Epidemiological investigation of pathogenic *Leptospira* spp. harbored by water buffalo (*Bubalis bubalis*) in the Philippines

（フィリピンの水牛(*Bubalis bubalis*)が保有する病原性レプトスピラの疫学的研究）

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CONTENTS

ABBREVIATIONS

EDTA	ethylenediaminetetraacetic acid
flaB	flagellin B
LAMP	loop-mediated isothermal amplification
MAT	microscopic agglutination test
MST	minimum spanning tree
OIE	World Organization for Animal Health (Spanish: Oficina Internacional de Epizootias)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
rrs	16S ribosomal RNA
WHO	World Health Organization

PREFACE

Leptospirosis is an important re-emerging zoonotic disease worldwide [1, 7, 26, 34], and is predominantly found in impoverished populations-inhabiting countries with tropical or subtropical climates [11, 45]. The disease is caused by spirochetes from the genus *Leptospira* [1, 34], which includes at least 21 species that can be divided into pathogenic, intermediate pathogenic and non-pathogenic groups [33]. Of significant importance is the pathogenic group that has been classified into over 250 distinct serotypes and cause disease in human and other animals of varying severity, ranging from subclinical infections to severe disease and death. Most, if not all, severe disease is caused by serovars belonging to the evolutionarily-related species *L. interrogans, L. kirschneri,* and *L. noguchii* [33], while *L. borgpetersenii* is responsible for the decreased animal production in livestock [14, 15, 33]. Transfer of infection usually occurs either by direct contact with the urine or tissues of an infected animal [1, 34] or indirectly through urine-contaminated environment such as water and soil (Figure 1). These pathogenic leptospires are carried by most mammalian species and are maintained by the persistent colonization of the proximal tubules of their kidney [7, 40]. Synanthropic rodents are the most important maintenance hosts; however, other animal species such as wildlife, domestic pets and farm animals can act as significant reservoirs of leptospires [14].

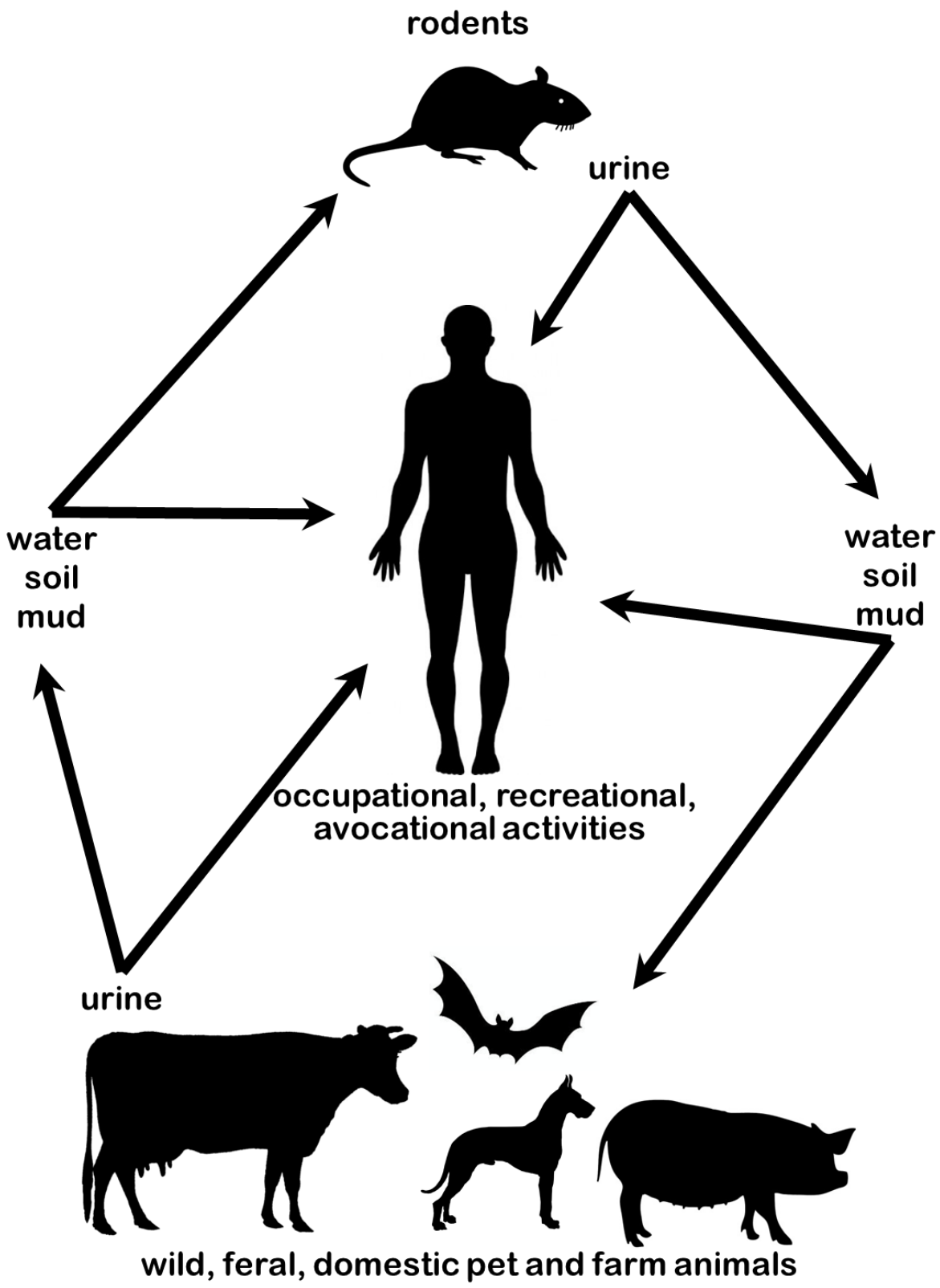

Figure 1. Transmission cycle of pathogenic *Leptospira*.

Leptospirosis in animals, especially in large ruminants continues to be a global economic problem (Figure 2) as a result of infection by a variety of *Leptospira* serovars [14, 15, 42]. Bovine leptospirosis causes significant reproductive problems such as abortion, increased number of services per conception, prolonged calving intervals, stillbirth, infertility, pre-mature birth, weak newborn calf with decreased daily weight gain and agalactia in lactating cows leading to important, but not quantified, economic hazards [1, 14, 15, 36, 41, 42]. Cattle are known to maintain serovar Hardjo (*L. borgpetersenii* serovar Hardjo subtype Hardjobovis and *L. interrogans* serovar Hardjo subtype Hardjoprajitno) that often leads to subclinical and persistent infection of the reproductive tract [14, 36, 41]. Infected animals become carriers harboring leptospires in the renal tubules and intermittently shedding into the environment for extended periods [1, 14, 34]. Acute leptospirosis in this animal is uncommon, and is characterized by pyrexia, hemolytic anemia, hemoglobinuria, jaundice, occasional meningitis, and death that are associated with infections from serogroups Icterohaemorrhagiae, Canicola, Hebdomadis, Sejroe, Pyrogenes, Autumnalis, Australis, Javanica, Tarassovi, Grippotyphosa and Pomona in several parts of the world [14, 34, 36]. Domestic water buffalo, another important livestock especially in several Asian, South American and Mediterranean countries, had reported cases of *Leptospira* infection [4, 5, 31, 38, 49]. However, limited studies on leptospirosis among water buffalo are available as it was claimed to have a similar scenario with that of cattle [14]. Nevertheless, it is important to investigate the disease situation in water buffaloes because the epidemiological scenario that is currently acknowledged may not represent what really occurs in many parts of the world, particularly on tropical regions where leptospirosis is endemic [36].

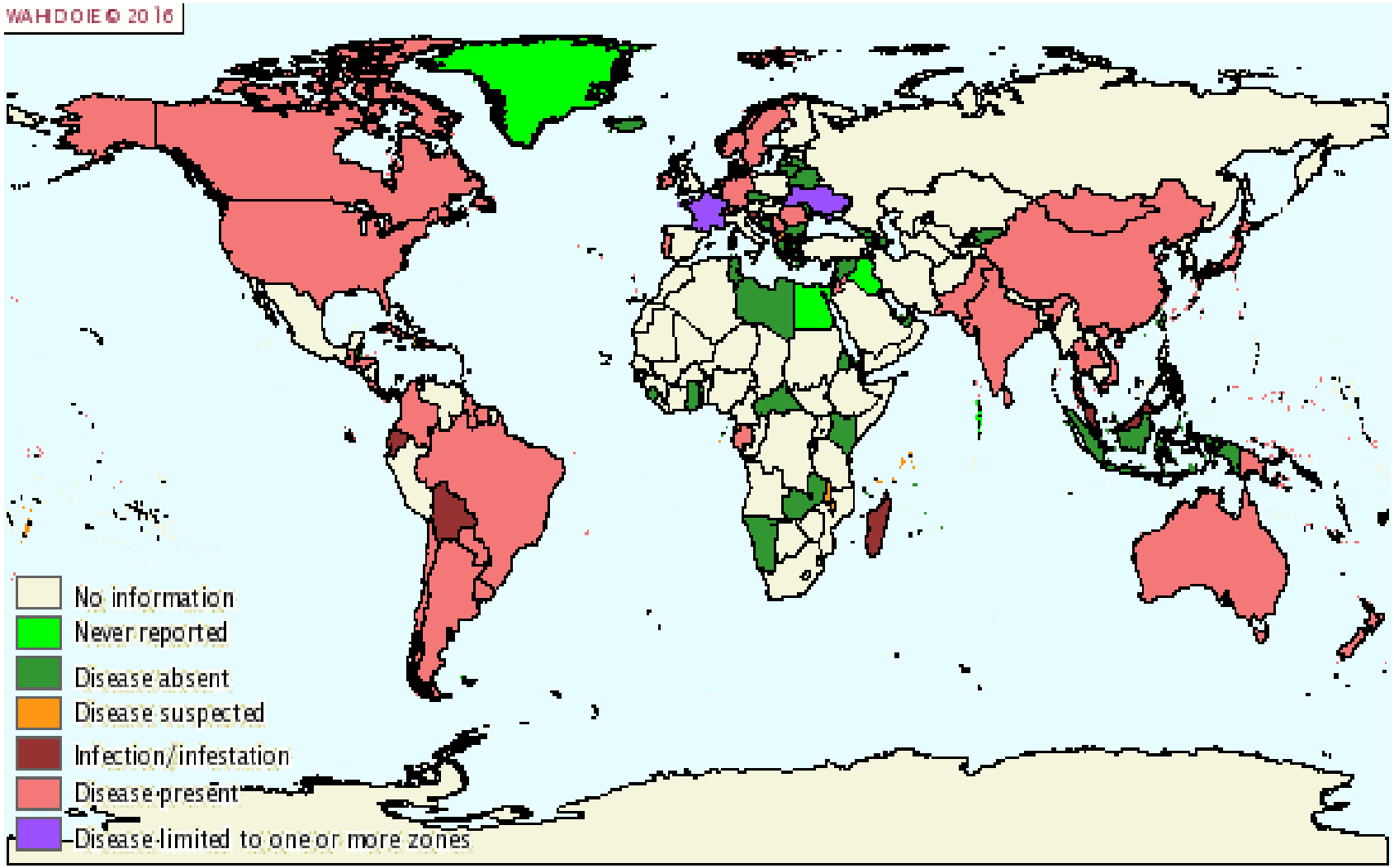

Figure 2. Reported cases of animal leptospirosis worldwide as of 2016 from OIE [43].

Domestic water buffalo (*Bubalus bubalis*) is an indispensable livestock in the Philippines and is favored over cattle as a source of milk since it is well adapted in hot weather, poor pasture area and requires low maintenance of management and as shown by a higher population number in the latest large ruminant inventory in 2016 [46] (Figure 3). Cases of abortion and mastitis or agalactia among water buffaloes were reported but were often attributed to protozoal [30, 53] and bacterial infections [47], excluding the possibility of leptospires as the causative agent. The Philippines is a leptospirosis-endemic country [3, 39, 50, 52, 55], however, *Leptospira* infection in water buffalo seems neglected as evidenced by few serological studies that were conducted since 1970's, where two independent studies showed anti-*Leptospira* antibodies in water buffaloes against serovars Tarassovi, Sejroe and Poi and against serovars Pyrogenes, Pomona and Grippotyphosa, respectively [6, 10]. It is believed the economic damage that leptospirosis causes to the livestock industry is considerable, but the extent of this damage is yet to be thoroughly assessed in the Philippines. In addition, the role of water buffalo as a potential reservoir host is a possibility considering its close contact with humans in the rural farming communities of the country [55]. Indeed, the effect of leptospirosis is underestimated due to lack of awareness of the disease and relatively inaccessible and insufficiently rapid diagnostic tests [7, 23].

