Serological and molecular identification of *Leptospira* spp. in swine and stray dogs from Malaysia

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Abstract. Leptospirosis is endemic in Malaysia with *Leptospira* species extensively isolated from domestic and wild animals. Rats were found to be the principal maintenance hosts followed by cattle, pigs, and dogs. The objectives of this study were to isolate and identify *Leptospira* serovars circulating among swine from three different farms and also from stray dogs and cats from Klang valley, Selangor, Malaysia. Urine and kidney samples collected from 150 stray dogs, 50 cats and 81 swine were inoculated into semi-solid Ellinghausen McCullough Johnson and Harris (EMJH) media supplemented with additional 5-Fluorouracil. Dark field microscopy revealed only one positive culture of *Leptospira* from dog and swine samples, but all cat samples were negative. The PCR technique using published primers detected 11 positives in urine samples of dogs and 5 positives from swine. The microscopic agglutination test (MAT) confirmed the presence of two serovars in both dog and swine populations namely, *L. interrogans* serovar Canicola and *L. interrogans* serovar Pomona (MAT ≥ 100), with *Not* I-PFGE analyses separating these two serovars into distinct profiles. Despite the low prevalence in stray dogs, the latter may play an important role in the contamination of the environment. Swine can also pose a potential risk of infection to humans and other domestic animals, especially those living close to swine farms. Thus improving hygiene and eradication of rodents in swine farms are likely to reduce the risk of infection.

INTRODUCTION

Leptospirosis is one of the most prevalent zoonotic disease found worldwide (Levett, 2001) that is caused by pathogenic species within the genus *Leptospira*. This disease has an major public health impact in view of its occurrence in human as well as in domestic and wild animal hosts. Pathogenic species of *Leptospira* colonize the renal tubules and urine of various mammals including rodents, domestic and wild animals. Humans can be infected indirectly from exposure to water or soil contaminated with urine of infected animals or through direct contact with animal reservoirs through skin or mucous membranes (eyes, nose, or mouth),

especially if the skin is broken from a cut or scratch (Bharti *et al.*, 2003). The clinical manifestation of leptospirosis is biphasic with acute or septicaemic phases lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine (Levett, 2001). The incidence of leptospirosis is highest in tropical and sub-tropical countries where environmental conditions for transmission and survival are optimal (Pappas *et al.*, 2008). Leptospires can survive for long periods in warm, moist soil and in water (Ullmann & Langoni, 2011). Some occupations especially those with recreational activities, farmers, veterinarians, fishermen, livestock and

abattoir workers constitute high risk populations (Levett, 2001).

Leptospirosis is an endemic disease in Malaysia and in recent years there has been a dramatic increase in the number of reported cases. According to Ministry of Health, Malaysia a marked increase in the disease occurred from 12.5 per 100,000 population in 2012 to 15.0 per 100,000 population in 2013, with 71 of 4,457 cases resulting in mortality (Benacer et al., 2016a).

Within the farming community, leptospirosis can potentially lead to economic losses, causing abortion, stillbirth, infertility, loss of milk production and death (Tilahun et al., 2013; Maleki et al., 2013). In Malaysia, Leptospira spp. have been isolated from a wide variety of animals (Bahaman & Ibrahim, 1988) including rodents, cattle, pigs, and dogs, which serve as potential carriers (Thayaparan et al., 2013).

Biodiversity within tropical ecosystems of Malaysia results in the divergence of transmission with isolates of Leptospira being detected from a variety of animals living in low land habitats to forests swamps and rivers (Bahaman & Ibrahim, 1988). To date 37 serovars of Leptospira from 13 different serogroups have been identified in Malaysia with more than half from rodents (Bahaman & Ibrahim, 1988; Benacer et al., 2013a). Both these studies showed that urban rats in Malaysia harbour two serovars, namely Bataviae and Javanica (Benacer et al., 2013a, Benacer et al., 2016b). The first report in Malaysia occurred in dogs (Fletcher, 1928), although both dogs and cats have been implicated as sources of infection because they mark territory by defecating and urinating indescrimiately and are closely associated with humans (Phumoonna et al., 2009). L. Canicola and L. Icterohaemorrhagiae are the main serovars infecting dogs although Pomona serotypes have also been reported (Joseph, 1979; Phumoonna et al., 2009). Leptospirosis in cats is rare (Bahaman & Ibrahim, 1988), but the importance of cats shedding Leptospira and serving as a source of infection has recently gained attention. An extensive study carried out by Gordon-Smith et al. (1961) showed that the serovar Pomona was found in 10.3% of 68 cats examined with non-observable clinical signs.

