

# The Use of Commercial Soil Nucleic Acid Extraction Kit and Nested PCR for Detection of *Leptospira* in Farm Environment after Flooding in Taiwan

Ying-Bin Fuh<sup>1</sup> Wei-Yau Shia<sup>2</sup> Wei-Ming Lee<sup>2</sup> Ching-Lin Shyu<sup>2</sup>  
Chi-Young Wang<sup>2</sup> Chang-Young Fei<sup>1\*</sup>

## Abstract

Leptospirosis is a rapidly transmitted and flood-related disease. The number of cases usually increase in the countries of Southeast Asia after flood damage during typhoon seasons. This disease also poses a potential threat to Taiwan due to its geographic characteristics. Therefore, it is necessary to establish a practical technique for an estimation of the distribution of *Leptospira* in natural environment, especially after flood damage. The aim of this study was to demonstrate the use of commercial soil nucleic acid extraction kits with nested PCR in the detection of *Leptospira* spp. in the farm environment. The detection limit for *Leptospira* DNA was 24.24 pg. A total of 108 soil samples were collected from farms located within flood-damaged areas; the overall positive rate was 30.6%. Based on the sequence analysis, positive samples were identified as *Leptospira interrogans* and *Leptospira biflexa*. This developed approach might be applied in a surveillance of leptospirosis in different countries or in the detection of other pathogens from soils.

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**Keywords:** flood, leptospirosis, nested PCR, soil

<sup>1</sup>Graduate Institute of Veterinary Medicine, School of Veterinary Medicine, National Taiwan University, Taipei, Taiwan

<sup>2</sup>Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan

\*Correspondence author E-mail: fei@ntu.edu.tw

## บทคัดย่อ

### การใช้ชุดสกัดกรดนิวคลีอิกจากดิน และ Nested PCR ในการตรวจหาเชื้อ เลปโตสไปรา จากสิ่งแวดล้อมในฟาร์มภายหลังน้ำท่วมในประเทศไต้หวัน

Ying-Bin Fuh<sup>1</sup> Wei-Yau Shia<sup>2</sup> Wei-Ming Lee<sup>2</sup> Ching-Lin Shyu<sup>2</sup> Chi-Young Wang<sup>2</sup> Chang-Young Fei<sup>1\*</sup>

เลปโตสไปโรซิสเป็นเชื้อที่ติดต่อได้รวดเร็วและเป็นโรคที่สัมพันธ์กับภาวะน้ำท่วม จำนวนสัตว์ป่วยที่เพิ่มขึ้นมักจะอยู่ในประเทศในเอเชียตะวันออกเฉียงใต้หลังจากที่เสียหายจากน้ำท่วมในช่วงฤดูพายุไต้ฝุ่น โรคนี้ยังถูกวางเป็นภัยคุกคามที่อาจเกิดขึ้นกับไต้หวัน เนื่องจากลักษณะทางภูมิศาสตร์ของประเทศ ดังนั้นจึงจำเป็นต้องสร้างเทคนิคการตรวจสำหรับการประมาณค่าการกระจายตัวของเชื้อ *Leptospira* ในสภาพแวดล้อมทางธรรมชาติโดยเฉพาะอย่างยิ่งหลังจากที่เสียหายจากน้ำท่วม จุดมุ่งหมายของการศึกษานี้เพื่อทดสอบการใช้ชุดสกัดกรดนิวคลีอิกจากดิน และ Nested PCR ในการตรวจหาเชื้อเลปโตสไปราจากสิ่งแวดล้อมในฟาร์ม ขีดจำกัดของการตรวจจับดีเอ็นเอของเลปโตสไปรา คือ 24.24 พิโคกรัม จากตัวอย่างดิน 108 ตัวอย่างที่เก็บจากฟาร์มที่ตั้งอยู่ภายในพื้นที่น้ำท่วมเสียหาย ผลตรวจที่เป็นบวกโดยรวมคิดเป็นร้อยละ 30.6 และจากการวิเคราะห์ตัวอย่างที่ให้ผลบวกด้วยลำดับเบสระบุได้ว่าเป็น *Leptospira interrogans* และ *Leptospira biflexa* ดังนั้นวิธีนี้อาจนำมาใช้เพื่อการเฝ้าระวังเลปโตสไปโรซิสในประเทศอื่นหรือในการตรวจสอบของโรคอื่น ๆ จากดิน

**คำสำคัญ:** น้ำท่วม เลปโตสไปโรซิส nested PCR ดิน

<sup>1</sup>Graduate Institute of Veterinary Medicine, School of Veterinary Medicine, National Taiwan University, Taipei, Taiwan

