IDENTIFICATION OF PREVALENT *LEPTOSPIRA* SEROVARS INFECTING WATER BUFFALOES, COWS, DOGS, PIGS, AND RATS IN THE PHILIPPINES

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Abstract. Leptospirosis is an infectious disease that affects humans and a wide range of wild and domestic animals. Pathogenic leptospires dwell in the renal tubules of carrier animals, especially rodents, and may shed the pathogens in their urine. In this study, 222 animals (50 water buffaloes, 25 cows, 84 dogs, 12 pigs, and 51 rats) from Metro Manila and Nueva Ecija, Philippines were tested for seropositivity against *Leptospira* and culture isolation from blood, urine, and kidney. A total of 213 animals (95.9%) had antibodies against leptospires. The topmost prevailing serovar infecting the animals were: Tarassovi for water buffaloes (18/50); Patoc for cows (9/25); Manilae for dogs (23/84); Poi for pigs (7/12); and, Copenhageni for rats (20/51). Blood cultures were negative. However, 7 rats were culture-positive, 4 were positive for both kidney and urine cultures, 2 were positive for kidney culture, and 1 was positive for urine culture. Leptospirosis in domestic animals poses not only health problems but also a serious economic burden. It is endemic in the Philippines and identification of locally relevant serovars infecting animals can aid in formulating prevention and control measures of this zoonosis in the country such as vaccine development and methods of carrier control.

Keywords: domestic and wild animals, leptospirosis, seropositivity, serovars, Philippines

INTRODUCTION

Leptospirosis is an infectious disease caused by pathogenic spirochetes of the genus *Leptospira* (Faine, 1999; Levett, 2001; Bharti *et al*, 2003; ILS-WHO, 2003). It affects humans and a wide range of domestic and wild animals, such as rats, dogs, cattle, horses, swine, etc. The disease is acquired through direct contact with the urine of an infected animal or by exposure to urine-contaminated environment such as soil and floodwater.

Animals that maintain pathogenic leptospires in their system without exhibiting signs of illness are called carriers or natural maintenance hosts (ILS-WHO, 2003). When infected, these animals show little or no symptoms at all. Certain animals may also be maintenance host to

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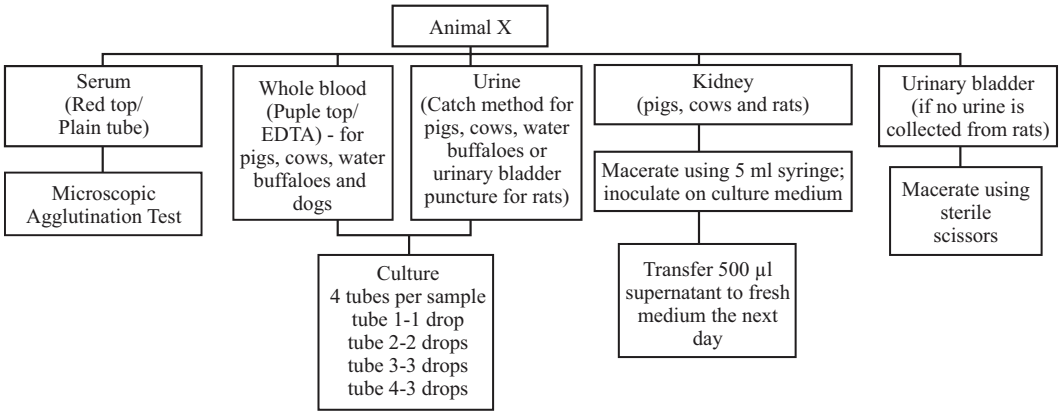

Fig 1–Schematic diagram of sample processing.

specific serovars but incidental host to others (Levett, 2001).

