

## Asian Pacific Journal of Tropical Disease

journal homepage: <http://www.apjtc.com>Original article <https://doi.org/10.12980/apjtd.7.2017D7-220> ©2017 by the Asian Pacific Journal of Tropical Disease. All rights reserved.Isolation and genetic detection of pathogenic *Leptospira* spp. from environmental soils and water in Central Luzon, PhilippinesOurlad Alzeus Gaddi Tantengco<sup>1</sup>, Nina Gonzales Gloriani<sup>2\*</sup><sup>1</sup>College of Medicine, University of the Philippines Manila, Pedro Gil Street, Ermita, Manila, Philippines<sup>2</sup>Department of Medical Microbiology, College of Public Health, University of the Philippines Manila, Pedro Gil Street, Ermita, Manila, Philippines

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## ABSTRACT

**Objective:** To determine the prevalence, environmental distribution and genetic identity of *Leptospira* species in the provinces of Central Luzon, Philippines.**Methods:** A total of 135 soil and water samples were collected from all provinces in Central Luzon, Philippines. Soil samples were soaked in HEPES buffer and the aqueous phase was transferred to liquid 2× Korthof's medium supplemented with 10× STAFF to prevent the growth of microbial contaminants. Leptospire were isolated using a 0.2 µm syringe filter. 23S rRNA PCR test was used to detect the isolates belonging to genus *Leptospira*, *flaB* PCR test was used to detect pathogenic *Leptospira* and 16S rRNA gene sequencing was used to determine the genomospecies of isolates.**Results:** 23S rRNA PCR test revealed that 77 samples belonged to genus *Leptospira*. Three isolates showed positive results for the *flaB*-PCR assay. This result suggests that the isolates Ta8, Au4 and Au8 were pathogenic *Leptospira*. Phylogenetic analysis using the 16S rRNA gene revealed that one isolate from Aurora might belong to the pathogenic species *Leptospira kmetyi*.**Conclusions:** This study showed the presence of saprophytic and pathogenic leptospire in the environmental soils and water of Central Luzon, Philippines.

## 1. Introduction

Leptospirosis is a serious bacterial disease that is considered a global public health concern. This important zoonotic disease is most commonly present in tropical or sub-tropical countries because leptospire have longer survival in warm and humid conditions[1]. It is highly endemic to the Philippines and most frequently reported during typhoon season, especially during the months of July to October[2]. The Philippine Department of Health reported a total of 406 leptospirosis cases in the Philippines from January 1 to August 1, 2016.

Leptospirosis can be acquired through occupational and recreational exposures. Farmers and veterinarians, abattoir workers, rodent control workers and occupations that involve handling animals are at greater risk of being infected with leptospirosis. The

most common animal reservoir hosts of *Leptospira* are rodents and domestic mammals such as cattle, pigs and dogs. Infected animals can excrete leptospire in their urine and contaminate the environment. Indirect contact via contaminated soil and waters poses danger of infection to sewer workers, miners, soldiers, septic tank cleaners, fish farmers, canal workers and rice field workers[1].

Leptospirosis is a highly prevalent zoonotic infection in the Asia Pacific region especially in the Philippines. Aside from the animal host reservoir, the environment also serves as an important reservoir for pathogenic *Leptospira*[2]. Despite the important role of the environment in the transmission of *Leptospira* in human population, there are only few published studies on the prevalent *Leptospira* species present in the soil and water in the Philippines[4,5]. There was only one study on the isolation of leptospire from soils and water in the provinces of Central Luzon which was done in the province of Nueva Ecija.

Isolation and molecular studies of leptospire from the environment will greatly help in further understanding the epidemiology of leptospirosis. The isolates from the environment can be compared with isolates from humans and animals. This can infer sources of transmission based on the isolates from human,

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animals and environment. It will also contribute to the body of knowledge on prevention and control of the disease[5]. It will also help in development of diagnostic tests that will capture the serovars most prevalent in the country. Identifying *Leptospira* serovars prevalent in the Philippines will help in formulating vaccines most relevant to our setting. Thus, this study determined the presence of pathogenic leptospires in environmental soils and water in all of the provinces of Central Luzon using culture isolation and genetic detection techniques.