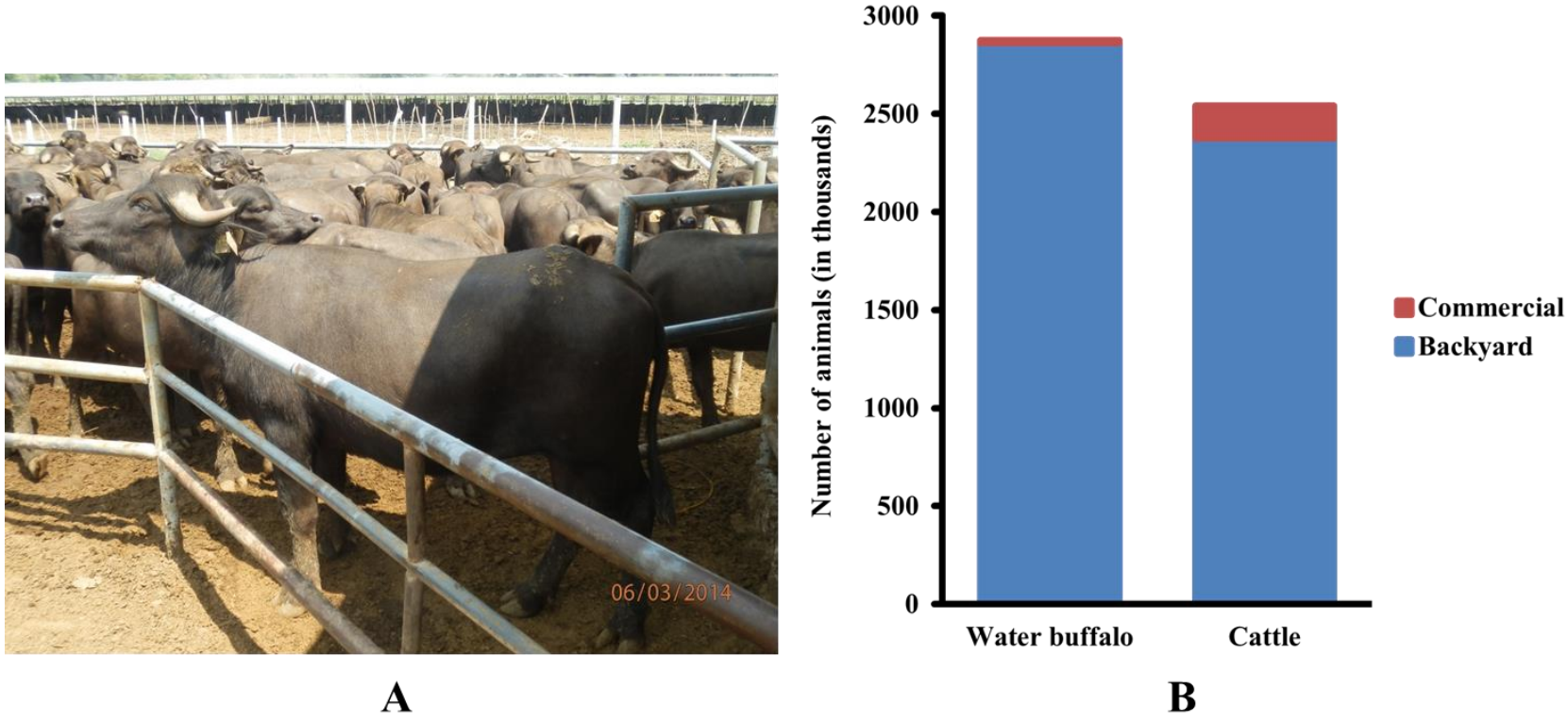

Figure 3. A: A riverine water buffalo in the Philippines that is used for milking purposes; B: The current inventory of water buffalo and cattle in the Philippines which showed higher populations of water buffaloes, particularly those managed in backyard setting [46].

The present thesis consists of three chapters; in chapter I, I did the serological investigation of *Leptospira* infection and its circulation in one intensive-type water buffalo farm in the Philippines. Then in chapter II, I did the molecular investigation of the similar animals tested in chapter I and compared and analyzed the results I obtained from both tests. Finally, in chapter III, I elucidated the molecular epidemiology of pathogenic *Leptospira* spp. among large ruminants from various water buffalo and cattle farms in the country.

Chapter I

Serological investigation of *Leptospira* infection and its circulation in one intensive-type water buffalo farm in the Philippines

Introduction

Leptospirosis in water buffalo has been neglected in the Philippines. Limited serological studies on this animal dated back in the 1970's demonstrated the presence of anti-*Leptospira* antibodies against serovars that may cause reproductive failures in water buffaloes as well as a possible source of infection to humans [6, 10]. Leptospirosis in human and other animals is often attributed to rodents as the source of infection and the presence of reproductive problems in water buffalo such as abortion and agalactia are associated with brucellosis, trypanosomiasis and staphylococcal/ streptococcal infections, where these disease signs can also be seen in leptospirosis. Our previous study applied the loop-mediated isothermal amplification (*Lepto-rrs* LAMP) assay for the rapid detection of pathogenic *Leptopira* DNA in animal urine, where we detected 20% (10/51) of positive water buffaloes [29]. Interestingly, I conducted a preliminary study of screening the urine samples from water buffaloes, cattle, pigs and goats in close confinement setting using *Lepto-rrs* LAMP and found that only water buffalo samples were positive (unpublished work).

A basic and updated knowledge of *Leptospira* serovars or serogroups that infect animals is important to a better understanding of the epidemiology of leptospirosis in a determined region [36]. Therefore, I aimed to investigate *Leptospira* infection and its circulation in an intensive-type water buffalo farm, which is the major source of riverine-type water buffaloes for smallholder farming communities in the country. I determined by microscopic agglutination test (MAT) the prevailing *Leptospira* serogroups in rats infesting a

farm as well as the water buffalo herd. I also assessed the variables associated with *Leptospira* seropositivity in the study.

Materials and Methods

Sample collection

A total of 170 serum samples from several groups (pre-weaned calves: 29 samples; post-weaned calves: 35; yearlings: 35; bulls: 24, heifer/pregnant cows: 20; lactating cows: 27) of water buffaloes were collected at an intensive-type farm in the Philippines in June, 2014. During sample collection, it was confirmed that the farm did not carry out vaccination against leptospirosis, although visual inspection did not detect obvious health problems in the animals. Blood samples from field rats (n=21) captured alive in the vicinity of the farm were also collected. Under aseptic condition, blood samples were collected from rats through cardiac venipuncture with sterile 3 ml syringes, and from water buffaloes via the jugular vein using regular 10 ml glass evacuated blood collection tubes. Collected blood samples were incubated at room temperature until coagulation was completed, and then they were centrifuged at 3,000× *g* for 10 minutes. Sera were then separated from samples, aliquoted in 2.0 ml microtubes and stored at -80 ºC until further use. For the statistical analysis of infection risks, information on risk variables such as age, sex and management (i.e., animal grouping within the farm) of water buffaloes was recorded.

Microscopic agglutination test

The MAT was conducted according to WHO guidelines [54], at the College of Public Health, University of the Philippines-Manila, the Philippines. The test consisted of two stages in succession, namely, the screening test for determining reactive serogroups and the quantitative test for determining the maximum serum titer for the resulting positive serogroups. For the screening test, heat-inactivated serum samples were first diluted to 1:10 in phosphate buffered saline (PBS; pH 7.6). Next, 25 µl of each serum solution and aliquots of

approximately 1-2 × 10^8 leptospires/ml from the culture of a battery of 39 *Leptospira* reference and local strains (Table 1) were mixed in a sterile 96 flat-bottomed well plate (Becton Dickinson, USA) to a final serum dilution of 1:20. After 2-hr incubation at 30 ℃ in a dark room, serum-antigen mixtures were examined under dark field microscopy. Serum samples with >50% agglutination were subjected to the quantitative MAT. The quantitative MAT was carried out by preparing 2-fold serial dilutions of sera to determine the highest antibody titer for each positive antigen. The endpoint titer was defined as the highest serum dilution reacted with at least one strain. The cutoff titer of the MAT for water buffalo samples was set at 1:40 [48, 49].

Statistical analysis

Associations of MAT results with age, sex and animal grouping were estimated by either the Chi-square test or the Fisher's exact test. The calculations were carried out with SPSS software version 23.0 (IBM Corp., Armonk, NY, USA).

Table 1. The 39 *Leptospira* strains used for MAT[a] in the study.

Species	Serogroup	Serovar	Strain
Leptospira borgpetersenii	Javanica	Javanica	K6
		Javanica	Veldrat Batavia 46
		Poi	Poi
	Tarassovi	Tarassovi	Perepelitsin
	Autumnalis	Ballum	Fort Bragg
	Celledoni	Anhoa	LT 90-68
	Mini	Mini	Sari
	Sejroe	Sejroe	M 84
Leptospira fainei	Hurstbridge	Hurstbridge	BUT 6T
Leptospira noguchii	Louisiana	Louisiana	LSU 1945
	Panama	Panama	CZ 214 K
Leptospira alexanderi	Manhao	Manhao 3	L60
Leptospira weilii	Sarmin	Sarmin	Sarmin
Leptospira santarosai	Shermani	Shermani	1342 K
Leptospira interrogans	Pyrogenes	Manilae	LT 398
		Manilae	K64
		Pyrogenes	Salinem
	Canicola	Canicola	Hond Utrecht IV
	Autumnalis	Autumnalis	Akiyami A
	Bataviae	Los banos	LT101-69
		Los banos	K37
	Hebdomadis	Hebdomadis	Akiyami B
		Hebdomadis	Hebdomadis
	Australis	Australis	Akiyami C
		Australis	Ballico
	Icterohaemorrhagiae	Copenhageni	M20
		Icterohaemorrhagiae	RGA
		Icterohaemorrhagiae	Ictero No. 1
	Pomona	Pomona	Pomona
	Sejroe	Hardjo	Hardjoprajitno
	Djasiman	Djasiman	Djasiman
Leptospira kirschneri	Grippotyphosa	Grippotyphosa	K5
		Ratnapura	UP-BL-FR13
		Grippotyphosa	Moskva V
	Cynopteri	Cynopteri	3522 C
Leptospira meyeri	Semaranga	Semaranga	Veldrat Semarang 173
Leptospira biflexa	Semaranga	Patoc	Patoc I
	Andamana	Andamana	CH 11
Leptospira yanagawae	Semaranga	Sao Paolo	Sao Paolo

[a]Microscopic agglutination test.

Results

Although 21 blood samples were initially collected from rats, only 10 were subjected to the MAT due to the insufficient volume of the other 11 samples. Only three (30%) rat serum samples reacted with single serogroups Pomona (1:320), Canicola (1:1280) and Icterohaemorrhagiae (1:160).

Eighty one out of 170 (48%) water buffalo serum samples contained anti-*Leptospira* antibodies against at least one of the 39 strains investigated (Table 2). The predominant reactive serogroups were Mini (25/81, 30.9%), Hebdomadis (25/81, 30.9%), Tarassovi (13/81, 16%) and Pyrogenes (11/81, 13.6%) (Table 3). The number of seropositive water buffaloes younger than one year of age, i.e., pre- and post-weaned calves, was lower than that of older seropositive water buffaloes (Tables 2 and 4). While younger water buffaloes presented low MAT titers to *Leptospira* strains (Figure 4), older animals showed variable MAT titers, including the highest titer against serogroup Mini (Figure 4 and Table 3). Fifty five (68%) and 26 (32%) seropositive serum samples agglutinated single and multiple serotypes, respectively (Tables 2 and 4). Samples from pre- and post-weaned calves contained anti-*Leptospira* antibodies against single serogroups (Tables 2 and 4). In contrast, 30-40% of older water buffaloes exhibited seropositivity against multiple serogroups (Figure 5, Tables 2 and 4). In addition, antibodies against serogroups Icterohaemorrhagiae and Pomona were detected in sera of both rats and water buffaloes.

Table 2. Seroprevalence in sera of rats and buffaloes from different groups.