Leptospirosis in livestock particularly in swine is of great concern to farmers because the disease is usually chronic, occasionally resulting in abortions and still-births with poor survival rates in newborn piglets. An outbreak of Leptospira Icterohaemorrhagiae was reported as the cause of abortion in sows from Selangor (Bradenburg & Too, 1981) although serovars Pomona, Icterohaemorrhagiae and Tarassovi are also prominent in swine (Bahaman & Ibrahim, 1987).

The present study was undertaken to identify and assess the prevalence of Leptospira in swine and stray dog populations from the Klang Valley, especially as little research had been undertaken in Malaysia since the 1980's except for one study conducted by Phumoonna et al. (2009).

MATERIALS AND METHODS

Ethics statement
This study was approved with the ethics reference no. ISB/31/01/2013/SNMZ (R) by the Institutional Animal Care and Use Committee, University of Malaya, Malaysia (UM IACUC).

Study sites
Selected sites occurred in Klang Valley (latitude 3.139003 and longitude 101.686855), where large populations of stray cats and dogs roamed in close contact with human communities. The animals were captured by the workers (dog-catchers) of the Kuala Lumpur City Council from different sites in Klang Valley as part of an animal control program. A total of 3 swine farms located in Selangor State in central of Peninsular Malaysia, during 2012 and 2013. All sites were characterized by a tropical climate and high humidity throughout the year with temperatures ranging from 30°C to 36°C.

Isolation of Leptospira
All stray dogs and cats were screened for leptospirosis and in view of unknown

vaccination history, the general physical condition of each animal was noted at the time of sampling and approximate ages were determined by dental examination. Following euthanization, all dogs and cats underwent a post-mortem examination. Urine samples were collected via direct puncture of the bladder and then cultured in a modified semi-solid EMJH medium. Following removal of kidney tissue with sterile blades, small pieces of tissue were each placed in a sterile syringe and placed in EMJH medium (Sigma), supplemented with 5-fluorouracil (Merck, Germany). Cultures were incubated for 30 days at 30°C and examined at intervals of 10 days using a dark field microscope. For swine urine samples up to 2 to 3 drops were inoculated into in 5 ml of EMJH medium, followed by the addition of 10% BSA, incubated at 30°C and then examined every 10 days.

Serology

Serological identification of *Leptospira* isolates was performed using the microscopic agglutination test (MAT) as described by the World Health Organization (WHO, 2003). A set of 10 reference antisera were provided by the Institute of Medical Research (IMR), Malaysia. The antisera raised used in this study were against serovars Javanica (Veldrat Batavia 46), Canicola (Hond Ultrecht IV), Hebdomadis (Hebdomadis), Pomona (Pomona), Hardjo (Hardjoprajitno), Australis (Ballico), Bataviae (Swart), Icterohaemorrhagiae (RGA), Tarassovi (Perepelicin) and Bratislava (Jez Bratislava). Leptospiral isolates were cultured into liquid media with the addition of 1.0% rabbit serum to increase bacterial density. Agglutination of anti-leptospiral antibodies with living leptospires were viewed using dark field microscopy (Benacer *et al.*, 2013a).

PCR analysis

Fresh urine samples collected from dogs, cats and swine were further centrifuged and the sediments were used to detect leptospiral DNA. PCR primers, LA/LB [(5´-GGC GGC GCG TCT TAA ACA TG-3´) and (5´-TTC CCC CCA TTG AGC AAG ATT-3´)] which target the 16S rDNA gene were used to confirm the genus *Leptospira* (Merien *et al.*, 1992). To determine the pathogenic status of isolates, G1/G2 primers [(5´-CTG AAT CGC TGT ATA AAA GT-3´) and (5´-GGA AAA CAA ATG GTC GGA AG-3´)] were used as these target the secY gene among isolates except for *L. kirschneri* (Gravekamp *el al.*, 1993). Amplified DNA products from representative isolates were verified by DNA sequencing. Amplicons were purified using a DNA purification kit (Qiagen, Germany) and sequenced at a commercial facility (First BASE, Pte. Ltd., Singapore). The resulting DNA sequence data were compared with the GenBank database using a BLAST algorithm available at web site (http://www.ncbi.nlm.nih.gov).