Leptospirosis poses a serious threat to human and animal health, which may lead to economic losses (Zuerner, 2005). Leptospirosis in humans and susceptible animals may cause mild to severe infection, and may even cause death (Hässig and Lubsen, 1998; Levett, 2001; ILS-WHO, 2003). Leptospirosis may cause abortion or stillbirth in adult cattle and swine. Calves, on the other hand may experience fever, hematuria, and jaundice, which may lead to death. Depending on the age and immunity, clinical signs of leptospirosis in dogs vary (Hassig and Lubsen, 1998). Infected dogs may experience fever, vomiting, pulmonary hemorrhage and shock. The dogs may also have anorexia, depression, and conjunctivitis. Severe infections in dogs may lead to renal failure, weight loss, polyuria, hepatic failure, and even death.

Conducting leptospirosis surveillance studies in animals is very important, especially in countries where it is prevalent. Through such studies, the prevailing *Leptospira* serovars may be identified. And, identifying the commonly occurring serovars in a specific location is very important, especially in developing prevention and control measures against the said zoonosis. One measure is the development of an effective vaccine against locally relevant serovars in the country. This study, therefore, aimed to determine the prevalent *Leptospira* serovars infecting water buffaloes, cows, dogs, pigs, and rats in selected areas in the Philippines.

MATERIALS AND METHODS

Study area and study population

Animal samples were collected from different areas in Metro Manila and Nueva Ecija where leptospirosis patients or rodent sightings were previously reported. Water buffalo samples were collected from an institute in Nueva Ecija, Philippines. Dog samples were obtained from two dog pounds in Metro Manila while the pig and cow samples were from one of the government-owned slaughterhouses in Manila City. Rat samples were collected from selected Metro Manila markets (three from Manila City and one from Makati City), two office compounds in the city of Manila, and rice fields in Nueva Ecija.

Sample collection and processing

A schematic diagram of the sample collection and processing is shown in Fig 1. Two blood samples were collected from

Table 1
Leptospira strains used in MAT.

Species	Serogroup	Serovar	Strain
L. interrogans	Pyrogenes	Manilae	LT 398
		Pyrogenes	Salinem
	Canicola	Canicola	Hond Utrecht IV
	Autumnalis	Autumnalis	Akiyami A
	Bataviae	Losbanos	LT 101-69
	Hebdomadis	Hebdomadis	Akiyami B
	Australis	Australis	Akiyami C
	Icterohaemorrhagiae	Copenhageni	M20
		Icterohaemorrhagiae	Ictero No. 1
			RGA
	Pomona	Pomona	Pomona
	Sejroe	Hardjo	Hardjoprajitno
L. borgpetersenii	Tarassovi	Tarassovi	Perepelitsin
	Javanica	Poi	Poi
L. kirschneri	Grippotyphosa	Ratnapura	UP-BL-FR13
		Grippotyphosa	Moskva V
L. meyeri	Semaranga	Semaranga	Veldrat Semarang 173
L. biflexa		Patoc	Patoc I

each big animal (cows, dogs, pigs, water buffaloes): one tube with EDTA for culture isolation from whole blood and one for serum in plain/red top tube for microscopic agglutination test (MAT). For rats, only serum samples were collected, which were used for MAT. Urine samples for culture isolation were collected by clean catch or urinary bladder puncture. If there was no urine, the urinary bladders of rats were also collected aseptically. Whole blood and urine samples were cultured based on previous methods (Villanueva *et al*, 2010, 2014). Briefly, 1-3 drops of blood or urine were cultured in modified Korthof's medium containing 5-fluorouracil (5-FU). The cultures were incubated at 30ºC and were checked every week for 3 months for the presence of leptospires.

Kidney samples were also obtained from cows, pigs, and rats for isolation of leptospires. Kidneys from cows and pigs were collected after they were slaughtered and were placed in a sterile container prior to culturing. The kidneys of rats were also collected aseptically. A portion of the kidneys of big animals and the whole kidney of rats (Villanueva *et al*, 2010) were placed in sterile syringe and macerated into Korthof's medium with 5-FU. These were incubated overnight at 30ºC and, the following day, 500 µl of the supernatant was transferred into fresh Korthof's medium and kept at 30ºC. The kidney and urinary bladder cultures were also checked weekly and kept for 3 months like the blood and urine cultures.

