MOLECULAR DETECTION OF *LEPTOSPIRA* FROM ENVIRONMENTAL SAMPLES AROUND ABATTOIRS OF CAVITE PROVINCE, PHILIPPINES

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Abstract. Leptospirosis is endemic in the Philippines with disease incidences, which peak during rainy season or after heavy rains in flood-prone areas. This study screened for *Leptospira* spp from abattoir surroundings (soil and water samples) in Cavite Province, Philippines. Seventy-two samples were cultured in Kolthoff's medium, 25% of which were *Leptospira*-positive based on presence of PCR-amplified *rll* and sequences of 16S rDNA, with five pathogenic (carriage of *fla*B) isolates from Bacoor City, Imus City and Noveleta Municipality. Of these, one isolate was serogroup Grippotyphosa while the remaining isolates did not react with any of the monoclonal and polyclonal antibodies tested. The presence of *Leptospira* in abattoir surroundings could constitute continual sources of leptospires and pose health problems to humans and animals. These *Leptospira* isolates could be potential candidates for the development of diagnostic tests and vaccines.

Keywords: *Leptospira* spp, 16S rDNA, abattoir, *fla*B, *rrl*, Philippines

INTRODUCTION

Leptospirosis is an important zoonotic disease common in both developing and developed countries (WHO and ILS, 2003). It is caused by spirochetes belonging to pathogenic *Leptospira* sp (Inada *et al*, 1916). These leptospires are carried in the convoluted tubules of the kidneys of reservoir animals and via urine contaminate the environment, where they can survive in alkaline soil, mud, swamp and stream (Smith and Self, 1955). Survival of these pathogenic leptospires in the environment is dependent on several factors including pH, temperature and presence of inhibitory compounds (Chang *et al*, 1948; Smith and Turner, 1961). Isolation of leptospires from the environment is important for epidemiological studies. However, the exact factors remain to be proven due to slow growth of the pathogenic forms and overgrowth of saprophytic strains (Saito *et al*, 2013). Environmental contamination by leptospires contributes to the prevalence of the disease in endemic areas as a result of leptospiral penetration via open wounds (Faine *et al*, 1999; Evangelista and Coburn, 2010).

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Leptospirosis is highly endemic in the Philippines with disease prevalence estimated at 4.8 per million population (DOH, 2014). From 2011 to 2014, an average of 786 leptospirosis/leptospirosis-like illnesses were recorded (DOH, 2014), with the majority of the cases occurring during the rainy months (August to November) or after typhoons (Victoriano *et al*, 2009). In Cavite, Philippines, clusters of cases were reported in lowland areas where flooding is common (RESU, 2014). This can be attributed to exposure of people to environmental sources contaminated with urine of infected animals, conditions suitable for transmission of leptospires and exacerbating the risk of infection. Recently Tabo *et al* (2018) reported abattoir workers are more likely to be exposed to leptospires because of the wet environmental conditions and rat infestations in the work place.

In order to identify the potential threat of leptospirosis from abattoir surroundings, this study aimed to detect *Leptospira* spp from the environment of selected abattoirs in Cavite, Philippines.

MATERIALS AND METHODS

Research and sampling design

The study utilized a prevalence study design to determine the proportion of *Leptospira* spp in various abattoir environments. PCR amplification of *rrl* and *fla*B were used to characterize the *Leptospira* isolates.

Stratified sampling design was utilized in the survey of 23 cities/municipalities of Cavite Province, and six were selected based on the number of leptospirosis cases in the previous five years, namely, the cities of Bacoor, Cavite, Dasmariñas and Imus, and the municipalities of Noveleta, and Tanza (RESU, 2014). As only one public abattoir was registered with the National Meat Inspection Services of the Philippines for each of the localities of Cavite City, Dasmariñas City, Imus City, and Tanza, they were automatically included in the study. On the other hand, no public abattoir was registered in the municipality of Noveleta and the City of Bacoor, hence a private abattoir was randomly included from each locality. Soil and water samples were collected within a 100-meter radius during February - August 2015.