## 2. Materials and methods

### 2.1. Isolation of *Leptospira* species from environmental samples

The environmental samples were collected from seven provinces of Central Luzon Region, Philippines, namely, Aurora, Bataan, Bulacan, Nueva Ecija, Pampanga, Tarlac and Zambales from February 2015 to June 2016. A map containing the geographical coordinates of all sampling sites was generated using ArcGIS 2.0 (Figure 1).

For each province, the city or municipality with the highest reported cases of leptospirosis for 2013 and 2014 based on the data from Provincial Health Office served as sampling site. Then 15 to 25 environmental samples were collected from each sampling site. The geographical coordinates of each sampling site were recorded using GPS. Soil and water samples were collected from home backyards, irrigation areas, water canals near market places, rice fields, dry canals, and rivers.

50 g of soil and 50 mL of water samples were collected in sterile 50 mL screw-capped tubes. Then 10 mL of HEPES buffer was added to the tubes containing 5 g of soil sample and mixed. The tubes were allowed to stand for 1 h to facilitate settling down of the soil particles. A total of 2 mL of aqueous phase from the soil-buffer suspension was added to 2.5 mL of 2× concentrated Korthof's medium containing 500 µL of 10× STAFF (400 g/mL sulfamethoxazole, 200 g/mL trimethoprim, 50 g/mL amphotericin

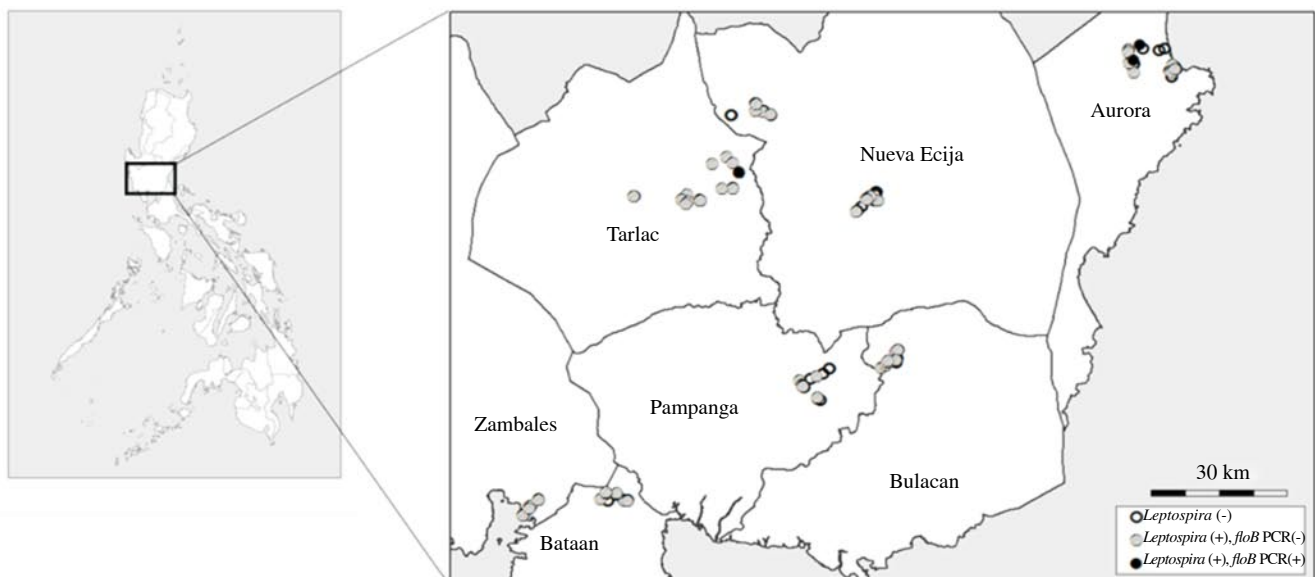
B, 4 mg/mL fosfomycin, 1 mg/mL 5-fluorouracil)[6]. The cultures were incubated at 30 °C. The growth of leptospires in the cultures was observed every day using a dark-field microscope. The presence of thin bacteria, rapidly rotating around its long axis and with hooked ends confirmed the growth of leptospires. Samples without leptospires detected under dark-field microscope after 28 days of incubation were considered as negative for leptospires. The samples with positive growth of *Leptospira* were sterilized using a 0.2 µm-pore-size membrane filter and 0.5 mL of the filtrate was transferred to new tubes containing 4.5 mL of fresh 1× liquid Korthof's medium without STAFF.

