Identification of *Leptospira* spp. from environmental sources in areas with high human leptospirosis incidence in the Philippines

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ABSTRACT

Leptospira is the causative agent of leptospirosis, which is considered an emerging major threat to public health due to its increasing frequency reported worldwide. In the Philippines, the prevalence of the disease continuously rises, particularly in urban areas. Because leptospirosis is commonly transmitted through contact with contaminated environment, water and soil samples were collected in regions in the Philippines where high incidence of human leptospirosis cases was reported recently. Of the 54 samples screened for the presence of *Leptospira*, 35% were found positive through 23S rRNA gene PCR-based detection. None were found positive when primers targeting *lipL32*, *lipL41*, and *ompL1* genes were used. Most of these isolates were collected from rural areas. However, 16S rRNA gene sequencing identified all isolates to be *L. yanagawae* and *L. meyeri*, which are nonpathogenic. Despite the lack of evidence of the presence of pathogenic species in the environmental sources, the results still suggest that leptospires persist in these areas. These data are crucial for environmental monitoring and identification of contaminated areas where humans may be at risk of infection.

KEYWORDS

Leptospira; leptospirosis; environmental sources; Philippines; PCR detection; 16S rRNA gene sequencing

Introduction

Leptospirosis is considered a globally widespread public health disease which can infect both humans and animals. It mostly occurs in humid subtropical and tropical regions with an overall estimate of 900,000 human cases and 49,000 deaths recorded each year [1,2]. Outbreaks were observed particularly in developed and developing countries where population growth is increasing [3,4]. Although the actual number of human leptospirosis is underestimated due to ineffective surveillance systems, large outbreaks have been documented in Southeast Asia, Central and South America with fatality reaching up to 30% [4–6]. In the Philippines, high incidence in rural areas was reported predominantly from 1998 to 2001 wherein morbidity was at 147 cases per 100,000 population and fatality was at 12–14% [7,8]. In 2017, the Philippine Department of Health disease surveillance (doh.gov.ph) has recorded a total of 2,495 suspected cases, which is 49.1% higher compared to the number of cases in 2016 (1,673).

In humans, leptospirosis can cause chronic or acute infection with clinical manifestations ranging from mild to severe form. The latter may progress rapidly with fatal outcomes [9–11]. Since transmission occurs commonly through direct or indirect contact with contaminated soil or water, urine, and animal carcasses infected with *Leptospira* [12], exposure to

unsanitary living conditions and flood increases the risk of having leptospirosis [1]. Occupational exposure also increases the likelihood of infection. People at risk include those who work in sewers, garbage collection, agricultural fields, and abattoir where there is a high possibility of contact with contaminated environment [10,13]. The incidence of such infections, however, still depends on several factors such as the presence of host reservoir species (rats, horses, pigs, cattle, dogs, bats, and even frogs) [14–16], the bacteria carrying load of its host, the environmental dilution once it is shed by its animal carrier, and the ecological conditions which favor the growth and survival of *Leptospira* [13,17]. Such factors have been investigated extensively; nevertheless, molecular evidence showing direct transmission of *Leptospira* from the environment to humans still remains lacking particularly in the Philippines.

Molecular and serological investigations in the Philippines showed rats and large ruminants such as water buffalo and cattle as possible host reservoirs of pathogenic *Leptospira* species suggesting their possible contribution to the incidence of human infection [8,18,19]. In fact, one study already revealed similarities in serotypes of human and rat isolates from the same area [8]. However, the distribution and transmission of pathogenic species in the environment to humans have yet to be established.

Since prolonged rainy season and increased flooding conditions are expected in the coming years, there is a need to investigate the potential environmental reservoirs of leptospires. The present study investigated the presence of *Leptospira* in regions where human leptospirosis cases had been reported. Using standard PCR which targets 23S rRNA gene and virulence-associated genes, *lipL32*, *lipL41*, and *ompL1*, soil and water samples were screened to identify possible sources of infection. Also, the 16S rRNA gene of each isolate was sequenced for species classification. These data are important for surveillance purposes in areas with high risk of infection, for determination of circulating species in the country, and for the establishment of preventive controls to avert the occurrence of further outbreaks.