Collection and processing of soil and water samples

Approximately 10 ml of water samples were collected in duplicate. One sample was placed in a sterile 50-ml conical tube for isolation of *Leptospira* spp, while the other sample was placed in a sterile 50-ml beaker for pH measurement. Approximately 10 g of soil sample were collected in triplicate: one was placed in a 50-ml conical tube for leptospiral enrichment, the second in a sterile 50-ml beaker for pH measurement and the third in a sterile Petri dish for moisture content analysis. The samples were stored at 15°C and transported to UPM CPH Leptospirosis Laboratory, Manila within 4-6 hours.

Measurement of pH and moisture content

The pH of water samples was directly measured using a digital pH meter (Orion™ Star A321, Beverly, MA). For pH measurement of soil samples, 10 g of sample were added to 40 ml of sterile distilled water, rapidly mixed and pH of the soil suspension immediately measured twice. Moisture content of soil samples was determined using methods adapted from those of Henry and Johnson (1978) and Saito *et al* (2013). In brief, 10 g of soil sample were dried at 110°C for 2 hours or until the dry weight remained constant

and recorded as percent moisture content.

Enrichment of collected soil and water samples

Enrichment of samples was adapted from the method of WHO and ILS (2003) with modifications. In brief, 10 ml aliquot of 20 mM HEPES buffer was added to each soil sample, which was allowed to stand for one hour and then 2 ml aliquot of supernatant was added to 2.5 ml of 2× modified Korthof medium supplemented with 500 μl of 10× STAFF (sulfamethoxazole, 400 μg/ml; trimethoprim, 200 μg/ml; amphotericin B, 50 μg/ml; fosfomycin, 4 mg/ml; 5-fluorouracil, 1 mg/ml) (HiMedia Labs, Mumbai, India). The solution was incubated at 30°C for one month. For water samples, 2 ml aliquot of each sample was added to 2.5 ml of the abovementioned Korthof medium and incubated at 30°C for one month.

Detection of leptospires

The enriched soil and water samples were observed for presence of leptospires weekly under a dark field microscope (20× magnification). The presence of leptospires was based on motile thin helical structures with prominent hooked ends (Levett, 2001). If contaminants were present in the Korthof medium at the start of the enrichment procedure, 1 ml aliquot of the suspension was filtered using a 0.2 μm pore-size syringe filter (Acrodisc® PALL, West Chester, PA). The filtrate was added to 4 ml of fresh Korthof medium without STAFF and incubated at 30°C for one month (Saito *et al*, 2013). Samples absent of leptospires after one month are reported as negative.

Identification of serotypes

Serotyping of isolates was conducted using microscopic agglutination test (MAT) together with monoclonal antibodies (mAb) against serovars Grippotyphosa, Javanica, Losbanos, and Manilae and polyclonal antibodies (pAb) against serovars Grippotyphosa, Losbanos, Manilae, Patoc, Poi, and Ratnapura (UPM Leptospirology Laboratory, Manila City, Philippines) as previously described (Villanueva *et al*, 2010). In short, 20 μl aliquot of each isolate was mixed with 20 μl of each of the antibodies in a 96-well microtiter plate, incubated at 30°C for 2-4 hours and examined under dark field microscope. Antibodies against a serovar demonstrating ≥90% agglutination compared to negative control is considered indicative of that serotype. If two or more serotypes were identified for the same isolate, titrations of the pertinent antibodies were performed and the antibodies with the highest serum dilution producing ≥90% agglutination is considered positive for that serotype.

PCR-based detection of *Leptospira rrl* and *fla*B

Genomic DNA was extracted from the isolates using Illustra™ Bacteria GenomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) and stored at -20°C until used. PCR was carried out employing primers targeting *Leptospira rrl* rrl-F (5′-GACCCGAAGCCTGTCGAG-3′) and rrl-R (5′-GCCATGCTTAGTCCCGATTAC-3′) (Woo *et al*, 1997), *Leptospira fla*B L-flaB-F1 (5′-CTCACCGTTCTCTAAAGTTCAAC-3′) and L-flaB-R1 (5′-TGAATTCGGTTTCATATTTGCC-3′) (Kawabata *et al*, 2001). Reaction solution (50 μl) was composed of 1× ExTaq buffer (TaKaRa, Shiga, Japan), 100 μM dNTPs, 0.25 μM of primers, 1.25 U ExTaq polymerase (TaKaRa), 100 ng of DNA and deionized sterile distilled water. Thermocycling was carried out in a thermal cycler (Biometra TProfessional Standard, Göttingen, Germany) as follows: 30 cycles of 94°C for 20 seconds, 54°C for 30 seconds and 72°C for 60 seconds; with a final heating of

