Tropical Biomedicine 33(2): 276–284 (2016)

Seroprevalence and molecular detection of leptospirosis from a dog shelter

Khor Kuan Hua[1], Tan Wei Xian[1], Lau Seng Fong[1], Mohd Azri Roslan[1], Rozanaliza Radzi[1], Siti Khairani Bejo[1] and Abdul Rani Bahaman[1]
[1]Faculty of Veterinary Medicine, University Putra Malaysia, UPM Serdang, Selangor, Malaysia
*Corresponding author e-mail:
Received 28 September 2015; received in revised form 20 November 2015, accepted 25 November 2015

Abstract. A study on seroprevalence and molecular detection of canine leptospirosis was carried out in a dog population (randomly selected *n*=80 dogs) from an animal shelter X. All the dogs in Shelter X appeared clinically healthy. Eighty blood samples were obtained and their serum were serologically examined using Microscopic Agglutination Test (MAT) against 10 Leptospira serovars. Plasma samples obtained were subjected to Polymerase Chain Reaction (PCR) assay. Three out of 80 dogs (3.8%) tested positive for *L. bataviae* based on MAT at a titer of 1:80. The seroprevalence of 9 other Leptospira serovars was not evident in this study. All the dogs tested negative against leptospirosis with PCR assay. In conclusion, canine leptospirosis was detected in dogs in this animal shelter. *L. bataviae* was identified as the infecting serovar. To our knowledge, this is the second report of serovar Bataviae infection in dogs in Malaysia. The 3 dogs in our study could possibly be a source of leptospiral infection to other dogs and may shed the bacteria into the environment. This serovar is not available in canine vaccination programs, therefore the dogs are not protected from this disease. Further investigation is warranted to determine whether the infected dogs are carriers of this serovar.

INTRODUCTION

Leptospirosis, an emerging worldwide zoonosis is caused by thin helical spirochetes belonging to the genus *Leptospira* (Adler & Moctezuma, 2010; Kikuti *et al*., 2012). It is further classified into pathogenic (nearly 300 serovars) and saprophytic (more than 60 serovars) species (Adler & Moctezuma, 2010; Hartskeerl *et al*., 2011). *Leptospira* poses a great public health concern as humans are at risk of infection due to close contact with carrier rodents, dogs, wild and domestic animals which may be the maintenance hosts of various leptospiral serovars (Venkataraman & Nedunchelliyan, 1992; Goldstein *et al*., 2006). According to Miller (1991), most cases of human leptospirosis around the world were due to transmission by rodents. Epidemics reported in Nicaragua (2007), in Sri Lanka (2008), and in Philippines (2009) were examples of leptospirosis outbreaks which resulted in hundreds of deaths and affected thousands of people (Agampodi *et al*., 2009; Koizumi *et al*., 2009; McCurry, 2009). Although leptospirosis has been reported in Malaysia, the actual disease burden in this country remains underestimated.

Canine leprospirosis was first described in 1899 and serovars Icterohaemorrhagiae and Canicola were believed to be responsible for most clinical cases of canine leptospirosis (Brown *et al.*, 1996.). However, the predominant *leptospira* serovars have shifted to others such as Grippotyphosa, Pomona, Bratislava, and Autumnalis as a result of the widely used bivalent vaccines (Goldstein *et al*., 2006). Canine leptospirosis is a worldwide zoonosis and its distribution pattern within the canine population can greatly varied according to different countries (Venkataraman & Nedunchelliyan, 1992; Jimenez-Coello *et al*., 2008; Lavinsky

