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A novel genotype of *Leptospira interrogans* recovered from leptospirosis outbreak in southern Thailand

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**Key words:** *Leptospira*, genotype, leptospirosis, outbreak

**Running head:** Novel *Leptospira* genotype from Thailand outbreak
Summary

We performed *Leptospira* culture of 76 clinical samples collected from animals and 6 soil samples for the investigation of a leptospirosis outbreak in southern Thailand, 2017. Recovery of leptospires was found in a kidney sample of a fatal canine leptospirosis case and all of the soil samples. 16S rRNA sequence analysis demonstrated that the clinical isolate was closely related to the pathogenic *L. interrogans* while the soil isolates were related to different species including the pathogenic *L. ellisii*, the intermediate *L. wolffii* and the non-pathogenic *L. yanagawae*. Multilocus sequence typing (MLST) identified a novel sequence type (ST) of the isolate of *L. interrogans* in ST263, suggesting the causative agent of canine leptospirosis in the southern Thailand outbreak has a unique genetic profile.

Text

Leptospirosis is an important zoonotic disease caused by spirochete bacteria in the genus of *Leptospira* (1). The disease is globally distributed but it is more prevalent in tropical and subtropical areas where temperature and humidity are favorable for *Leptospira* survival and persistent in the environment (2, 3). Several outbreaks of leptospirosis had occurred after an extensive heavy rainfall and flooding (4, 5, 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 reported from this outbreak. Of those, three fatal cases including two humans and one canine were confirmed in the third week after the flooding had finished (7). Outbreak investigation by Joint Investigation Team of Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand was conducted to identify the causative agent of human and canine leptospirosis (7). Within the same week after the dead cases of human leptospirosis were confirmed, the potential sources of infectious *Leptospira* were investigated in animals and soil presented in the outbreak areas particularly in the areas of the
leptospirosis patients’ houses. The suspected animal reservoirs exhibiting either noticeable clinical symptoms 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 to use as the anesthetic agent. Animal and soil samples collected from the outbreak investigation were submitted for laboratory diagnosis at The Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals (MoZWE), Faculty of Veterinary Science, Mahidol University.

Detection of *Leptospira* was carried out by culture method. Blood, urine and tissue samples including heart, kidney, liver and pancreas obtained from animals were used for culturing leptospires. The clinical samples were processed immediately after collection. For blood and urine samples, two drops of fresh individual samples were inoculated into a 1 mL of the liquid Ellinghausen McCullough Johnson Harris (EMJH) medium containing 100 µg/mL of 5-fluorouracil (5-FU). 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 kidneys of the fatal canine related to the leptospirosis outbreak were inoculated into a 2 mL of the semi-solid EMJH medium containing 0.2% of Noble agar base and 100 µg/ml of 5-FU. On the other hand, the soil samples were transported under an ambient condition to the laboratory for sample processing. Isolation procedure of *Leptospira* spp. from soil samples was described in our previous study (9). Cultures were maintained in 28°C and the presence of leptospires was observed under dark field microscope weekly. The culture without detectable leptospires after 4 months of incubation was considered to be negative.

The culturable *Leptospira* obtained from either animal or soil samples was further characterized using molecular-based methods. Genomic DNA of culturable leptospires was prepared from one milliliter of a confluent liquid culture by the centrifugation at 20,000 x g for
10 minutes and the pellet was extracted for DNA using Genomic DNA Mini Kit (blood and cultured cell) (Geneaid, New Taipei City, Taiwan). To detect the pathogenic or intermediate group of leptospires, amplification of partial 16S rRNA gene from the extracted DNA was performed according to a published nested PCR assay (10). A DNA sample with an absence of PCR product was subsequently confirmed by *Leptospira* genus-specific PCR using another set of 16S rRNA primers (11) to amplify the non-pathogenic group of leptospires. PCR products were purified from agarose gel using GenepHlow™ Gel/PCR Kit (Geneaid). DNA sequencing of 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 16S rRNA sequences was constructed using the maximum likelihood method by MEGA version 7.0.