72°C for 6 minutes. Amplicons (482 and 793 bp of *rrl* and *fla*B, respectively) were analyzed by 1.5% agarose gel-electrophoresis, stained with ethidium bromide (1.0 μg/l) and recorded using a DigiDoc-It® Imaging System (UVP, Upland, CA). Preparation of PCR solution, DNA amplification and electrophoresis were performed in the same way for both primer sets.

Identification of *Leptospira* from 16S rDNA sequence

An internal fragment of *Leptospira* 16S rRNA gene (~1,480 bp) was amplified using a bacterial universal primer set, P16S-8UA (5′-AGAGTTTGATCMTGGCTCAG-3′) and P16S-1485R (5′-TACGGYTACCTTGTTACGACTT-3′) where M is A or C and Y is C or T (Frank *et al*, 2008; Saito *et al*, 2013). PCR mixture was prepared as described above and thermocycling performed as follows: 30 cycles of 96°C for 60 seconds, 55°C for 60 seconds and 72°C for 90 seconds; and a final step of 72°C for 6 minutes. Amplicons were separated as described above, gel-purified and sequenced (1st Base Asia, The Gemini, Singapore). Sequences were aligned using Multalin version 5.4.1 software (Corpet, 1998) and subjected to BLAST search of *Leptospira* spp sequences deposited at GenBank.

RESULTS

Physical properties of abattoir soil and water samples

A total of 72 soil (dry, $n = 6$; wet, $n = 6$; and water-logged, $n = 6$) and water (puddle, $n = 6$; canal, $n = 6$; river/creek, $n = 6$); flood, $n = 18$); and muddy, $n = 18$) samples were collected within a 100-meter radius at each of the six abattoirs from four cities and two municipalities in Cavite Province. Water-logged soils and water samples were collected in April and May 2015, and muddy and flood-water samples one hour to one day after heavy rains in July and August 2015. Median pH of the 72 samples was 7.6, ranging from 6.4 to 8.9 [95% confidence interval (CI) 7.5-7.7] (Table 1). Wet soils had the highest median pH value, followed by rivers/creeks and dry soils. Median moisture content of the soil samples was 28%, ranging from 2% to 60% (95% CI: 24-33), with water-logged soils having (as expected) the highest median moisture content followed by wet soils (Table 1).

Identification and serotyping of leptospires isolates

Of the 72 water and soil samples collected, 26 (36%) were culture-positive for leptospires (Table 2). The majority of the 15 (58%) culture-positive soil samples were from mud, while the majority of culture-positive water samples were from flood samples, with none from rivers/creeks. *Leptospira*-specific *rrl* [encoding 23S rDNA was present in 18 (69%)] leptospires cultures, among which 5 (28%) also carried the pathogenic gene marker *fla*B (encoding flagellin B protein) (Fig 1). The five pathogenic *Leptospira* isolates were collected from water-logged soil (B1S3) and mud (B1M3) in the vicinity of an abattoir in Bacoor City, from wet soil (N1S2) and canal water (N1W2) of a private abattoir in Noveleta Municipality, and mud (I1M1) of an abattoir in Imus City. Of the five pathogenic leptospires isolated, one isolate (I1M1) reacted to serogroup Grippotyphosa, while the other four did not react to any of the monoclonal and polyclonal antibodies tested.

Based on the 16S rDNA gene sequence, 99% sequence similarity was observed for isolate I1M1 to *Leptospira interrogans* serovar Grippotyphosa (GenBank accession no. EF536975.1), and

Table 1
Mean pH and moisture content of samples from vicinities of abattoirs in Cavite Province, the Philippines collected during February-August 2015.