### 2.2. DNA extraction

DNA were extracted from the supernatant of the confluent pure culture of *Leptospira* isolates. A confluent culture of *Leptospira* isolates was collected by centrifugation with 11952 r/min for 3 min at 4 °C. Illustra Bacteria GenomicPrep mini spin kit (GE Healthcare, Buckinghamshire, United Kingdom) was used to extract the genomic DNA of the isolated *Leptospira* species.

### 2.3. 23S rRNA and *flaB* genes PCR amplification

Amplification of 23S rRNA gene was used to detect all isolates belonging to the genus *Leptospira*[7]. The primers used was rrl-F (5'-GACCCGAAGCCTGTGCGAG-3') and rrl-R (5'-GCCATGCTTAGTCCCGATTAC-3'). After confirming the isolates belonging to genus *Leptospira*, amplification of *flaB* gene using PCR was used for the detection of pathogenic *Leptospira* strains[8]. The primers used were L-*flaB*-F1 (5'-CTCACCGTTCTCTAAAGTTCAAC-3') and L-*flaB*-R1 (5'-TGAATTCGGTTTCATATTTGCC-3'). The PCR solution (50 µL) was composed of 1 Ex Taq buffer (TaKaRa), 100 µmol/L concentration of each deoxynucleoside triphosphate, 0.25 µmol/L concentration of the primer, 100 ng of extracted bacterial DNA, and 1.25 IU of Ex Taq HS DNA polymerase (TaKaRa). These genes were separately amplified using the following conditions: 30 cycles



**Figure 1.** Locations where the soil and water samples were collected. A total of 135 samples were collected from all provinces in Central Luzon. The geographical coordinates of the sites where the soil and water samples were collected are plotted on the map.

of 94 °C for 20 s, 54 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 6 min. Gene amplification was confirmed by performing 1.5% agarose gel electrophoresis.

#### 2.4. 16S rRNA gene sequence determination and phylogenetic analysis

16S rRNA gene sequence was used to determine the species of the *Leptospira* isolates. 16S rRNA gene (1 480 bp) was amplified by PCR using the bacterial universal primer set P16S-8UA (5'-AGAGTTTGTATCMTGGCTCAG-3'), P16S-1485R (5'-TACGGYTACCTTGTACGACTT-3'), P16S-519A (5'-CAG CMG CCG CGG TAA T-3'), P16S-519B (5'-ATT ACC GCG GCK GCT G-3'), P16S-907A (5'-AAA CTY AAA KGA ATT GAC GG-3'), and P16S-907B (5'-CCG TCA ATT CMT TTR AGT TT-3'). Amplification was performed under the following conditions: 30 cycles of 96 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min. The sequence of the amplicons of the 16S rRNA was determined using a 96-capillary 3730xl DNA Analyzer. The sequences of the other *Leptospira* species used for the construction of phylogenetic tree were obtained from GenBank. Staden Package was used in the sequence assembly of the 16S rRNA gene and BioEdit was used for sequence alignment[9,10]. Neighbor-joining method in MEGA 6.06 software was used to calculate phylogenetic distances and construct phylogenetic tree[11].

#### 2.5. Nucleotide sequence accession number

The sequence of the isolate Au8 from this study was deposited in the GenBank and can be retrieved under the accession number KY385446.

### 3. Results

#### 3.1. Environmental isolation of leptospires

This study demonstrated the presence of pathogenic leptospires in environmental soils as suggested by PCR detection of *flaB* gene present in pathogenic *Leptospira* species. A total of 135 samples were collected from all the provinces of Central Luzon in the Philippines. Using dark-field microscopy, spirochetes with hooked ends rotating rapidly in its long axis were observed in the primary cultures of 82 out of 135 samples. 23S rRNA PCR results showed that five of the 82 positive samples through dark-field microscopy did not belong to the genus *Leptospira*. Thus, 77 samples (60%) were found to be under the genus *Leptospira*: 15 in Aurora, 7 in Bataan, 9 in Bulacan, 12 in Nueva Ecija, 10 in Pampanga, 17 in Tarlac and 10 in Zambales. The results are presented in Table 1.

