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Molecular detection and isolation of pathogenic *Leptospira* from asymptomatic humans, domestic animals and water sources in Nan province, a rural area of Thailand

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ABSTRACT

Leptospirosis is an important zoonotic disease that is often associated with animal carriers and contamination of the environment via infected urine. This study aimed to assess pathogenic leptospiral carriage in Nan province, a rural area of Thailand where leptospirosis is endemic. Samples from 20 villages were obtained during the period 2013 to 2016, comprising urine samples collected from asymptomatic people (n = 37) and domestic animals (n = 342), and environmental water samples (n = 14). *Leptospira* were cultured in Ellinghauson McCullough Johnson and Harris (EMJH) media. An *rrs* nested PCR identified 9.92% (95% confidence interval (CI) 6.96–12.88) of the urine and water samples as being positive for *Leptospira* spp., and phylogenetic analysis was conducted on the 443 bp amplicons. *Leptospira weilii*, which has not previously been identified in Thailand, was recovered from 13 cattle, 9 pigs, 2 dogs, 2 water samples and 1 goat. *L. interrogans* was found in 4 dogs, 3 pigs, 3 cattle, 1 human and 1 water sample. Four leptospiral strains were isolated and multilocus sequence typing (MLST) analysis was performed on these. Three novel sequence types were identified, including two singletons of *L. interrogans* in ST26 and ST33, and one of *L. weilii* in ST94, with this having a close relationship to previous isolates from cases of human leptospirosis in Laos and China. Our results revealed that pathogenic *Leptospira* occur commonly in asymptomatic domestic animals, humans and environmental water samples in Nan Province, and emphasize the high potential for zoonotic transmission in the province.

1. Introduction

Leptospirosis is an important zoonosis caused by infection with pathogenic *Leptospira* species. The disease occurs worldwide, but is especially common in warm and moist tropical regions where survival of the spirochetes in the environment is favored (Evangelista and Coburn, 2010). The spirochetes are carried in the convoluted tubules of the kidney and contaminate the environment via urine. Rodents are important reservoirs of the bacteria (Adler and de la Pena Moctezuma, 2010), but infected domestic animals including cattle, pigs and dogs also can be source of disease transmission (Sykes et al., 2011). Environmental contamination by leptospires increases the prevalence of the disease in endemic areas as a result of subsequent ingestion and leptospiral penetration via open wounds (Evangelista and Coburn, 2010; Faine et al., 1999).

In Thailand, the Bureau of Epidemiology reported leptospirosis in humans in 2015 at a rate of 3.47 per 100,000 population with the occurrence mainly being in rural areas including Udon Thani, Rayong, Maha Sarakham, Lumpang and Nan provinces (Della Rossa et al., 2015; Wuthiekanun et al., 2007). In addition, many large-scale serological surveys of leptospirosis have showed positive results in animals including pigs (10%), dogs (10%), rodents (5.6%) and the livestock population (11.5%) (Kositanont et al., 2003; Lilenbaum and Martins, 2014; Meeyam et al., 2006; Niwetpathomwat et al., 2006; Suwancharoen et al., 2013). Even though the microscopic agglutination test (MAT) was used disease screening as recommended by the Office International des Epizooties (OIE) (OIE, 2014), the MAT lacks reliability for detecting individual carriers or chronically infected animals (Lilenbaum and Martins, 2014). On the other hand, molecular techniques such as polymerase chain reaction (PCR) and DNA sequencing are helpful for detection and for epidemiological studies (Balamurugan et al., 2013; Boonsilp et al., 2011; Cosson et al., 2014; La Scola et al., 2006). In addition, multilocus sequence typing (MLST) analysis has been used to explore the clonal linkage of *Leptospira* spp. isolates from

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humans and rodents in Thailand (Thaipadungpanit et al., 2007).

Although Nan province is an endemic area for leptospirosis (Cosson et al., 2014; Della Rossa et al., 2015), it should not be assumed that rodents are the main reservoir as recent studies have shown that this is not always the case (Della Rossa et al., 2015). Consequently this study aimed to investigate the occurrence of pathogenic *Leptospira* spp. in the urine of asymptomatic domestic animals and environmental water samples in Nan province, to give an insight into the extent to which these could be sources of human infection. All positive samples were identified to species level and isolates were examined using molecular typing methods to compare them to those in the databases.

2. Materials and methods

2.1. Ethics statement

All animal and human sampling was approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC; Protocol NO 1531075), Chulalongkorn University and the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University (Protocol NO.2556/294).

2.2. Study site and sample collection

Nan province is a rural region located in the North East of Thailand, bordering Laos, and has an area of approximately 11,472.07 km^2 containing hills, forests and cultivated land. The climate ranges from warm to cold and there is a high rainfall during May to September. The human population is 483,641 and mainly is employed in agriculture and animal husbandry. The landscape, climate and abundant presence of animal hosts may facilitate circulation of leptospirosis in this area. According to the 2015 report of the Bureau of Epidemiology, the incidence of human leptospirosis in Nan province was 6.73 per 100,000 population, and rodents were regarded as being a maintenance host of the disease (Della Rossa et al., 2015). Hence, in this study, we restricted investigation of leptospirosis to potential domestic animal hosts including cattle, pigs and dogs, and focused on the linkage with humans and the local environment.

