Short Communication

Potentially Pathogenic *Leptospira* Species Isolated from a Waterfall in Thailand

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SUMMARY: We collected water and soil samples from a waterfall in Thailand to investigate the presence of potentially pathogenic *Leptospira*. Isolation of *Leptospira* from all the 17 environmental samples was successful. Based on 16S rRNA gene sequence analysis, a diverse group of *Leptospira* species was recovered from waterfall samples including 2 pathogenic species (*Leptospira alstonii* [5/17, 29%] and *Leptospira kmetyi* [1/17, 6%]); 1 intermediate species (*Leptospira wolffii* [9/17, 53%]); and 2 non-pathogenic species (*Leptospira meyeri* [1/17, 6%] and *Leptospira idonii* [1/17, 6%]). The high prevalence of pathogenic and intermediate *Leptospira* indicates that a waterfall may serve as a natural reservoir of possible pathogens of leptospirosis.

Leptospirosis is an important zoonotic disease that affects humans and other animals worldwide. The disease is caused by spirochete bacteria of the genus *Leptospira*. Infection can occur by direct contact with urine of infected animals or via indirect contact with a contaminated environment. Lately, leptospirosis transmission via recreational activities has been increasingly recognized, particularly via water-based activities (1,2). In many countries including Thailand, a waterfall is a popular place for tourism and outdoor recreational activities. There is evidence of human leptospirosis that is related to contact with water bodies of waterfalls, e.g., several leptospirosis outbreaks in Malaysia (1). Similarly, 2 leptospirosis cases associated with swimming in a waterfall have been reported (French travelers who returned from Koh Samui, Thailand) (3). No causative *Leptospira* spp. could be confirmed in those reported cases. Because a waterfall seems to be a possible source of leptospirosis, in our study, we attempted to investigate the presence of potentially pathogenic *Leptospira* in environmental samples obtained from a waterfall in Thailand.

The study was carried out in November 2016 in the western part of Thailand where a waterfall we chose as a sampling site is located. A total of 17 environmental samples including 13 water samples and 4 soil samples were collected along the route from the top to the base of the waterfall (Fig. 1). Sampling was performed in the water stagnation zone at a specific sampling point. The geographical position of each sampling point was recorded. Temperature and pH of water were averaged from 3 measurements at each sampling point. The

samples were transported under ambient condition to the laboratory.

Approximately 30 mL of each water sample was passed through a 0.2- μ m filter, and 0.5 mL of the filtrate was inoculated, in duplicate, into 2 mL of the liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium containing 100 μ g/mL 5-fluorouracil (5-FU). The rest of the filtrate was centrifuged, and the pellet was inoculated into 2 mL of the semisolid EMJH medium containing 0.2% of Noble agar base and 100 μ g/mL 5-FU. As for soil, approximately 50 g of each soil sample was added into 100 mL phosphate buffered saline to create a soil suspension and was mixed by manual shaking before incubation for 30 min to ensure sedimentation at room temperature. Water at the top was passed through 0.2- μ m filter, and the filtrate was inoculated into the liquid and semisolid EMJH medium as mentioned above. The cultures were maintained in 28°C, and the possible presence of leptospires was examined under a dark-field microscope weekly. One milliliter of a stationary-phase liquid culture was centrifuged at 20,000 $\times$ g for 10 min, and DNA was extracted from the pellet using the Genomic DNA Mini Kit (blood and cultured cells) (Geneaid, New Taipei City, Taiwan). To detect the pathogenic or intermediate group of leptospires, amplification of the partial 16S rRNA gene from the extracted DNA was performed using outer primers of a published nested PCR assay (4). A DNA sample with the absence of a PCR product was then confirmed by *Leptospira* genus-specific PCR involving another set of 16S rRNA gene primers (5) to detect non-pathogenic groups of leptospires. Amplified DNA products were purified from an agarose gel using the GenepHlow Gel/PCR Kit (Geneaid). DNA sequencing of purified DNA products was carried out at AITbiotech (West Coast, Singapore), and trimmed nucleotide sequences were deposited in the GenBank database. Nucleotide sequence analysis was conducted using National Center for Biotechnology Information (NCBI) BLAST, and a phylogenetic tree of 16S rRNA gene sequences was constructed by the neighbor-joining method in MEGA ver. 7.0.

