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FULL PAPER

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

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Abstract

Water buffalo is an indispensable livestock in the Philippines. Leptospirosis is a serious zoonosis that can be fatal to humans and cause reproductive problems in livestock. Leptospirosis has been reported in some countries where water buffaloes are commercially raised, highlighting the *Leptospira* prevalence in this farming system, but information on leptospirosis in water buffalo farms in the Philippines is limited. In this study, we 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

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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. Further investigation and appropriate control strategies are required to prevent leptospirosis from causing risks to public health and economic losses to the water buffalo farming industry.

Key Words: intensive-type farming, *Leptospira*, leptospirosis, the Philippines, water buffalo

Introduction

Domestic water buffalo (*Bubalus bubalis*) is one of the important livestock in many developing countries due to its adaptability to hot and humid tropical areas[17], minimal requirements to produce good quality milk and meat, and its use as draft power. Water buffalo is an underutilized animal, but improved breeding and good husbandry practices[17] have enhanced its contribution to the livestock industry. In the Philippines, for instance, with a population of 2.86 million head in the latest large ruminant inventory[24], water buffalo is favored over cattle for animal production. Moreover, benefits from on-going efforts by the government through the Philippine Carabao Center ("carabao", local term for water buffalo) are being translated into better nutrition and greater income for Filipino people. This is especially true for smallholder farming communities where almost 99% of water buffaloes are distributed[23].

It is well known that water buffaloes are afflicted by numerous infectious diseases including zoonoses[2,7,9,11,13,34]. With the increasing popularity of the "One Health" concept, zoonotic diseases have been getting more attention as they concern both animal and human health. One troubling zoonosis is leptospirosis, a re-emerging disease caused by pathogenic *Leptospira* spp., which is an important zoonotic problem in world regions with tropical and subtropical climates. Particular concern about leptospirosis has especially increased in countries where the water buffalo industry is an important economic activity[17,21], as leptospires can cause serious reproductive problems such as abortion in livestock[10]. Although a wide variety of wild and domestic animals can act as maintenance hosts for this pathogen, *Leptospira* infection in human or other accidental hosts such as water buffaloes is acquired mainly through direct or indirect contact with contaminated urine or environmental factors[1,10,22,25].

Furthermore, in addition to accidental infection, water buffaloes are also considered maintenance hosts for several *Leptospira* serovar strains[5]. Leptospirosis can be either an asymptomatic, mild and self-limiting illness, or a severe and potentially fatal systemic condition in humans and accidental hosts. Clinical symptoms largely depend on host susceptibility, the degree of infection and virulence of the infecting *Leptospira* strain[15].

The Philippines is considered to be endemic for leptospirosis[3,14,26,30,36]. Several studies have focused on leptospirosis in Filipino cities where poor sanitation, increased urban slum communities, frequent occurrence of typhoons and expansion of flood-prone areas are present that increased the risk of infection[3,30,31,32,33,36]. Occupational activities that had contact with surface water (e.i. floodwater, sanitary sewage) predispose to contract *Leptospira* infection[30,36]. On the other

hand, the status of the disease in rural areas is underestimated due to lack of systematic monitoring in the country. Indeed, although rats are known to be major reservoir animals in urban settings in the Philippines, there are only limited reports on leptospire carriage in rural areas by other animals such as water buffaloes. Moreover, although economic losses for the livestock industry caused by leptospirosis are well documented in other countries[10], the impact of leptospirosis on the economy of animal farming in the Philippines remains unknown.

This study aimed to investigate *Leptospira* infection in the Philippines 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. We determined by the microscopic agglutination test (MAT) the prevailing *Leptospira* serogroups in rats infesting a farm as well as its water buffalo herd. We also assessed the variables in the study associated with *Leptospira* seropositivity.

Methods

1. 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°C 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.

2. Microscopic agglutination test: The MAT was conducted according to WHO guidelines[35], 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 (Supplementary 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°C 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[28,29].

