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

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ABSTRACT. 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.
KEY WORDS: cattle, *flaB*, *Leptospira*, the Philippines, water buffalo

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Leptospirosis is an important re-emerging zoonotic disease worldwide and is predominantly found in impoverished populations inhabiting developing countries with tropical or subtropical climates [28]. The disease is caused by gram-negative spirochetes from the genus *Leptospira* [1] which is divided into pathogenic and non-pathogenic species and with more than 250 recognized pathogenic serovars and further clustered in 24 serogroups [7]. Pathogenic leptospires are carried by most mammalian species (wild, domestic and farm animals) and can transmit infection to humans and other animals either by direct contact with the urine of a carrier animal or indirectly through urine-contaminated environment [1, 21].

In livestock, leptospirosis is an important cause of decreased animal production as a result of infection by a variety of *Leptospira* serovars [10]. Bovine leptospirosis creates serious economic losses in the livestock industry, causing abortions, stillbirths, infertility, reduced milk yield, mortality in calves and decreased daily weight gain [1, 9, 26, 27]. 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 [10, 22, 26]. Infected animals become carriers harboring leptospires in the renal tubules and intermittently shedding into the environment for extended periods [1, 10, 21]. Acute leptospirosis in this animal is uncommon and characterized by pyrexia, hemolytic anemia, hemoglobinuria, jaundice, occasionally meningitis and death that is associated with infections from serogroups Pomona, Icterohaemorrhagiae and Grippotyphosa in young animals [10, 12, 26]. Domestic water buffalo is also infected with leptospirosis, however, the information is scarce as it was thought to have similar scenario with that of cattle [10].

Water buffalo and cattle are indispensable livestock in the Philippines, particularly the former, as it is well adapted in tropical climate of the country. Cases of abortion and mastitis or agalactia among water buffaloes were reported, but were often attributed to protozoal [19, 32] and other bacterial infections [29], excluding the possibility of leptospires as the causative agent. The Philippines is a leptospirosis-endemic

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Table 1. 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.

country [25, 31, 33], however, infection in large ruminants 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 [3, 6]. A similar study from Basaca-Sevilla *et al*. (1986) showed antibodies against serovars Pomona, Pyrogenes and Cynopteri from cattle sera [3].Our recent findings also found evidence of high seroprevalence (48%) and MAT titers against serogroups Mini, Hebdomadis, Tarassovi and Pyrogenes among adult animals, demonstrating a widespread occurrence of leptospirosis in a water buffalo communal farm [30].

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.

Therefore, the present study aimed to determine the local prevalence and carrier status of leptospirosis among water buffalo and cattle using molecular techniques.

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 1, Fig. 1). 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 1) 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 m*l* 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°C 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°C for 10 min. Extracted DNA was stored at −30°C before use.

Leptospira flagellin B (*flaB*)-nested PCR: Nested PCR was performed as previously described with minor modifications [18]. Briefly, the reaction mixture (20 μl) consisted of LA *Taq* buffer (TaKaRa Bio, Otsu, Japan) with 25 mM $MgCl_2$, 2.5 mM each of dNTPs, 0.2 μM of each primer, 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 as previously described [18]. The nucleotide sequences of the partial *flaB* (691 bp) were determined using

Table 2. 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 (0.16–12.7)
Cattle	4	2	50	
Animal source				
Resident or local	19	12	63.2	0.33[a)] (0.01–7.92)
Imported	2	0	0	
Farm management				
Intensive	8	5	62.5	1.43 (0.24–8.64)
Semi-intensive	13	7	53.8	
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 (0.11–6.62)
No	5	3	60	

a) Modified odds ratio calculation was performed.

the ABI Prism BigDye Terminator v3.1 cycle sequencing kit (ThermoFisher Scientific, Carlsbad, CA, U.S.A.).

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, U.S.A.) 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, TX, U.S.A.). 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. The DDBJ accession numbers of the representative *flaB* sequences from uncultured *Leptospira* spp. detected from large ruminants comprised of LC164000 to LC164012.