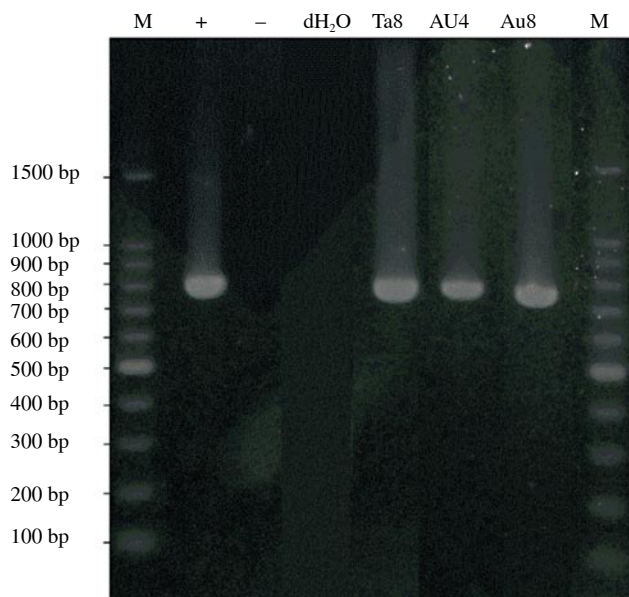
**Table 1**

*Leptospira* environmental isolation data from Central Luzon, Philippines.

Area	No. of positive samples/ total number of samples		
	Water	Soil	Total
Baler City, Aurora	13/23	2/2	15/25
Dinalupihan, Bataan	1/6	6/8	7/14
San Miguel, Bulacan	4/6	5/9	9/15
Cabanatuan, Nueva Ecija	12/25	N/A	14/25
Magalang, Pampanga	5/7	5/9	10/16
Tarlac City, Tarlac	14/22	3/3	17/25
Olongapo City, Zambales	4/8	6/7	10/15
Total	53/97	27/38	82/135

#### 3.2. *flaB*-PCR of the isolates

Three isolates showed positive results for the *flaB*-PCR test. These results confirmed that the isolates Ta8, Au4 and Au8 were pathogenic *Leptospira* (Figure 2). The other 74 isolates were determined to be saprophytic species of *Leptospira* since they tested negative for *flaB*-PCR. The Au4 pathogenic leptospire was isolated from soil sample near a garbage area in Maria Aurora, Aurora. A pig is located in the exact spot where the soil sample was taken. The Au8 and Ta8 pathogenic leptospires were isolated from rice fields in Alcala, Maria Aurora, Aurora and Balayang, Victoria, Tarlac, respectively. Different livestock such as cattle and water buffalo were present in these rice fields.