<sup>2</sup>Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan

\*ผู้รับผิดชอบบทความ E-mail: fei@ntu.edu.tw

## Introduction

The incidence of many zoonotic diseases, including *Leptospira*, *Plasmodium*, *Vibrio cholerae*, *Rotavirus*, *Escherichia coli*, *Shigella*, and *Salmonella* species, often increase after flood damage (Schwartz et al., 2006; Gaynor et al., 2007). For the countries of Southeast Asia, including China, Taiwan, the Philippines, the northern part of Indonesia, Malaysia, Singapore, Thailand, Vietnam, and Myanmar, flood damage resulting from typhoons is commonly seen due to their geographic distribution. Therefore, the prevention of zoonotic diseases has been an important issue for these countries.

Leptospirosis, an acute bacterial infectious disease caused by the spirochete, has been recognized as a significant global public health problem due to its high prevalence and wide distribution (Meites et al., 2004; McBride et al., 2005). Leptospiral pathogens are identified in tropical, subtropical, and temperate zones with the most prominence in tropical areas with heavy rainfall (Levett, 2001). Leptospirosis belongs to an important pathogen that caused decreased milk production, infertility, stillbirths, and abortions in cattle (Natarajaseenivasan et al., 2011). In sheep and goats, chronic renal infection and persistent leptospiuria lead to shedding serving as a carrier for the dissemination of bacteria to other animals and humans after exposure to infected urine, animal reservoirs or contaminated environment (Slack, 2010). To date, only limited information as to the prevalence

of *Leptospira* in animals in Taiwan is available. Based on the laboratory-confirmed cases, the mean annual incidence for humans, which increased following heavy rains, was 0.21 cases/100,000 population in Taiwan (Chou et al., 2008).

Both direct and indirect methods have been developed for the extraction of environmental nucleic acid (Radnedge et al., 2003). In direct methods, microbial cells are simultaneously lysed within soil particles for subsequent extraction. This leads to higher nucleic acid yields. Due to the possible binding of extracted nucleic acid to humic acid substances, a well-known inhibitor for PCR step from soils, several techniques were refined to alleviate such inhibitory effects (Roh et al., 2006; Rajendhran and Gunasekaran, 2008). In indirect methods, microbial cells are first separated from soil particles and then subjected to further extraction. Although indirect methods give purer *Leptospira* DNA contents, performance is weakened by a low yield (Roh et al., 2006). To date, various kits have been developed for the extraction of nucleic acids from different types of samples. Saving time and standard procedures potentiate the commercial kits as powerful tools to prevent disease outbreak or bioterrorism attack (Mumy and Findlay, 2004). However, no application of commercial kits for the extraction of environmental *Leptospira* nucleic acid related to the flood hazard has been reported. Therefore, the aim of this study was to demonstrate the use of a commercial soil nucleic acid extraction kit in the detection of *Leptospira* spp. from the soil. This

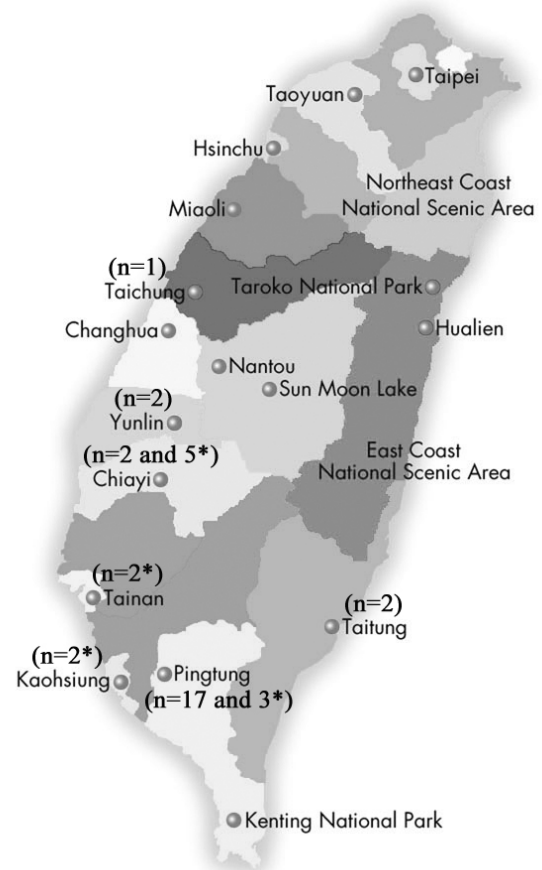
could be employed in the detection of other zoonotic pathogens in soil.