et al., 2012; Roqueplo *et al.*, 2014). A higher occurrence of canine leptospirosis was observed in tropical countries (Weekes *et al.*, 1997), especially during rainy days (Meeyam *et al.*, 2006; Jimenez-Coello *et al.*, 2008) and many studies of human leptospirosis caused by transmission from dogs to humans has been reported in the temperate regions (Feigin *et al.*, 1973; Fraser *et al.*, 1973; Everard *et al.*, 1987; Gautam *et al.*, 2010; Levett, 2001). In Malaysia, the first case of canine leptospirosis was reported by Fletcher (1928) and limited studies (Bahaman & Ibrahim, 1987; 1988) were carried out after that. According to the most recent study by Low (2014), the seroprevalence were 5.3% for serovar Canicola and 1.8% for serovar Icterohaemorrhagiae in the 57 pet dogs. This indicates that dogs can be an important reservoir for human leptospirosis (Lau, 2016).

A study of canine leptospirosis in Australia which focused on dogs from animal shelter revealed the seroprevalence of 1.9% (n=956) (Zwijnenberg *et al.*, 2008). According to Oliveira (2012), leptospiruria was detected in 20% of the 65 urine samples from shelter dogs tested molecularly. In another study by Cruz-romero (2013), eight dogs were tested positive for leptospirosis among 92 dogs (8.6%) from dog shelters in Mexico using serological method. These indicate that shelter dogs may play an important role in public health zoonosis as the apparently healthy dogs are actively shedding leptospires to the environment.

Thus, the prevalence of the disease among dog shelters in Malaysia should be investigated in order to reduce the possible risk of leptospirosis transmission among the dogs and also to the caretakers or adopters of these dogs. This study was a preliminary study investigating whether an animal shelter located in Selangor was infected with canine leptospirosis and the epidemiological information was documented.

MATERIALS AND METHODS

Sample Collection

Prior to sample collection, consent was obtained from the representative of the animal shelter. This study conducted obtained approval from the Institutional Animal Care and Use Committee (IACUC, UPM/IACUC/FYP-2014/FPV.041).

Eighty dogs were randomly selected from the animal shelter (Shelter X) as a representative of the total population approximately 350 dogs. The dogs were manually restrained for blood collection. Approximately 3 mL of blood collected via cephalic venipuncture were immediately transferred into ethylenediamine tetraacetic acid (EDTA) tubes and plain tubes. All the blood samples tubes were maintained at 4°C and immediately transported to the Bacteriology Laboratory in Faculty of Veterinary Medicine, Universiti Putra Malaysia. The blood samples were immediately centrifuged at 5000 rpm for 10 minutes. Blood serum and plasma isolated from the plain tubes and EDTA tubes respectively were then transferred into 1.5 mL Eppendorf tubes and stored at -20°C until further analysis. Information such as name, sex, age, and vaccination status of each dog was obtained.

Microscopic Agglutination Test (MAT)

Serum samples were tested against ten leptospiral serovars antigen including Canicola, Icterohaemorrhagiae, Grippotyphosa, Pomona, Andaman, Tarassovi, Hebdomadis, Australis, Lai, and Bataviae. Preparation of the leptosoiral serovars (live antigens) for MAT required the antigens cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium and incubated at 30°C for 5 to 7 days. All the live antigens were checked before used.

The microtiter plates which contained positive control, negative control and serial dilution of the sample tested were prepared as follows. The wells of first column were set as negative control while the wells of last row were set as positive control. All wells of first row of a microtiter plate were filled with 50 µL of phosphate buffer saline (PBS) of pH 7.2. Additional 40 µL of PBS was then added to the well of second column. Ten microliter of serum sample was then added to the well of second column. It was followed by serial dilution of 50 µL starting from second well

until the last well. The last 50 µL of dilution from the last well was discarded. These steps were repeated for all the serum samples. Fifty microliter of leptospiral antigen was then added to all the wells and mixed thoroughly using an incubator shaker at 37ºC for 2 minutes. The samples were then incubated for 2 hours at 37ºC. After incubation, a drop of the solution from each well was placed on a glass slide and examined under dark field microscope. Any evidence of microscopic agglutination was noted and the highest antibody titre for each sample was recorded.