Microscopic Agglutination Test

The reference method for serological diagnosis of leptospirosis is the MAT (ILS-WHO, 2003). In the present study, MAT was carried out using a panel of 18

Table 2
Topmost prevailing *Leptospira* serovars per animal based on MAT.

Serovar	Animal species				
	50 Water buffaloes *n* (%)	23 Cows *n* (%)	84 Dogs *n* (%)	11 Pigs *n* (%)	45 Rats *n* (%)
Tarassovi	18 (36.0)	6 (24.0)			
Pomona	7 (14.0)			3 (25.0)	
Patoc	6 (12.0)	9 (36.0)	16 (19.1)		
Poi		6 (24.0)	16 (19.1)	7 (58.3)	13 (25.5)
Manilae			23 (27.4)		
Copenhageni			15 (17.9)	3 (25.0)	20 (39.2)
Grippotyphosa			12 (14.3)		6 (11.7)
Ratnapura					3 (5.9)

antigens (Table 1), which included three local isolates [LT 398 (Kmety and Dikken, 1993), LT 101-69 (Kmety and Diken, 1993), and UP-BL-FR13]. Briefly, the animal sera were mixed with the live antigens for 2 to 4 hours. Serum samples that had >50% agglutination at a titer of ≥1:20 were considered MAT-positive (Villanueva *et al*, 2010). The *Leptospira* serovar with the highest titer was considered to be the presumptive infecting serovar for the tested animal.

Animal ethical considerations

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Manila-National Institutes of Health. A thorough explanation of the study was also given to the owners of the big animals or the owners of the slaughterhouses. After explanations were given, informed consent was obtained prior to sample collection.

RESULTS

Blood, urine, urinary bladder, and kidney samples were collected from a total of 222 animal samples. The sources of the samples were: 50 water buffaloes, 25 cows, 84 dogs, 12 pigs, and 51 rats.

Microscopic Agglutination Test

MAT was used in detecting leptospiral antibodies in the animal sera. Two hundred thieteen of the 222 (96%) animals tested were seropositive (Table 2).

All of the water buffalo sera examined were MAT-positive. Serovars Tarassovi (36.0%), Pomona (14.0%) and Patoc (12.0%) were found to be the most common *Leptospira* serovars infecting the said animals (Table 2).

Similar to water buffaloes, cows were also found to have antibodies against serovars Patoc (36.0%) and Tarassovi (24.0%) (Table 2). Ninety-two percent of these animals were MAT-positive.

Like the water buffaloes, all of the dog sera collected in this study were found to have antibodies against leptospires. Most of the dogs reacted to serovar Manilae (27.4%), followed by Poi (19.1%), Patoc (19.1%), Copenhageni (17.9%), and Grippotyphosa (14.3%).

Eleven of the 12 pig samples (91.7%) were found positive for *Leptospira* antibodies whereas only one pig sample did not react against the panel of antigens (Table 2). Serovars Poi (58.3%), Copenhageni (25.0%), and Pomona (25.0%) were found to be the most common infecting serovars in pigs.

Antibodies were detected in 45 of the 51 (88.2%) rats collected. Twenty-four (53.3%) of which were from Nueva Ecija while 21 (46.7%) were from Metro Manila. Of these samples, 25 reacted with only one serovar, the majority of which were from Nueva Ecija (19 rats). The rest reacted with multiple serovars, with titers ranging from 1:20 to 1:5,120. Serovar Copenhageni was the most common serovar (39.2%) in rats, followed by serovar Poi (25.5%). A wider variety of serovars were detected in rats from Metro Manila and several serovars such as Manilae, Pyrogenes, Canicola, and Icterohemorrhagiae were only detected in rats collected from Metro Manila. Likewise, serovar Ratnapura was detected only in rats from Nueva Ecija.

