[3,4]. It is possible that Cambodia has already had leptospirosis as a great public health risk among agriculturists. Those at greatest risk are rice and cattle farmers and sugarcane workers because they have extended contact with water and mud contaminated by the urine of rats and other infected animals, including most warm-blooded species. However, the existence of leptospirosis in Cambodia has not been clearly documented because of the lack of a surveillance system and the limitations of their laboratory diagnosis. The authors conducted the present study with the objective to address the existence of leptospirosis and to determine antibodies to serovars of leptospires in Cambodia.

Material and Method

Study population

Study site: Takeo Province is located in northwestern Cambodia with only one administrative district. It has a population of 854,727. There are 236 villages of 20 communes. Takeo Provincial Hospital is based in Doun Keo District, 78 km from Phnom Penh City. It is a large government referral hospital in Cambodia. The hospital and 15 associated health centers are responsible for 35,525 people in 40 villages of 4 communes. Takeo Hospital has 230 beds for inpatient care. Normally, 100 outpatient cases receive treatment from the hospital and 40 inpatient cases are admitted every day. This hospital was chosen as a study site because it is located in the rural area of Cambodia and there is high feasibility of enrolling many cases for the present study.

Definition: Case definition was adapted from CDC, 1997 case definition[5]. A suspected case was a patient having at least one symptom (fever, headache, or myalgia), or sign (body temperature more than 38.3 Celsius, conjunctival suffusion, and muscle tenderness) with an epidemiological history of exposure to infected animals or an environment contaminated with animal urine. A confirmed case was a suspected case with positive PCR detection of leptospires, or an antibody titer $\geq$ 200 detected by MAT (microscopic agglutination test)[5]. Patients older than 10 years old who came to visit Takeo Provincial Hospital and met case definition were included in this cross-sectional study (both inpatient and outpatient). Patients who were eligible but refused to participate or have blood drawn were excluded. Patients were enrolled from 8 September to 30 November 2003.

Sample size was calculated by Epi-Info software version 6[6]. The expected incidence (P)*: 6.00%, half of confidence interval (D): 4.20%, confidence Level: 95%. The estimated sample size was equal to 116. Data collection started from interviewers training on recruitment interview methods. When there was an eligible patient, the interviewer read the information in the consent form for that patient. After the patient gave oral consent, they were interviewed face-to-face. Standardized questionnaires were used to collect information from the patients on age, gender, risk factors, clinical manifestations etc. After interview, the patients were sent to the laboratory, where five milliliters of blood were drawn. Venous blood was collected by aseptic technique. One to three drops of blood were inoculated into three transport medium tubes. Blood samples were centrifuged to separate the serum (1.5 ml), and were frozen at-20^{o} C. Then all specimens were sent to the Thai National Institute of Health. Serum specimens were packed in a box with dry ice, separately from transport medium tubes.

Laboratory testing

The serum was tested by the gold standard MAT. It was performed at the Thai National Institute of Health by standard methods[7] using a battery of 24 serovars representing 23 serogroups of *L.interrogans* and 1 serogroup of *L.biflexa*. Each serum sample was screened at the dilution of 1:25 in 96 well-microtiter plate. Fifty microliters of both diluted serum and 10^8 cells/ml of each live antigen were incubated for 90 minutes at 30^{o}C. After incubation, 10 ml of the serum-antigen mixture was placed on a slide and observed for agglutination under a dark-field microscope. The serum showing agglutination at this dilution was further tested at serial two-fold dilution up to 1:12800. A MAT test was considered positive at the titer of

equal or more than 100 for a single sample or four-fold rising for paired sera.

Culture of *leptospires* was performed at the National Institute of Health, Thailand. Blood was inoculated into three transport medium tubes containing 5 ml of EMJH (Ellinghausen and McCullough, modified by Johnson and Harris) medium containing 200 mg/ml of 5 fluorouracil. The inoculated tubes were kept at room temperature until transferred to the Thai National Institute of Health. All culture was subcultured into EMJH medium and observed for growth of *leptospires* at two week intervals for 4 months by dark-field microscopy[8].