## Materials and Methods

**Flood hazard and soil sampling:** Two flood hazards occurred in August 2009 and September 2010 as results of typhoons Morakot and Fanapi, respectively. Typhoon Morakot caused severe flood damage in the south of Taiwan, including Kaohsiung, Pingtung and Taitung. The rainfall reached more than 1000 mm (more than 2000 mm in mountainous area) in many rain-gauge stations of the affected cities in a given day. Similarly, typhoon Fanapi caused severe flood damage in Kaohsiung and Pingtung. In the flood that occurred in September 2010, the affected area was mainly in the south of Taiwan. Soil samples were collected from May to December 2010 from twenty-four dairy and twelve chicken farms that had all suffered flood damage. The sampling area comprised farms in cities described above and some nearby cities, including Yunlin, Chiayi, and Tainan. Since the distribution of animal farms was concentrated in southern Taiwan, the farms sampled in the study (Fig 1) represented the major region of the farmlands frequently attacked by floods during typhoon seasons. The survey included a total of thirty-six farms (including dairy and chicken farms). For each farm, three soil samples were separately collected from the surface of the front, intermediate, and distal part of the farm. Hence, 108 soil samples in total were collected for detection of the existence of DNA of *Leptospira* spp. A farm was considered a *Leptospira*-positive farm if at least one sample from the premises tested positive for *Leptospira*.

**Soil DNA extraction:** The genomic DNA of soil samples was extracted and purified using a SoilMaster™ DNA Extraction Kit (EPICENTRE® Biotechnologies, Philadelphia, USA) according to the manufacturer's protocols with proper modifications. Twenty microliters instead of 300 µl were used to resolve DNA. Briefly, soil samples (100 mg) were mixed with soil DNA extraction buffer and 100 µg of proteinase K and then incubated at 37°C for 10 min. Lysis buffer was added, and the samples were vortexed and incubated at 65°C for spore lysis. The incubated sample was centrifuged, and its supernatant was collected. After protein precipitation on ice for 8 min, the supernatant was subsequently transferred into the prepared spin column to remove inhibitory substances. The DNA of flow through was precipitated at room temperature for 5 min. The precipitant was then centrifuged to remove supernatants, and the purified DNA was washed and suspended in Tris-EDTA Buffer.

**Detection limit of *Leptospira* examination:** The detection limit for *Leptospira* was determined using the genomic DNA of *Leptospira interrogans* serovar Pomona as the standard DNA (kindly provided by Professor Chang CC, Graduate Institute of Microbiology and Public Health, College of Veterinary Medicine, National Chung-Hsing University, Taiwan). Fifteen microliters of two-fold



**Figure 1** Sampling regions and number of sampled farms in the study. \*Number of sampled chicken farms.

serially diluted standard DNA (17.7 µg/ml) were added to the soil that was free of DNA of *Leptospira* spp. The genomic DNA of the premixed soil samples was extracted using a SoilMaster™ DNA Extraction Kit following the same protocol as previously described. A series of five-fold dilution of extracted DNA was subjected to PCR. The primers used and PCR condition are described in the next section. The soil sample with the lowest concentration of *Leptospira* DNA and the highest dilution factor of extracted DNA sample was defined as the detection limit.

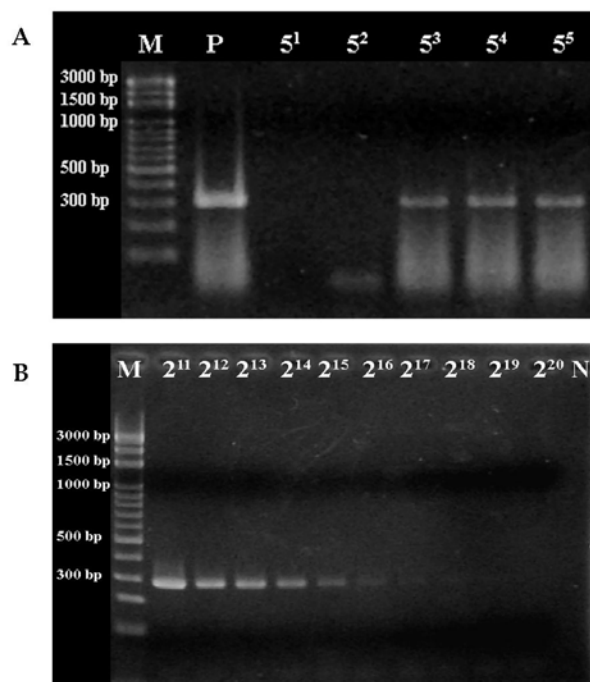
**Nested PCR for 16S rRNA gene detection:** Detection of pathogenic and nonpathogenic *Leptospira* spp. from extracted soil DNA samples was conducted using nested PCR targeting 16S rRNA sequence (EU497658.1, 497659.1, 497660, 497661.1, 497662.1, 497663.1 and 1EU497664.1). Two sets of PCR primers including LP1 (LP1F 5'-GGCGGCGCGTCTTAAACA TG-3' and LP1R 5'-GTCCGCCTACGCACCCTTTA CG-3') (Djadid et al., 2009) as well as LP2 (LP2F 5'-CAAGTCAAGCGGAGTAGCAA-3' and LP2R 5'-CTT AACCTGCTGCCTCCCGTA-3') (Merien et al., 1992) were respectively used for the first (525-bp) and second (289-bp) rounds of PCR. The reaction mixture and PCR condition were performed as previously published protocols (Djadid et al., 2009). Briefly, PCR