Animals/ buffalo groups	Number of animals	Seropositive animals	Seropositivity rate (%) (95% CI)	Seropositive animals reacting with single serotype (%)	Seropositive animals reacting with multiple serotypes (%)
Water buffalo (total)	170	81	48 (40-55.1)	55 (68)	26 (32)
Pre-weaned calves	29	3	10 (2.7-26.4)	3 (100)	0 (0)
Post-weaned calves	35	3	9 (2.2-22.4)	3 (100)	0 (0)
Yearlings	35	20	57 (39.5-72)	14 (70)	6 (30)
Bulls	24	21	88 (66.5-95.7)	14 (67)	7 (33)
Heifer/ pregnant cows	20	15	75 (50.6-88.8)	9 (60)	6 (40)
Lactating cows	27	19	70 (49.7-84.1)	12 (63)	7 (37)
Rat	10	3	30 (8.1-60.3)	3 (100)	0 (0)

Table 3. Distribution of water buffaloes exhibiting different MAT[a] titers against *Leptospira* strains[b].

Serogroup	Serovar	Strain	Number of serum samples exhibiting the following reciprocal MAT titers[c]									Total number of positive samples[d]
			40	80	160	320	640	1280	2560	5120	10240	
Mini	Mini	Sari	2	5	6	3	2	2	1	2	2	25
Hebdomadis	Hebdomadis	Hebdomadis	1	8	7	3	1	2				22
Tarassovi	Tarassovi	Perepelitsin	2	2	3	2	1	3				13
Pyrogenes	Manilae	LT 398	2	3		1						6
Cynopteri	Cynopteri	3522 C	2	3	1							6
Sejroe	Hardjo	Hardjoprajitno		2	3							5
Bataviae	Los banos	K37	1	1		2						4
Icterohaemorrhagiae	Copenhageni	M20	2	1	1							4
Shermani	Shermani	1342 K	1	1	2							4
Hurstbridge	Hurstbridge	BUT 6T	2		1	1						4
Pyrogenes	Manilae	K64	1	1		1						3
Hebdomadis	Hebdomadis	Akiyami B		1	2							3
Pomona	Pomona	Pomona	1				1	1				3
Sejroe	Sejroe	M 84			2		1					3
Semaranga	Patoc	Patoc I	1	1	1							3
Pyrogenes	Pyrogenes	Salinem	1	1								2
Autumnalis	Autumnalis	Akiyami A		1				1				2
Grippotyphosa	Grippotyphosa	K5		1			1					2
Bataviae	Los banos	LT101-69				1						1
Grippotyphosa	Ratnapura	UP-BL-FR13					1					1
Louisiana	Louisiana	LSU 1945			1							1
Semaranga	Sao Paolo	Sao Paolo	1									1

[a]Microscopic agglutination test.

[b]Negative *Leptospira* strains are not listed in this table.

[c]Highest reactive serovars of each positive sample are described.

[d]Twenty six of 81 positive samples reacted equally with multiple serovars.

Table 4. Number per group of water buffaloes of which sera reacted with *Leptospira* strains[a] used in MAT[b].

Serogroup	Serovar	Strain	Number of serum samples per group containing antibodies against *Leptospira* serovars[c]					
			Pre-weaned calves	Post-weaned calves	Yearlings	Bulls	Heifer/ pregnant cows	Lactating cows
Mini	Mini	Sari	1		7	9	4	4
Hebdomadis	Hebdomadis	Hebdomadis			5	8	8	1
Tarassovi	Tarassovi	Perepelitsin					2	11
Pyrogenes	Manilae	LT 398			4	1	1	
Cynopteri	Cynopteri	3522 C			1	3	1	1
Sejroe	Hardjo	Hardjoprajitno			1	2	2	
Bataviae	Los banos	K37			1		1	2
Icterohaemorrhagiae	Copenhageni	M20		1	2	1		
Shermani	Shermani	1342 K	1			1		2
Hurstbridge	Hurstbridge	BUT 6T		2				2
Pyrogenes	Manilae	K64			2		1	
Hebdomadis	Hebdomadis	Akiyami B				2	1	
Pomona	Pomona	Pomona			1	1	1	
Sejroe	Sejroe	M 84			2		1	
Semaranga	Patoc	Patoc I				2		1
Pyrogenes	Pyrogenes	Salinem			2			
Autumnalis	Autumnalis	Akiyami A				2		
Grippotyphosa	Grippotyphosa	K5	1		1			
Bataviae	Los banos	LT101-69						1
Grippotyphosa	Ratnapura	UP-BL-FR13			1			
Louisiana	Louisiana	LSU 1945						1
Semaranga	Sao Paolo	Sao Paolo			1			

[a]Negative *Leptospira* strains are not listed in this table.

[b]Microscopic agglutination test.

[c]Highest reactive serovars of each positive sample are described.

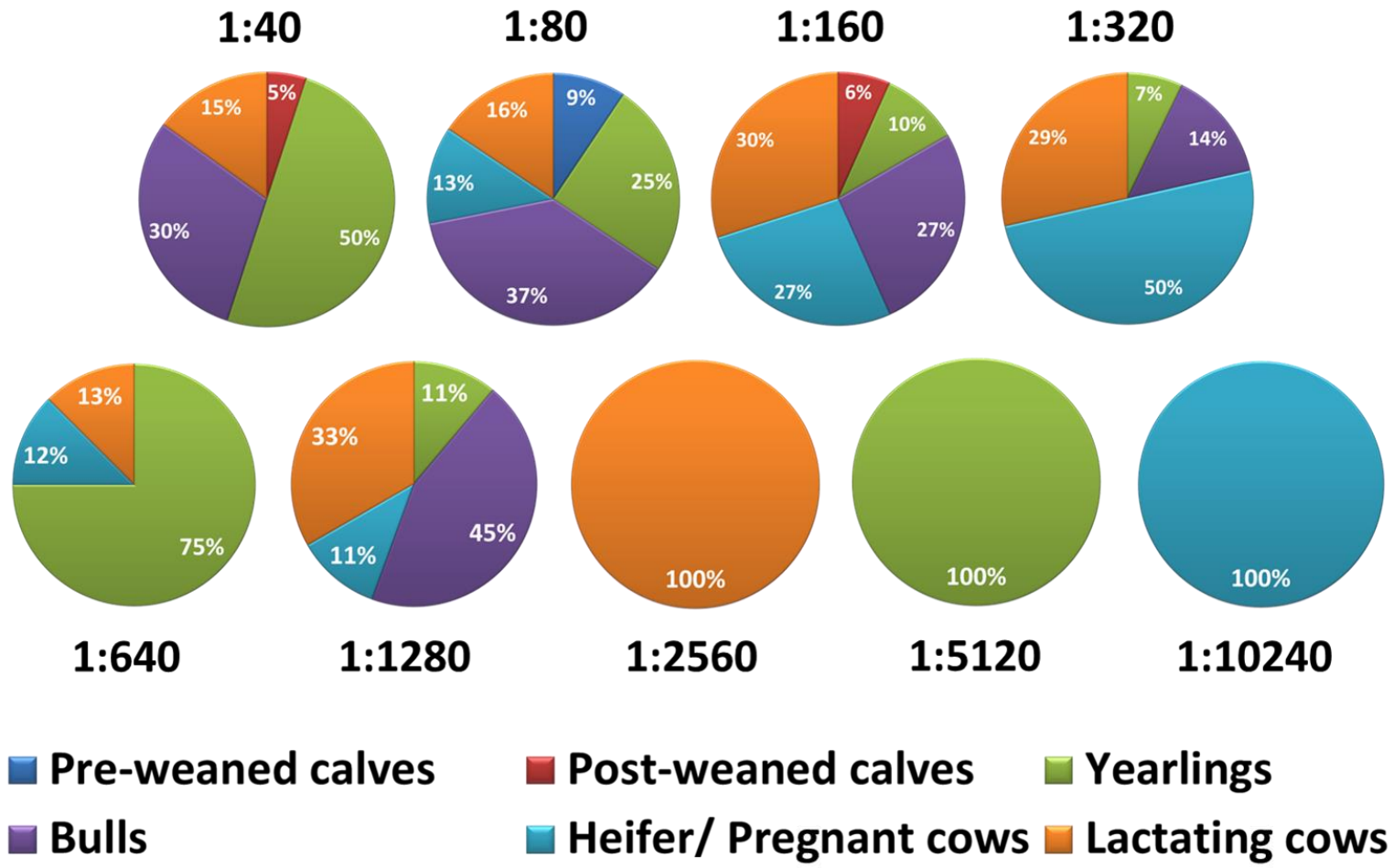

Figure 4. Proportions of seropositive water buffalo groups with respect to MAT titers.

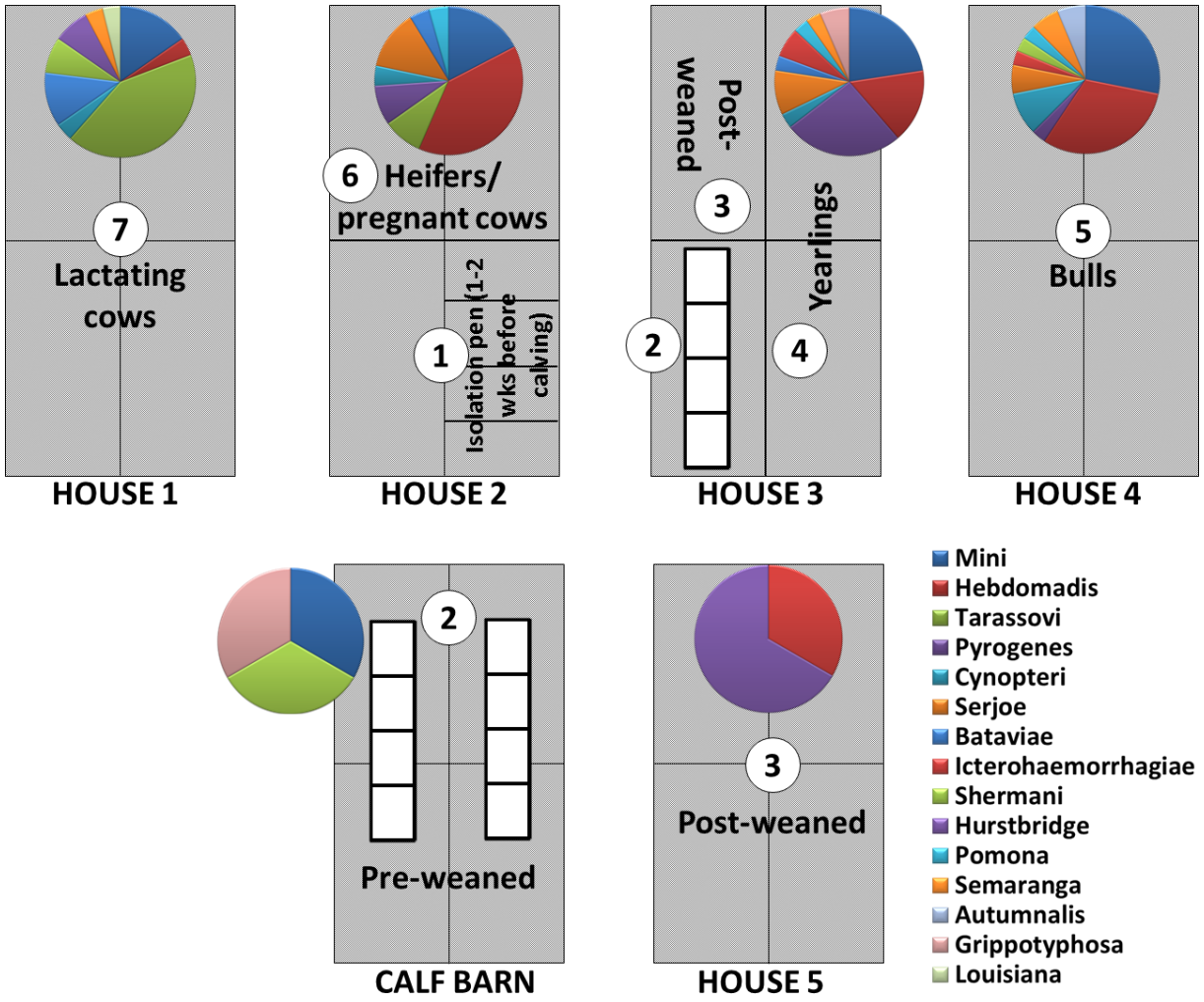

Figure 5. Layout of the farm, including the movement of animals starting at birth in ① followed by succeeding movement as indicated by numbers. Pie graphs indicate the proportion of reacting serogroups in each group of buffaloes.

The results from the univariable analysis of seropositive samples and epidemiological variables such as age, sex and animal grouping are summarized in Table 5. Seropositivity was significantly associated with age and animal grouping variables ($P < 0.0001$), but not with the sex variable.

Table 5. Association between *Leptospira* serostatus and identified risk variables.