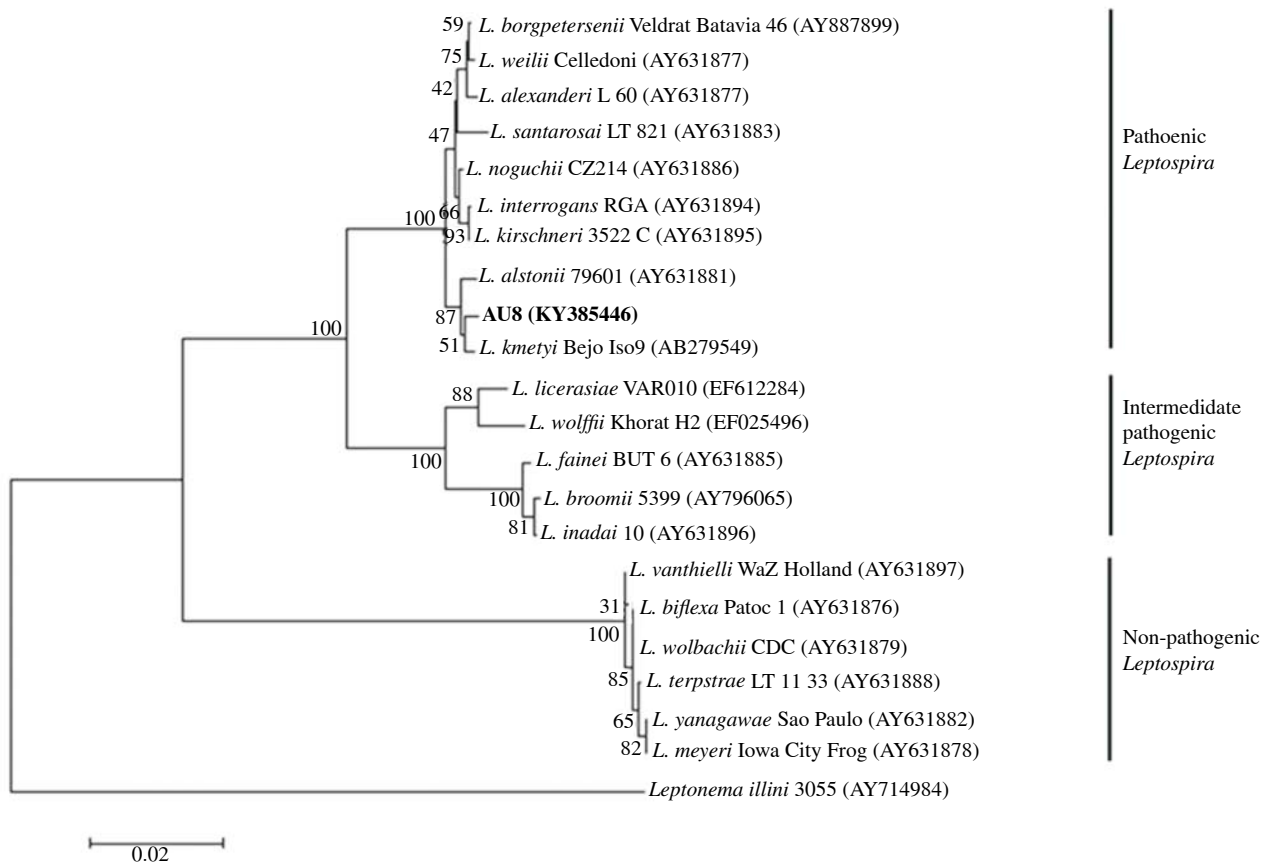
**Figure 2.** Agarose gel electrophoresis (1.5×) of *flaB*-PCR positive isolates from environmental samples from Central Luzon, Philippines. M: 100 bp molecular ladder; +: *Leptospira interrogans*; -: *Leptospira biflexa*; dH<sub>2</sub>O: Distilled water; Ta: Samples from Tarlac; Au: Samples from Aurora.

#### 3.3. 16S rRNA gene sequencing and phylogenetic analysis

The almost full-length 16S rRNA gene sequence of Au8 isolate of pathogenic species was determined. The neighbor-joining phylogenetic tree was constructed using the 16S rRNA sequences of this isolate together with 21 *Leptospira* genomospecies deposited in GenBank. AU8 clustered in the clade of pathogenic *Leptospira* species and exhibited the highest similarity with *Leptospira kmetyi* (Figure 3).

### 4. Discussion

In the Philippines, there is a limited amount of information regarding the prevalence and circulating species of saprophytic and pathogenic leptospires in the environment. In this study, three pathogenic *Leptospira* were isolated from the environment. This was the second environmental isolation of *Leptospira* in the Philippines. Saito *et al.*[12] isolated an intermediate pathogenic species *L. licerasiae* from the environmental soil and water in the province of Nueva Ecija. This was the first study on the environmental isolation of leptospires in Central Luzon, Philippines. The environment is an important *Leptospira* reservoir. Leptospires can be transmitted to



**Figure 3.** Neighbor-joining phylogenetic tree using 16S rRNA gene sequence analysis showing the phylogenetic position of one pathogenic isolate (AU8) from Aurora, Philippines. GenBank accession numbers are shown in parentheses.

humans through exposures to contaminated soil or water, especially in times of flood. Pathogenic and saprophytic strains of *Leptospira* from urine-contaminated soils can be washed-off and survive in bodies of water such as rivers and lakes[1].

Isolation of leptospires from Central Luzon is important because of the recent leptospirosis outbreaks declared by the Department of Health in Central Luzon. Olongapo City in the province of Zambales experienced continuous torrential rain due to southwest monsoon and this resulted in high level of flood all over the city. Leptospirosis cases reached 580 with 11 deaths. Based on the latest leptospirosis morbidity report of the Public Health Surveillance Division of the Philippine Department of Health, Central Luzon has the second highest cases of leptospirosis with a total of 59 cases from January 1 to August 1, 2015. Central Luzon and National Capital Region have the highest number of deaths due to this disease during the same period[3].