Materials and methods

Sample collection, isolation and detection of *Leptospira* from environmental samples

Water and soil samples were collected from different geographical sites in the Philippines chosen based on the incidence of human leptospirosis cases reported in 2017. Investigated sites include rural and urban areas located in Luzon, the largest and most populous island in the Philippines (Figure 1). Reported incidence of leptospirosis in these areas were among the highest that was recorded from January to June 2017. Three study sites (Regions I, III, and IV-A) were mostly in rural areas where there are numerous recreational and agricultural activities such as farming, raising livestock, and fishing. Samples from these sites were randomly obtained from rice fields, canals,

creeks, irrigation systems, ponds, as well as water sources near households, backyard barns, and pens. Two river systems in Northern Luzon, the Bued River, which covers Benguet and Pangasinan provinces in Region I and Angat River in Region III, which passes through Bulacan province to Manila Bay, were also included. These rivers were selected because of the recreational activities in the area and its connection to major watersheds and other tributaries. The fourth sampling site is the National Capital Region (NCR), which is composed of several urbanized cities, hence, the most densely populated region in the country. Samples from this region were obtained from rivers, creeks, canals, flood water and stagnant water near establishments, landfills, and informal settlements or slums. Identified flood hazards were also included in the list of sampling sites, specifically the Estero de Sunog Apog, Estero de Tripa de Gallina, Tullahan River, San Juan River, and Maricaban Creek. These sites, which drain water from Manila to Manila Bay or Laguna Lake, are generally surrounded by informal settler communities living along NCR's major waterways.

Rainy season in the Philippines often begins in June and lasts until November. The southwest monsoon occurs from May to October often bringing heavy rains resulting in major flooding in the above-mentioned regions. Samples were obtained in June 2017 at the start of the rainy season. Collection proceeded following the procedures described by Saito et al. [20]. Briefly, 10 mL water and 10 g soil samples were collected and stored in 15 mL sterile screw-capped tubes at room temperature. For soil samples, each was mixed with 10 mL sterile water and kept upright for an hour at room temperature.

Figure 1. Map showing sampling sites (MapCustomizer, 2014). Colored markers (green, Region I; red, Region III; yellow, NCR; blue, Region IV-A) represent the sources of samples collected.

Two mL supernatant was transferred into tube containing 4 mL 2X modified Korthof's medium supplemented with 400 µL of 10X-antibiotic cocktail consisting of 400 g/mL sulfamethoxazole, 80 g/mL trimethoprim, 4 mg/mL fosfomycin, and 1 mg/mL 5-fluorouracil. Tubes were incubated at 30°C and checked every three days for the presence of *Leptospira* via microscopic examination for a month.

DNA was extracted directly from the soil and water samples and 7-day-old cultures using GF-1 bacterial DNA extraction kit (Vivantis). Total DNA was quantified using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). *Leptospira* DNA was detected based on the 23S rRNA gene using the rrl-F and rrl-R primers which identify all known species of *Leptospira* [21]. Virulence-associated genes were also amplified to detect pathogenic species. PCR mixtures were prepared using the GoTaq Green Master Mix (Promega) and amplification was carried out in T100™ Thermal Cycler (BioRad) following these conditions: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 1 min; and a 10-min final extension at 72°C for the 23S gene target. Cycling conditions for the *lipL32*, *lipL41*, and *ompL1* genes include an initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 sec, annealing at 54–56°C for 30 sec, extension at 72°C for 1 min. PCR products were checked by electrophoresis on 1.5% agarose gel. Selected 23S rRNA gene amplicons from representative soil and water isolates were sequenced to verify the gene target. All primer sequences used in the study are seen in Table 1.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene was sequenced to confirm the identity of *Leptospira* isolates. Initial PCR amplification was performed using 16S universal primers, fD1 and rD1 [22–24]. PCR conditions consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for

30 sec, extension at 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were subjected to a second round of amplification using the primers, *lepto16S11f* and *lepto16S1388r* [22–24]. Thermal cycling conditions were as follows: 16-min initial denaturation at 95°C and 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Amplicons were confirmed by electrophoresis on 1.2% agarose gels. PCR products were then sent to Macrogen Inc. (Seoul, Korea) for sequencing using additional internal sequencing primers, *lepto16S505f* and *leptos16S1006f*.