Isolation and characterization of isolated leptospires

All the blood cultures from the water buffaloes, cows, dogs, and pigs were negative for leptospires. However, leptospires were successfully isolated from the urine and kidneys of 4 rats; kidneys of 2 rats; and, urine of 1 rat. Five of these rats were caught in Metro Manila while two were from Nueva Ecija. All of the isolates were *fla*B-PCR-positive and 6 were found to be virulent in golden Syrian hamsters as reported by Villanueva *et al* (2014). It was also reported that the 2 isolates from Nueva Ecija belong to serogroup Grippotyphosa, while those from Metro Manila were serovars Manilae and Losbanos.

DISCUSSION

Leptospirosis is a very important infectious disease since it affects both humans and animals (O'Keefe *et al*, 2002). It may cause mild to severe infections and even death in both humans and animals (Levett, 2001; Bharti *et al*, 2003; ILS-WHO, 2003). Leptospirosis in animals, especially livestock may also cause economic losses.

Animal samples were collected from sites where they are likely to represent infection or contamination with leptospires causing infection. This study utilized the reference test for serodiagnosis of leptospirosis, MAT. MAT is used to detect the presence of leptospiral antibodies in the hosts. The frequencies of MAT positivity were highest among water buffaloes and dogs (both had 100% positivity), followed by cows (92.0%), pigs (91.7%), and lastly rats (88.2%). In Thailand, the prevalence of leptospirosis in the livestock studied was as follows: 30.5% in buffaloes, 10.1% in pigs, 9.9% in cattle, 8.0% in goats, and 4.7% in sheep (Suwancharoen *et al*, 2013). The frequency of seropositivity in the current study in buffaloes, cows, and pigs seemed higher than the study in Thailand. The difference in the MAT-positivity among the animals studied between Thailand and Philippines may be due to the difference in the prevalence of leptospirosis among the animals or because of the difference in sample size. Thus, it is difficult to conclude whether seropositivity among these animals is higher in Philippines compared to Thailand.

In 1971, a study reported the seropositivity of 20-out-of-27 carabaos to serovars Tarassovi and Sejroe (Carlos *et al*, 1971). In the same study, 3 carabaos were culture-positive for serovar Tarassovi. In the present study, although no leptospires were isolated from the water buffaloes

(carabaos), antibodies against serovar Tarassovi were also detected in the said animals similar to what was reported by Carlos *et al* in 1971. This suggests that this serovar persists as an infecting serovar among these animals for several decades already.

Among the seropositive rats, 24 were from Nueva Ecjia while 21 were from Metro Manila. The most prevalent serovars infecting the animals were: Tarassovi for water buffaloes (18 out of 50, 36.0%); Patoc for cows (9 out of 25, 36.0%); serovar Manilae for dogs (23 out of 84, 27.4%); Poi for pigs (7 out of 12, 58.3%); and, Copenhageni for rats (20 out of 51, 39.2%).

In a previous study on rats in the Philippines (Villanueva *et al*, 2010), antibodies against serovar Copenhageni were not detected. However, in the present study, it was the most common serovar among the rats. One reason may be the sites where the rats were trapped in Villanueva *et al*'s study may have been different from the current study, thus the difference in some of the infecting serovars in rats. It may also be possible that there is an emergence of infections due to serovar Copenhageni.

Results of the study further revealed that Poi was the most common prevailing serovar among the animals studied (cows, dogs, pigs, and rats). Copenhageni was also commonly found in dogs, pigs, and rats. Serovar Patoc is a saprophytic leptospire and does not cause infection in humans and animals. In our study, antibodies against this saprophyte were also commonly detected in water buffaloes, cows, and dogs. The commonness of the serovars found among the animals in this study suggests that there is a possible transmission of these serovars among the said animal species. It may also be possible that there is a common source of infection among these animals. Although Patoc does not cause infection, it is usually included in the MAT panel of antigens since it has common antigens with the pathogens (ILS-WHO, 2003). There are more than 260 serovars of pathogenic leptospires and it is impossible to include all these antigens in one MAT panel. Thus, saprophytes, such as Patoc, are usually included in the panel.