One pathogenic leptospire was isolated in Aurora from a garbage area with pigs. There is a possibility that pigs contaminated the environment with the leptospires. Pigs are known source of leptospirosis. Several pathogenic serovars of *L. interrogans* such as Australis and Pomona have been isolated from blood serum, kidney, liver and genital tract of female pigs[13]. In the Philippines, pigs have been shown to be seropositive for *L. interrogans* serovar Poi[14]. Pigs can shed leptospires in the environment and can increase the risk of acquiring infection to humans and other domestic animals. The pig located in the environment where the soil sample was obtained can be the potential source of the isolated pathogenic leptospires.

Two pathogenic leptospires were isolated from rice field with livestock such as cattle and water buffalo. Gamage *et al.*[15] reported that cattle could be one of the important leptospirosis reservoirs for

human infection. *L. borgpetersenii*, *L. kirschneri* and *L. interrogans* were found to be present in the cattle. These pathogenic species were isolated from kidney tissue samples of the cattle suggesting that these infected cattle must have excreted the leptospires in the environment.

There are also other studies that have isolated pathogenic leptospires from the rice fields. Pathogenic species of *Leptospira* have been detected from the rice fields of Tonekabon Township in Northern Iran[16]. Different serovars of *L. interrogans* have been isolated from the rice fields in Guilan Province in Iran. The presence of leptospires in the rice field puts the farmers at higher risk of getting leptospirosis. This was supported by several studies that have observed high leptospirosis seropositivity among rice field workers. A local study in the Philippines revealed that male farmers are the most affected group in symptomatic and asymptomatic cases of leptospirosis after the onslaught of typhoons Nesat, Nalage and Washi in the country[17]. Rice field workers are more prone to skin abrasion and cuts especially when working on the rice field on bare foot. Rice fields have large rodent populations and various livestock such as cattle and water buffalo living within the area. It also has wet environment especially during planting season and abundant sources of food for rodents and livestock. These conditions create a very ideal setting for the transmission of leptospirosis[1].

AU8 pathogenic *Leptospira* isolate showed the highest similarity with *L. kmetyi*. This isolate might be *L. kmetyi* or a novel species of *Leptospira*. This was the second time that this species of *Leptospira* was isolated from environmental sample in the Philippines. Saito *et al.*[5] isolated two pathogenic species of *Leptospira* from coastal soil in Leyte, Philippines after a storm surge due to Super Typhoon

Haiyan. These two isolates showed highest similarity with *L. kmetyi* using 16S rRNA gene sequencing.

16S rRNA gene sequences of Ta8 and Au4 were not identified because of difficulty in the assembly of the gene sequence. The results of the sequencing showed inconsistent pattern overall. This can probably be attributed to systematic errors in sequencing. Another reason for the inconsistency might be the lack of single-colony isolation of the pathogenic bacterial cultures from this study. Saito *et al.*[5] reported that *Leptospira*-positive environmental samples sometimes contain more than one *Leptospira* strain thus requiring the need for single-colony isolation.

One isolate from Aurora was identified as the pathogenic species with highest 16S rRNA gene similarity with *L. kmetyi*. This study has proven the presence of pathogenic leptospires in environmental samples from Central Luzon especially in rice fields and garbage area. This puts greater emphasis on the role of the environment in the transmission of leptospirosis in the community. It is recommended to examine the pathogenicity of the three pathogenic *Leptospira* isolates in golden Syrian hamsters to determine whether the isolates can kill the hamster and if leptospires can be recovered from internal organs and tissues of the challenged hamsters.

The presence of pathogenic leptospires in soils and water of Central Luzon puts the rice field workers and people living near rice fields at a greater risk of exposure to contaminated soils and water. Isolation of these pathogenic leptospires from the environment in Central Luzon is an indication of the presence of mammalian species that contaminate the environment with leptospires through their urine. Better control of the animal reservoir, frequent monitoring of water bodies, proper garbage management and educating the public of *Leptospira*-contaminated areas should be considered by the authorities.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

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