Short Report: *Leptospira* Species in Floodwater during the 2011 Floods in the Bangkok Metropolitan Region, Thailand

Janjira Thaipadungpanit,* Vanaporn Wuthiekanun, Narisara Chantratita, Surapon Yimsamran, Premjit Amornchai,
Siriphan Boonsilp, Wanchai Maneeboonyang, Prapin Tharnpoophasiam, Natnaree Saiprom, Yuvadee Mahakunkijcharoen,
Nicholas P. J. Day, Pratap Singhasivanon, Sharon J. Peacock, and Direk Limmathurotsakul

*Mahidol-Oxford Tropical Medicine Research Unit and Departments of Microbiology and Immunology, Tropical Hygiene, and Social
and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; Centre for Clinical Vaccinology
and Tropical Medicine, Nuffield Department of Clinical Medicine, University of Oxford, Churchill Hospital, Oxford, United Kingdom;
Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom*

Abstract. Floodwater samples ($N = 110$) collected during the 2011 Bangkok floods were tested for *Leptospira* using culture and polymerase chain reaction (PCR); 65 samples were PCR-positive for putatively non-pathogenic *Leptospira* species, 1 sample contained a putatively pathogenic *Leptospira*, and 6 samples contained *Leptospira* clustering phylogenetically with the intermediate group. The low prevalence of pathogenic and intermediate *Leptospira* in floodwater was consistent with the low number of human leptospirosis cases reported to the Bureau of Epidemiology in Thailand. This study provides baseline information on environmental *Leptospira* in Bangkok together with a set of laboratory tests that could be readily deployed in the event of future flooding.

Leptospirosis is an endemic infection throughout the tropics, where outbreaks are well-described, often in the context of heavy freshwater flooding.[1,2] Flooding of the Bangkok Metropolitan Region between late October of 2011 and January of 2012 posed a potential risk for a leptospirosis outbreak, and case reports were monitored by the Bureau of Epidemiology of Thailand. The purpose of this article is to describe two adjunctive methods (direct polymerase chain reaction [PCR] and culture) to determine whether pathogenic *Leptospira* spp. was present in the floodwater.

A total of 110 floodwater samples was collected between November 14 and December 6 of 2011; 70 samples were taken within a radius of approximately 2 km around the Salaya Campus of Mahidol University in Nakhon Pathom province (sample code NP), and 40 samples were taken within a radius of approximately 8 km in Don Muang district, Bangkok province (sample code DM) (Figure 1). At each sampling point, 100 mL floodwater were collected at a depth of 20 cm into a sterile glass bottle. Samples were maintained at ambient temperature (25–32°C) during transportation to the laboratory; 50 mL sample were used for direct PCR assay, and the remaining 50 mL sample were used for culture.

A published PCR assay based on amplification and sequencing of a region of the 16S rRNA gene (*rrs*)[3] was used to test all water samples. This testing was performed on a 50-mL sample that was first centrifuged at $3,000 \times g$ for 30 minutes. The deposit was resuspended in 140 µL sterile water, and DNA was extracted from the suspension using the QIAamp Viral RNA Mini Kit (QIAGEN, Santa Clarita, CA) Five microliters DNA extract were used in the reaction, which was performed as described previously.[3] The 433-bp amplicons were sequenced, and the species of *Leptospira* was inferred based on position in a maximum likelihood (ML) phylogenetic tree, which included 36 *rrs* sequences from GenBank for *Leptospira* species belonging to the pathogenic, intermediate

(of intermediate pathogenicity), non-pathogenic, or unculturable groups Supplemental Table 1. The tree was constructed using the algorithm implemented in PhyML version 3.0.1,[4] and the model of sequence evolution used was the generalized time-reversible (GTR) model with γ-distributed rate variation. An ML tree was constructed using the nearest neighbor interchange (NNI) method.[4] The MEGA program[5] was used to display and edit the tree. The *rrs* sequences generated during this study were submitted to GenBank, and they are provided in Supplemental Table 2.