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The environmental samples were collected along the waterfall route within an approximate distance of 1.5 km as shown in Fig. 1. During the sampling period, the average temperature of waterfall water ranged from 24.3°C to 25.1°C, and the average pH ranged from 7.49 to 8.63. *Leptospira* isolation from all 17 environmental samples was successful with 70% (12/17) and 100% (17/17) culture yield in the liquid and semisolid EMJH medium, respectively. The moderately warm temperature and relatively high pH of the waterfall water in this study were within the range that has been previously reported to promote leptospiral viability (6). This result implied that physical factors such as temperature and pH of waterfall water should support the survival of leptospires although information on those parameters in soil samples was not available in this study. The 16S rRNA gene PCR analyses of 17 *Leptospira*-positive cultures showed that 15 (88%) cultures contained intermediate or pathogenic *Leptospira*, and the remaining 2 (12%) cultures contained non-pathogenic *Leptospira*. The phylogenetic tree was constructed based on partial 16S rRNA gene nucleotide sequences of our 17 *Leptospira* isolates (GenBank accession Nos. MF380456–MF380472) (Fig. 2).

Our isolates recovered from waterfall environmental samples were categorized into all 3 clades of *Leptospira* spp. Six water isolates ended up in the pathogenic leptospiral clade. Of these, 5 isolates (5/17, 29%) were found to be closely related to *L. alstonii* with 99% to 100% identity, whereas 1 isolate (1/17, 6%) is closely related to *L. kmetyi*, with 99% identity. The presence of *L. alstonii* and *L. kmetyi* in the environment has been reported in many countries (7–10). Recently, *L. alstonii* was isolated from greater white toothed-shrews in Ireland (11) and from an asymptomatic cow in a slaughter house in Brazil (12). On the other hand, *L. kmetyi* was identified in a blood sample of a patient with suspected leptospirosis who participated in canyoning activities on the Caribbean island of Martinique (2). Although there is evidence of human and animal infection by either *L. alstonii* or *L. kmetyi*, pathogenicity of both species still needs to be elucidated because the previous hamster experiments have revealed that no hamsters show any signs of leptospirosis or die after inoculation with either *L. alstonii* or *L. kmetyi* isolates (9–11).

The majority of waterfall isolates (9/17, 53%) including 6 water isolates and 3 soil isolates, clustered within the intermediate leptospiral clade, and those isolates manifested 98% to 99% identity with reported sequences of *L. wolffii*. The intermediate *L. wolffii* was originally isolated from the urine of a male patient with suspected leptospirosis from Nakornrachasima province, Thailand (13). Some studies have shown that *L. wolffii* can be detected in clinical samples from human patients with suspected leptospirosis and various kinds of animals in Iran (14) and India (15). The circulation of *L. wolffii* among different host species is suggestive of its role in the transmission cycle and its potential to cause the disease in human and animal hosts: possibly either symptomatic or asymptomatic diseases. In addition, the remaining of 2 waterfall isolates (2/17, 12%) were classified into the non-pathogenic leptospiral clade and had 99% identity to *L. meyeri* and *L. idonii*.

The diversity of *Leptospira* spp. found in our study may indicate that waterfall affords physical factors such

Fig. 1. Map of the waterfall route shows sampling points and their waterfall *Leptospira* isolates.

Fig. 2. Phylogenetic tree analysis of partial 16S rRNA gene sequences of *Leptospira* isolated from waterfall samples. Reference species and their GenBank accession Nos. are included.

as temperature and pH that are suitable for survival of diverse species of *Leptospira*. Moreover, our study revealed that waterfall samples have high prevalence of pathogenic and intermediate *Leptospira*, which may be responsible for leptospirosis. Our findings are expected to raise public awareness and address the important function of waterfalls as a natural reservoir of potential pathogens of leptospirosis.

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Conflict of interest None to declare.

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