Survey of Pathogenic *Leptospira* in Rats by Polymerase Chain Reaction in Sisaket Province

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Background: *Leptospirosis, a zoonotic disease caused by Leptospira, has been a health problem in Thailand for several years. It can be transmitted to humans via rats' urine, which may contaminate the environment. The prevalence rate of Leptospira infection in rats may result in the spread of leptospirosis in humans.*
Objective: *The present study aimed to determine the prevalence rate of Leptospira infection in a total of 87 rats from areas where patients with leptospirosis had been reported compared to areas with no reports of leptospirosis in Sisaket Province.*
Material and Method: *DNA samples were isolated from rats' kidneys. The polymerase chain reaction (PCR) technique was used for the detection of 16s rRNA and LipL32 genes specific to genus and pathogenic Leptospira, respectively.*
Results: *In areas where patients with leptospirosis had been reported, 8.7% (4/46) of rats were infected with pathogenic Leptospira; no infected rats were found in non-endemic areas.*
Conclusion: *This indicated the prevalence rate of Leptospira infection in rats between endemic and non-endemic areas of human leptospirosis. The prevalence rate of Leptospira infection in rats may result in the spread of leptospirosis to humans. These results may be of benefit in the prevention and/or control of the spread of leptospirosis in humans due to Leptospira-infected rats.*

Keywords: *Leptospirosis, Leptospira, 16s rRNA, LipL32, Polymerase Chain Reaction, Rats*

J Med Assoc Thai 2014; 97 (Suppl. 4): S20-S24
Full text. e-Journal: *http://www.jmatonline.com*

Leptospirosis is an infectious disease caused by *Leptospira* that affects humans and a wide variety of animal species in several countries[1,2]. In Thailand, leptospirosis is an emerging disease. Data from disease notification reports (Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand) showed a dramatic increase in the incidence rate of leptospirosis from less than 0.3 per 100,000 population in 1995 to 23.7 in 2000. The highest incidence rate of leptospirosis from 2006 to 2010 was found in North-East Thailand, especially in Buriram, Surin, and Sisaket Province[3,4].

The common reservoir hosts of *Leptospira* are several species of rodents and domestic animals, such as cattle, dogs, and pigs[5]. The carrier animals retain *Leptospira* in their kidneys and excrete the bacteria into the environment via urine. Direct contact with contaminated areas increases the risk of *Leptospira* infection. A survey of leptospirosis among rodents in Northeastern, Northern, Central, and Southern Thailand during the period October 1998 to April 2000 found that the rates of positive infection were 7.1%, 4.9%, 4.3% and 3.0%, respectively[6]. The prevalence rate of *Leptospira* infection in rats may result in the spread of leptospirosis to humans. Therefore, the objective of the present study was to determine the prevalence rate of *Leptospira* infection in rats in areas where patients with leptospirosis had been reported compared to areas with no reports in Sisaket Provine.

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Material and Method

Study areas

Bak Dong subdistrict, Kuhn Han district in Srisaket Province was chosen for the study because of the high prevalence rate of leptospirosis in the three years (2008-2010) prior reported by Srisaket Provincial Health Office, Ministry of Public Health, Thailand[7-9]. Two villages with reports of patients with leptospirosis, Lak Hin and Bak Dong Tawan Tok, and two villages with no reports, Sam Rong Mai Thai Charoen and Sam Rong Kiat Tai, were selected for the study (Fig. 1).

Trapping of rats and sample collection

Trapping of rats was completed from June 2011 to January 2012 in household areas and rice fields by

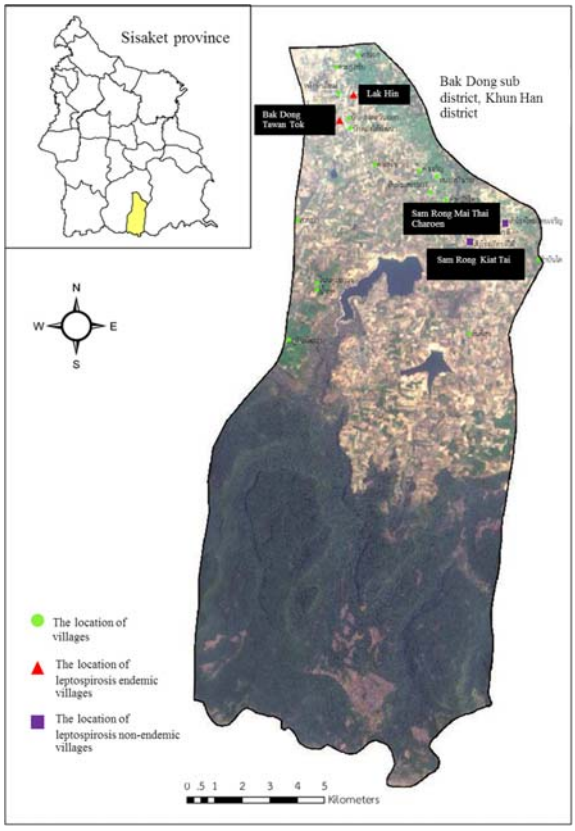

Fig. 1 Bak Dong district, Khun Han district, Sisaket Province indicating the location of endemic and non-endemic areas of leptospirosis.