Fifty milliliters each water sample were passed through a sterile 0.2-µm filter (Sartorius AG, Gottingen, Lower Saxony, Germany); 0.5 mL filtrate were inoculated into a tube containing 3 mL *Leptospira* Vanaporn Wuthikanun (LVW) solid agar slant containing 1% Noble agar base and 10% rabbit serum.[6] Slants were incubated at 30°C in 5% CO_2 for 2 days followed by 30°C in air for a total of 28 days.[6] The surface fluid on each agar slant was examined two times weekly by darkfield microscopy. If spirochetes were observed, 100 µL surface fluid were spread-plated onto LVW agar[6] supplemented with 2,6-dichlorophenolindophenol (10 mg/mL; Sigma-Aldrich) to enhance visibility of *Leptospira* colonies and incubated at 30°C in 5% CO_2 for 2 days followed by 30°C in air for up to 28 days. Plates were examined two times weekly for visible colonies. We hypothesized that water samples might contain more than one strain of *Leptospira* species, which may manifest as different colony morphologies on solid agar. In view of this possibility, a single colony of each morphology type on a given plate was picked for additional analysis. These colonies were inoculated into 3 mL Ellinghausen-McCullough-Johnson-Harris (EMJH) broth and incubated at 30°C in air for 5–7 days to achieve a *Leptospira* concentration of 10^8 cfu/mL. DNA was then extracted using a boiling method and screened using a multiplex PCR assay developed during this study (using both *rrs* and *lipL32* genes as targets) (Supplemental Text 1). Colonies were assigned to pathogenic, intermediate, or non-pathogenic groups (Supplemental Text 1). Samples that were positive for pathogenic and intermediate species were further evaluated using the *rrs* assay.[3]

Direct *rrs* PCR of 110 floodwater samples yielded an amplicon that could be sequenced in 65 cases (59%), of which

––––––––––––––
*Address correspondence to Janjira Thaipadungpanit, Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajthevee, Bangkok 10400, Thailand. E-mail: janjira@tropmedres.ac
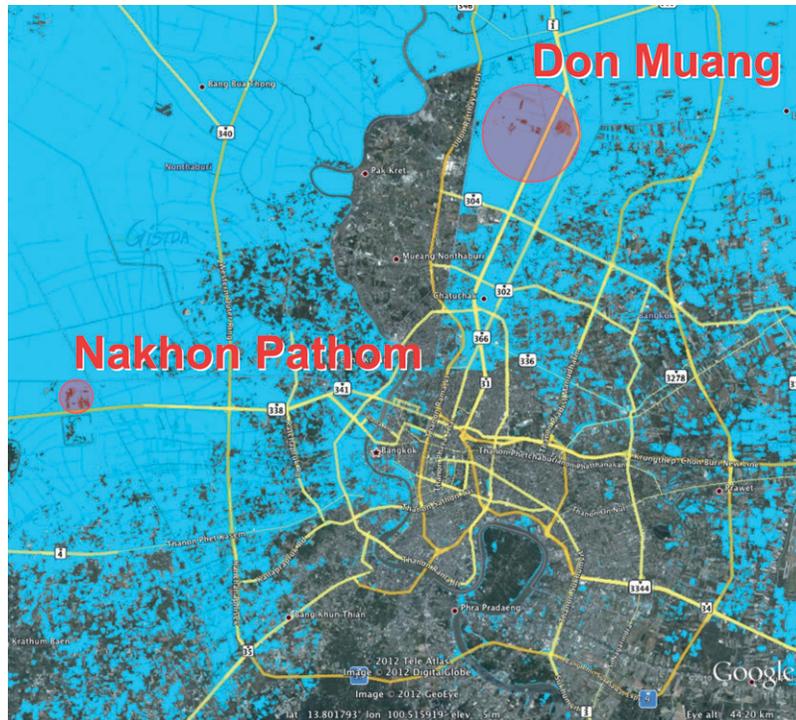
FIGURE 1. Map of the Bangkok Metropolitan Region showing the extent of flooding in 2011 and the two sampling zones. Flood data were obtained from the Geo-Informatics and Space Technology Development Agency in Thailand, and the map was generated using Google Earth (Google Inc.). The area flooded in 2011 is shown in blue, and the sampling zones are shown as red circles.