Sample type	Property	Number of samples	pH		95% Confidence interval	Moisture content (%)		95% Confidence interval
			Median	Range		Median	Range	
Soil	Dry	6	7.6	6.9-8.3	7.0 - 8.3	7	2 -18	1 - 13
	Wet	6	8.2	7.4-8.9	7.6 - 8.8	30	20 - 37	24 - 36
	Water-logged	6	7.3	6.4-8.2	6.5 -8.1	48	40 - 60	40 - 56
	Mud	18	7.6	7.0-8.4	7.4 - 7.7	28	17 - 38	25 - 31
	Total	36	7.6	6.4-8.9	7.4 - 7.8	28	2 - 60	24 - 33
Water	Puddle	6	7.4	6.8-8.4	6.8 - 8.0			
	Canal	6	7.5	7.2-8.3	7.1 - 8.0			
	River/Creek	6	7.7	6.4-8.5	7.0 - 8.5			
	Flood	18	7.6	7.0-8.8	7.4 - 7.8			
	Total	36	7.6	6.4 - 8.8	7.4 - 7.7			

Table 2
Leptospires positivity in culture, and carriage of *Leptospira*-specific *rrl* and *fla*B of samples from vicinities of abattoirs in Cavite Province, the Philippines collected during February - August 2015.

Sample type	Property	Number of samples	Culture positivity (%)[a]	*rrl* (%)[b]	*fla*B (%)[c]
Soil	Dry	6	1 (17)	1 (100)	0 (0)
	Wet	6	3 (50)	2 (67)	1 (50)
	Water-logged	6	3 (50)	3 (100)	1 (33)
	Mud	18	8 (44)	5 (63)	2 (40)
Water	Puddle	6	1 (17)	0 (0)	0 (0)
	Canal	6	4 (67)	3 (75)	1 (33)
	River/Creek	6	0 (0)	0 (0)	0 (0)
	Flood	18	6 (33)	4 (67)	0 (0)
Total		72	26 (36)	18 (69)	5 (28)

[a]Relative to number of samples. [b]Relative to number of culture-positive samples.
[c]Relative to number of *rrl*-positive samples.