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE analysis was carried out as described by Galloway & Levett (2008), but with minor modifications (Benacer *et al.*, 2013a). The standard size marker strain *S*. Braenderup H9812 was used as the PFGE size marker. Equal volumes of cell suspension were mixed with agarose and dispensed into wells of PFGE plug molds and allowed to solidify at room temperature for 20 min. Plugs were transferred into 50 ml flacon tubes containing 2 ml of cell lysis buffer (CLB) and Proteinase K (20 mg/ml). Plugs were then incubated at 55°C for 2 hours and after lysis, each plug was washed thoroughly in 10-15 ml of sterile deionized water for 2 times and then washed up to 6 times with 1X TE buffer (Benacer *et al.*, 2013a).

RESULTS

Within the study location, a total of 150 dogs (n=106 males; n=44 females) with an average age of 30 months and 50 cats (n=22 males; n= 28 females) with an average age of 18 months were examined for leptospires (Table 1). Molecular characterization detected a low prevalence (7.3%; 11/150) of *Leptospira* in the urine of adult dogs aged between 2 and 5 years. Only one isolate successfully grew on EMJH medium and this was further identified as *L. interrogans* serogoup Canicola by the microscopic

Table 1. The number of urine and kidney samples examined for *Leptospira* isolates from dogs, cats and swine in Peninsular Malaysia

Number of hosts	Number of samples		Host gender		Positive samples	
	Urine	Kidney	Male	Female	PCR	Culture
Dogs (150)	128	22	106	44	11	1
Cats (50)	36	14	22	28	0	0
Swine (81)	81	0	0	81		

agglutination test (MAT). DNA sequencing of 11 positive samples confirmed that 9 were identified as *L. interrogans* serovars Canicola (98% similarity), with the remaining two samples being Icterohaemorrhagiae (99% similarity). Nine positive dogs (82%) were males while the remaining 2 (18%) were females. Six of the positive dogs (54.5%) presented with skin lessions and the entire cat samples were negative using both culture and PCR.

Of 81 urine samples examined from female swine, only 5 (6%) samples were positive based on the PCR method, and one sample was clearly identified as *L. interrogans* serogroup Pomona using MAT. Using PCR and DNA sequencing resulted in 4 positive samples being identified as *L. interrogans* serovar Pomona and the remaining sample as *L. interrogans* serovar Bratislava, all with up to 99% similarity (Table 1).

PFGE of *Not*I-digested chromosomal DNA subtyped 2 isolates into 2 PFGE profiles (LS01 and LD01) and whereas 10 to 25 DNA fragments ranging from 28 to 706 kb were generated. Both PFGE profiles were compared with the *Not*I patterns from the database of the Center for Disease Control and Prevention (CDC), USA, confirming that LS01 was similar to *L. interrogans* serovar Pomona, and LD01 to *L. interrogans* serovar Canicola (Figure 1).

DISCUSSION

Human leptospirosis is on the rise in Malaysia and consequently there is an urgent need to update the role of domestic animals in the
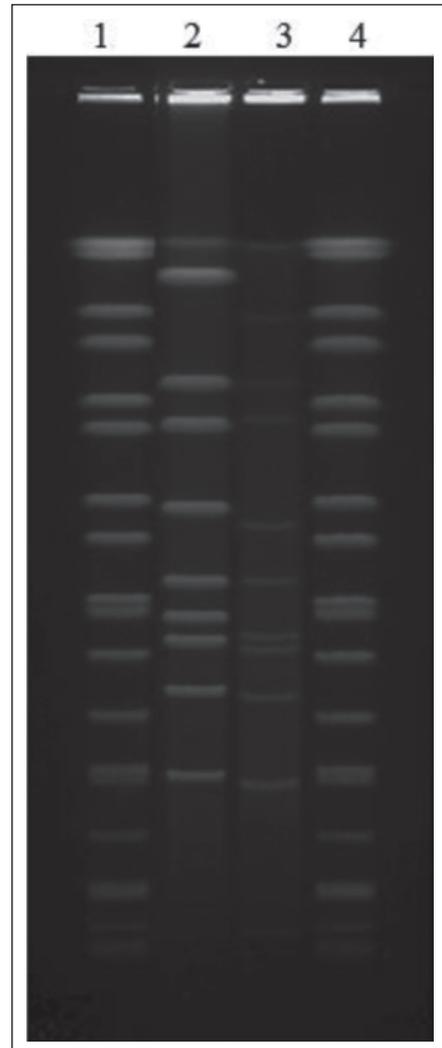

