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Prevalence of leptospirosis in healthy dogs and dogs with kidney disease in Klang Valley, Malaysia

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Abstract. Leptospirosis is under-reported and remarkably neglected in Malaysia, especially in companion animals. In recent years, dogs have become popular pets and potentially act as one of the risk factors for human leptospiral infection. The purpose of this study was to determine the serological and molecular status of leptospirosis in healthy and dogs with kidney disease in Klang Valley, Malaysia and to gain insight of the possible serovars involved in the dog population in Klang Valley, Malaysia. Blood samples were obtained from 57 dogs (19 kidney disease patient; 38 healthy dogs, respectively). Serum samples obtained from these animals were screened for leptospiral antibodies by the microscopic agglutination test (MAT). Polymerase chain reaction (PCR) assay was performed on plasma samples to detect leptospiral DNA. By MAT, three out of 19 (15.8%) dogs with kidney disease were positive for *L. canicola*. One out of 38 (2.6%) healthy dogs was positive for *L. icterohemorrhagiae*. The overall seroprevalence for leptospirosis in dogs in Klang Valley, Malaysia was 7.0% (n=4/57). Only one out of the 19 dogs (5.3%) with kidney disease was tested positive to pathogenic *Leptospira* by PCR assay. All the 38 healthy dogs were negative. Positive results in healthy dogs and dogs with kidney disease for leptospirosis warrant further investigation of leptospirosis in dog population in Malaysia. The prevalence and incidence of this disease in the dog population in this country need further investigation.

INTRODUCTION

Klang Valley is the heartland of the Malaysia's metropolitan area densely populated with over six million humans covering an area of approximately 2800 square kilometres (Department of Statistics, Malaysia, 2010). Despite its rapid transformation into a wide urban region for the past decade, not only there was a tremendous increased in human population, the dog population has also increased as dogs have become popular pets among pet owners. Therefore, the potential role of dogs in disease transmission has become an important issue.

As one of the globally important zoonotic diseases (Bharti *et al.*, 2003), leptospirosis is under-reported and remarkably neglected in Malaysia, especially in companion animals for the past decades. The first case of leptospirosis in domestic animals in Malaysia was reported in dog by Fletcher in 1928 (Fletcher, 1928). Since then, investigation on leptospirosis in dog population in Malaysia is limited as dogs are presumably related with low prevalence of leptospirosis although dogs have become popular pets which act as one of the risk factors for human leptospiral infection (Bahaman & Ibrahim, 1988; Chandrasekaran *et al.*, 1995).

Clinical signs of leptospirosis in dogs have often been associated with acute multisystemic febrile illness, such as fever,

anorexia, coagulopathies, hepatic disease, and renal failure (Goldstein, 2010). Most cases of leptospirosis can be detected serologically as antibodies in the blood increased approximately five to seven days after onset of symptoms. Microscopic agglutination test (MAT) remains as the definitive and gold standard of serological investigation for leptospirosis. The presence of agglutinating antibodies indicates the evidence of current infection or past exposure, however it cannot distinguish between antibodies caused by natural infection from those caused by vaccination. Therefore, paired sera test with fourfold or greater rise in titer has been used to confirm leptospiral infection with MAT (Levett, 2004; Ooteman *et al.*, 2006; Shivakumar and Krishnakumar, 2006). Molecular detection with polymerase chain reaction (PCR) has been widely used for the early diagnosis of leptospirosis (Ooteman *et al.*, 2006). It can detect the circulating leptospiral DNA before the development of the antibodies. Despite its high sensitivity (up to 100%) and specificity (88.3%) (Harkin *et al.*, 2003), PCR assays do not identify the infecting serovars, therefore a further step of DNA sequencing need to be performed in order to conclusively diagnose the serovars.

The main aim of this study was to determine the prevalence of leptospirosis and to compare the serological and molecular status of leptospirosis between healthy and dogs with kidney disease in Klang Valley, Malaysia. The second aim was to determine the possible serovars affecting the dog population in Klang Valley, Malaysia. The results of this preliminary study could provide some information on the current status of canine leptospirosis in these selected areas of Klang Valley, Malaysia, which can be useful to the small animal practitioners.

MATERIALS AND METHODS

Data collection

Dogs diagnosed with acute or chronic kidney disease with unknown etiology and healthy dogs were selected from the Society for the Prevention of Cruelty to Animals (SPCA) Selangor, Paws Animal Welfare Society (PAWS), and ten private veterinary clinics located in Klang valley. The selection criteria for the dogs diagnosed with kidney disease included; 1) abnormal physical examination findings such as lethargy, dehydration 5-12% with +/- painful or enlarged kidney upon abdominal palpation, 2) history and clinical signs of vomiting, inappetance, polyuria and polydipsia, 3) abnormal complete blood count and serum biochemistry profiles especially increased serum BUN (>7.5 mmol/L) and creatinine (>176 µmol/L) levels and 4) abnormal urinalysis findings with a urine specific gravity <1.035. As for the healthy dogs, the dogs were recruited based on; 1) normal physical examination and 2) absence of clinical signs of kidney disease.