Variables	Categories	No. of samples	No. of positive (%)	*P* value
Age	< 1 year	69	8 (11.6)	0.0001
	> 1-2 years	56	40 (71.4)	
	> 2 years	45	33 (73.3)	
Sex	Male	72	32 (44.4)	0.474
	Female	98	49 (50)	
Animal groups	Pre-weaned calves	29	3 (10.3)	0.0001
	Post-weaned calves	35	3 (8.6)	
	Yearlings	35	20 (57.1)	
	Bulls	24	21 (87.5)	
	Heifer/ pregnant cows	20	15 (75)	
	Lactating cows	27	19 (70.4)	

Discussion

MAT is considered the gold standard serological test for leptospirosis. It is commonly used as a reference test for epidemiological studies, although is often tagged as tedious and hazardous as it requires maintenance of live pathogenic leptospires. In this study, MAT was carried out using 39 *Leptospira* strains that were composed of reference and local strains. There is no established cutoff point on MAT titer for water buffalo. The 1:100 titer recommended by the World Organization for Animal Health (OIE) is considered positive for international trade, but lower MAT titers may indicate previous exposure to *Leptospira* [43]. Thus, I decided to use 1:40 as the cutoff titer, which has been considered positive in previous studies [48, 49]. Serum samples from older water buffaloes (> 1 year old) exhibited higher MAT titers against multiple serogroups than younger water buffaloes (Figure 4, Tables 2-4). These findings were consistent with those previously observed by Suwancharoen et al. (2013), which showed that seroprevalence in water buffaloes was directly proportional with age. The higher MAT titers and variability of reacting serogroups can be explained by the greater and repetitive chance of older animals to being exposed to different *Leptospira* serogroups (serovars). Moreover, reaction to multiple serogroups in adult water buffaloes can be explained by cross-reaction within related serogroups [21], paradoxical reaction [34, 35] or mixed infections [14].

Higher *Leptospira* prevalence in older water buffalo groups regardless of sex suggested that management (i.e., animal grouping) was crucial for the transmission of *Leptospira* in the farm, which was statistically supported (Table 5). Pre-weaned calves are housed in elevated and slatted individual pens soon after birth while older water buffaloes are kept in group pens. Thus, it is believed that introduction of naïve (pre-weaned) animals into group pens causes them to acquire leptospires. Pools for wallowing of adult water buffaloes

may also allow infection to disseminate and, unlike cattle, the characteristic wallowing behavior by water buffalo is probably associated with 3-fold higher seropositivity [49]. These management settings, especially in intensive dairy farming, are estimated to be the main source of the disease in farms, which results in continuous infection of susceptible animals [14]. In addition, intensive farm settings lead themselves to high seroprevalence due to high animal stocking rates that result in easy transmission of *Leptospira* [4]. Although the current study may serve as a basic model for studying *Leptospira* infection and circulation in intensive-type animal farms, it may be worth comparing it with other management systems in the Philippines, because circulation of leptospires may differ in each management type. Finally, appraisal of the wastewater flow system as a source of infection in this study was deemed unnecessary because waste from each housing facility was drained separately.

The predominant reactive serogroups in water buffaloes in this study were Mini, Hebdomadis, Tarassovi and Pyrogenes (Figure 5, Tables 2-4). Two independent studies demonstrated anti-*Leptospira* antibodies in water buffaloes in the Philippines against serovars Tarassovi, Sejroe and Poi and against serovars Pyrogenes, Pomona and Grippotyphosa, respectively [6, 10]. Discrepancy between the positive serogroups detected in previous studies and the present work may be attributed to the fact that our study focused only on a single farm. Nevertheless, positive serogroups in this study were similar to those previously detected in neighboring countries such as Thailand and Malaysia [4, 49]. In contrast, predominant *Leptospira* serogroups (serovars) were different than those reported in water buffaloes in South America. Indeed, antibodies against serovars Pomona, Canicola, Grippotyphosa, Pyrogenes, Wolffi and Hardjo were predominantly detected in the northeastern part of Argentina [31], whereas that was not the case in the present work (Figure 5, Tables 2-4). Current differences in the predominant serogroups in each region can be attributed to external environmental factors and the presence of different animals as reservoir hosts for *Leptospira*

[14]. However, importation of riverine-type water buffaloes from South American and Mediterranean countries is permitted in the Philippines, which may cause the introduction of different *Leptospira* serogroups (serovars) if proper quarantine procedures are not implemented. Therefore, it is recommended that strict monitoring on the possible entry of this bacterium be established in the Philippines.

Rats are major reservoir animals of *Leptospira* and hence are expected to have low or static antibody titer [14], which is consistent with what was observed in the current study. The detection of antibodies against the same serogroups in water buffaloes and rats may indicate the contact of water buffaloes with forage, drinking water or environmental elements contaminated with rat urine or carcasses of rats killed by agriculture machinery on the field. Nevertheless, due to the limited number of samples used in this study, the involvement of rats in the *Leptospira* transmission within the farm was not conclusive. Further studies are needed to determine the actual role of rodents in the maintenance of *Leptospira* infection in the farm.

Impaired reproductive performance is one of the significant impacts of leptospirosis on large ruminants. Bulls, pregnant and lactating cows showed high seroprevalence in this study. Previous studies showed the presence of *L. interrogans* serovar Hardjo in the genital tract of infected cows and bulls, which suggest importance of venereal transmission and artificial insemination in the epidemiology of bovine leptospirosis [14, 37]. *Leptospira* infection in bulls is often subclinical [37], and clinical manifestation is inapparent. Pathogenic leptospires were found to reside in seminal vesicle [37], sustaining the venereal spread of infection. For example, intrauterine infection with leptospires can cause abortion, stillbirth and fetal problems in the late term of gestation [14]. In addition, leptospirosis has a profound influence on lactating animals as it causes mastitis [49] and agalactia [14]. It is well known the involvement of *Trypanosoma evansi* in abortion, and *Streptococcus* and *Staphylococcus* spp. in mastitis/agalactia in water buffaloes in the Philippines [30, 47, 53]. However, little

information is available on the involvement of leptospirosis in reproductive failures in water buffaloes and it was not investigated in the present study. Nonetheless, infertility cases in water buffaloes have been reported in the region of study [30]. Thus, future work needs to investigate whether or not reproductive problems in water buffaloes are caused by *Leptospira* infection.

Similar to cattle, water buffaloes can be potential carriers of pathogenic leptospires, although information on water buffaloes as persistent carriers remains scarce [14]. Nevertheless, evidence of the status of water buffaloes as carriers and a possible source of infection to humans has been found in India [5]. Indeed, based on partial *rpoB* sequences, previous evidence showed that buffaloes harbored pathogenic and intermediate *Leptospira* species that were similar to those found in human patients. The present study focused only on serological prevalence in a single water buffalo herd. Previous serological studies on human leptospirosis in the Philippines and surrounding areas where the present study was conducted found that serogroups Pyrogenes, Bataviae, Grippotyphosa and Tarassovi were predominant which were also found in this study [52, 55]. Therefore, accurate characterization of the role of water buffaloes as either accidental hosts or carrier animals in the Philippines is yet to be established. To that end, isolation and/or molecular detection techniques are likely to be required in the future to evaluate the zoonotic potential of water buffaloes.

Summary

A basic and updated knowledge of *Leptospira* serovars or serogroups that infect water buffalo is important to better understand the epidemiology of leptospirosis in the Philippines where available information is limited. In this study, I collected blood samples from rats (*n*=21), and water buffaloes (*n*=170) from different groups and locations in one intensive-type buffalo farm in the Philippines. Serum was analyzed by microscopic agglutination test (MAT). Anti-*Leptospira* antibodies reacting with serogroups Canicola, Icterohaemorrhagiae and Pomona were found in sera of 30% tested rats, and 48% of water buffalo sera tested positive for at least one *Leptospira* strain, in which serogroups Mini, Hebdomadis, Tarassovi and Pyrogenes were predominantly agglutinated. The number of seropositive young water buffaloes (<1 year-old) was lower than that of older seropositive ones. Furthermore, sera from younger water buffaloes were reactive with single serotypes with low MAT titers, but older animals were reactive with multiple *Leptospira* strains with variable MAT titers. In addition, antibodies against serogroups Icterohaemorrhagiae and Pomona were detected in both animals. Finally, *Leptospira* infection was found associated with age and animal grouping, highlighting the impact of management in the persistence of leptospirosis at intensive-type buffalo farm settings in the Philippines.

Chapter II

Molecular investigation of pathogenic *Leptospira* spp. circulation in a water buffalo communal farm in the Philippines

Introduction

My previous study demonstrated the widespread occurrence of leptospirosis in water buffalo herd in an intensive-type farm as shown by the presence anti-*Leptospira* antibodies reacting with serogroups Canicola, Icterohaemorrhagiae and Pomona in sera of 30% tested rats, and 48% of water buffalo sera tested positive for at least one *Leptospira* strain, in which serogroups Mini, Hebdomadis, Tarassovi and Pyrogenes were predominantly agglutinated [51]. However, although MAT is considered a reference method for serological diagnosis of leptospirosis, it is mainly used for epidemiological serosurveys and has low sensitivity in detecting the actual infected animals in the herd [1, 34]. Therefore, in connection with the seroprevalence of leptospires in water buffalo farm [51], this follow-up study aimed to identify the species and prevalence of pathogenic leptospires among water buffaloes in a communal farm in the Philippines using molecular techniques. Agreement between the results obtained from this study with the existing serological data was also evaluated.

Materials and Methods

Sample collection

Urine samples of all 170 water buffaloes from several age groups were collected in an intensive-type farm in the Philippines on June 2014. In addition, urine samples from 21 field rats that were captured alive in the vicinity of the farm were also collected. Serum samples from all of these animals were previously collected and analyzed for *Leptospira* seroprevalence study [51].

DNA extraction and leptospiral *flaB* nested PCR

The DNA from urine samples were extracted and performed within 24 hr after collection. The samples were centrifuged at 16,000 × *g* for 10 min at 10 ºC to collect leptospires in the sediments. The sediments were suspended with 37 µl of 10 mM Tris 1 mM EDTA (TE, pH 8.0) and boiled at 95 ºC for 10 min. A nested PCR with primers specifically designed to amplify the flagellin B gene (*flaB*) of pathogenic *Leptospira* spp. was applied to the extracted DNA samples (*n*=191) and performed as previously described [28].

Sequencing and phylogenetic tree analysis

The PCR products from the *flaB* nested PCR (691 bp partial *flaB* nucleotide sequences) were purified and subjected to direct sequencing using the ABI Prism BigDye Terminator v3.1 cycle sequencing kit (ThermoFisher Scientific, Carlsbad, CA, USA) and 3500 Genetic Analyzer (ThermoFisher Scientific). The data sequences were aligned and a maximum-likelihood phylogenetic tree with the JC69 model was constructed with 1,000 bootstrap replications using BioNumerics software v.6.0 (Applied Maths, Austin, Texas, USA). Representative *flaB* sequences from different pathogenic *Leptospira* species together

with the local isolates from the Philippines downloaded from the NCBI database were included in the phylogenetic tree. Minimum spanning tree (MST) was employed for illustration of the results.

Cloning

The amplicons in which overlapping peaks were observed by direct sequencing were subjected to DNA cloning. PCR products were cloned into a vector using the TOPO® TA Cloning kit (Invitrogen/ Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Five colonies were selected from each sample and the cloned *flaB* was amplified according to the manufacturer's instructions followed by DNA sequencing as described above.

Statistical analysis

Concordance between MAT and *Leptospira flaB* nested PCR results was performed by calculating the Cohen's Kappa value using SPSS software version 23.0 (IBM Corp., Armonk, NY, USA) with a 95% confidence interval.

Results

Detection of pathogenic *Leptospira* in water buffalo urine

A 619 bp fragment of *Leptospira flaB* gene was detected in 49 out of 170 (28.8%) water buffalo samples and in two out of 21 (9.5%) rat samples using *flaB*-nested PCR (Table 6). The animal groups' bull, heifer/ pregnant cow and yearling have the highest detection rates with 58.3%, 55% and 48.6%, respectively, while no positive samples were found among pre-weaned calf groups (Table 6).

Analysis of leptospiral *flaB* gene sequence

From the 49 *flaB* nested PCR-positive water buffalo samples, 33 have a determined nucleotide sequences by direct sequencing while 16 contained overlapping sequence peaks (Table 6). Interestingly, percentages of positive samples from bull, heifer/ pregnant cow and lactating cow with mixed *flaB* sequences range from 43-50% (Table 6). In addition, rat-positive samples have one with determined nucleotide sequence whereas the other has mixed sequence (Table 6). Mixed sequences where subjected to subcloning, and sequences from five colonies of each sample were analyzed for its similarity or difference.