Figure 1. Representative PFGE patterns of *Not*I digested chromosomal DNA of *Leptospira* isolates from dog and swine hosts in Peninsular Malaysia.

Lanes 1,4: *XbaI* digested chromosomal DNA of *Salmonella* H9812 marker strain; lane 2: *L.* Pomona; lane 3: *L.* Canicola.

potential spread of the disease locally and nationally. New information on prevalence and serovar types circulating in the population can facilitate better understanding of how these animal reservoirs contribute to disease transmission to humans, thus improving research on existing diagnostic methods i.e MAT and ELISA and contribute to the development of an effective vaccine against the pathogen.

Recently Rafizah *et al*. (2013) determined the seroprevalence of leptospirosis among febrile inpatient cases in northeastern Malaysia, where 88 of 999 cases admitted to 10 hospitals were positive with over 50% being in direct contact with domesticated animals either through outdoor activity or agriculture. Rodents, cattle, dogs and swine are known to be primary reservoirs of *Leptospira* in Malaysia (Thayaparan *et al*., 2013) and the present study confirmed that infections do occur in stray dogs and swine but not in cats. Therefore the identification of serovars in stray and domesticated animals in Malaysia will help to extrapolate epidemiological patterns of infection of leptospirosis.

In the present study the PCR technique recorded low infections in the stray dog population compared with Phumoonna *et al*. (2009), who found that 17 of 142 IgM seropositive samples were identified as the serovar Pomona. These authors reported that all dogs were in the early stages of the disease with only one being IgG positive, and also confirmed by PCR with low MAT titres (50 to 100). In contrast the present results indicate that infected dogs were in the later stages of the disease or were reservoir hosts for the pathogen. The latter assumption is in accordance with sequencing results of the PCR products which showed 9 of 11 positive samples belong to the serovar Canicola. Dogs with subclinical infections can be reservoir hosts for the serovar Canicola with the ability to shed the bacteria in urine into the environment over long periods (Aedo & Smits, 2014).

In the present study positive samples occurred in dogs older than 1 year, which is similar to the findings of Batista *et al*. (2005)

and Aguiar *et al*. (2007) where dogs in this age category were at higher risk. On the other hand neither age nor gender of dogs influenced infection with leptospires as also shown in studies undertaken by Alton *et al*. (2009) and Kikuti *et al*. (2012). Other risk factors may be involved as 6 of the infected dogs in the present study showed skin lesions, facilitating transmission of the spirochete bacteria through the damaged skin.

Two serovars identified in dogs the present study included Canicola from 9 and Icterohaemorrhagiae from 3 samples and these appeared to be the most prevalent in Malaysia (Gordon-Smith *et al*., 1961; Phumoonna *et al*., 2009) and worldwide (Bolin, 1996; Kikuti *et al*., 2012; Vojinovic *et al*., 2015). Dogs are the main hosts for Canicola, although the occurrence of this serovar has declined in many European countries (Vojinovic *et al*., 2015) mainly attributable to the use of vaccines in recent years. Similarly, from 1970 to the mid 1990's, a decline in the prevalence of both Canicola and Icterohaemorrhagiae serovars in North America was reported following the introduction of vaccination, but infections have since remerged (Bolin, 1996). The serovar Pomona previously reported by Phumoona *et al*. (2009) did not occur in dogs in the present study.

Wild rats are he sources of the infection with Icterohaemorrhagiae in dogs and consequently urban dogs are at a higher risk of infection than rural dogs due to the abundance of rodents in the urban environment (Ampily, 2013; Alton *et al*. 2009). Peripheral urban and slum areas with inadequate sanitation, open sewers and poor garbage management will attract large numbers of rodents and consequently expose dogs to infection (Lelu *et al*., 2015).

