Short Communication

A Novel Genotype of *Leptospira interrogans* Recovered from Leptospirosis Outbreak Samples from Southern Thailand

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SUMMARY: We performed *Leptospira* culture analysis of 76 clinical samples collected from animals and of six soil samples for the investigation of a leptospirosis outbreak in southern Thailand in 2017. Leptospire were recovered from a kidney sample (a fatal canine leptospirosis case) and from all the soil samples. Next, 16S rRNA sequence analysis demonstrated that the clinical isolate was closely related to the pathogenic *L. interrogans*, whereas the soil isolates represented different species, including pathogenic *L. ellisii*, intermediate *L. wolffii*, and nonpathogenic *L. yanagawae*. Multilocus sequence typing identified an isolate of *L. interrogans* as a novel sequence type (ST263), suggesting that the causative agent of the canine leptospirosis in the southern Thailand outbreak has a unique genetic profile.

Leptospirosis is an important zoonotic disease caused by spirochete bacteria from the genus *Leptospira* (1). The disease is globally distributed but is more prevalent in tropical and subtropical areas, where temperature and humidity are favorable for *Leptospira* survival and persistence in the environment (2,3). Several outbreaks of leptospirosis have occurred after an extensive heavy rainfall and flooding (4–6), including a recent outbreak in Krabi province, southern Thailand, in January 2017. Sixty-two cases of human leptospirosis and two cases of canine leptospirosis were detected in samples from this outbreak. Of those, three fatal cases, including two humans and one canine, were confirmed in the third week after the flooding had receded (7). An outbreak investigation was conducted by the Joint Investigation Team of the Bureau of Epidemiology, the Department of Disease Control, the Ministry of Public Health, Thailand, to identify the causative agent of human and canine leptospirosis (7). Within the same week after the fatal cases of human leptospirosis were confirmed, the potential sources of infectious *Leptospira* were investigated in animals and soil from the outbreak areas, particularly in the areas of the leptospirosis patients' houses. The suspected animal reservoirs exhibiting either noticeable or unnoticeable clinical symptoms were subjected to sample collection. All procedures of animal collection and handling followed the Ethical Principles and Guidelines for the Use of Animals (8). In case of rodent processing, isoflurane was chosen

as the anesthetic agent. The animal and soil samples collected for the outbreak investigation were subjected to laboratory diagnostic analysis at the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, the Faculty of Veterinary Science, Mahidol University.

Detection of *Leptospira* was carried out by the culture method. Blood, urine, and tissue samples, including heart, kidney, liver, and pancreas samples, obtained from the animals were used for culturing leptospire. The clinical samples were processed immediately after collection. As for blood and urine samples, two drops of fresh individual samples were inoculated into 1 mL of the liquid Ellinghausen McCullough Johnson Harris (EMJH) medium containing 100 µg/mL 5-fluorouracil. As for tissue samples, an approximately 1 mm thick transversal slice was dissected from each sample and crushed before inoculation into the same type of culture medium except that the kidney samples of the dead canine related to the leptospirosis outbreak were inoculated into 2 mL of the semisolid EMJH medium containing 0.2% of Noble agar base and 100 µg/mL 5-fluorouracil. Conversely, the soil samples were transported under ambient conditions to the laboratory for sample processing. The procedure for isolation of *Leptospira* spp. from soil samples has been described in our previous study (9). Cultures were maintained at 28°C, and the presence of leptospire was examined under a dark-field microscope weekly. A culture without detectable leptospire after four months of incubation was considered *Leptospira*-negative.

The culturable *Leptospira* obtained from either animal or soil samples was next characterized using molecular methods. Genomic DNA of culturable leptospire was prepared from 1 mL of a confluent liquid culture by centrifugation at 20,000 × *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

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Table 1. Summary of clinical samples collected from animals during the leptospirosis outbreak investigation in southern Thailand

Animal species	No. of individuals	Type of clinical samples						Total
		Blood	Urine	Heart	Kidney	Liver	Pancreas	
Dog	4 ¹⁾	3	0	0	1 ²⁾	0	0	4
Cat	2	2	0	0	0	0	0	2
Rat	14	9	1	13	14	8	7	52
Cattle	8	7	1	0	0	0	0	8
Pig	10	7	3	0	0	0	0	10
Total	38	28	5	13	15	8	7	76

¹⁾: Two of four dogs were leptospirosis cases associated with the outbreak. Both cases exhibited clinical symptoms and were laboratory confirmed with *Leptospira* infection, which one of them died.

²⁾: The kidney sample was derived from the fatal canine leptospirosis case.