All sequences obtained in this study were submitted to the NCBI GenBank database. Genbank accession numbers of isolates are listed in Table 2. Identity of isolates was determined using the Basic Local Alignment Search Tool in the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). To construct phylogenetic trees, reference sequences from known *Leptospira* spp. and *Turneriella parva* (which belongs to the same Family Leptospiraceae) were extracted from GenBank. Sequences of other Philippine isolates from previous studies were also included in the analysis. Partial 16S rRNA gene sequences of *L. yanagawae* (AB758728.1) and *L. licerasiae* (AB758738.1), both from Region III, *L. meyeri* (AB758717.1) from NCR, and *L. kmetyi* gene sequences (AB937994.1 and AB937993.1) from Region VIII were used. These isolates were also obtained from soil and water samples [20,25]. Sequences were aligned and trimmed using the ClustalW algorithm in Bioedit v 7.2.5 [26]. The optimal DNA substitution model was identified based on Bayesian Information Criterion (BIC) in jModeltest v 2.1.10 software [27]. Oversaturation in sequences was also measured using the Xia test implemented in DAMBE v 5.2.5. Phylogenetic trees were reconstructed using Maximum Likelihood (ML), Neighbor-Joining (NJ), and Bayesian Inference (BI) methods. ML was done with 1,000 bootstrap replicates in Phym 3.1 program [28], NJ was done in PAUP* v 4.0b10 program, and BI was done with 10,000,000 generations in MrBayes v 3.1.2 program [29]. Tree constructions were based on the parameters of the optimal

Table 1. Primer sequences used in the study.

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	References
23S rRNA	rrl-F	GACCCGAAGCCTGTCGAG	482	[21]
	rrl-R	GCCATGCTTAGTCCCGATTAC		[21]
16S rRNA	fD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	1200	[22–24]
	rD1	CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC		[22–24]
	<i>lepto16S11f</i>	GGCGGCGCTCTTAACATGC		[22–24]
	<i>lepto16S1338r</i>	TGTGTACAAGGTCCGGGAAC		[22–24]
	<i>lepto16S505f</i>	TCATTGGGCGTAAAGGGTG		[22–24]
	<i>leptos16S1006f</i>	TCAGCTCGTGTCTGAGATG		[22–24]
	<i>ompL1</i>	TTGATTGAATTCTTAGAGTTTCGTGTTTATA	960	[43]
<i>ompL1</i>	<i>ompL1</i> R	AAGGAGAAGCTTATGATCCGTAACATAAGT		[43]
<i>lipL32</i>	<i>lipL32</i> F	TTACCGCTCGAGGTGCTTCGGTGGTCTGC	782	[43]
<i>lipL32</i>	<i>lipL32</i> R	TGTTAACCCGGGTACTTAGTCGCGTCAGA		[43]
<i>lipL41</i>	<i>lipL41</i> F	AAAGGACTCGAGTTACTTTGCGTTGCTTTC	1065	[43]
<i>lipL41</i>	<i>lipL41</i> R	TGTTACCATGGGGAGAAAATTATCTTCTCT		[43]

Table 2. Source location of *Leptospira* isolates.

Sample code	Sample type	Province/origin	Region	Description	GenBank accession numbers
ESS01a	Soil	Angat River, Bulacan	III	Recreation area	MG979781
ESS03B	Soil	Ecopark, Quezon City	NCR	Lagoon	MG979782
ESS04	Soil	Anao, Tarlac	III	Rice field	MG979783
ESS05	Soil	Pura, Tarlac	III	Pond	MG979784
EWS01a	Water	Angat River, Bulacan	III	Recreation area	MG979769
EWS07c	Water	Ecopark, Quezon City	NCR	Lagoon	MG979770
EWS10	Water	Broadway, San Juan	NCR	Near establishments, urban slums	MG979771
EWS11	Water	Ermitaño Creek, San Juan	NCR	Near establishments, urban slums	MG979772
EWS12	Water	San Juan River	NCR	Informal settlers	MG979773
EWS24	Water	Paniqui, Tarlac	III	Rice field	MG979774
EWS25	Water	Balite, Pura, Tarlac	III	Rice field	MG979775
EWS26	Water	Maungib, Pura, Tarlac	III	Rice field	MG979776
EWS27	Water	Nilasin, Pura, Tarlac	III	Pond	MG979777
EWS30	Water	Anao, Tarlac	III	Rice field	MG979778
EWS34	Water	Ramos, Tarlac	III	Stream connected to Tarlac River	MG979779
EWS39	Water	Payatas, Quezon City	NCR	Canal, urban slums	MG979780

DNA substitution model identified, TrN+G, with unequal substitution rate and 0.1780 gamma distribution factor. NJ and ML trees were drawn in TreeExplorer v 2.12 software program [30]. BI tree was drawn in Treeview v 1.6.6 [31].