Information such as age, sex, body weight, vaccination status, and general health status were recorded. Owner consent forms and consents from animal shelters were obtained prior to recruitment of the dogs into the study. This study was carried out with the approval by Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUP-080-FPV-SYP-2013).

Blood sample collection

The dogs were restrained and 3 ml of blood were collected from the cephalic vein. Blood samples obtained were kept in EDTA anticoagulant and plain tubes and were immediately subjected to centrifugation. The serum and plasma obtained were transferred into 1.5 ml eppendorf tubes and stored at -30°C until further analysis. Both the MAT and PCR assays were performed at the Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM.

Microscopic Agglutination Test (MAT)

The dog serum samples were tested for antibodies against ten Leptospiral serovars, namely, *canicola*, *pomona*, *icterohaemorrhagiae*, *australis*, *andaman*, *bataviae*, *hebdomadis*, *tarassovi*, *grippothyphosa*, and *shermani*. The MAT

was performed as described by Everard (1995). For each serovar, MAT result was reported as the highest dilution of serum at which more than 50% agglutination of organism occurred. The antibody titers of $\geq$ 1:80 were considered as seropositive (Soman *et al.*, 2014).

Primer selection

Genus-specific primer (GGC GGC GCG TCT TAA ACA TG) (Scarcelli *et al.*, 2003) for the PCR assay to detect pathogenic *Leptospira* was selected by using specific primer targeting the 531 bp fragment of the pathogenic *Leptospira* gene.

DNA Extraction and Polymerase Chain Reaction Assay

Extraction and isolation of genomic DNA from blood plasma were carried out using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction. The preparation of PCR product was carried out using TopTaq Master Mix cocktail solution (Qiagen, USA). The master PCR cocktail was prepared, containing 25 µl of TopTaq Master Mix 2x, 1.25 µl of Genus Specific Forward Primer, 1.25 µl of Genus Specific Reverse Primer, and 15 µl of RNase free water. Five microliters of DNA template was placed into a PCR tube and 45 µl of PCR cocktail was then transferred into the PCR tube and mixed with the DNA template, making up to a total volume of 50 µl. For negative control, 5 µl of distilled water was used instead of the DNA template and purified DNA from the stock culture of *L. canicola* was used as positive control. The PCR tubes were then placed in the Thermal Cycler (Cyclogene, UK) programmed for the amplification of the specific leptospira genes. The reaction was performed for 30 cycles of 94°C for 4 minutes, 58°C for 90 seconds and a final extension step of 72°C for 10 minutes. After amplification, 5 µl of each sample, including the positive and negative controls were evaluated by use of electrophoresis on a 1.5% agarose gel.

RESULTS

A total of 57 blood samples were collected from dogs. Nineteen dogs diagnosed with kidney disease (ten males, nine females) were recruited; aged more than 12 months, except for two dogs which were five months old. Of the 19 dogs diagnosed with kidney disease, 15 were vaccinated against *L. icterohemorrhagiae* and *L. canicola*. All the dogs had increased BUN and creatinine levels in serum and five were accompanied with jaundice.

Thirty eight healthy (14 males, 24 females) dogs were recruited. Of the 38 dogs, seven were less than 12 months whereas the other 31 dogs were older than 12 months of age. All the dogs except three dogs in the healthy group have been vaccinated against *L. icterohemorrhagiae* and *L. canicola*.

Microscopic Agglutination Test (MAT)

In our study, three out of 19 (15.8%) dogs with kidney disease were positive for *L. canicola*. Of 38 healthy dogs, one (2.6%) was positive for *L. icterohemorrhagiae*. The overall seroprevalence of leptospirosis in dogs in Klang Valley, Malaysia was 7.0% (n=4/57) by MAT with titers of 1:80 (Table 1). All the animals tested negative for eight other serovars. None of the dogs showed seropositivity for multiple serovars.

Polymerase Chain Reaction (PCR)

Plasma samples from the dogs (n=57) were subjected to PCR and only one of the 19 dogs (5.3%) (Table 1) with kidney disease tested positive to pathogenic *Leptospira* spp. DNA sequencing was performed using the PCR product amplified from DNA extracted from positive plasma sample and the sequence analysis indicated the result corresponding to the *L. canicola*. This infected dog was diagnosed with acute kidney failure and jaundice. History obtained from the owner revealed that the dog had rat hunting behavior and had not been vaccinated. None of the healthy dogs was positive on PCR for pathogenic leptospirosis.

Table 1. Prevalence of Leptospirosis in dogs in Klang Valley, Malaysia

Category of dogs	Number of dogs tested positive with MAT	Number of dogs tested positive with PCR
Gender		
Male (n=24)	3	1
Female (n=33)	1	0
Age (years)		
≤1 (n=9)	0	0
>1 (n=48)	4	1
Health status		
Healthy (n=38)	1	0
Kidney disease (n=19)	3	1

MAT, Microscopic agglutination test; PCR, Polymerase chain reaction

DISCUSSION

The findings of seropositivity and molecular presence of Leptospirosis in dogs in Klang Valley supports the role of dogs in the transmission of *Leptospira* spp. to humans from animals. In this preliminary study, dogs from Klang Valley had a low leptospiral prevalance of 7.0% compared to those prevalence reported in New Zealand (14.2%) and India (36.4%) (O'Keefe *et al.*, 2002; Soman *et al.*, 2014). The present study showed evidence that dogs in Malaysia are exposed to and are regularly sub or clinically infected by pathogenic *Leptospira* spp. Since humans and dogs share the same environment, humans could be exposed.