The presence of antibodies in the animal sera in this study means that the animals were infected with leptospires (Babudieri, 1961). Although MAT is considered the gold standard of leptospirosis diagnosis, a disadvantage of the procedure is that it cannot differentiate between past or current infection and antibodies caused by vaccination (Hanson, 1976; Geisen *et al*, 2007).

Isolation of leptospires from humans and animals is difficult. Culturing samples from these hosts always has low yield (ILS-WHO, 2003). Among the 222 animal samples, leptospires were successfully isolated only from 7 rats. Leptospiral antigens can persist in the blood of animals up to seven days after infection (Adler and de la Pena-Moctezuma, 2010). It is possible that the blood samples were collected after the leptospiremic phase of the infection. On the other hand, the spirochetes appear in the urine only after two weeks post-infection, may persist in the kidney of the host from a few days to a few years, and shed continuously or intermittently throughout its carrier state (Babudieri, 1961). In this case, sample collection may have possibly fallen on days when the animal was not shedding leptospires in the urine. The zero isolation of leptospires especially from the big animals may also be because the animals may have already been infected in the past. Therefore, only

antibodies, not antigens, were detected. Another factor that may affect the isolation of leptospires is the acidity of the urine of the animals. Leptospires do not survive well in acidic urine but remain viable in alkaline urine (Adler and de la Pena-Moctezuma, 2010). Antibiotic treatment and vaccination of the animals can also kill the bacteria and prevent the shedding of viable leptospires. Domestic animals in the study might have been treated with antibiotics against leptospires prior to sample collection, which killed the leptospires and prevented their growth in culture.

All the isolates from rats were positive in *fla*B PCR, signifying that the isolates were pathogenic. However, it is interesting to note that the serotype of the *Leptospira* isolates from rats tested through sequencing and serotyping methods (Villanueva *et al*, 2014) were found to be different from the serotype of the antibodies detected in MAT. When compared with the MAT results of the rat samples, it can be seen that Copenhageni, despite being the most common serovar from rat samples (Table 2), was not isolated in any of the rats. However, of the two rats caught in Nueva Ecija, one rat was found to be carrying serovar Ratnapura (serogroup Grippotyphosa) and was also found to have homologous antibodies against this serovar. For rats trapped in Metro Manila, local serovar Manilae was mostly isolated. The serovars of the anti-*Leptospira* antibodies seen in the MAT of some of the rats from this area were mostly the same as the serovar of the isolates (Manilae). It means that the rats had antibodies against serovar Manilae and at the same time, are shedders/carriers of serovar Manilae.

Pathogenic leptospires can infect a wide variety of animals, the golden Syrian hamster is the preferred animal model for animal experiments because of its susceptibility to *Leptospira* infection, the reproducibility of results, and acute leptospirosis in this animals reproduce the severe form of leptospirosis in human (Haake, 2006). Although all of the rat isolates were pathogenic based on the *flaB* PCR and serotyping results, not all were lethal to hamsters (Villanueva *et al*, 2014). Possible reasons for this may be the infective dose of the isolate should be greater than 10^7 for hamsters, the 21 day observation period was too short, or the animal turned into carrier of leptospires as evidenced by the presence of the organisms in the kidneys and/or urine cultures even after 21 days of infection.

Although difficult, eradication of rodents is the best form of controlling leptospirosis. Another option would be to prevent transmission of infection by vaccination of animals that may be possible carriers of leptospires or high-risk groups.

There is a need to develop a vaccine containing the prevalent local serovars. The serovars that were isolated from this study may be used as vaccine components. Continuous epidemiological studies to monitor the circulation of serovars in the country should be conducted as well.

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