Results

Isolation and detection of *Leptospira* from environmental samples

A total of 54 samples were collected from areas where human leptospirosis cases were reported prevalent during the year 2017. Twenty-three (23) samples were from urban slum areas in the NCR, while the other 31 samples were from rural areas in Regions I, III, and IV-A (Figure 1). Nine were obtained from soils near rivers and ponds, and rice paddy fields. PCR targeting the 23S rRNA gene identified 35% (19/54) of the samples positive for *Leptospira* (Figure 2). Isolation rate from soil was found higher than that from water with 44% (4/9) and 27% (12/45) positive rates, respectively. No amplification was seen in other gene targets. Sequencing of the 16S rRNA gene, however, identified three isolates (EWS03, EWS04, ESS03A) as *Leptonema*, a nonpathogenic bacterium of the

Leptospiraceae family. Table 2 shows the source location of samples positive for *Leptospira*.

Molecular differentiation of *Leptospira* spp. based on 16S rRNA gene

BLAST reports for each generated 16S rRNA gene sequences showed 99–100% identity with known *Leptospira* species. Alignment of these sequences (1,230 nt length) confirmed high sequence homology among isolates. Saturation analyses showed significantly low level of saturation in gene sequences (Iss < Iss.c). Despite the difference in the location where they were isolated, phylogenetic trees constructed based on NJ, ML, and BI methods showed clustering of the Philippine isolates in a single clade together with reference nonpathogenic species, *L. yanagawae*, *L. meyeri*, *L. wolbachii*, *L. biflexa*, *L. terpestrae*, *L. vanthiellii*, and *L. idonii*, with 100% bootstrap support (Figure 2). Reference intermediate pathogenic species (*L. licerasiae*, *L. wolffii*, *L. inadai*, *L. fainei* and *L. broomii*) and pathogenic species (*L. santarosai*, *L. borgpetersenii*, *L. kirschneri*, *L. interrogans*, *L. noguchii*, *L. weilii*, *L. mayottensis*, and *L. kmetyi*) grouped together with bootstrap supports of at least 88% and 73%, respectively. Figure 3.

No pathogenic species was isolated in this study. All isolates were from the NCR and Region III. None were isolated from Regions I and IV-A. Nine of the 16 isolates were identified as *L. yanagawae*. The remaining seven isolates were *L. meyeri*. It was noted that soil isolates except for ESS03B, which was obtained from the NCR, clustered with *L. meyeri*.

Figure 2. Agarose gel electrophoresis for the PCR-based detection of *Leptospira* targeting 23S rRNA gene. Amplicon size is 482 bp. NTC, negative control; + control, positive control from clinical isolate *Leptospira interrogans*; *Leptonema* isolates marked in red.

Discussion

Samples in this study were obtained from regions in the Philippines with high number of cases reported during the year 2017. Specifically, the Philippine Department of Health documented 478 cases from

Figure 3. Neighbor-Joining tree of *Leptospira* species constructed using the sequence alignment of the 16S rRNA gene with 1,230 nt length. The tree was constructed based on the TrN+G DNA substitution model and was rooted with the outgroup, *Turneriella parva*. Bootstrap values inferred from NJ, ML, and BI are shown in each node. Values <50% are not shown. Scale bar represents 0.05 substitution per site. Pathogenic, intermediate, and nonpathogenic groups are identified. Environmental isolates are in bold. Philippine isolates obtained from previous studies included in the analysis is marked with (*). Each color represents regions where isolates were collected: NCR (blue), Region III (red), and Region VIII (yellow).

NCR, 369 cases from Region I, 207 cases from Region III, and 94 cases from Region IV-A out of the 2495 reported total number of cases in the country (<http://www.doh.gov.ph/statistics>). Despite the prevalence of human leptospirosis and the presence of possible host reservoirs that may aid in the transmission of *Leptospira* in these areas, this study failed to isolate pathogenic or intermediate pathogenic *Leptospira* species. This result is similar with previous surveillance conducted in the country in 2013, in which most isolates detected were nonpathogenic species [20]. The difficulty in isolating pathogenic *Leptospira* from environmental sources is attributed to the presence of contaminants in the culture as well as the fact that nonpathogenic *Leptospira* tend to outgrow the pathogenic species [17]. Also, an investigation on the survival of *L. interrogans*, a pathogenic species commonly detected in human clinical samples, suggested that though they may remain viable for a certain period in soil or in water, they cannot multiply in such conditions. Their low concentration in the environment thus makes it difficult for them to be detected or isolated [32]. Another study, however, showed evidence of persistence in the environment of pathogenic *Leptospira* which was also detected in human

cases reported within close proximity from the environmental source. In fact, molecular typing revealed the presence of the same strain in the soil for weeks after the reported human infections [17]. Unfortunately, there is still no evidence directly linking the environment as potential source of human infection in the Philippines.