Based on serological test in this study, only two leptospiral serovars were detected. Among four out of the 57 dogs, three dogs was tested positive for *L. canicola* (5.3%) and one dog tested positive for *L. icterohaemorrhagiae* (1.8%). All the other animals tested negative to other eight serovars. According to Patil *et al.* (2014), it was reported that in India, the major prevalent leptospiral serovars found in dogs and rodents were similar to human leptospirosis, which was *L. canicola*. Similarly in this study, *L. canicola* was shown to have the highest prevalence of 5.3% followed by *L. icterohaemorrhagiae*.

The serological data on *Leptospira* exposure in dogs in this study must be interpreted with caution as it can be complicated by the presence of antibodies due to active response to field infection or due to previous vaccination (Harkin *et al.*, 2003; Iwamoto *et al.*, 2009). Two out of the three dogs found seropositive in this study for *L. canicola* were vaccinated annually. These two dogs should have been protected against common leptospiral infection but both dogs showed evidence of acute kidney failure and jaundice with unknown cause. Hence, the potential role of the leptospirosis causing the kidney and liver failure in these dogs remains to be elucidated. There could still be a possibility that these dogs were infected by other serovars that were not tested. Another dog which was positive for *L. canicola* was not vaccinated and was presented with clinical signs of vomiting and hematology results indicative of kidney disease. One dog that showed seropositivity against *L. icterohaemorrhagiae* had been vaccinated and the dog was recruited from PAWS. This dog was active and alert during blood sampling and showed no evidence of leptospiral infection. Without second blood sampling, we cannot rule out leptospirosis as this dog might be at the subclinical stage of the disease.

In our study, the dogs with seropositivity against *Leptospira* serovars were negative on the PCR test. The dog with kidney disease and positive on PCR was negative serologically. The different findings between these two diagnostic methods performed on the same group of animals could be due to different stages of the disease, acute versus convalescent stage. The clinical presentation of leptospirosis is biphasic (Levett, 2004). The acute stage of clinical infection lasts for one week, followed by the immune or convalescent stage lasting from two weeks to months and years, characterized by the production of antibodies and excretion of leptospires from urine by the animal (Levett and Haake, 2010). MAT is a serological test that detects the antibodies against the specific leptospiral serovars, which is useful if it was carried out on animals during the convalescent stage when antibodies were massively produced. PCR assay is a molecular test that detects the DNA of the leptospires, which could be helpful if it was carried out on animals having acute leptospiral infection. This study, PCR assay was only performed on the blood plasma and not on urine samples. The opportunity to detect leptospiral DNA in the urine of the animals is further diminished. Combining of the both techniques should be done in prevalence study with unknown endemic status; this is to prevent the likelihood of the false negative of either method.

As this was a preliminary study of canine leptospirosis with unknown epidemic status in the selected areas of Klang Valley, a cut-off titer of 1:80 was used. Different MAT cut-off titers have been used in different surveys (Vijayachari *et al.*, 2001; Moore *et al.*, 2006; Soman *et al.*, 2014). Although a four-fold rise in titer of paired sera is the most definitive criterion for diagnosis of current leptospiral infection (Levett and Haake, 2010), second sample of blood from same animal is difficult to obtain in the prevalence study. Studies have shown that the best cut-off titer to be used is 1:100, with sensitivity of 70.7% and specificity of 95.0% in high endemic areas, and sensitivity of 68.3% and specificity of 96.5% in low endemic areas (Petkanchanapong *et al.*, 2009). Further investigations are required to determine an appropriate cut-off titer to be used for future prevalence study of leptospirosis in our local setting.

The prevalence of leptospirosis in this study (7.0%) was lower compared to those reported in New Zealand and India (O'Keefe *et al.*, 2002; Soman *et al.*, 2014). Environmental factors, namely, climate change, amount of rainfall, floods, and presence of outbreak need to be taken into consideration for results interpretation. Lau *et al.* (2010) stated that the combination of climate change, flooding, population growth and urbanization have led to a drastic escalation in the global burden of leptospirosis. In this study, blood samples were obtained from urban dogs in the Klang Valley in January 2014 which has low amount of rainfall and absence of flooding.

Incidence of leptospirosis in both human and animal population in Malaysia could be underreported, due to inadequate health and surveillance networks. The results from our study helped to shed some light on the importance of further investigation the disease's current epidemiology, especially in dogs and its' role in disease transmission. In the future studies, large sample size should be involved in order to better understanding and assisting in the planning of more targeted control programs. Planned, region based studies could provide valuable insights into the epidemiology of leptospirosis in dog populations in Malaysia.

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