was performed in a 25 µl reaction mixture containing 0.2 mM concentration of each deoxyribonucleotide, 1.5 mM MgCl<sub>2</sub>, 1 U of Taq polymerase, and 10 pmol of each primer. During the examination of the detection limit, 3 µl of the template were used. After the minimal concentration of the template DNA in the PCR reaction was confirmed, an appropriate concentration of the template was used to examine the existence of *Leptospira* from soil samples. The PCR reactions were subjected to 94°C for 5 min and then 30 cycles consisting of 94°C for 1 min, 60°C for 2 min, and 72°C for 1.5 min, and a final extension at 72°C for 15 min. The second amplification of the nested PCR was performed with the same program. Nested PCR products were resolved on 2% agarose gel in 1 x TBE buffer with ethidium bromide (0.5 µg/ml) stain and visualized on a UV transilluminator. PCR products of expected sizes were purified and sequenced using an LP2 primer set (Mission Biotech, Taipei, Taiwan). The sequences were aligned using BLAST databases on the National Center for Biotechnology Information (NCBI) website. The *Apo I* restriction site, which existed only in PCR products amplified from non-pathogenic *Leptospira*, was used for differential diagnosis (Djadid et al., 2009).

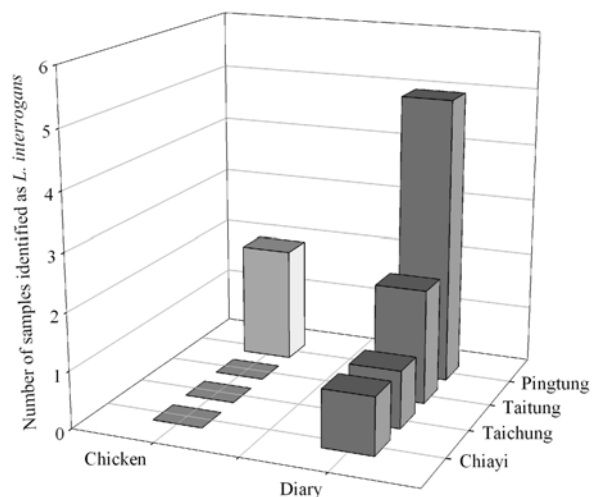
### Results

For the optimal dilution fold of extracted DNA from soil, at least a 125-fold of dilution was needed from 20 µl of extracted DNA (about 40 µg/ml) as templates to obtain a successful PCR reaction (Fig 2A). The soil containing 15 µl of 218-fold dilution of standard DNA (equal to 1.01 ng) could still be detected using nested PCR reaction (Fig 2B). After careful calculation, the minimum amount of *Leptospira* could be detected in combination with a commercial soil DNA extraction kit and nested PCR reaction equal to 24.24 pg. After the sequencing of the PCR product, the sequence was consistent with the original standard *Leptospira* DNA. Miscellaneous signals were not noticed in the sequencing reaction of the PCR product amplified from the extracted soil DNA that contained *Leptospira* standard DNA. The PCR reaction was confirmed not to have been interfered by other *Leptospira* species during the examination of the detection limit in the study.