Table 6. Details of animals' urine samples and their *flaB*-nested PCR results.

Animals	Urine samples	*flaB* nPCR positive	direct *flaB* sequencing	
		No. of samples (%)	Single	Mixed[a]
Water buffalo (total)	170	49 (28.8)	33	16
Pre-weaned calf	29	0 (0)		
Post-weaned calf	35	1 (2.9)		1
Yearling	35	17 (48.6)	16	1
Bull	24	14 (58.3)	8	6
Heifer/ pregnant cow	20	11 (55)	6	5
Lactating cow	27	6 (22.2)	3	3
Rat	21	2 (9.5)	1	1

[a]These samples showed overlapping peaks and subjected to DNA cloning.

Four major groups were formed upon creating the MST of the *Leptospira* partial *flaB* sequence. A cluster designated as Group I (G I) belonged to *L. borpetersenii* and is related with the sequence from local isolate CBPCC11-13 with Accession No. AB698542 (Figure 6-A) and is composed of water buffaloes samples from all positive animal groups (Figure 5-B). These samples also contained single and mixed leptospires (Figure 6-C). Group II (G II) is smaller that is also belonged to *L. borgpetersenii*. The samples came from field rat and all water buffalo groups of except yearling containing mixed *flaB* sequences (Figure 6). Group III (G III) is also included in the *L. borgpetersenii* cluster and has a related sequence with *L. borgpetersenii* (AB027169), (AB027173) and a local isolate, CBPCC11-4 (AB698541). The samples from this group are composed of all positive animal groups except post-weaned calf, and contain water buffalo samples with single and mixed sequences (Figure 6). Group IV (G IV), on the other hand is clustered with *L. kirschneri* (AB027170) (Figure 6-A) and is composed of samples from field rat and all water buffalo animal groups (Figure 6-B). The samples from this group contained single and mixed *Leptospira* sequence (Figure 6-C).

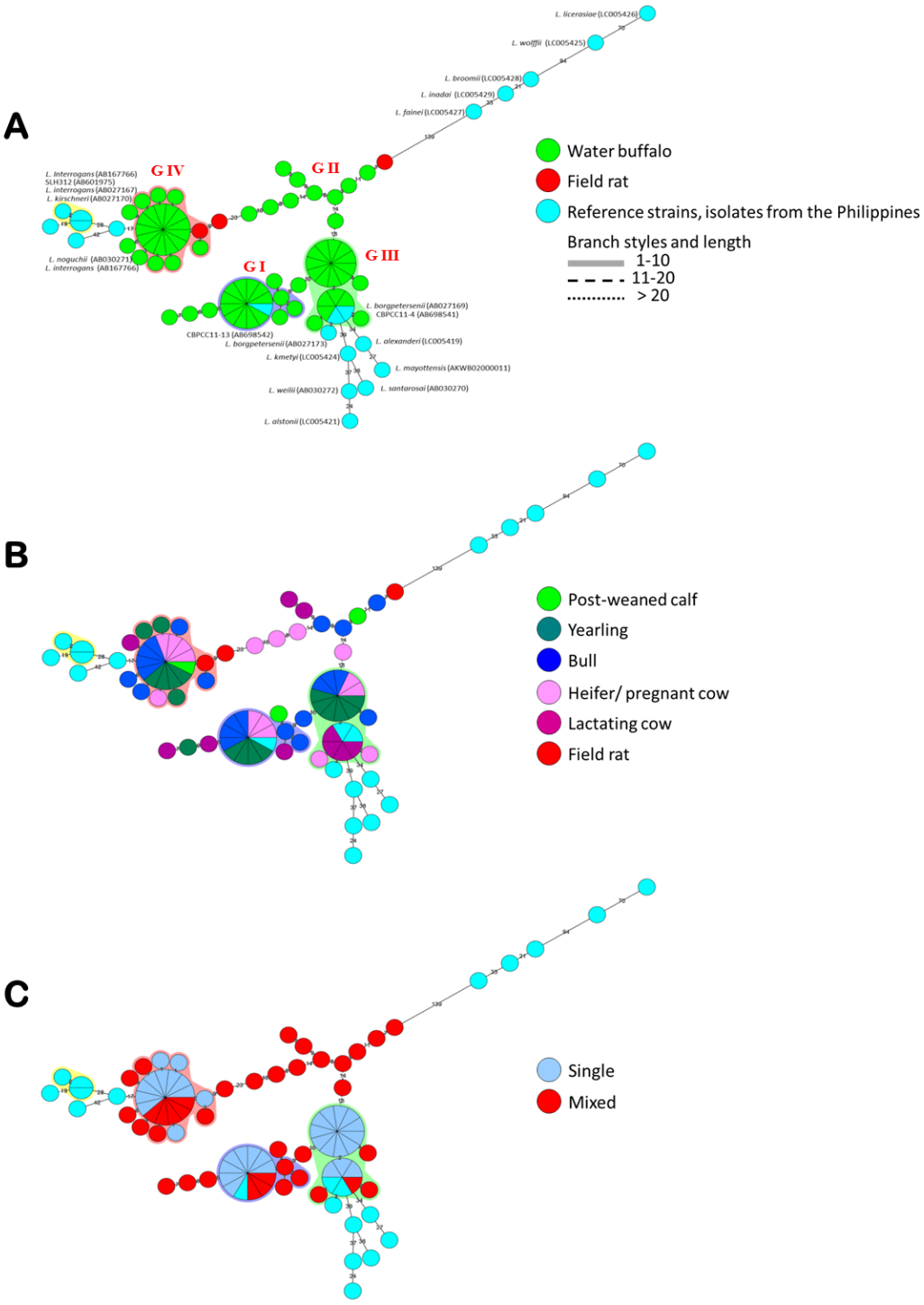

Figure 6. Minimum spanning tree (MST) based on the partial *flaB* sequences of *Leptospira* spp. detected from water buffalo and field rat. Four clusters were identified and designated as Group I (G I), Group II (G II), Group III (G III) and Group IV (G IV). A: MST based on the animal source; B: MST based on the water buffalo age groups, including rat samples, and; C: MST based on number of *flaB* sequences detected from animals.

From 16 water buffaloes with mixed sequences, six were from bulls, five from heifer/ pregnant cows, three from lactating cows and one each from post-weaned calf and yearling (Table 7). Two bulls, a lactating cow and a weaned calf were found to have three mixed *flaB* sequences while the remaining water buffalo samples including a field rat sample had two mixed sequences (Table 7). Group IV has the highest identified clones from samples with mixed sequences (12), followed by Group II with 11 clones, Group I with 10 clones and Group III with five clones.

Table 7. Details of 17 rat and water buffalo samples with mixed *flaB* sequences.

Animal ID	Animal group	No. of sequences	*L. borgpetersenii*			*L. kirschneri*
			Group I	Group II	Group III	Group IV
PWB14-325	Weaned calf	3	1	1		1
PWB14-368	Yearling	2	1			1
PWB14-378	Bull	2	1			1
PWB14-380	Bull	2	1		1	
PWB14-382	Bull	2		1		1
PWB14-384	Bull	2	1			1
PWB14-395	Bull	3	1	1		1
PWB14-396	Bull	3		1	1	1
PWB14-403	Heifer/pregnant cow	2		1	1	
PWB14-412	Heifer/pregnant cow	2		1		1
PWB14-413	Heifer/pregnant cow	2		1		1
PWB14-415	Heifer/pregnant cow	2	1		1	
PWB14-417	Heifer/pregnant cow	2		1		1
PWB14-435	Lactating cow	2	1	1		
PWB14-436	Lactating cow	2	1		1	
PWB14-439	Lactating cow	3	1	1		1
PRAT14-1		2		1		1
TOTAL			10	11	5	12

Concordance between MAT and *flaB* nested PCR results

Agreement between MAT and *flaB* nested PCR results showed a Cohen's Kappa value of 0.37 (95% CI: 0.22-0.52) which is considered fair, with 39 positive and 79 negative water buffalo samples detected by both tests, while 10 and 42 were positive only by *flaB* nested PCR and MAT, respectively (Tables 8 and 9). Meanwhile the result from rat samples from both tests showed no concordance (data not shown).

Table 8. Concordance between MAT and *flaB* nested PCR results.

	Microscopic agglutination test (MAT)		Total
	Positive	Negative	
Leptospira flaB nested PCR			
Positive	39 (0.23)	10 (0.06)	49 (0.29)
Negative	42 (0.25)	79 (0.46)	121 (0.71)
Total	81 (0.48)	89 (0.52)	170 (1.00)

Cohen's Kappa test - 0.37 (95% CI: 0.22-0.52)

Table 9. Diagnostic test results of detected *Leptospira* species and serogroups.

Animal ID[a]	Positive serogroups determined by MAT[b]	DNA detection and sequencing of *flaB* amplicon[c]
PWB14-278	Shermani (1:80)	
PWB14-290	Mini (1:80)	
PWB14-292	Grippotyphosa (1:80)	
PWB14-308	Hurstbridge (1:40)	
PWB14-325		*L. kirschneri, L. borgpetersenii*
PWB14-327	Icterohaemorrhagiae (1:160)	
PWB14-332	Hurstbridge (1:160)	
PWB14-341	Cynopteri (1:40)	*L. borgpetersenii*
PWB14-342	Grippotyphosa, Pomona (1:640)	
PWB14-343		*L. borgpetersenii*
PWB14-347	Icterohaemorrhagiae (1:40)	
PWB14-350	Icterohaemorrhagiae (1:40)	
PWB14-353	Bataviae (1:40)	
PWB14-356	Mini (1:40)	*L. borgpetersenii*
PWB14-357	Mini (1:320)	*L. borgpetersenii*
PWB14-358	Pyrogenes (1:40)	*L. borgpetersenii*
PWB14-359		*L. kirschneri*
PWB14-361		*L. borgpetersenii*
PWB14-362	Mini (1:1280)	
PWB14-363	Hebdomadis (1:80)	*L. kirschneri*
PWB14-364	Sejroe, Mini (1:160)	*L. borgpetersenii*
PWB14-365	Mini (1:5120)	
PWB14-366	Hebdomadis (1:640)	*L. kirschneri*
PWB14-368	Pyrogenes (1:80)	*L. kirschneri, L. borgpetersenii*
PWB14-370	Mini (1:640)	*L. kirschneri*
PWB14-371	Pyrogenes, Hebdomadis (1:80)	*L. kirschneri*
PWB14-372	Mini (1:5120)	*L. borgpetersenii*
PWB14-373	Pyrogenes, Hebdomadis (1:80)	*L. kirschneri*
PWB14-374	Hebdomadis, Semaranga (1:40)	*L. borgpetersenii*
PWB14-375	Serjoe (1:640)	*L. borgpetersenii*
PWB14-376	Shermani (1:40)	
PWB14-377	Mini (1:320)	*L. kirschneri*
PWB14-378	Mini (1:160)	*L. kirschneri, L. borgpetersenii*
PWB14-379	Hebdomadis, Mini, Cynopteri (1:80)	
PWB14-380	Autumnalis (1:80)	*L. borgpetersenii*
PWB14-381	Hebdomadis, Mini (1:160)	*L. kirschneri*
PWB14-382	Cynopteri (1:160)	*L. kirschneri, L. borgpetersenii*
PWB14-383	Hebdomadis, Sejroe (1:160)	
PWB14-384	Autumnalis, Hebdomadis (1:1280)	*L. kirschneri, L. borgpetersenii*
PWB14-385		*L. borgpetersenii*
PWB14-387	Hebdomadis (1:160)	*L. borgpetersenii*
PWB14-388	Hebdomadis, Mini (1:80)	*L. borgpetersenii*
PWB14-389	Pyrogenes (1:40)	
PWB14-390	Mini (1:80)	
PWB14-391	Mini (1:1280)	*L. borgpetersenii*
PWB14-392	Cynopteri (1:40)	*L. kirschneri*
PWB14-393	Semaranga, Pomona, Mini (1:40)	*L. borgpetersenii*
PWB14-395	Icterohaemorrhagiae (1:80)	*L. kirschneri, L. borgpetersenii*
PWB14-396	Hebdomadis (1:1280)	*L. kirschneri, L. borgpetersenii*
PWB14-397	Mini (1:320)	
PWB14-398	Sejroe (1:80)	

Table 9. (Continued)