Unlike dogs, cats in this study were free from infection although, the serovar Pomona was previously identified in Malaysia but with no observable clinical signs of leptospirosis (Gordon-Smith *et al.*, 1961). In a more recent serological survey, Mosallanezhad *et al.*, (2011) found only 5 of 105 cats infected with the serovars Ballum and Australis. Cats can be incidental hosts to a variety of *Leptospira*

serovars prevalent in wildlife or domestic hosts. In Southern India, Natarajaseenivasan *et al.* (2002) showed that up to 33% of cats were infected with theserovar Autumnalis and 16.6% both with Canicola and Icterohaemorrhagiae. The serovar Autumnalis was also detected in rats from rice fields in the same region. In the metropolitan area of Goiania, Brazil, Parreira *et al.* (2010) found that 23 of 330 cats were infected with up to five serovars including Cynopteri, Djasiman, Butembo, Castellonis and Patoc.

Wisseman *et al.* (1995) conducted the earliest study on swine leptospirosis and found 3 of 5 pigs positive for Autumnalis, Pomona and Sentot. In the present study, low infections (6.2%) of the serovar Pomona and Bratislava occurred in female pigs over 2 years of age. The presence of Pomona in swine is in agreement with previous studies (Bahaman *et al.*, 1987; Tan, 1981) although Icterohaemorrhagiae, Cynopteri, Pomona and Pyrogenes were also previously reported by Joseph (1979).

The decline in infections may be due to improved husbandry practices and the extensive use of antibiotics in feeding regimes in swine farms (Bahaman *et al.*, 1987). Icterohaemorrhagiae, which primarily occurs in rats acting as reservoir hosts, was previously found in swine in Selangor resulting in an abortion epidemic (Brandenburg & Too, 1981).

Culturing *Leptospira* is tedious and time consuming as this slow growing bacterium is normally displaced by other contaminating microorganisms in enrichment media (Benacer *et al.*, 2013b). The PCR technique accompanied with sequencing proved to be the more sensitive method for serovar identification compared with culture techniques. This method was also used in the early detection of serovars of *Leptospira* especially in clinical, animal and environment samples (Fonseca *et al.*, 2006; Bomfim *et al.*, 2008; Benacer *et al.*, 2013b). However, one limitation of the present study is the use of urine as the only source available for PCR screening. The occasional negative result can occur mainly because infections in susceptable hosts are influenced by several factors such as the type of infection and the timing of sampling or if reservoir or accidental hosts are involved. PCR screening of urine samples from reservoir hosts can occasionally be negative and not always accurate due to the intermittent shedding of leptospires, whereas in accidental hosts, leptospires can only be detected in urine at the later stages of the disease. The use of kidney tissues for conventional PCR screening may also be limiting when material with a high concentration of host's DNA is used. Such a limitation could be overcome by using real time PCR to investigate kidney tissue for the colonization of leptospires.

In the present study, PFGE of *Not*I-digested *Leptospira* DNA showed two profiles which were in accordance with MAT when referencing PFGE profiles in the leptospiral database at CDC. Consequently the serovars *L. interrogans* Canicola in dogs and *L. borgpetersenii* Pomona in swine were identified, thus confirming PFGE as a promising and useful tool in the identification of leptospire isolates.

The present findings indicate that stray dogs rather than cats are potential carriers of this zoonotic disease and contribute to the spread and maintenance of *Leptospira* spp. in Malaysia. Further studies using larger samples of hosts from a variety of sites and habitats will contribute further to our understanding of the epidemiology of leptospirosis infections in stray canine and feline hosts, but combined with methods of disease prevention and control including vaccination.

Swine can also be potential carriers of *Leptospira* serovars, resulting in the spread of disease to farmers, livestock handlers and other animals. Therefore, apart from vaccination, preventive measures should be used in situations where spirochete bacteria are likely to thrive and these include the exclusion of rodents from swine farms and avoiding where possible habitats such as stagnant water, ponds, marshes and muddy areas.

Conflict of interest:
The authors declare that they have no competing interests.

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