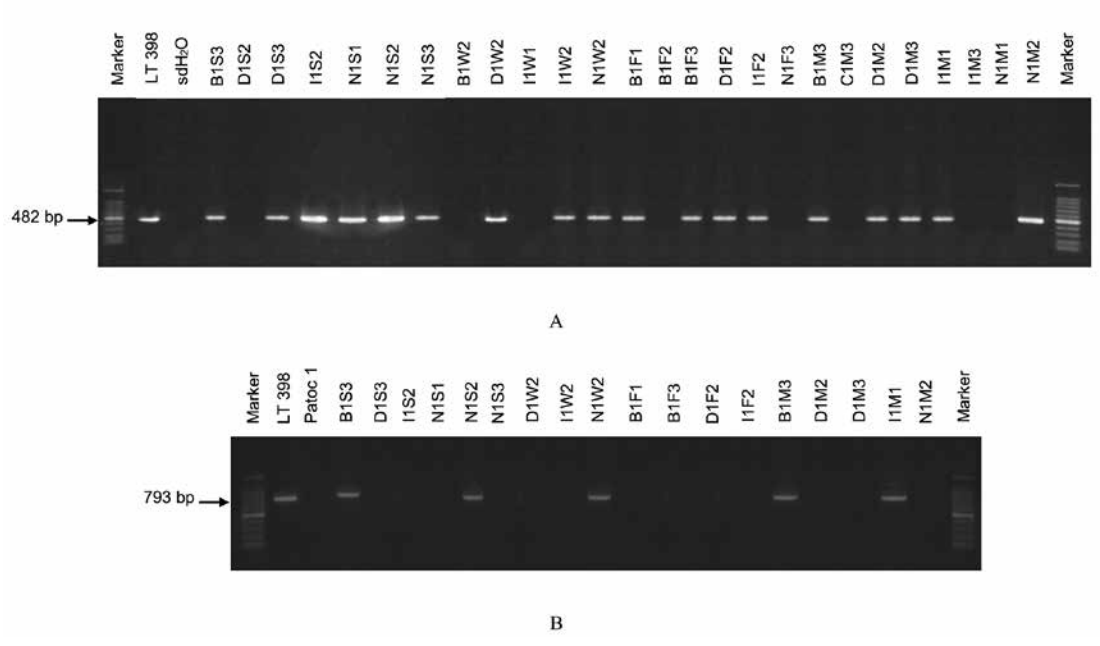

Fig 1 – Amplicons of *Leptospira rrl* (A) and *fla*B (B) among twenty-six leptospires culture-positive samples from vicinities of abattoirs in Cavite Province, the Philippines collected during February - August 2015. Water and soil samples were cultured in Kolthof medium for one month, and leptospires isolates were analyzed for carriage of *Leptospira rrl* and *fla*B (latter only among *rrl*-positive isolates) by PCR. Markers, DNA 100 bp size standards.

isolate B1M3 to *Leptonema illini* (GenBank accession no. JQ988853.1). On the other hand, 100% sequence similarity was observed for isolate B1S3 to *Leptospira* sp MS194 (GenBank accession no. AB758724.1); isolate N1S2 to *Leptospira interrogans* serovars Hardjoprajitno (GenBank accession no. CP013147.1), Hardjo (GenBank accession no. CP012603.1), Saxkoebing (GenBank accession no. KR107202.1), Bratislava (GenBank accession no. CP011410.1), Linhai (GenBank accession no. CP006723.1), Lai (GenBank accession no. NR074481.1), Sejroe (GenBank accession no. FJ154558.1), Australis (GenBank accession no. FJ154557.1), Icterohaemorrhagiae (GenBank accession no. NR116542.1), Bratislava (GenBank accession no. FJ154547.1), Bulgarica (GenBank accession no. AY996792.1) and Copenhageni (GenBank accession no. AE016823.1); and isolate N1W2 to *Leptospira* sp MS379 (GenBank accession no. AB758737.1) (Table 3).

DISCUSSION

Pathogenic leptospires in renal tubules of infected animal reservoir are shed through urine into the environment where they can survive in moist soil and surface water for up to several months (Smith and Self, 1955). Humans and other animals become infected mainly through their skin when they encounter leptospires-contaminated environment. Gloriani *et al* (2016) reported a significant association between wading in flood waters and leptospiro-

Table 3
Sequence similarities of isolates obtained from GenBank.

Isolate	*fla*B PCR	Serotype	16S rDNA	Percent similarity	GenBank accession number
B1S3	+	No reaction	MS194	100	AB758724.1
N1S2	+	No reaction	Hardjoprajitno	100	CP013147.1
			Hardjo	100	CP012603.1
			Saxkoebing	100	KR107202.1
			Bratislava	100	CP011410.1
			Linhai	100	CP006723.1
			Lai	100	NR074481.1
			Sejroe	100	FJ154558.1
			Australis	100	FJ154557.1
			Icterohaemorrhagiae	100	NR116542.1
			Bratislava	100	FJ154547.1
			Bulgarica	100	AY996792.1
			Copenhageni	100	AE016823.1
N1W2	+	No reaction	MS379	100	AB758737.1
B1M3	+	No reaction	*Leptonema illini*	99	JQ988853.1
I1M1	+	Serogroup Grippotyphosa	Grippotyphosa	99	EF536975.1

sis. Due to this association, isolation of leptospires in environmental sources is essential in epidemiological studies. In the present study, 25% of the environmental samples (water and soil) in the vicinities of abattoirs in Cavite Province were culture-positive for *Leptospira* spp.

Soil serves as an important reservoir for pathogenic leptospires. Factors affecting their growth are inhibitory compounds, acids, basic disinfectants, and detergents (Faine *et al*, 1999). In Cavite City, workers clean the abattoir using water from a deep well, which has a salinity of up to 3.0%, not allowing leptospires growth (Saito *et al*, 2013). This could explain why soil and water samples from the vicinity of Cavite City abattoir tested negative for leptospires cultures. However, Saito *et al* (2014) found 96% of soil samples obtained in the coastal areas of Leyte, Philippines after a storm surge during the super typhoon Yolanda were leptospires culture-positive and concluded that the isolates are able to survive in seawater for four days. In abattoirs of the cities of Bacoor, Dasmarinas and Imus, and the municipalities of Noveleta and Tanza, Cavite Province, tap water is used for cleaning. Most of the samples positive for leptospires culture were observed in these areas especially in Noveleta Municipality and Imus City; however, abattoirs of Dasmarinas City and Tanza Municipality use detergent for cleaning their abattoirs and hence leptospires were not recovered from cultures of these environmental samples.