Genotyping of the pathogenic *Leptospira* isolates was carried out by MLST according to the published method (12). Briefly, seven housekeeping genes consisting of *glmU*, *pntA*, *pfkB*, *sucA*, *tpiA*, *caiB*, *mreA*, were amplified from the extracted DNA of the pathogenic *Leptospira* isolate and the gel-purified PCR products were DNA sequenced. The trimmed nucleotide sequences of each gene were submitted into 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 the sequence type (ST) to the isolate, which the novel combinations of existing alleles was assigned a novel ST. The relationship between the *Leptospira* spp. was analyzed using eBURST algorithm (http://eburst.mlst.net/).

A total of 76 clinical samples derived from 2 symptomatic dogs and 36 asymptomatic animals; including 2 dogs, 2 cats, 14 rats, 8 cattle and 10 pigs (Table 1), and 6 soil samples were submitted for *Leptospira* isolation. The culture isolation was successful from 1 out of 76
(1.3%) clinical samples and 6 out of 6 (100%) soil samples. The only one clinical isolate could be recovered from the kidney of the fatal canine leptospirosis case. The 16S rRNA PCRs of the 7 positive cultures showed that 5 cultures from 1 clinical sample and 4 soil samples contained pathogenic or intermediate *Leptospira* and the remaining 2 cultures from soil samples contained non-pathogenic *Leptospira*. The phylogenetic tree was constructed based on partial 16S rRNA nucleotide sequences of our 7 *Leptospira* isolates (GenBank accession number MK370582-MK370588) (Fig. 1).

The clinical isolate (MW159) was classified into the pathogenic leptospiral clade with the highest similarity to *L. interrogans*, the major cause of human and animal leptospirosis (1, 10). One of the soil isolates (MW162) presented on the branch close to the newly described pathogenic species; *L. ellisii*. Infection of this novel species in the hamster model did not induce signs or symptoms of leptospirosis (13). The other three soil isolates (MW163, MW166 and MW286) were clustered within the intermediate leptospiral clade on the branch related to *L. wolffii* which was previously found in the environment of the western part of Thailand (9). This species could contribute to either symptomatic or asymptomatic disease in humans and animals (14, 15). In addition, the remaining of two soil isolates (MW165 and MW167) resided on a branch of the non-pathogenic *L. yanagawae*. MLST-based genotyping of the isolate of *L. interrogans* generated a new allelic profile of the seven housekeeping genes (*glmU*-*pntA*-*pfkB*-*sucA*-*tpiA*-*caiB*-*mreA*: 1-1-1-2-7-5-6), hence the new ST was accordingly assigned as ST263. Based on eBURST analysis of the entire 275 sequence types listed on the *Leptospira* PubMLST database (January 2019), the ST263 was displayed as a singleton on the eBURST diagram (Fig. 2) indicating that the strain had no relationship with the other *Leptospira* spp.

The evidence of a unique genotype of *L. interrogans* recovered from the outbreak indicated the existence of this bacterial strain in this particular area and its ability to cause
disease in the infected animals while the evidence of human infection was not available in this study. Although the 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 could imply the high risk of leptospirosis exposure. The information provided by our study will be useful for the disease control and enhance the future epidemiological studies.

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**Conflict of interest**

None to declare.

**References**


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

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. of individuals</th>
<th>Type of clinical samples</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blood</td>
<td>Urine</td>
</tr>
<tr>
<td>Dog</td>
<td>4 ¹)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Cat</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Rat</td>
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<td>9</td>
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</tr>
<tr>
<td>Cattle</td>
<td>8</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Pig</td>
<td>10</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>28</td>
<td>5</td>
</tr>
</tbody>
</table>

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 a leptospirosis outbreak in southern Thailand. Reference species and their GenBank accession numbers are included.
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.