Animal ID[a]	Positive serogroups determined by MAT[b]	DNA detection and sequencing of *flaB* amplicon[c]
PWB14-399	Semaranga, Hebdomadis (1:80)	
PWB14-400	Tarassovi (1:80)	
PWB14-402	Tarassovi, Hebdomadis (1:320)	*L. kirschneri*
PWB14-403	Tarassovi, Hebdomadis, Bataviae (1:320)	*L. borgpetersenii*
PWB14-404	Mini (1:640)	
PWB14-405	Mini (1:10240)	
PWB14-406	Hebdomadis (1:160)	
PWB14-407	Sejroe, Hebdomadis, Mini (1:160)	*L. kirschneri*
PWB14-408	Hebdomadis (1:160)	
PWB14-409	Mini (1:10240)	*L. borgpetersenii*
PWB14-411	Pomona (1:1280)	
PWB14-412		*L. kirschneri, L. borgpetersenii*
PWB14-413	Cynopteri (1:80)	*L. kirschneri, L. borgpetersenii*
PWB14-414	Hebdomadis (1:160)	*L. borgpetersenii*
PWB14-415	Pyrogenes (1:320)	*L. borgpetersenii*
PWB14-416	Sejroe, Hebdomadis (1:80)	
PWB14-417	Hebdomadis (1:320)	*L. kirschneri, L. borgpetersenii*
PWB14-418		*L. borgpetersenii*
PWB14-419		*L. borgpetersenii*
PWB14-422	Tarassovi (1:640)	
PWB14-423	Tarassovi, Mini (1:160)	
PWB14-424	Tarassovi (1:1280)	
PWB14-426	Hebdomadis (1:80)	
PWB14-429	Mini (1:160)	
PWB14-430	Batavie (1:320)	
PWB14-431	Tarassovi (1:160)	
PWB14-432	Tarassovi (1:40)	*L. borgpetersenii*
PWB14-433	Tarassovi, Shermani (1:160)	*L. borgpetersenii*
PWB14-434	Bataviae, Cynopteri (1:80)	
PWB14-435	Tarassovi (1:1280)	*L. borgpetersenii*
PWB14-436	Tarassovi (1:320)	*L. borgpetersenii*
PWB14-437	Tarassovi (1:40)	
PWB14-438	Semaranga (1:160)	
PWB14-439		*L. kirschneri, L. borgpetersenii*
PWB14-440	Louisiana, Shermani (1:160)	
PWB14-441	Hurstbridge (1:320)	
PWB14-442		*L. borgpetersenii*
PWB14-443	Tarassovi (1:1280)	
PWB14-444	Mini (1:2560)	
PWB14-445	Tarassovi, Mini (1:80)	
PRAT14-1		*L. kirschneri, L. borgpetersenii*
PRAT14-6		*L. kirschneri*
PRAT14-8	Pomona (1:320)	
PRAT14-17	Canicola (1:1280)	
PRAT14-20	Icterohaemorrhagiae (1:160)	

[a]Negative samples from this table are not listed in this table.
[b]Highest reactive serogroups of each positive sample are described.
[c]Results of direct sequencing and sequencing after cloning of amplicons with mixed peaks.

Discussion

The detection rate of 28.8% in water buffalo through leptospiral *flaB* nested PCR can be considered comparable with previous studies on bovine leptospirosis in other tropical countries like in Brazil with 21.6 – 25.6% [22, 44], 12.5% in Sri Lanka [18] and 37% in Colombia [24], since no molecular studies have been conducted in water buffaloes in the Philippines to date except for limited and old serological studies, and is therefore neglected. High prevalence of pathogenic leptospires in water buffaloes at least in the communal farm setting is indeed alarming and demands a more extensive surveillance of leptospirosis in the country in relation to its possible negative impact on the water buffalo industry.

I identified four groups of leptospires detected from water buffalo clustered with either *L. borgpetersenii* or *L. kirschneri* or both, and found that almost all infected age groups harbor each of the identified groups of leptospires, suggesting an on-going transmission of pathogenic leptospires inside the farm. Farm management practices as explained in Chapter I may play an important role in the persistence of pathogenic leptospires in the farm [14, 51]. Since vaccination against leptospirosis was not practiced in the study area, temporary and immediate control measures, including isolation and treatment of infected animals, proper cleaning and disinfection of corrals and wallowing pools are needed. Meanwhile, the presence of three different groups of *L. borgpetersenii* may suggest an on-going evolution or diversification of leptospires within the farm, as claimed in previous reports [12, 27, 56]. *L. borgpetersenii* is known to be restricted to animal-to-animal transmission [9] and has a broad host range [2, 27], and therefore is prone to diversification. Presence of *L. kirschneri* in water buffaloes is also significant as it showed similar results with cattle in Sri Lanka [18] and India [5] with a potential link to human infections. Considering the variety of detected leptospires in

one water buffalo farm highlighted the importance of intensive farming system in the persistence of leptospirosis at least in the Philippines' setting.

Clustering of *Leptospira flaB* sequences from water buffalo and field rat was observed in G II and G IV while G I and G III contained only those from water buffaloes. The presence of leptospires detected only from water buffaloes may indicate host specificity. Leptospirosis is thought to be a disease of natural nidality such as serovar Hardjo that tends to be maintained by cattle and/ or water buffalo [14, 15, 36]. Meanwhile, the evidence of pathogenic leptospires detected from field rats may suggests an active circulation of this leptospires in the vicinity of the farm. Rodents, specifically rat are considered the main reservoir host of *Leptospira*, and the source of pathogenic leptospires that often infect humans [1] as well as large ruminants causing acute leptospirosis [36]. However, other animals may also be involved as carriers of infection in the study area. Identifying the main source of infection whether water buffalo, rats or other possible reservoir hosts that may be involved are beyond the scope of this study, therefore a more elaborate study design is needed to elucidate the actual transmission route of infection at least in the communal farm setting.

This study demonstrated that adult water buffaloes and field rats can be infected by a single or multiple pathogenic leptospires as shown by several *Leptospira* partial *flaB* gene sequences. This confirmed my previous serological findings that mixed infection is present as evidenced by reaction to multiple *Leptospira* serogroups in adult water buffaloes [51]. High stocking rate in the farm [4, 51] together with less efficient biosecurity measures, i.e., preventing the possible entry of other contaminated vehicles and infected animals inside the farm are thought to be the possible reasons for the presence of mixed infection. This phenomenon needs a more extensive investigation in exploring the *Leptospira* species present in the Philippines that may cause disease in humans and other incidental animals. Isolation

and studying the virulence and pathogenesis of these leptospires will be helpful in clarifying the role of water buffaloes and other animals in human leptospirosis.

The concordance between MAT and PCR results by Cohen's Kappa test was categorized as fair [32], which was expected since each test has its different targets, where the former measures the antibody titers against pathogenic leptospires through the presence of IgM and IgG in blood circulation for months to years after infection or in the case of re-infection [34, 43, 54], while the latter detects the excreted leptospires in urine which tends to be intermittent [1, 13]. MAT is a gold standard test for the herd level epidemiological serosurveys, however, it is laborious and inadequate for individual detection of carrier animals [18, 22, 44]. Meanwhile, PCR has several advantages such as being simple, rapid and highly sensitive test that directly detects shedding animals, with clear implications for the success of the control programs [22]. However, a PCR-negative result cannot exclude the possibility that the animal is infected since shedding of leptospires in urine is referred as intermittent in livestock [1, 22]. The present study collected both urine and serum samples at the same time, which illustrates the situation of a water buffalo located in leptospirosis-endemic country using both tests. It is worth mentioning that MAT-positive/ PCR-negative results was more common (42 animals) than the contrary (10 animals), as shown in Table 8. The result from 42 animals can be explained the intermittent shedding of leptospires in urine while those 10 animals may have cleared the agglutinating antibodies in their blood or other *Leptospira* serovars that were not included in the battery of strains used in MAT were involved. Nevertheless, both tests are important as each compensates on its limitations, therefore it is recommended that MAT be used as a screening test and further urine analysis by PCR is considered suitable for detection of renal carriers of leptospires in water buffalo [44].

Summary

I investigated the circulating *Leptospira* species and its prevalence from water buffaloes managed in an intensive farm setting in the Philippines using molecular techniques and evaluated these results in concordance with the previously published serological analysis data from similar samples. Results of *flaB* nested PCR detected pathogenic leptospires in 49 out of 170 water buffaloes and in two out of 21 rats, with a prevalence of 28.8% and 9.5%, respectively. Older water buffaloes and those housed in groups like yearlings, bulls, heifer/ pregnant and lactating cows were detected with high percentages of pathogenic *Leptospira* DNA, a finding similar with MAT results. Sequencing analysis of amplicons revealed clustering with *L. borgpetersenii* or *L. kirschneri* or amplicons containing both sequences. Moreover, clustering of sequences from both water buffalo and rat was observed. Agreement between MAT and *flaB* nested PCR results were fair, but recognized the importance of both tests in covering the majority of water buffaloes shedding leptospires in the farm.

Chapter III

Molecular epidemiology of pathogenic *Leptospira* spp. among large ruminants in the Philippines

Introduction

Water buffalo industry in the Philippines belonged to two farm types, the commercial farms that practiced intensive farming system and the backyard farms practicing the semi-intensive or extensive farming system where 99% of water buffalo population is situated [46] as shown on Figure 2. The two former studies clearly demonstrated the high prevalence of leptospirosis in an intensive-type water buffalo farm which has potential negative economic and public health implications in the country. However, leptospirosis situation in water buffaloes managed in backyard farms is still unknown. Furthermore, importation of large ruminants from other countries is increasing where new pathogenic leptospires can be introduced and infect local animals or vice versa. Therefore, this present study aimed to determine the local prevalence and carrier status of leptospirosis among water buffalo and several cattle, and the potential of these animals to act as a reservoir for human infection.

Materials and Methods

Sample collection

A total of 831 urine samples from 102 cattle and 729 water buffalo were collected from 21 farms located in three regions of Luzon (north, central and south) and eastern Visayas during the period from 2013 to 2015 (Table 10, Figure 7). Seventeen farms were located in the central Luzon area, two farms in north Luzon and one farm in each of the remaining regions. From the total number of samples, more than half (417 samples) were derived from water buffalo on Farm A (Table 10) from which samples were collected in each of the three years studied. A midstream sample of voided urine from each animal was collected into a sterile 15 ml conical tube. Collected samples were immediately placed on ice until processed for DNA extraction. Additional information such as animal source, farm management setting, source of drinking water and contact with other animals was collected using a questionnaire to assess the risk factors for acquiring leptospirosis.

DNA extraction from urine samples

Extraction of DNA from urine samples was performed within 24 hr after collection. The samples were centrifuged at 16,000 × *g* for 10 min at 10 ºC to collect leptospires in the sediments. The sediments were suspended with 37 µl of 10 mM Tris 1 mM EDTA (TE, pH 8.0) and boiled at 95 ºC for 10 min. Extracted DNA was stored at -30 ºC before use.