The moisture content of soil could also influence the frequency of positive leptospires culture isolation. Moisture content of >20% allows leptospires to grow and to move and reach the hosts (Saito *et al*, 2013). In addition, soil moisture can provide leptospires the needed minerals and growth factors. The soil moisture content in the present study was comparable to that (3.5-42.8%) reported by Saito *et al* (2013).

Aside from moisture content, pH can also affect growth of leptospires (Adler and dela Pena Moctezuma, 2010). With the aid of HEPES buffer, the pH of leptospires samples maintained at 7.0 allows for growth (Saito *et al*, 2013), a pH value within the range measured in our culture-positive environmental samples.

Although Korthof medium is a selective medium for *Leptospira*, it is possible that other members of family Leptospiraceae, such as *Leptonema*, can grow in this medium (Hovind-Hougen, 1979), even in the presence of the antimicrobials STAFF, as evidenced in our study (isolate B1M3 collected from mud). Carriage of *rrl* was reported to be capable of detecting all *Leptospira* spp and differentiate strains of pathogenic *Leptospira* genospecies from *Leptonema illini*, *Escherichia coli*, and strains of *Leptospira biflexa* (Woo *et al*, 1997), this was the case in the current study. Kawabata *et al* (2001) applied RFLP-PCR to detect *fla*B in pathogenic *Leptospira*. Saito *et al* (2013) found all *Leptospira* isolates from water samples in Metro Manila and from the soil and water samples in the province of Nueva Ecija are *fla*B-negative indicating the leptospires are non-pathogenic, although one isolate from Nueva Ecija Province is an intermediate pathogenic *Leptospira licerasiae*. Of the five pathogenic leptospires isolates in the present study, one isolate (I1M1) was found to belong to serogroup Grippotyphosa, while the other four did not react to any of the monoclonal and polyclonal antibodies tested.

Three basic factors are involved in the transmission of leptospiral infection, namely, animal carrier, suitable environment for leptospires survival and hu-

mans/animal exposure to infected environment (WHO and ILS, 2003). Rat is well known to be the most important chronic carrier of leptospirosis (Levett, 2001). In the Philippines, the most common infecting serovars in rat based on MAT are Hebdomadis, Losbanos, Manilae, Poi, and Ratnapura (Villanueva *et al*, 2010). In the recent study of Tabo *et al* (2018) in the Philippines, serovars Losbanos, Manilae and Poi were detected in slaughtered animals and serovars Losbanos, Poi, and Ratnapura in workers near the sampling sites. The presence of five pathogenic leptospiral isolates in soil and water in the vicinities of abattoirs suggests the possibility that some of the abattoir workers and slaughtered animals were infected. As mentioned earlier, rats were observed in the abattoirs and hence slaughtered animals were most likely exposed to rat excreta and urine.

Human leptospirosis results from direct exposure to carrier animals or from exposure to environment contaminated by urine of infected animals. In the present study, the presence of pathogenic leptospires in abattoir environmental samples suggests the possibility of indirect transmission from abattoir surroundings to slaughtered animals and abattoir workers. This knowledge could be used in improving control measures designed to inhibit leptospires viability, such as cleaning abattoirs with sodium hypochlorite solution or detergents, appropriate treatment of waste water, requirement for abattoir workers to wear protective gear, particularly rubber boots, during slaughtering of livestock. Diagnostic tests can be developed using serovar Grippotyphosa and used for early diagnosis of leptospirosis and proper case management.

In summary, this study demonstrates that abattoir environment (soil and water) in Noveleta Municipality and cities of Bacoor and Imus in Cavite Province harbored pathogenic leptospires. Soil pH and moisture content as well as water pH favored growth of these leptospires.

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