DNA Extraction and Polymerase Chain Reaction (PCR) Assay

DNA extraction and isolation of genomic DNA from blood plasma were carried out using DNeasy Blood and Tissue Kit (QIAGEN, Germany). Twenty microliter of proteinase K and 100 µL of anticoagulant-treated blood sample were transferred into a 1.5 mL Eppendorf tube. The total volume was then adjusted to 220 µL by adding 100 µL of PBS. Cultured leptospiral antigen of serovar Canicola was prepared as below and used as the positive control. One milliliter of leptospiral antigen was transferred into a 1.5 mL Eppendorf tube and centrifuged at 5000 rpm for 5 minutes. Supernatant of the positive control was discarded and re-suspended with 200 µL of PBS and 20 µL of proteinase K. Two hundred microliter of Buffer AL added into the mixture, mixed thoroughly by vortexing for 10 seconds and incubated at 56ºC for 10 minutes. After incubation, 200 µL of ethanol (96-100%) was then added and mixed by vortexing for 10 seconds. Five hundred microliter of the mixture was pipetted and transferred to a DNeasy Mini spin column placed in a 2 mL collection tube. The mixture was then centrifuged at 8000 rpm for 1 minute and the flow-through in the collection tube was discarded. Spin column was placed into a new collection tube and 500 µL of Buffer AW1 was added. The mixture was then centrifuged at 8000 rpm for 1 minute. Previous steps were repeated by adding 500 µL of Buffer AW2 and centrifuged at 14,000 rpm for 3 minutes. After the flow-through and collection tube were discarded, the spin column was transferred to a new 1.5 mL Eppendorf tube. The DNA was eluted by adding 200 µL of Buffer AE to the center of the spin column membrane and incubated for 1 minute at room temperature. The mixture was again centrifuged at 8000 rpm for one minute. The spin column was then discarded and the flow-through served as the DNA template for PCR assay.

PCR product was prepared by using Top Taq Master Mix cocktail solution. The PCR cocktail solution consist of 12.5 µL of Top Taq Master Mix 2x, 1.25 µL of Genus Specific Forward Primer, 1.25 µL of Genus Specific Reverse Primer, 1.25 µL Pathogenic Specific Forward Primer, 1.25 µL of Pathogenic Specific Reverse Primer, and 2.5 µL of RNase-free water. Twenty microliter of PCR cocktail was transferred into a PCR tube together with 5 µL of sample DNA template. The mixture was mixed thoroughly by vortexing and followed by short spinning. Five microliter of RNase-free water was used as negative control. Master cycler Pro S (Eppendorf, Germany) was used to amplify the specific leptospira genes. All the samples were processed by following the steps mentioned earlier to produce PCR products from the DNA templates for gel electrophoresis.

Agarose gel (1.5%) was selected for separation of small DNA molecules (100-1000 base-pairs in length). The gel was prepared by mixing 1.5 g of HyAgarose™ powder with 100 mL of fresh 0.5% Tris-Borate-EDTA (TBE) solution. The solution was then microwaved for 2 minutes and cooled under running tap water for a few minutes. The cassette was set up. The gel was poured into the cassette and left to solidify which contained 14 wells to be loaded with DNA products for electrophoresis. The electrophoresis tank was filled with 0.5% TBE solution until the gel was covered. First well of the agarose gel was filled with 2 µL of 100 bp DNA ladder. Five microliter of PCR product was mixed with 2 µL of loading dye before the mixture was loaded into the well of agarose gel. The same steps were repeated for other PCR products and loaded into the wells of agarose gel. Electrophoresis was then run for 70 minutes with the setting of

100 V and 50 mA. After electrophoresis was done, agarose gel was stained with Ethidium Bromide solution for 10 minutes before being soaked with distilled water for 5 minutes. The agarose gel was viewed under UV light using the agarose gel viewer.