45 cases were from Nakhon Pathom and 20 cases were from Bangkok. These 65 sequences were resolved into eight *rrs* alleles with two polymorphic sites. Analysis of eight alleles using the blastn algorithm implemented in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the highest matches (nucleotide identities ranging from 98% to 99%) were *rrs* sequences cloned from unculturable bacteria in freshwater samples from several countries, including the United States, Korea, and China in 2003, 2008, and 2010, respectively (GenBank ID codes DQ065390, FJ164045, and FJ820401, respectively). On phylogenetic analysis, all eight alleles resided in the non-pathogenic *Leptospira* spp. cluster (Figure 2).

Culture yielded colonies from 87 of 110 samples (79.1%), which on dark-field microscopy, consisted of spirochetes (Supplemental Table 4). Picking one representative colony for each morphology type on a given plate yielded 140 single colonies for evaluation by the multiplex PCR assay. Results are presented using sample as the denominator, with additional information provided for samples containing more than one species. A single sample (sample code NP-29) was positive for pathogenic *Leptospira* species, and phylogenetic analysis of *rrs* placed this sample in the pathogenic clade on a discrete branch that did not contain any other isolates (Figure 2). Six samples were positive for intermediate *Leptospira* species. Using BLAST analysis, two samples were designated as *L. licerasiae* (NP-21 and NP-46), and two samples were designated as *L. wolffii* (NP-49 and NP-63). The remaining two samples (NP-30 and NP-64) were most closely related to *L. licerasiae*, with 99% and 97% nucleotide identity, respectively Supplemental Table 3, but they resided on their own branch in the intermediate *Leptospira* cluster (Figure 2). Samples NP-30 and NP-49 also contained non-pathogenic *Leptospira* along with an addi-

tional 74 samples. Six samples contained non-*Leptospiraceae* spirochetes. There was overlap between samples that were both culture-positive and positive on direct PCR for unculturable non-pathogenic *Leptospira* ($N = 47$).

In summary, we found a low prevalence of pathogenic and intermediate *Leptospira* species. The culture and PCR assays used here require additional validation to determine their accuracy for environmental use, but our findings are consistent with the low number of human leptospirosis cases reported to the Bureau of Epidemiology of Thailand, with only 11 notified cases from the Bangkok Metropolitan Region between November of 2011 and January of 2012 (Annual Epidemiological Surveillance Report 2011; http://www.boe.moph.go.th/Annual/AESR2011/index.html). Although outbreaks of leptospirosis during periods of flooding are well-documented in Thailand and elsewhere in the world,[1,2] our evidence indicates that an outbreak was not the case during the 2011 Bangkok flood.

Authors' addresses: Janjira Thaipadungpanit, Vanaporn Wuthiekanun, Premjit Amornchai, Siriphan Boonsilp, and Nicholas P. J. Day,
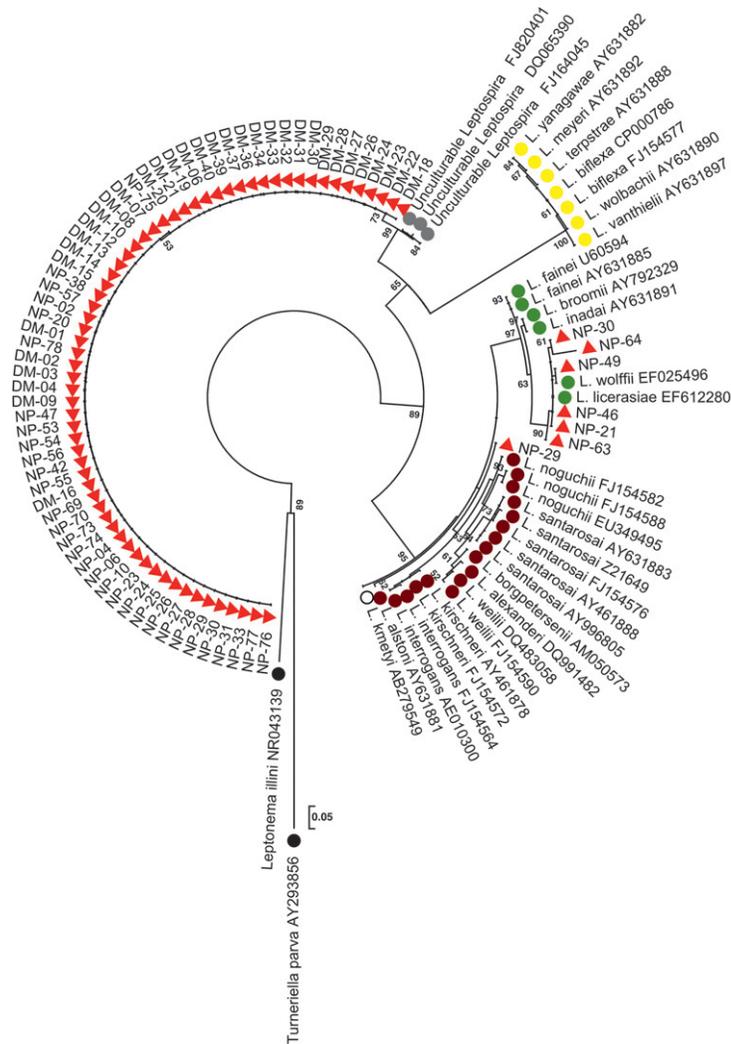
Figure 2. Phylogenetic analysis of partial *rrs* sequences. An ML tree was based on a 443-nt region of *rrs*. Thirty-six reference sequences from GenBank are shown as color-coded circles that denote their species group: pathogenic (brown), intermediate pathogenicity (green), non-pathogenic (yellow), uncertain pathogenicity (white), or unculturable (grey). Black circles denote two members of the family *Leptospiracae*. *Leptospira* species sequences obtained from floodwater samples are shown as red triangles. Two samples are shown two times, because they each contained two different species.