There were three inclusion criteria for selecting the study sites. The first was the occurrence of cases of human leptospirosis (index patients) in the previous 1–2 months, with diagnosis based on medical history, physical examination and routine diagnostic testing during screened by physicians from regional hospitals, as previously described (Myint et al., 2007). The second was the site having a history of cases of human leptospirosis during 2013 to 2016, as recorded by the Bureau of Epidemiology, Thailand. The third criterion was that local people should be employed in agriculture and animal husbandry. Those areas where it was not practical to access samples were excluded from this study. Twenty villages from the three districts of Muang Nan, Tha Wang Pha and Chiang Klang were chosen for study (Fig. 1).

Urine samples of at least 15 mL were collected from animals without a history of vaccination and/or clinical signs of leptospirosis such as jaundice and/or reproductive failure, and were derived from 131 cattle, 152 pigs, 58 dogs, and one goat. A total of 37 human samples were obtained from individuals in 7 villages (in 3 districts) where there had either been index patients or a history of death associated with leptospirosis (Supplementary Table S1). Urine was obtained from index patients, or from individuals in the neighborhood where fatal cases of leptospirosis had occurred. For the latter, there was no other evidence of them being infected. The samples from cattle and pigs were collected in 18 of 20 villages that had traditional housing and where the animals were present around the households. The sampled animals were located in different epidemiological units and, in some cases, from pigs or cattle in the same household. In addition, at least 50 mL of 14 underground water sources used in animal husbandry for drinking or cleaning pens, and/or rice field water samples were collected for analysis.

All asymptomatic animals and humans were assessed for their clinical appearance, and an oral history was taken for the humans, including any previous history of fever, headache, muscle pain, vomiting or jaundice (Koizumi et al., 2013; Wuthiekanun et al., 2007). Voided urine samples were collected from humans, cattle, pigs and a goat, whilst in dogs urine was collected by catheterization. All urine and water samples were stored in 50 mL plug-seal capped containers (Corning Incorporated, Mexico) and kept at 4 °C before further laboratory processing within three hours.

2.3. Leptospira culture and isolation

Approximately 500 µL of each urine and environmental water sample was inoculated into semisolid modified Ellinghausen McCullough Johnson and Harris (EMJH) medium containing 5-fluorouracil, rifampicin and neomycin (Adler et al., 1986; Faine et al., 1999). The remaining urine and water samples were centrifuged at 3500*g* for 15 min. Approximately 1 mL of the pellet was subjected to 10-fold serial dilution in modified liquid EMJH medium (WHO, 2003). The inoculated EMJH media were incubated at 28 to 30 °C for 28 days, and observed for *Leptospira*-like microorganisms under a dark field microscope once a week. Positive samples were passed through a 0.2-µM filter (Corning Incorporated, Germany) and 100 µL was spread on a Leptospira Vanaporn Wuthiekanun (LVW) agar plate for isolation, as previously described (Wuthiekanun et al., 2013).

2.4. Leptospira detection by rrs nested PCR

Pellets obtained by centrifuging 2 mL of urine or environmental water samples were used for DNA extraction with the Nucleospin® extraction kit (Macherey-Nagel, Germany) following the manufacturer's instructions, and stored at − 20 °C before use. To detect pathogenic and intermediate pathogenic *Leptospira* spp., a single-tube nested PCR was performed to amplify 547 bp of the ribosomal 16S RNA gene (*rrs*) using two sets of primer comprising rrs-outer-F (5′-CTCAGAACTAACGCTGGCGGCGCG-3′), rrs-outer-R (5′-GGTTCGTTACTGAGGG TTAAAACCCCC-3′), rrs-inner-F (5′-CTGGCGGCGCGTCTTA-3′), and rrs-inner-R (5′-GTTTTCACACCTGACTTAC-A-3′) in a total volume of 25 µL PCR reaction, as previously described (Boonsilp et al., 2011). The *rrs* amplicons were purified by Nucleospin®Gel and PCR clean up (Macherey-Nagel, Germany) and submitted to a commercial service provider for DNA sequencing (1st BASE Pte Ltd., Singapore).

2.5. Multilocus sequence typing (MLST)

DNA was extracted from purified leptospiral isolates using the Nucleospin® extraction kit (Macherey-Nagel, Germany), and MLST was performed with seven housekeeping genes comprising *glmU*, *pntA*, *sucA*, *tpiA*, *pfkB*, *mreA* and *caiB* (Boonsilp et al., 2013). The allelic profile of each gene and novel sequence types (ST26, ST33 and ST94) was submitted to the *Leptospira* MLST database (https://pubmlst.org/leptospira/).

2.6. Phylogenetic analysis and clonality analysis of Leptospira isolates

Thirty-nine *rrs* nucleotide sequences were obtained and submitted to the GenBank database (accession numbers: KU854349-KU854387). The 547 bp nucleotide sequences were trimmed to 443 bp and this region was aligned and used to define species by comparison with 14 pathogenic and intermediate *Leptospira* spp. *rrs* genes obtained from the GenBank database, as previously described (Boonsilp et al., 2011). A maximum likelihood tree was constructed using PhyML version 3.0.1 based on a generalized time-reversible (GTR) model and optimized parameters of gamma distribution and proportion of invariant sites, as previously described (Guindon et al., 2010). A BioNJ tree was constructed using the Nearest Neighbor Interchange (NNI) method