Polymerase chain reaction (PCR) was performed at Siriraj Hospital, Bangkok, Thailand. Extracted DNA from serum samples were tested for leptospiral DNA by PCR assay at the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Oligonucleotide primers specific for 23S rDNA sequences of pathogenic and non-pathogenic *leptospires* were used to detect pathogenic *leptospires* by generating 615 bp product whereas primers NLU1 and NPL2 were employed to amplify 23S rDNA sequence of non-pathogenic leptospires by generating 316 bp product (unpublished data). Ten ml of extracted DNA were amplified in 50 ml of a reaction mixture containing 5 µl of 10X PCR buffer, 8 µl of 25 mM $MgCl_2$, 1 µl of 20 pM of each primer, 200 µM of each four deoxynucleotide triphosphates (dATP, dCTP, dTTP, and dGTP), 1 unit of Thermus aquaticus (Taq) polymerase (Promega, USA). The amplification reaction was performed with a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, USA). DNA of *leptospirae* serovar Bataviae and Patoc were used as positive controls for pathogenic and non-pathogenic *leptospires*, respectively. MQ water without DNA was used as negative control.

Data analyses were performed under Epi-Info 2002 application. The prevalence of leptospirosis among suspected cases was calculated by the number of specimens positive (MAT) over number of specimens tested, and the prevalence of leptospirosis in Takeo Province was calculated by the number of specimens positive over the number of Takeo population (632,298).

Results

From September 8 to November 30, 2003, 1,158 patients visited Takeo Hospital. Ten percent (121 cases) were eligible cases. Of these 121 suspected cases, mean age was 35 years old (median 35 years, range 16-60 years). Most of the suspected cases (86.8%) were in the working age group (20-49 years). Male to female case ratio was 1.2:1 (67:54). Eighty-five of the 121 cases (70.2%) were farmers, 13 cases (10.7%) were drivers, 5 cases (4.1%) were merchants, and 9 cases (7.4%) were other occupations. Most cases (97.5%) were out patient cases, 3 cases were admitted. Duration of symptoms prior to hospital visit was around 2 weeks (median 14 days).

Most frequent symptoms included fever, headache, and myalgia. The most frequent signs were fever >38 ^{o} C, myalgia, and rash. Patients reported dark urine more frequently than the physician whereas the physician reported red eye more frequently than patients. When the authors considered symptoms and signs, the most frequent were fever, myalgia, headache, rash, dark urine, jaundice, red eyes, and oliguria, respectively (Table 1). There was no statistically significant difference on clinical manifestations among the 110 suspected cases and 11 confirmed cases.

Frequently reported risk factors included walking through water (89%), throwing garbage on the ground (84%), pulling out sprouts in the wet field for less than six hours (77%), keeping animals (51%), and fertilizing in the wet field for less than six hours (49%). Cows were kept by suspected cases more than dogs and pigs (Table 2). There was no statistically significant difference on potential risk factors among the 110 suspected cases and 11 confirmed cases. Patients reported that they went to visit other health posts and they had already received medication. Thirteen cases (30%) took only antibiotics, 21 cases (49%) took antipyretics, and 9 cases (21%) took both antibiotic & antipyretics from outside Takeo Hospital.

Table 1. Symptoms and signs of suspected and confirmed leptospirosis cases, Takeo hospital, Cambodia 8 Sep. to 30 Nov. 2003 (n = 121)

Symptoms and Signs	Confirmed Cases (%) (n = 11)	Suspected Cases (%) (n = 110)
Fever >= 38.3	72.7	87.3
Muscle Tenderness	81.8	76.4
Severe Headache	18.2	28.2
Persistant Rash	45.5	35.5
Reporting Dark Urine	18.2	31.8
Jaundice	0.0	5.5
Red Eye	0.0	2.7
Reporting Oliguria	0.0	7.3