Statistical analysis: Univariate analysis by Fisher's exact test followed by estimation of odds ratio was 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 previously described [11], 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, U.S.A.).

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 1). 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 1).

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 2). 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 2).

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. From that, five different clones were obtained.

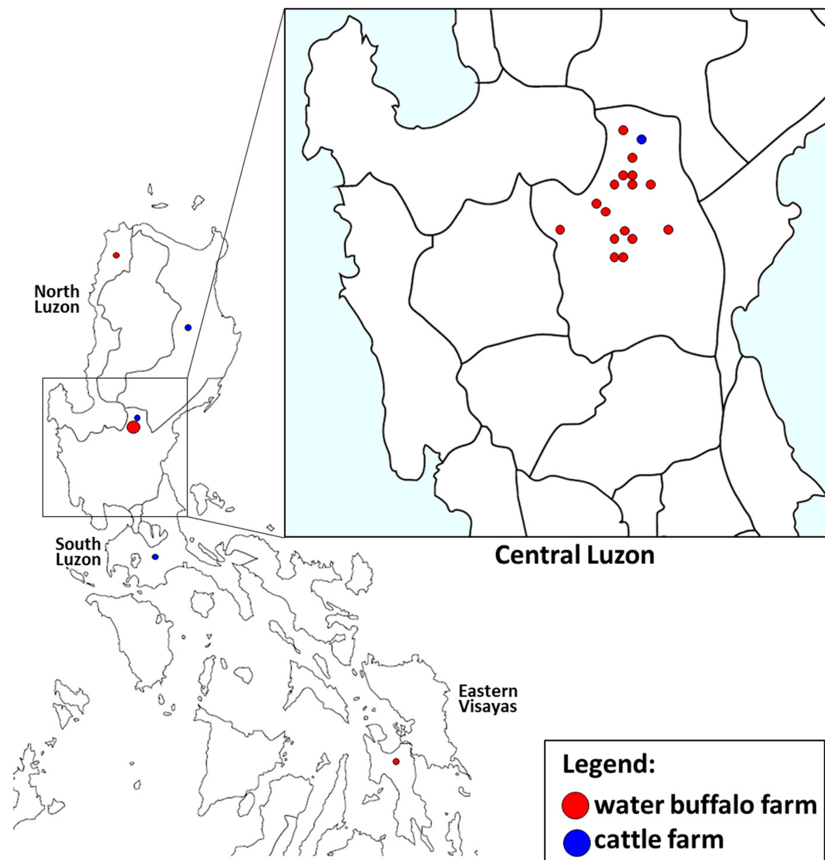

Fig. 1. Location of the farms where the samples were obtained. Red and blue circles represent water buffalo and cattle farms, respectively.