the use of rat traps left out overnight. Live rats were anesthetized by the use of diethyl ether and kidney samples were collected for DNA extraction. Animal protocols were approved by Ubon Ratchathani University-Animal Care and Use Committee.

DNA extraction from rats' kidneys

Fresh rats' kidneys were washed with sterile phosphate buffer saline (PBS) pH 7.2. Whole kidneys were meshed in a petri-dish. The kidney cell debris was discarded by centrifugation at 1,000 x g for 5 min. The supernatant was removed to a new tube and then centrifuged at 10,000 x g for 5 min. The pellet was collected for DNA extraction using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction. Extracted DNA was stored at -20°C.

Detection of Leptospira DNA by PCR

The diagnosis of *Leptospira* in humans and animals was achieved by the use of *16srRNA* and *LipL32* genes[10-14]. *LipL32* gene was reported as specific for pathogenic *Leptospira*[15,16]. All samples were detected for the *16srRNA* gene specific for genus *Leptospira* using primers reported by Merien et al[10] (*16srRNA*-F: 5'-GGCGGCGCGTCTTAAACATG-3' and *16srRNA*-R: 5'-TTCCCCCCATTGAGCAAGATT-3'). In addition, *LipL32* primers specific for pathogenic *Leptospira* (*LipL32*-F: 5'-GGACGGTTTAGTCGATG GAA-3' and *LipL32*-R: 5'-GCATAATCGCCGACATT CTT-3') were designed using nucleotide sequences of *Leptospira interrogans* serovar Autumnalis (Accession number AY609324.1) from GenBank.

Preparation for PCR started with mixing an amount of 11.5 µL of each DNA sample with 10 µM forward and reverse primers, 1X PCR reaction buffer, 0.2 mM of each dNTP, 1 mM of $MgCl_2$, and 2.5 units of Taq DNA polymerase (Invitrogen, USA) to a final volume of 25 µL. DNA amplification was performed under conditions as follows: pre-amplification for one cycle at 95°C for 2 min, and PCR amplification for 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec. Control amplification templates included water as a negative control and *L. interrogans* serovar Pomona DNA as a positive control. Amplification products were analyzed by 2 % agarose gel electrophoresis.

Results

DNA isolation from rats' kidneys

DNA samples were extracted from 87 rats from the four villages in Sisaket Province, forty-six (18 from rice fields and 28 from houses) from the two villages where patients with leptospirosis had been reported and 41 (17 from rice fields and 24 from houses) from the two villages with no reports of patients with leptospirosis.

Detection of Leptospira in rats' kidneys by PCR

All DNA samples isolated from the 87 rats' kidneys were subjected to PCR to amplify *16srRNA* and *LipL32*, the target genes for *Leptospira* detection. The *16sr RNA* (331 bp) and *LipL32* (227 bp) genes specific to *Leptospira* were found only in DNA samples isolated from four rats from Lak Hin (L15) and Bak Dong Tawan Tok (B2, B4, and B5) (Fig. 2), the villages where patients with leptospirosis had been reported. The result indicated that the four rats (8.7%) from both villages were infected with pathogenic *Leptospira* and all these infected rats came from rice fields. No *Leptospira*-infected rats were found in both villages

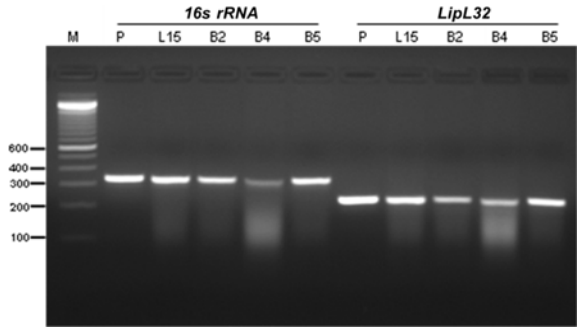

Fig. 2 The agarose gel electrophoresis analysis of *16sr RNA* (331 bp) and *LipL32* (227 bp) genes specific to *Leptospira* in DNA samples isolated from rats' kidneys. The positive bands of both genes appeared in sample number L15 (DNA sample from trapped rats in Lak Hin village), B2, B4, and B5 (DNA samples from trapped rats inWest Bak Dong village). P was a positive control (DNA sample from *Leptospira interrogans* serovar Pomona). M, 100 bp DNA ladder.

with no reports of patients with leptospirosis.