Table 2. Risk Factors of leptospirosis among cases, Takeo Hospital, Cambodia, 8 September to 30 November 2003 (n = 121)

Risk Factors	Number (%)
Walking through water	108 (89)
Pulling out sprouts in a wet field < 6 hr.	93 (77)
Fertilizing in a wet field < 6 hr.	59 (49)
Plowing in a wet field < 6 hr.	57 (47)
Fishing	44 (36)
Pulling out sprouts in a wet field > 6 hr.	7 (6)
Killing or Hunting Rats	5 (4)
Plowing in a wet field > 6 hr.	4 (3)
Fertilizing in a wet field > 6hr.	4 (3)
Keeping animals	
Yes	62 (55)
Cow	46 (74)
Dog	25 (40)
Pig	24 (39)
Garbage management:	
Throw on the ground	101 (84)
Burying in the ground	11 (9)
Burn	7 (6)
Throw in water	2 (2)

First serum samples from all eligible cases were collected at the first visit to Takeo Hospital. The authors could collect 121 first serum samples but less than 50% (42 cases) of second serum samples. Average duration between the first and the second serum was around 3 weeks (median 21 days). Among 121 samples tested, four first serum specimens were positive by MAT (2 cases positive to serogroup Javanica (serovar Javanica) at titer 1:400 and 1:100 and 2 cases positive to serogroup Australis (serovar Australis) at titer 1:200. There was no MAT positive sample among second serum samples. Cultures of all serum samples were negative. The positive results for leptospiral DNA detection by PCR were found in 10 samples. Of 10 cases with positive PCR, 5 cases were positive in both first and second samples while the other 2 and 3 samples were positive from the first and second samples, respectively. Overall, the authors identified 11 cases as confirmed leptospirosis, 3 cases were positive for both MAT and PCR.

Discussion

Cambodia is in a tropical region, which is climatologically appropriate for survival of leptospires. Although the surrounding countryside is prevalent with leptospirosis, literature about leptospirosis in Cambodia is scarce. An examination of sera obtained from hemorrhagic fever disease case episodes in Cambodia, 1999-2001, showed that 3% (5 of 194) were positive by MAT or PCR results[9]. The result of the present study is again proving the existence of leptospirosis in areas that ecological parameters help to maintain *leptospires* in the environment and local spreading can happen. The Cambodian population has many environmental and occupational risk factors similar to Thais[4]. Therefore, an outbreak of human leptospirosis can occur at any time when other predisposing factors such as flooding, increasing rodent population, water resources development, etc., trigger transmission of *leptospires* to human or increased opportunity of getting infection in humans as Thailand experienced in a recent epidemic[9,10].

The authors calculated the annual incidence of leptospirosis in Takeo province to be 7.65 per 100,000 population. This incidence is in the same range of the annual incidence of leptospirosis in Thailand in 2003, 7.88 per 100,000 total populations[12]. According to the finding that a significant proportion of patients (30%) took antibiotics before visiting the hospital, it may be estimated that a large group of people with leptospirosis might not come to the hospital and not be diagnosed. In addition, the study period (September to November) was the end of the rainy season and might be the high season for leptospirosis. These reasons might affect the estimated prevalence in the present study.

Although the case definition of leptospirosis in the present study was quite broad, the prevalence computed from the confirmed leptospirosis cases should be able to estimate the lower bound of leptospirosis problem in Cambodia. Symptoms and signs of leptospirosis cases in Cambodia also need a prospective clinical study because of the low number of confirmed cases and most of the enrolled patients were outpatients with mild clinical manifestations. Other etiologies of febrile illness, for example, scrub typhus, influenza, dengue fever, or murine typhus should be further examined as previous studies found many causative organisms among febrile patients who were Thais or Khmers in Thailand[13,14].