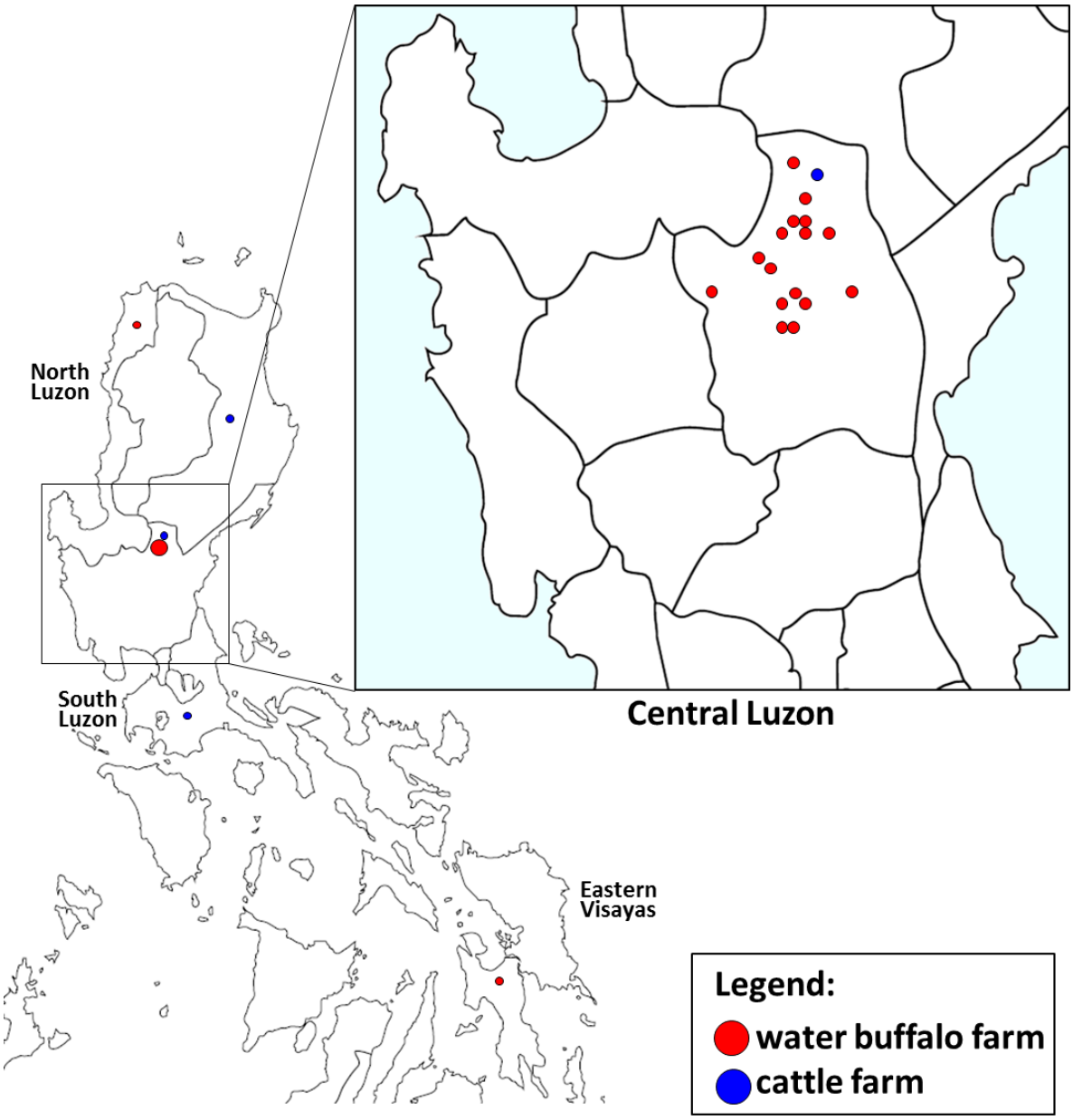

Figure 7. Location of the farms where the samples were obtained. Red and blue circles represent water buffalo and cattle farms, respectively.

Leptospira flagellin B (*flaB*)-nested PCR

Nested PCR were performed as described with minor modifications [28]. Briefly, the reaction mixture (20 µl) consisted of LA *Taq* buffer (TaKaRa Bio, Shiga, Japan) with 25 mM $MgCl_2$, 2.5 mM each of dNTPs, 0.2 µM of each primers, 1.2 U of LA *Taq* DNA polymerase (TaKaRa Bio) and 2 µl/ 1 µl of sample DNA for the first/second PCR. The PCR amplification condition was followed as previously described [28]. The nucleotide sequences of the partial *flaB* (691 bp) were determined using the ABI Prism BigDye Terminator v3.1 cycle sequencing kit (ThermoFisher Scientific, Carlsbad, CA).

Cloning

The amplicons in which overlapping peaks were observed by direct sequencing were subjected to DNA cloning. PCR products were cloned into a vector using the TOPO® TA Cloning kit (Invitrogen/ Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Five colonies were selected from each sample and the cloned *flaB* was amplified according to the manufacturer's instructions followed by DNA sequencing as described above.

Phylogenetic and minimum spanning tree analysis

The *flaB* sequences were aligned and subjected to construction of a maximum-likelihood phylogenetic tree with the JC69 model and with 1,000 bootstrap replications using BioNumerics software v.6.0 (Applied Maths, Austin, Texas, USA). Representative *flaB* sequences from different pathogenic *Leptospira* species together with the local isolates from the Philippines downloaded from the NCBI database were included in the phylogenetic tree. Minimum spanning tree (MST) was employed for illustration of the results.

Statistical analysis

Univariate analysis by Fisher's exact test followed by estimation of odds ratio were used in assessing the associations between the presence of *Leptospira* DNA in large ruminants with animal species and sources, farming management systems, sources of drinking water or contact with other animals (aside from rodents) with a 95% confidence interval. A modified odds ratio calculation for the source of drinking water was performed as described [16] because one cell of the corresponding contingency table contained the value of zero. The calculations were carried out using the Statistical Package for Social Sciences (SPSS) software version 23.0 (IBM Corp., Armonk, NY, USA).

Results

Detection of pathogenic *Leptospira* spp. in large ruminant urine

Among the 831 samples, 134 (16.1%) were found to be positive for pathogenic *Leptospira* spp. by *flaB*-nested PCR (Table 10). The detection rate for water buffalo and cattle was 17.3% (126/729) and 7.8% (8/102), respectively. A total of 57.1% of the farms (12/21), including 10 water buffalo farms and two cattle farms, were positive. Water buffalo farms A, H and G had the three highest detection rates at 28.8%, 46.7%, and 27.6%, respectively. The total detection rate in Farm A within the three-year period was 17.5% (73/417) (Table 10).

Table 10. Details of animal sampling and their urine *flaB*-nested PCR results.

Year	Region	Farm	Species	Farm management	Urine (n = 831)	*flaB* nPCR positive (n = 134)	No. of multiple *flaB* sequences[b]
					No. of samples (%)	No. of samples (%)	
2013	Central Luzon	A[a]	water buffalo	intensive	195 (23.5)	19 (9.7)	4
		B	water buffalo	intensive	51 (6.1)	11 (21.6)	3
	South Luzon	C	cattle	intensive	30 (3.6)	0 (0)	
2014	Central Luzon	A	water buffalo	intensive	170 (20.5)	49 (28.8)	16
		D	water buffalo	intensive	55 (6.6)	0 (0)	
		E	water buffalo	semi-intensive	32 (3.9)	4 (12.5)	
2015	Central Luzon	A	water buffalo	intensive	52 (6.3)	5 (9.6)	1
		F	cattle	intensive	32 (3.9)	7 (21.9)	
		G	water buffalo	intensive	58 (7)	16 (27.6)	2
		H	water buffalo	semi-intensive	30 (3.6)	14 (46.7)	
		I	water buffalo	semi-intensive	8 (1)	1 (12.5)	
		J	water buffalo	semi-intensive	12 (1.4)	3 (25)	
		K	water buffalo	semi-intensive	8 (1)	0 (0)	
		L	water buffalo	semi-intensive	8 (1)	1 (12.5)	
		M	water buffalo	semi-intensive	9 (1.1)	0 (0)	
		N	water buffalo	semi-intensive	8 (1)	0 (0)	
		O	water buffalo	semi-intensive	7 (0.8)	0 (0)	
		P	water buffalo	semi-intensive	2 (0.2)	0 (0)	
		Q	cattle	semi-intensive	13 (1.6)	1 (7.7)	
		R	water buffalo	semi-intensive	4 (0.5)	1 (25)	
	North Luzon	S	cattle	semi-intensive	27 (3.2)	0 (0)	
	North Luzon	T	water buffalo	intensive	12 (1.4)	0 (0)	
	Eastern Visayas	U	water buffalo	intensive	8 (1)	2 (25)	

[a]Urine samples were collected from Farm A every year.

[b]These samples showed overlapping peaks in direct sequencing and were subjected to DNA cloning.

Analysis of farm characteristics in relation to pathogenic *Leptospira* detection

Among the 21 farms, 17 (81%) and four (19%) managed water buffalo and cattle, respectively (Table 11). Regardless of species, the majority of the farms (90.5%) reared resident or local animals while the rest had animals imported from overseas. Eight farms (38.1%) practiced an intensive farm management whereas the others had a semi-intensive farm management. Fourteen farms (66.7%) used a deep well as a source of drinking water while the rest mostly utilized an open water source. Furthermore, sixteen farms (76.2%) had other animals in close contact with the herd while the remaining five farms (23.8%) did not. Comparison by estimated odds ratio revealed that there were no significant differences in these parameters among farms with or without the detection of *Leptospira* DNA (Table 11).

Table 11. Analysis of risk factors to leptospirosis infection on cattle and water buffalo farms.

Variables	Total number of farms (n = 21)	Infected farms (n = 12)		Odds ratio (95% CI)
		n	%	
Animal species				
Water buffalo	17	10	58.8	1.43
Cattle	4	2	50	(0.16–12.7)
Animal source				
Resident or local	19	12	63.2	0.33[a]
Imported	2	0	0	(0.01–7.92)
Farm management				
Intensive	8	5	62.5	1.43
Semi-intensive	13	7	53.8	(0.24–8.64)
Source of drinking water				
Deep well	14	10	71.4	6.25 (0.84–46.57)
Open water source (e.g. river, pond, creek, canal)	7	2	28.6	
Contact with other animals (other than rodents)				
Yes	16	9	56.3	0.86
No	5	3	60	(0.11–6.62)

[a]Modified odds ratio calculation was performed.

Sequence analysis of *Leptospira flaB*

From a total of 134 PCR-positive samples, we could determine nucleotide sequences of 108 samples by direct sequencing. The other 26 samples showed overlapping peaks in direct sequencing and were subjected to subcloning, and sequences from five colonies that were obtained for each sample were analyzed depending on the sequences' similarity or difference.

Four main groups were formed by MST based on partial *flaB* sequences (Figure 8, Table 12). Group I consisted of samples coming from water buffalo farms A, E, G, H, I, J, and R and cattle farms F and Q. These sequences were clustered with the uncultured *Leptospira* sp. clone (accession no. AB698542) that was previously detected in the same region (Table 12, Figure 8), and was under the *L. borgpetersenii* cluster. Group II, a smaller distinct group with proximity to Group I was from water buffalo farms A and B (Figure 8, Table 12). Group III consisted of samples from water buffalo farms A, B, E, G, H, and U and cattle farm F. The sequences from this group were clustered with *L. borgpetersenii* serovars Tarassovi (AB027169) and Sejroe (AB027173) and uncultured *Leptospira* sp. clone (AB698541) detected in the Philippines (Figure 8). Group IV was composed of samples from water buffalo farms A, B, G and L (Table 12). These sequences were related to *L. kirschneri* serovar Grippotyphosa (AB027170) (Figure 8). Overall, Farms A, B and G contained sequences belonging to all three groups while Farms E, F and H contained sequences from two groups and the remaining farms contained those from one group (Table 12, Figure 8).

All 26 samples containing mixed sequences came from water buffalo farms A, B and G (Table 10). Of the 26 samples, 20 (76.9%) and six (23.1%) contained two and three clones with variable *Leptospira flaB* sequences, respectively (Table 12). Nineteen samples (73.1%) were found to have sequences related to both *L. kirschneri* and *L. borgpetersenii* whereas the

remaining samples contained sequences identified in groups I, II and III related to *L. borgpetersenii* (Table 12).