3. 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. 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)

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 1). 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 2). 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 1 and 3). While younger water buffaloes presented low MAT titers to *Leptospira* strains (Supplementary Fig. 1), older animals showed variable MAT titers, including the highest titer against serogroup Mini (Supplementary Fig. 1 and Table 2). Fifty five (68%) and 26 (32%) seropositive serum samples agglutinated single and multiple serotypes, respectively (Tables 1 and 3). Samples from pre- and post-weaned calves contained anti-*Leptospira* antibodies against single serogroups (Tables 1 and 3). In contrast, 30–40% of older water buffaloes exhibited seropositivity against multiple serogroups (Supplementary Fig. 2, Tables 1 and 3). In addition, antibodies against serogroups Icterohaemorrhagiae and Pomona were detected in sera of both rats and water buffaloes.

The results from the univariable analysis of seropositive samples and epidemiological variables such as age, sex and animal grouping are summarized in Table 4. Seropositivity was significantly associated with age and animal grouping variables ($P < 0.0001$), but not with the sex variable.

Discussion

Water buffaloes are getting more attention in the Philippines because with proper management, milk and meat production by this livestock can translate into a stable source of income for rural farming communities. However, leptospirosis is endemic in the country, and underestimation of the actual situation of this disease in water buffaloes due to limited information may conceal a considerable threat to public health and the economy of the water buffalo industry. Hence, this study focused on elucidating the seroprevalence of *Leptospira* infection in water buffalo and the persistence of pathogenic *Leptospira* in intensive farm settings.

MAT is considered the gold standard

Table 2. 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.

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*[22]. Thus, we decided to use 1 : 40 as the cutoff titer, which has been considered positive in previous studies[28,29]. Serum samples from older water buffaloes (> 1 year old) exhibited higher MAT titers against multiple serogroups than younger water buffaloes (Supplementary Fig. 1, Tables 1–3). 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[12], paradoxical reaction[18,19] or mixed infections[10].

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 4). Pre-weaned calves are separated and housed in elevated and slatted individual pens soon after birth while older water buffaloes are kept in group pens. Immediate separation of the calf to

Table 3. 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.

the dam can stop or reduce the horizontal transmission of infection. It is also 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[29]. 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[10]. In addition, intensive farm settings lend 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 (Fig. 1, Tables 1–3). 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[6,8]. 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

Table 4. 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)	

serogroups in this study were similar to those previously detected in neighboring countries such as Thailand and Malaysia[4,29]. 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[17], whereas that was not the case in the present work (Fig. 1, Tables 1–3). Current differences in thepredominant serogroups in each region can be attributed to external environmental factors and the presence of different animals as reservoir hosts for *Leptospira*[10]. 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[10], 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. Thus, it is possible that this situation will occur in the investigated area as evidenced by the seropositive groups mainly from sexually matured water buffaloes. For example, intrauterine infection with leptospires can cause abortion, stillbirth and fetal problems in the late term of gestation[10]. Bulls may play an important role in the sustained spread of infection in the herd as previous studies have already established the semen as a vehicle for bovine leptospirosis transmission[10,20]. It has been shown that often subclinically infected bulls harbor leptospires in the genital tract, particularly in the seminal vesicle, highlighting the importance venereal transmission in the epidemiology of bovine leptospirosis[20]. In addition, leptospirosis

has a profound influence on lactating animals as it causes mastitis[29] and agalactia[10]. 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[16,27,34]. 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[16]. 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[10]. 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 nationwide as well as in the surrounding areas where the present study was conducted showed that serogroups Pyrogenes, Bataviae, Grippotyphosa and Tarassovi were predominantly detected which were also found in this study[32,36]. 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.

In conclusion, widespread occurrence of leptospirosis was demonstrated 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. Finally, further investigation on the effect of *Leptospira* virulence on the reproductive performance of water buffaloes and elucidation of the role of this livestock as either accidental hosts or carrier animals are needed to determine future actions to prevent leptospirosis from causing risks to public health and economic losses to the water buffalo farming industry in the Philippines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.64.1.15

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