With respect to the prevalence of *Leptospira* in the farm environment, DNA of *Leptospira* spp. was founded on eleven farms (11/36, 30.6%), including nine dairy (9/24, 37.5%) and two chicken farms (2/12, 16.7%). Most of these samples were collected from Pingtung County (in the south of Taiwan). The *Leptospira*-positive chicken farms were located in Pingtung only. According to the existence of *Apo I* restriction site, three *Leptospira* sequences of soil samples collected from Pingtung belonged to non-pathogenic *Leptospira* and were identified as *Leptospira biflexa* (identity from 98%) after aligning with the databank of NCBI. Meanwhile, eleven *Leptospira*-positive sequences were identified as *Leptospira interrogans* (*L. interrogans*) (identity from 92-98%). Three of them had the same sequence but



**Figure 2** Amplification of a series of five-fold dilution of extracted soil DNA using nested PCR (A). M: marker, P: positive control; nested PCR reaction of DNA extracted from soil premixed with two-fold serial diluted standard *Leptospira* DNA (B). M: marker, N: negative control.



**Figure 3** Geographic distribution of sequences identified as *L. interrogans* isolated from soil samples.

came from different farms (one dairy and two chicken farms). The distribution of sequences identified as *L. interrogans* surveyed from the soil samples is shown in figure 3. All sequences of PCR products amplified from the soil samples were different from the positive control. Artificial contamination can be ruled out.

## Discussion

In this study, DNA extraction from the soil using a commercial kit was tested, and the extracted DNA could be successfully amplified by nested PCR for the detection of *Leptospira*. According to the manufacturer's instructions, 300 µl TE buffer should be used to resolve the extracted DNA. However, this volume might not be enough to dilute the inhibitory substance present in extracted DNA. Based on our study, 2.5 ml of resolving solution were needed to complete the PCR reaction successfully. The result also indicated that despite the purification by the inhibitor removal resin, the remnant inhibitory substances might still be able to interfere with the PCR reaction. Accordingly, even though a further dilution will reduce the amount of the pathogen DNA in the soil sample, without an adequate dilution, a productive amplification of PCR reaction will not be achieved (Kirk et al., 2004).

In the observation of samples with identical sequences but originated from different farms, these areas were all heavily damaged by flood. The minimum and maximum distances of these three farms were 15.2 km and 57.5 km, respectively. Hence, these three *Leptospiras* might have originated from the same source and had been spread out by a given flood. Owing to the rapid spread of pathogens after flood damage, it was necessary to detect potential zoonotic pathogens from contaminated soil accurately and effectively. As we know, non-pathogenic *L. biflexa* is widespread in soil and water (Henry and Johnson, 1978). The combination of the reliable method of soil DNA extraction and identification of pathogens by PCR and *Apo* I restriction site, as used in the study, will assist in the expeditious survey of the zoonotic *Leptospira* species during flooding. Besides the prevention of epidemic diseases in animals, this also help veterinary staffs who handle the corpses and the service people who clean waste materials brought from flood disasters to avoid the occupational contraction of this disease. Human infection by leptospires occurs through contact with mucous membranes, including eyes, nose or the oral cavity, and via wounds on the skin (Cinco, 2010). Soaking the feet in *Leptospire*-contaminated water during flooding for long periods of time is likely to increase the possibility of infection. The hypothesis was also proved from the findings of two cases of the highest prevalence of human leptospirosis occurring in Taiwan in 2009 and 2010 (Centers for Disease Control, 2010).

*L. interrogans*, the predominant *Leptospira* species detected in this study, was also the main pathogen of human leptospirosis (Palaniappan et al., 2007; Patarakul et al., 2010; Scialfa et al., 2010). The outbreak of leptospirosis in humans was not reported during this flood damage. However, Taiwan did experience an outbreak of leptospirosis from August to September 2009 because of severe flood damage caused by the Morakot typhoon. The affected area included the southern and eastern regions of Taiwan where our survey focused. In 2009, a total of 203 people were diagnosed with leptospirosis; most of them came from Pintung County (Centers for Disease

Control, 2010). This finding was coincident with the high prevalence of *L. interrogans* found in Pintung in the study. As for animals, the information about leptospirosis after flooding is limited. Despite leptospirosis in chickens being quite unusual, the finding of *Leptospira* DNA on chicken farms remained worthy of note. Since rats, the principal reservoir for *Leptospira*, were occasional inhabitants of the chicken farms and were mostly attracted to the chicken feed and waste, the presence of *L. interrogans* may represent a risk factor for rat-related exposure (Reis et al., 2008).

In summary, a practical approach was demonstrated for the surveillance of *Leptospira* in the environment after flood damage. With further investigation of the prevalence of this disease in animals and humans in this area, a complete picture related to the prevalence of leptospirosis after floods in Taiwan will surely be provided.

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