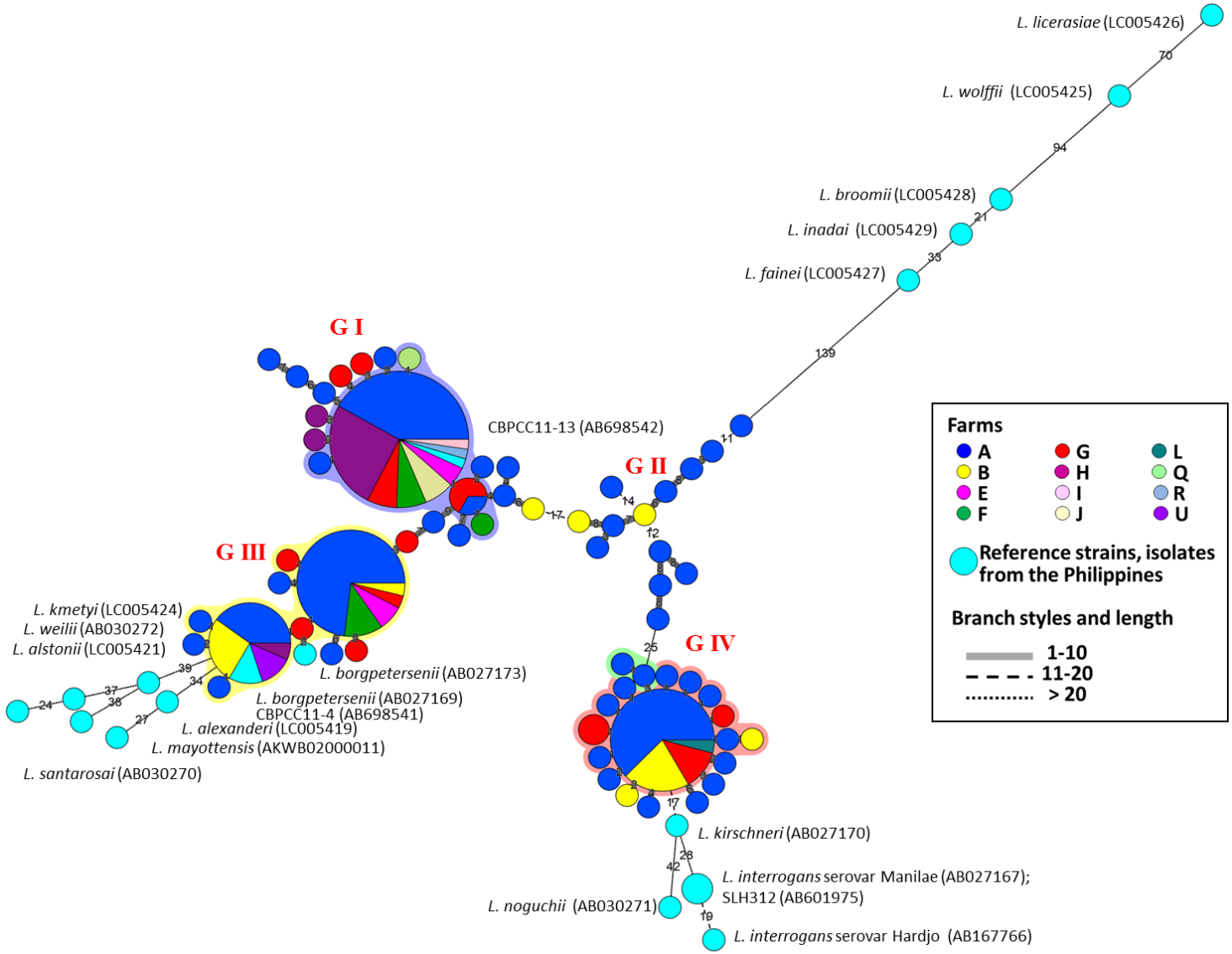

Figure 8. Minimum spanning tree based on the partial *flaB* sequences of *Leptospira* spp. in water buffalo and cattle. Partitioned groups are indicated by shaded area including Group I (G I), Group II (G II), Group III (G III) and Group IV (G IV).

Table 12. Identified *Leptospira* species and groups from positive animal samples with single and mixed *flaB* sequences.

Farms[a]	*L. borgpetersenii*						*L. kirschneri*		Total
	Group I		Group II		Group III		Group IV		
	DS[b]	CS[c]	DS	CS	DS	CS	DS	CS	
A	15	13		11	23	8	14	15	99
B		1		2	4	1	4	3	15
E	2				2				4
F	4				3				7
G	6	1			3	2	5	1	18
H	13				1				14
I	1								1
J	3								3
L							1		1
Q	1								1
R	1								1
U					2				2
Total	46	15	0	13	38	11	24	19	166

[a]Farms with negative samples were not included
[b]DS – direct sequencing from 108 samples with single sequence
[c]CS – mixed *flaB* sequences from clones of 26 samples

Discussion

The aim of the present study was to detect pathogenic leptospires from live large ruminants. Since culturing from urine samples often fails and collection of kidney samples are difficult, I decided to test urine samples for the presence of *Leptospira* DNA by *flaB*-nested PCR [25, 28]. I found an overall detection rate of 16.1%, with a higher detection rate in water buffalo compared with cattle. Many countries with tropical climates such as the Philippines raise water buffalo in preference to cattle since they are well adapted to poor quality forage without a substantial loss in productivity [31, 51]. In addition, the central Luzon area, where the majority of the samples were obtained, is a region with a high population of water buffalo that are used for dairy production and other agricultural activities [20, 47]. Therefore, a host-pathogen relationship is expected to be established in the area with high population density and favorable environmental conditions for the pesistence of leptospires [14, 42].

I could not find any statistically significant difference among the identified variables in relation to the presence of pathogenic *Leptospira* DNA in large ruminant farms. This may be explained by the small number of enrolled farms when the large scale of the confidence intervals is taken into consideration. In addition, the number of sampled herds could be reasonable if there was an equal distribution of cattle to water buffalo population in the studied region. However, I theorize that several identified variables likely contribute to the presence of *Leptospira* infection as these factors were reported in previous studies [14, 15]. For instance, the endemic nature of *Leptospira* and non-practice of vaccination among large ruminant herds in the Philippines are the probable causes of high carriage rate in these animals. *L. borgpetersenii* serovar Hardjo is adapted to and maintained by cattle as well as water buffalo where direct cow-to-cow transmission is probably of greatest importance, independent of regions or environmental conditions [15]. Resident animals can be constantly

exposed to a variety of leptospires as the country's warm and wet conditions favor the optimum survival of leptospires outside the host [15]. In addition, other domestic and free-living animals in most tropical countries also maintain leptospires and play an important role in causing incidental infection of large ruminants [15, 17, 34]. This situation raises the concern that imported non-domestic animals would be at high risk of acquiring leptospirosis. Although importation of animals from other countries requires strict regulations to prevent the possible entry of infectious diseases [55], imported (naïve) animals may also be at risk when exposed to the infected local stock. Imported animals are usually vaccinated in their country of origin, but as current vaccines are serovar-specific [1, 23, 34] and since *Leptospira* serovars or strains vary between countries or regions [34], vaccines may fail or provide only partial protection against leptospirosis. Moreover, the infection risk may be aggravated if imported animals drink water from open water sources such as ponds, rivers and creeks which *Leptospira*-endemic herds have also access to [1, 34]. In addition, other domestic and free-living animals in most tropical countries also maintain leptospires and play an important role in causing incidental infection of large ruminants [15, 17, 34]. Therefore, a more systematic study plan focusing on reported and other possibly unknown risk factors is necessary to understand the current *Leptospira* transmission routes among large ruminants in the Philippines.

Several samples, particularly from water buffalo managed in intensive farm settings, showed multiple infections of *Leptospira* species. Similar findings were found in aborted water buffalo fetuses in Italy [38], demonstrating their high susceptibility to *Leptospira* infection as well as their potential to act as a reservoir of infection for other animals. Although the animal can be infected under any farm management practices, a limited and close confinement setting could predispose the animal to continuous re-infection and possible introduction of various pathogenic leptospires from outside sources. This phenomenon

demands a more extensive investigation to explore the *Leptospira* species present in the Philippines. Isolation and study of their virulence and pathogenesis will be helpful in clarifying the role of large ruminants in human leptospirosis, and the potential negative impact of leptospirosis on the productivity of these animals, which is responsible for economic losses in the country.

DNA sequencing results revealed the formation of groups of *flaB* sequences from large ruminants clustered with *L. borgpetersenii* or *L. kirschneri*. Interestingly, Farms A, B and G contained all three distinct *flaB* sequence groups (Figure 8, Table 12). Farm A is a major source of milk-type water buffalo to other farms and farm cooperatives, while Farm B functions similarly with Farm A because this farm is responsible for supplying water buffalo in central Luzon region. Farm G is a bull farm and is a source of semen that is used for artificial insemination and proven bulls for natural mating. It has been identified that natural mating as a major risk factor for *Leptospira* transmission in the herd [14, 37]. Meanwhile, the majority of the animals in Farm E, F and H which is managed by a farmers' cooperative, come from Farm A. Considering Farm A as an established herd for distributing animals all in most parts the country suggests that this farm may be the main source of pathogenic leptospires to other farms. Bulls may also play an important role in the sustained spread of infection as previous studies have shown that subclinically infected bulls harbor *Leptospira* spp. serovar Hardjo in the genital tract, highlighting the importance of venereal transmission in the epidemiology of bovine leptospirosis [14, 37]. Cattle farms were also infected with *L. borgpetersenii*, as evidenced by the sequences from groups I and II, indicating that *L. borgpetersenii* genotypes have endemically spread in the study areas. *L. kirschneri* was found in water buffalo farms in this study. Previous studies have reported its presence in cattle in Africa [2], Sri Lanka [18], India [5] and Brazil [8] and were one of the predominant *Leptospira* species linked to human disease and animal infection [2].

I found the formation of two main groups of *flaB* sequences related to *L. borgpetersenii* in both water buffalo and cattle in similar and even in different geographical locations in the Philippines. Group II, a small cluster that is related to group I (Figure 8, Table 12) was also observed to be detected only in water buffalo managed in communal farms. Although the majority of my samples came from water buffalo and the molecular analysis was limited, my findings may further support the previous reports regarding the diversification of *Leptospira* species [12, 27, 56], wherein this phenomenon can be attributed to maintenance host animals (alteration of physiology or immune system) or environmental factors [19, 27]. *L. borgpetersenii* is known to have a broad host range [2, 27] and is restricted to animal-to-animal transmission [9], and therefore is more prone to diversification. A wider study coverage area along with the use of recent molecular typing methods such as multiple-locus variable-number of tandem repeat analysis and multilocus sequence typing should be employed in future studies to reveal the genetic diversity of *Leptospira* present in large ruminants in the Philippines.

Summary

The extent of *Leptospira* infection in large ruminants resulting to economic problems in livestock industry in a leptospirosis-endemic country like the Philippines has not been extensively explored. Therefore, we determined the prevalence and carrier status of leptospirosis in large ruminants using molecular techniques and assessed the risk factors of acquiring leptospirosis in these animals. Water buffalo and cattle urine samples (n=831) collected from 21 farms during 2013-2015 were subjected to *flaB*-nested PCR to detect pathogenic *Leptospira* spp. Leptospiral *flaB* was detected in both species with a detection rate of 16.1%. Leptospiral DNA was detected only in samples from animals managed in communal farms. Sequence analysis of *Leptospira flaB* in large ruminants revealed the formation of three major clusters with *L. borgpetersenii* or *L. kirschneri*. One farm contained *Leptospira flaB* sequences from all clusters identified in this study, suggesting this farm was the main source of leptospires for other farms. This study suggested that these large ruminants are infected with various pathogenic *Leptospira* species causing possible major economic loss in the livestock industry as well as potential *Leptospira* reservoirs that can transmit infection to humans and other animals in the Philippines.

CONCLUSION

Studies on *Leptospira* infection in water buffalo have not been extensively explored despite the endemic nature of the disease in the Philippines. As a source of milk, meat, aesthetic products and draft power, water buffalo is an important livestock in the country. Therefore, it is deemed necessary to understand the status of leptospirosis among this animal and clarify its role in the persistence of this zoonosis affecting animal and public health. This study analyzed the leptospires detected in water buffalo using both serological and molecular techniques in the Philippines, where previously limited serological data were available.

In Chapter I, I demonstrated the widespread occurrence of leptospirosis in a water buffalo herd in an intensive-type farm in the Philippines. Higher seroprevalence and MAT titers among water buffaloes of one year of age and older confirmed the active *Leptospira* transmission and the crucial role of animal management in sustained circulation of *Leptospira* infection at least at communal farming level. Although involvement of field rats as a source of infection was not conclusive, the role of these rodents in the maintenance of leptospires on the farm should be considered as a possibility pending further studies on external sources of leptospirosis.

In Chapter II, I confirmed the presence and high prevalence of pathogenic leptospires using molecular diagnostic test in an intensive-type water buffalo farm that was investigated in Chapter I. Continued and active circulation of pathogenic *Leptospira* either through animal-to-animal transmission as evidenced by infection from all animal groups or from environmental source may occur within the farm as shown by related *Leptospira* sequence from water buffalo and field rat. Molecular diagnostic test like nested PCR in combination with serological test such as MAT is recommended in investigating animal leptospirosis.

In Chapter III, I revealed the presence of various pathogenic *Leptospira* species in water buffalo and cattle in the Philippines. These local animals, particularly water buffalo, were infected with multiple *Leptospira* species that diversified as a possible consequence of interaction with host animals and environmental factors. This study emphasized the significance of one farm as a possible source of *Leptopira* infection distributed in different locations. My results suggest that these animals may act as a significant reservoir of leptospires in the area and pose a potential risk to local agricultural communities as well as a possible major economic loss in the livestock industry. Strict farm biosecurity measures, periodic testing and treatment of infected herds and quarantine to prevent the spread of infection from one farm to another are necessary to control leptospirosis.

The findings from this study will serve as a preliminary information and guide regarding the current prevalence and carrier status of leptospirosis among water buffalo and cattle in the Philippines. However, further investigations into the effect of *Leptospira* virulence on the reproductive performance of these animals and elucidation of the role of livestock as either accidental or maintenance hosts are needed to take future actions to prevent leptospirosis from causing risks to public health and economic losses to the large ruminant farming industry in the country.

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