The MAT results identified the existence of serogroup Javanica (serovar javanica) and Australis (serovar australis) in Cambodia. Australis serogroup especially serovar bratislava was a major serovar found in Thailand during the recent epidemic. It was also classified as a new serovar in Thailand. In Cambodia, it may be possible to have serovar bratislava as in Thailand. Javanica has been discovered in Thailand

and included in the MAT batteries for more than 40 years in Thailand[15]. MAT can detect both IgM and IgG antibodies, which IgM or recent immunity can persist for several months up to a year and IgG or specific immunity can persist from 6 months up to 20 years[1]. However, MAT results can only show the pattern of cross- reactivity among serovars represented serogroups in its batteries. It cannot identify the causative serovar of disease.

The finding that all specimens were negative cultures might be because the duration of blood drawn was too long (median 14 days) from the period of leptospiremia (7 days after the date of onset)[16], or this may be due to the long period of time between serum collection and laboratory receipt in Thailand (median 26 days). Taking antibiotics prior to collecting blood was also a negative factor to yield *leptospires*. Normally, culture is a low sensitivite method for leptospiral cultivation. MAT titer was not high because the time of blood drawn was not in the peak of antibody response (3rd to 4th week after date of onset)[16]. Low MAT titer might also be the effect of antibiotics. PCR results showed higher numbers of infected cases than MAT did because of its higher sensitivity[8].

Further studies are needed in Cambodia in many aspects such as: 1) A study to identify the exact magnitude of leptospirosis problem. 2) A study and/or implementation of a surveillance system for getting base line data to convince policy makers. 3) An evaluation study on laboratory capacities on leptospirosis diagnosis. 4) Health personnel and farmers' knowledge of leptospirosis. 5) Risk factors and appropriate preventive strategies for Cambodia. However, health education on leptospirosis disease and increasing awareness of leptospirosis in both health personnel and people in Cambodia should be implemented immediately to prevent new cases and complications of leptospirosis.

Acknowledgements

The authors wish to thank Dr. Pathom Sawanpanyalert (National Institute of Health, Thailand), Thomas Clarke (Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, United States of America) for their valuable support. We wish to thank Dr. Oum Sokhan (Director of the Takeo provincial health office, Cambodia), and staff of Takeo provincial hospital for their help in collecting data and specimens.

References

1. Faine S. Guidelines for the control of leptospirosis. Geneva: World Health Organization; 1982.
2. Tangkanakul W, Smits HL, Jatanasen S, Ashford DA. Leptospirosis: an emerging health problem in Thailand. Southeast Asian J Trop Med Public Health 2005; 36: 281-8.
3. Phraisuwan P, Whitney EA, Tharmaphornpilas P, Guharat S, Thongkamsamut S, Aresagig S, et al. Leptospirosis: skin wounds and control strategies, Thailand, 1999. Emerg Infect Dis 2002; 8: 1455-9.
4. Tangkanakul W, Tharmaphornpil P, Plikaytis BD, Bragg S, Poonsuksombat D, Choomkasien P, et al. Risk factors associated with leptospirosis in northeastern Thailand, 1998. Am J Trop Med Hyg 2000; 63: 204-8.
5. Case definitions for infectious conditions under public health surveillance. Centers for Disease Control and Prevention. MMWR Recomm Rep 1997; 46: 1-55.
6. Centers for Disease Control and Prevention (CDC). Epi-Info. Version 6. Atlanta, GA: CDC; 1993.
7. Faine S, Alder B, Bolin C, Perolat P. Leptospira and leptospirosis. 2nd ed. Melbourne: Medisci, Australia; 1999.
8. World Health Organization. Human leptospirosis guidance for diagnosis, surveillance and control. Geneva: WHO; 2003.
9. Laras K, Cao BV, Bounlu K, Nguyen TK, Olson JG, Thongchanh S, et al. The importance of leptospirosis in Southeast Asia. Am J Trop Med Hyg 2002; 67: 278-86.
10. Kositanont U, Naigowit P, Imvithaya A, Singchai C, Puthavathana P. Prevalence of antibodies to Leptospira serovars in rodents and shrews trapped in low and high endemic areas in Thailand. J Med Assoc Thai 2003; 86: 136-42.
11. Bunnag T, Sornmani S, Impand P, Harinasuta C. Potential health hazards of the water resources development: a health survey in the Phitsanulok Irrigation Project, Nan River Basin, Northern Thailand. Southeast Asian J Trop Med Public Health 1980; 11: 559-65.
12. Annual epidemiological surveillance report 2003. Nonthaburi: Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health; 2005.
13. Duffy PE, Le Guillouzic H, Gass RF, Innis BL. Murine typhus identified as a major cause of febrile illness in a camp for displaced Khmers in Thailand. Am J Trop Med Hyg 1990; 43: 520-6.
14. Leelarasamee A, Chupaprawan C, Chenchittikul M, Udompanthurat S. Etiologies of acute undifferentiated febrile illness in Thailand. J Med Assoc Thai