Discussion

Leptospirosis is a serious health problem in Thailand, especially in many provinces in the North-East. In Sisaket Province, incidence rates of patients with leptospirosis were 35.95, 29.4, and 24.53 per 100,000 population in 2010, 2011, and 2012, respectively[17-19]. Rats are common reservoir hosts of *Leptospira*, which are an important source of transmission to humans[5,20]. The prevalence rate of pathogenic *Leptospira* infection in rats was 8.7% (four of 46 of the rats) in Lak Hin and Bak Dong Tawan Tok, the two villages with reports of patients with leptospirosis. The study period was performed during June 2011 to January 2012, while the incidence rates of patients with leptospirosis in Sisaket Province in 2011 and 2012 were 29.4 and 24.53 per 100,000 population, respectively[18]. The prevalence rate of *Leptospira* infected rats in Thailand was previously reported in 2002 by Wangroongsarb et al, during the period October 1998 to April 2000. The prevalence rates of *Leptospira*-infected rats in Buriram, Udon Thani, and Nakhon Ratchasima Province were 12.5% (8/64), 6.7% (4/60), and 3.9% (4/102), respectively[6]. The highest incidence rates of leptospirosis in human were found in Buriram and Nakhon Ratchasima Provine (over 50 per 100,000 population) during 1999-2001[22].

These results indicated the prevalence rate of *Leptospira* infection in rats between endemic and non-endemic areas of human leptospirosis. The results also showed that all *Leptospira*-infected rats came from rice fields. The prevalence rate of these infected rats may affect the transmission rate of the disease in people who are usually in contact with rice field environments. This data supported that farmers are at risk of exposure to *Leptospira*[5,21].

Both saprophytic and pathogenic *Leptospira* can be found in natural settings[12] but only pathogenic *Leptospira* were found in this study. This was because of the presence of the positive bands of *LipL32* gene (Fig. 2), which were used to detect pathogenic *Leptospira*[15,16].

In conclusion, the prevalence rate of pathogenic *Leptospira* infection in rats was 8.7% in areas in which patients with leptospirosis had been reported. The rate of infection in rats may result in the spread of leptospirosis in humans. It is anticipated that the findings of this study may be of benefit in the prevention and/or control of the spread of leptospirosis to humans by *Leptospira*-infected rats.

Acknowledgement

This research was supported by grants from the Higher Education Commission, Thailand. The authors thank the staff of the Office of International Relations at Ubon Ratchathani University for assistance with English. The authors wish to thank Wacharapong Saengnill, a research assistant, for his support.

Potential conflicts of interest

None.

References

1. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 2003; 3: 757-71.
2. Levett PN. Leptospirosis. Clin Microbiol Rev 2001; 14: 296-326.
3. Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health. Situation of leptospirosis in Thailand. Weekly Epidemiological Surveillance Report, Thailand 2011; 42: 465-8.
4. 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.
5. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control.

Malta: WHO; 2003.
6. Wangroongsarb P, Petkanchanapong W, Yasaeng S, Imvithaya A, Naigowit P. Survey of leptospirosis among rodents in epidemic areas of Thailand. J Trop Med Parasitol 2002; 25: 55-8.
7. Sisaket Provincial Health Office. Annual epidemiological surveillance report (copy). Sisaket: Sisaket Provincial Health Office; 2008.
8. Sisaket Provincial Health Office. Annual epidemiological surveillance report (copy). Sisaket: Sisaket Provincial Health Office; 2009.
9. Sisaket Provincial Health Office. Annual epidemiological surveillance report (copy). Sisaket: Sisaket Provincial Health Office; 2010.
10. Merien F, Amouriaux P, Perolat P, Baranton G, Saint G, I. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol 1992; 30: 2219-24.
11. Bourhy P, Collet L, Clement S, Huerre M, Ave P, Giry C, et al. Isolation and characterization of new *Leptospira* genotypes from patients in Mayotte (Indian Ocean). PLoS Negl Trop Dis 2010; 4: e724.
12. Ganoza CA, Matthias MA, Collins-Richards D, Brouwer KC, Cunningham CB, Segura ER, et al. Determining risk for severe leptospirosis by molecular analysis of environmental surface waters for pathogenic *Leptospira*. PLoS Med 2006; 3: e308.
13. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, et al. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun 2000; 68: 2276-85.
14. Thaipadungpanit J, Chierakul W, Wuthiekanun V, Limmathurotsakul D, Amornchai P, Boonslip S, et al. Diagnostic accuracy of real-time PCR assays targeting *16S rRNA* and *lipL32* genes for human leptospirosis in Thailand: a case-control study. PLoS One 2011; 6: e16236.
15. Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG, Mayer LW. Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol 2005; 54: 45-9.
16. Stoddard RA, Gee JE, Wilkins PP, McCaustland K, Hoffmaster AR. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the *LipL32* gene. Diagn Microbiol Infect Dis 2009; 64: 247-55.
17. Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health. Leptospirosis. Annual epidemiological surveillance report. Nonthaburi, Thailand: Bureau of Epidemiology; 2010: 69-71.
18. Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health. Leptospirosis. Annual epidemiological surveillance report. Nonthaburi, Thailand: Bureau of Epidemiology; 2011: 61-4.
19. Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health. Leptospirosis. Annual epidemiological surveillance report. Nonthaburi, Thailand: Bureau of Epidemiology; 2012: 82-6.
20. Holt J, Davis S, Leirs H. A model of Leptospirosis infection in an African rodent to determine risk to humans: seasonal fluctuations and the impact of rodent control. Acta Trop 2006; 99: 218-25.
21. Tangkanakul W, Kingnate D. Leptospirosis epidemic in northeastern provinces of Thailand, 1997. J Health Sci 1998; 7: 386-95.
22. Tangkanakul W. Leptospirosis, Thailand. J Health Sci 2005; 14: 299-310.