Mahidol-Oxford Tropical Medicine Research Unit, Mahidol University, Bangkok, Thailand, E-mails: janjira@tropmedres.ac, lek@tropmedres.ac, premjit@tropmedres.ac, boonsilp.s@gmail.com, and nickd@tropmedres.ac. Narisara Chantratita, Surapon Yimsamran, Wanchai Maneeboonyang, Prapin Tharnpoophasiam, Natnaree Saiprom, Yuvadee Mahakunkijcharoen, Pratap Singhasivanon, and Direk Limmathurotsakul, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, E-mails: narisara@tropmedres.ac, tmpbw@mahidol.ac.th, surapon.yim@mahidol.ac.th, tmwmn@mahidol.ac.th, ningkhub@hotmail.com, yuvadee.mah@mahidol.ac.th, pratap.sin@mahidol.ac.th, and direk@tropmedres.ac. Sharon J. Peacock, Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK, E-mail: sjp97@medschl.cam.ac.uk.

REFERENCES

1. Trevejo RT, Rigau-Perez JG, Ashford DA, McClure EM, Jarquín-González C, Amador JJ, de los Reyes JO, Gonzalez A, Zaki SR, Shieh WJ, McLean RG, Nasci RS, Weyant RS, Bolin CA, Bragg SL, Perkins BA, Spiegel RA, 1998. Epidemic leptospirosis associated with pulmonary hemorrhage-Nicaragua, 1995. *J Infect Dis 178:* 1457–1463.

2. Amilasan AS, Ujiie M, Suzuki M, Salva E, Belo MC, Koizumi N, Yoshimatsu K, Schmidt WP, Marte S, Dimaano EM, Villarama JB, Ariyoshi K, 2012. Outbreak of leptospirosis after flood, the Philippines, 2009. *Emerg Infect Dis 18:* 91–94.

3. Boonsilp S, Thaipadungpanit J, Amornchai P, Wuthiekanun V, Chierakul W, Limmathurotsakul D, Day NP, Peacock SJ, 2011. Molecular detection and speciation of pathogenic *Leptospira* spp. in blood from patients with culture-negative leptospirosis. *BMC Infect Dis 11:* 338.

4. Guindon S, Gascuel O, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol 52:* 696–704.

5. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol 28:* 2731–2739.

6. Wuthiekanun V, Amornchai P, Paris DH, Langla S, Thaipadunpanit J, Chierakul W, Smythe LD, White NJ, Day NP, Limmathurotsakul D, Peacock SJ, 2012. Rapid isolation and susceptibility testing of *Leptospira* spp. using a new solid medium (LVW agar). *Antimicrob Agents Chemother 57:* 297–302.