2004; 87: 464-72.
15. Sundharagiati B, Harinasuta C. Studies on leptospirosis in Thailand: a review. J Med Assoc Thai 1964; 47: 662-79.
16. Levett PN. Leptospirosis. Clin Microbiol Rev 2001; 14: 296-326.

เลปโตสไปโรสิสที่จังหวัดตะแก้ว ประเทศกัมพูชา ปี พ.ศ. 2546

เฮง เสง, ทัช สก, วราลักษณ์ ตังคณะกุล, วิมล เพชรกาญจนาพงศ์, อุไรวรรณ โฆษิตานนท์, เฮ็ม สาริท, บุญเล็ง ฮอ, ชุลีพร จิระพงษา

ข้อมูลระบาดวิทยาและภาวะโรคเลปโตสไปโรสิสในประเทศกัมพูชา ไม่มีการบันทึกไว้อย่างชัดเจน เนื่องจากไม่มีระบบเฝ้าระวังเลปโตสไปโรสิส และข้อจำกัดทางห้องปฏิบัติการในการวินิจฉัยโรค ด้วยเหตุนี้จึงได้ทำการศึกษาแบบภาคตัดขวาง เพื่อระบุว่ามีเลปโตสไปโรสิสในประเทศกัมพูชาหรือไม่ ผู้ป่วยที่สงสัยว่าเป็นเลปโตสไปโรสิส จำนวน 121 คน เข้าร่วมการศึกษา ระหว่าง วันที่ 8 กันยายน พ.ศ. 2546 ถึง 30 พฤศจิกายน พ.ศ. 2546 อาการทางคลินิกที่พบบ่อยในผู้ป่วย ได้แก่ ไข้ ร้อยละ 96 ปวดศีรษะ ร้อยละ 92 ปวดกล้ามเนื้อ ร้อยละ 87 พฤติกรรมเสี่ยงที่สำคัญ ได้แก่ การทิ้งขยะลงบนพื้นดิน (ร้อยละ 84), ถอนกล้า (ร้อยละ 77), ใส่ปุ๋ย (ร้อยละ 49), ไถนา (ร้อยละ 47) ในพื้นนาที่เปียก ผู้ป่วย 11 ราย เป็นผู้ป่วยยืนยันจากผลการวินิจฉัยทางห้องปฏิบัติการ โดยวิธีมาตรฐาน (microscopic agglutination test) และวิธี polymerase chain reaction ใน 4 และ 7 ราย ตามลำดับ ผู้ป่วย 2 รายตรวจพบแอนติบอดีต่อซีโรวาร์ Javanica ผู้ป่วยอีก 2 รายตรวจพบแอนติบอดีต่อซีโรวาร์ Australis อุบัติการณ์รายปีของเลปโตสไปโรสิสในจังหวัดตะแก้ว เป็น 7.65 ต่อแสนประชากร ควรมีการศึกษาต่อเกี่ยวกับระบาดวิทยา และภาวะโรคเลปโตสไปโรสิส การให้ความรู้เกี่ยวกับ เลปโตสไปโรสิส และการเพิ่มความตระหนักต่อโรคมีความจำเป็นในการลดผลเสียหายจากเลปโตสไปโรสิสในประเทศกัมพูชา