การสำรวจเชื้อเลปโตสไปร่าสายพันธุ์ก่อโรคในหนูในจังหวัดศรีสะเกษโดยวิธีโพลิเมอเรสเชนรีแอกชัน

จุฑารัตน์ จิตติมณี, จารุวรรณ์ วงบุตดี

ภูมิหลัง: โรคเลปโตสไปโรสิส (leptospirosis) เป็นปัญหาสุขภาพของประเทศไทยมายาวนาน โรคนี้จัดเป็นโรคติดต่อจากสัตว์สู่มนุษย์ซึ่งมีสาเหตุมาจากการติดเชื้อเลปโตสไปร่า (Leptospira) เชื้อสามารถติดต่อไปยังมนุษย์ผ่านทางปัสสาวะของหนูซึ่งอาจจะปนเปื้อนในสิ่งแวดล้อม อัตราความชุกของการติดเชื้อเลปโตสไปร่าในหนูอาจจะส่งผลต่อการแพร่กระจายของโรคเลปโตสไปโรสิสในมนุษย์ได้

วัตถุประสงค์: เพื่อตรวจหาอัตราความชุกของเชื้อเลปโตสไปร่าในหนูทั้งหมด จำนวน 87 ตัว ที่จับได้จากบริเวณที่มีรายงานว่าพบผู้ป่วยโรคเลปโตสไปโรสิสเปรียบเทียบกับบริเวณที่มีรายงานว่าไม่พบผู้ป่วยโรคเลปโตสไปโรสิสในเขตจังหวัดศรีสะเกษ

วัสดุและวิธีการ: ตัวอย่าง DNA ซึ่งสกัดแยกออกมาจากไตของหนูถูกนำไปตรวจหาจีน 16sr RNA และ LipL32 ซึ่งเป็นจีนที่จำเพาะสำหรับเชื้อเลปโตสไปร่า โดยใช้วิธีโพลิเมอเรสเชนรีเอกชัน (PCR)

ผลการศึกษา: ในบริเวณที่มีรายงานว่าพบผู้ป่วยโรคเลปโตสไปโรสิส มีอัตราของการติดเชื้อเลปโตสไปร่าสายพันธุ์ก่อโรคในหนู ร้อยละ 8.7 (4/46) ในขณะที่ไม่พบการติดเชื้อในหนูในบริเวณที่ไม่มีการระบาดโรคเลปโตสไปโรสิส

สรุป: ผลการวิจัยแสดงให้เห็นอัตราความชุกของการติดเชื้อเลปโตสไปร่าในหนูระหว่างบริเวณที่มีการระบาด และบริเวณที่ไม่มีการระบาดของโรคเลปโตสไปโรสิสในมนุษย์ อัตราความชุกของการติดเชื้อเลปโตสไปร่าในหนูอาจจะส่งผลต่อการแพร่กระจายของโรคเลปโตสไปโรสิสในมนุษย์ ผลการวิจัยนี้น่าจะเป็นประโยชน์ในการป้องกันและควบคุมการแพร่กระจายของโรคเลปโตสไปโรสิสในมนุษย์ซึ่งมีสาเหตุอันเนื่องมาจากการติดเชื้อเลปโตสไปร่าในหนู