Fig. 1. Phylogenetic tree analysis of partial 16S rRNA sequences of *Leptospira* isolates from leptospirosis outbreak samples from southern Thailand. Reference species and their GenBank accession numbers are included.

group of leptospires, amplification of a part of the 16S rRNA gene from the extracted DNA was performed according to a published nested polymerase chain reaction (PCR) assay (10). A DNA sample showing no PCR product was subsequently confirmed by *Leptospira* genus-specific PCR using another set of 16S rRNA primers (11) to amplify the nonpathogenic group of leptospires. PCR products were purified from an agarose gel using the GenepHlow™ Gel/PCR Kit (Geneaid). DNA sequencing of the purified PCR products was carried out at BIONEER (Daejeon, South Korea), and trimmed nucleotide sequences were submitted to the GenBank database. Nucleotide sequence analysis was performed using the National Center for Biotechnology Information (NCBI) BLAST, and a phylogenetic tree of the 16S rRNA sequences was constructed using the maximum likelihood method in MEGA, version 7.0.

Genotyping of the pathogenic *Leptospira* isolates was carried out by multilocus sequence typing (MLST) according to a published method (12). Briefly, seven housekeeping genes—*glmU*, *pntA*, *pfkB*, *sucA*, *tpiA*, *caiB*, and *mreA*—were amplified from the extracted DNA of a pathogenic *Leptospira* isolate, and the gel-purified PCR products were sequenced. The trimmed nucleotide sequences of each gene were used for searching the *Leptospira* MLST database (<https://pubmlst.org/leptospira/>) to identify the allele numbers. Allelic profiles in the order *glmU*-*pntA*-*sucA*-*tpiA*-*pfkB*-*mreA*-*caiB* were employed to assign a sequence type (ST) to each isolate, where novel combinations of existing alleles were assigned to a novel ST. The relationship among the *Leptospira* spp. was analyzed using the eBURST algorithm (<http://eburst.mlst.net/>).

A total of 76 clinical samples derived from two symptomatic dogs and 36 asymptomatic animals,

including two dogs, two cats, 14 rats, eight cattle, and 10 pigs (Table 1) as well as six soil samples were subjected to *Leptospira* isolation. The culture isolation was successful for one out of the 76 (1.3%) clinical samples and for six out of the six (100%) soil samples. Only one clinical isolate could be recovered from the kidney of the dead canine with leptospirosis. The 16S rRNA PCR analyses of the seven *Leptospira*-positive cultures showed that five cultures from one clinical sample and four soil samples contained pathogenic or intermediate *Leptospira*, and the remaining two cultures from soil samples contained nonpathogenic *Leptospira*. A phylogenetic tree was constructed based on partial 16S rRNA nucleotide sequences of our seven *Leptospira* isolates (GenBank accession numbers MK370582–MK370588; Fig.1).

The clinical isolate (MW159) was classified into a pathogenic leptospiral clade with the highest similarity to *L. interrogans*: a major cause of human and animal leptospirosis (1, 10). One of the soil isolates (MW162) was present in the branch close to a newly described pathogenic species: *L. ellisii*. Infection with this novel species in a hamster model does not induce signs or symptoms of leptospirosis (13). The other three soil isolates (MW163, MW166, and MW286) clustered within the intermediate leptospiral clade in the branch associated with *L. wolffii*, which has previously been found in the environment in the western part of Thailand (9). This species can contribute to either symptomatic or asymptomatic disease in humans and animals (14,15). In addition, the remaining two soil isolates (MW165 and MW167) ended up in a branch of nonpathogenic *L. yanagawae*. MLST-based genotyping of the isolate of *L. interrogans* generated a new allelic profile of the seven housekeeping genes (*glmU*-*pntA*-*sucA*-*tpiA*-*pfkB*-*mreA*-

Fig. 2. eBURST analysis of the isolate of *L. interrogans* recovered from a leptospirosis outbreak in southern Thailand. The novel ST of the isolate is indicated by a black circle.

caiB: 1-1-1-2-7-5-6), hence the new ST was accordingly designated as ST263. In eBURST analysis of all the 275 STs listed in the *Leptospira* PubMLST database (as of January 2019), ST263 manifested itself as a singleton in the eBURST diagram (Fig.2), indicating that this strain had no relationship with the other *Leptospira* spp.

The evidence of a unique genotype of *L. interrogans* recovered from the outbreak samples indicated the presence of this bacterial strain in this specific area and the ability to cause disease in the infected animals, whereas the evidence of human infection was not found in this study. Although our investigation of *Leptospira* in the relevant environment was limited by the small sample size, the presence of the potentially pathogenic *Leptospira* in the majority of the soil samples collected in the outbreak area may imply the high risk of leptospirosis exposure. The information provided by our study will be useful for the disease control and will facilitate future epidemiological studies.

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Conflict of interest None to declare.

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