In the present study, it was noted that the most common species present in the regions surveyed, NCR and Region III, are *L. yanagawae* and *L. meyeri*, same with the species isolated in the previous study though these isolates were from different provinces. Nonetheless, previous studies were still able to isolate one *L. licerasiae*, an intermediate pathogenic species, from the province of Nueva Ecija in Region III and two *L. kmetzi* isolates from Leyte in Region VIII [20,25]. Thus, suggesting that soil and water sources are still a potential reservoir of different *Leptospira* species whether pathogenic or nonpathogenic.

Comparison of the detection rate showed higher positivity in soil than in water even with the limited number of soil samples collected. Low detection rate in water may be generally attributed to the bacterial dilution in water environment, the small volume of water (10 mL) collected, and the water flow rate in the

study sites. Of the 12 positive water samples detected, majority were collected from stagnant waters (EWS01a, EWS07c, EWS24, EWS25, EWS26, EWS27, EWS30, EWS39) possibly because of relatively low dilution in these sites. The absence of *Leptospira* in most water samples collected from contaminated sites, specifically in areas near establishments and slums, was also noted. Similar findings were observed in a previous study implying that these species cannot survive in the polluted environment [20]. The contributing factor for their persistence in soil, on the other hand, is associated with soil moisture content. Prevalence of leptospires was known to increase with higher soil moisture content [33]. Other factors that may influence their survival in the environment include pH, temperature, and presence of other bacteria [13,20,34,35].

Majority of the samples were collected from rural areas, hence, the higher number of isolates here compared to the urbanized areas. Rural isolates were mostly from ponds, rivers, and rice paddies with clean waters. Only a few nonpathogenic isolates were detected from water sources near areas with high numbers of informal settlers or urban slum community (EWS10, EWS11, EWS39). In studies conducted in South American countries, detection rate of *Leptospira* was higher in water samples from rural than urban settings. The diversity of isolates was discovered to be also higher in rural areas. The difference in the incidence of *Leptospira* in the two environmental settings was attributed to the presence of heavy loads of other bacteria and toxic chemicals in water sources in urban areas which decreases their chance of survival as well as the density of animal reservoirs present in rural areas [36,37]. However, because no pathogenic species were detected, the effect of the density of animal reservoirs, which only carries pathogenic species, in the persistence of leptospires cannot be confirmed in the present study.

The impact of water quality in urban and rural areas has also been assessed. In fact, investigation on human drinking water sources revealed that *Leptospira* can most likely survive in clean waters. In Chile, 20% of their drinking water were found to be contaminated with pathogenic species [38]. The survival and persistence of the organism were shown to reach more than a hundred days in natural freshwater through their ability to aggregate in viscous environment such as capsules and biofilms formed by other microorganisms, allowing them to endure harsh conditions [39].

Interestingly, water sources close to animal farms were found negative for pathogenic leptospires. Previous investigations on animal sources of leptospirosis in the Philippines confirmed that ruminants and rodents are potential animal reservoir of pathogenic species. The most predominant species

belonged to *L. interrogans* (rodents), and *L. borgpetersenii* and *L. kirschneri* (ruminants). Their data also revealed that Pyrogenes, Bataviae, and Grippotyphosa are the most common serovars found in the Philippines [8,18]. These findings are important in disease surveillance since cattle were found to shed significantly more *Leptospira* in urine than rats which suggest that contact with contaminated environment near cattle farms increases the likelihood of human transmission of the disease [40]. Since no pathogenic *Leptospira* were identified in water sources near animal farms, direct sampling from potential animal sources is suggested to check if transmission from animals to humans may have occurred in these regions.

Prevalence of leptospirosis outbreaks is not only linked to poor sanitation, rapid population growth, and urbanization, but also to the occurrence of flooding [1,7]. Heavy rainfall was already established to be correlated with leptospirosis transmission since such conditions favor the persistence and distribution of the pathogen in the environment [41,42]. In the current study, samples were obtained in the month of June which is the start of the rainy season in the country. However, no pathogenic species was isolated.

The lack of knowledge on disease transmission of leptospirosis in the Philippines highlights the need to conduct more studies to determine potential sources and understand the role of these sources in the epidemiology of the disease. The present study attempted to identify possible environment reservoir of pathogenic *Leptospira* in areas with high human leptospirosis cases. However, findings show that only nonpathogenic species are present. Because of the increasing incidence of the disease, investigating potential environment and host reservoirs of leptospires is deemed necessary. Evidence linking clinical cases with environmental source and the factors that contribute to the survival and persistence of pathogenic *Leptospira* species outside of host should also be further investigated. Such information is crucial to establish preventive controls that can avert the occurrence of further outbreaks.

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Disclosure statement

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