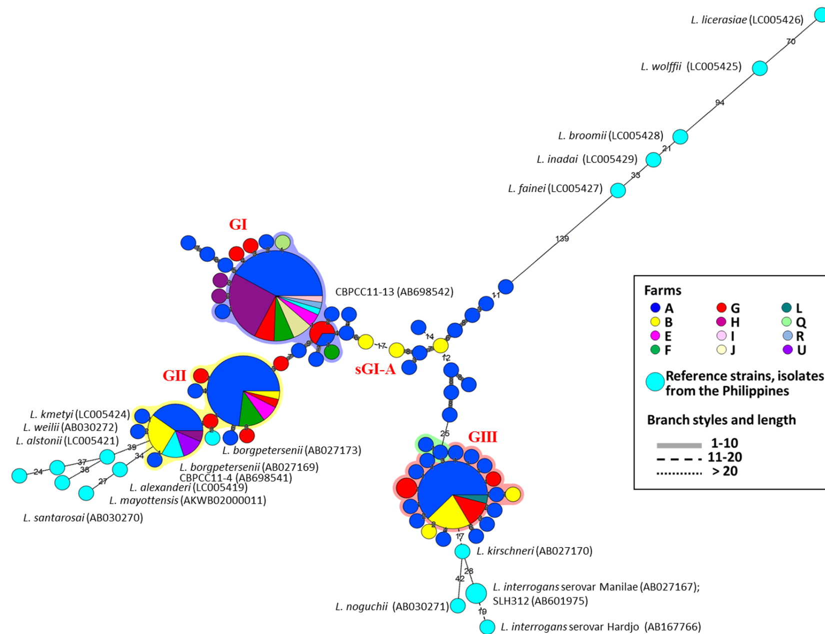

Fig. 2. Minimum spanning tree based on the partial *flaB* sequences of *Leptospira* spp. in water buffalo and cattle. Partitioned groups are indicated by shaded area including Group I (GI), subgroup I-A (sGI-A), Group II (GII) and Group III (GIII).

Table 3. Identified *Leptospira* species and groups from positive animal samples with single and mixed *flaB* sequences

Farms[a)]	*L. borgpetersenii*						*L. kirschneri*		Total
	Group I		Subgroup I-A		Group II		Group III		
	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.

Three main groups were formed by MST based on partial *flaB* sequences (Fig. 2, Table 3). 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 3, Fig. 2) and was under the *L. borgpetersenii* cluster. Subgroup I-A, a smaller distinct group with proximity to Group I was from water buffalo farms A and B (Fig. 2, Table 3). Group II 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 (Fig. 2). Group III was composed of samples from water buffalo farms A, B, G and L (Table 3). These sequences were related to *L. kirschneri* serovar Grippotyphosa (AB027170) (Fig. 2). 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 3, Fig. 2).

All 26 samples containing mixed sequences came from water buffalo farms A, B and G (Tables 1). Of the 26 samples, 20 (76.9%) and six (23.1%) contained two and three clones with variable *Leptospira flaB* sequences, respectively (Table 3). 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 subgroup I-A related to *L. borgpetersenii* (Table 3).

DISCUSSION

The aim of the present study was to detect pathogenic leptospires from live large ruminants. Since culture from urine often fails and collection of kidney samples is difficult, we decided to test urine samples for the presence of *Leptospira* DNA by *flaB*-nested PCR [16, 18]. We found an overall detection rate of 16.1%, with a higher detection rate in water buffalo when 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 [20, 30]. In addition, the central Luzon area, where the majority of the samples were obtained from, is a region with a high population of water buffalo that are used for dairy production and other agricultural activities [15, 29]. Therefore, a host-pathogen relationship is expected to be established in the area with high population density and favorable environmental conditions for the persistence of leptospires [10, 27].

We 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, we theorize that several identified variables likely contribute to the presence of *Leptospira* infection as these factors were reported in previous studies [9, 10]. 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 [9]. 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 [9, 12, 21]. 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 [24], 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 (Fig. 2, Table 3). 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 the natural mating as a major risk factor for *Leptospira* transmission in the herd [10, 23]. Meanwhile, the majority of the animals in farms E, F and H which are managed by a farmers' cooperative, come from Farm A. Considering Farm A as an established herd for distributing animals all in most parts of 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 serovar Hardjo in the genital tract, highlighting the importance of venereal transmission in the epidemiology of bovine leptospirosis [10, 23]. 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 [13] and Brazil [4] and were one of the predominant *Leptospira* species linked to human disease and animal infection [2].

We 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. A small cluster that is related to group I (designated as subgroup I-A) (Fig. 2, Table 3) was also observed to be detected only in water buffalo managed in communal farms. Although the majority of our samples came from water buffalo and the molecular analysis was limited, our findings may further support the previous reports regarding the diversification of *Leptospira* species [8, 17, 34], wherein this phenomenon can be attributed to maintenance of host animals (alteration of physiology or immune system) or environmental factors [14, 17]. *L. borgpetersenii* is known to have a broad host range [2, 17] and is restricted to animal-to-animal transmission [5], 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.

In conclusion, this study 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. Our 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. Finally, 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 industry in the Philippines.

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