RESULTS

In this study, 80 adult dogs were randomly sampled from Shelter X consists of 36 male and 44 female dogs. The animal shelter has a history of a suspected leptospirosis outbreak in one year ago (December 2013). A canine vaccination program for the dogs was conducted approximately 8 months later (in August 2014) includes vaccination against serovar Canicola, Icterohaemorrhagiae, Pomona, and Grippotyphosa.

Microscopic Agglutination Test (MAT)
Of the 80 serum samples, the seroprevalence of Leptospirosis was 3.8% (n=80) where three of the samples showed positive results towards serovar Bataviae using MAT with the titre of 1:80. On the other hand, all the samples showed negative results towards the other nine serovars (Figure 1).

Polymerase Chain Reaction (PCR) Assay
Eighty blood samples from dogs in an animal shelter were subjected to PCR assay to detect pathogenic and non-pathogenic leprospira species. Primer targeting 531 bp of pathogenic leptospira gene and 331 bp of non-pathogenic leptospira gene were used. All the blood samples were tested negative for both pathogenic and non-pathogenic leptospira.

DISCUSSION

The seroprevalence of canine leptospirosis in the animal shelter in this study was 3.8%. Three of the 80 dogs showed positive results against serovar Bataviae with a cut-off titre of 1:80 based on MAT. Comparison of the seroprevalence of canine leptospirosis from this particular animal shelter (3.8%) with other countries showed a much lower seroprevalence i.e. Porto Alegre, Brazil (20.0%; n=65) and Veracruz, Mexico (8.6%; n=93) (Oliveira *et al.*, 2012; Cruz-Romero *et al.*, 2013). Several factors could lead to the differences in the result.

The first factor could be due to the different tests used to diagnose canine leptospirosis with the blood sample obtained.

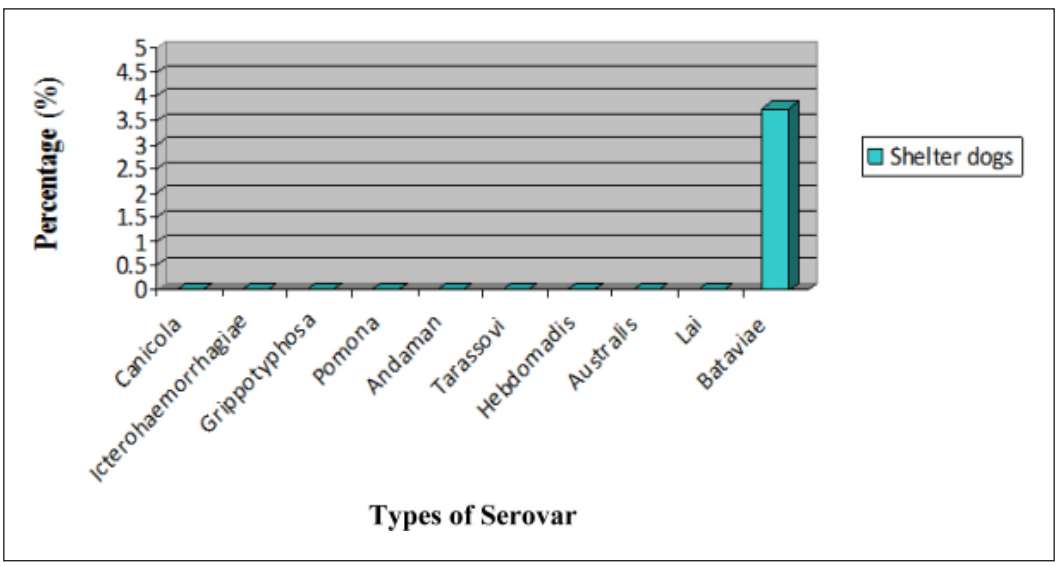

Figure 1. Seroprevalence of Leptospirosis from the blood sample obtained and tested against 10 leptospiral serovars using MAT with the cut-off titre of 1:80.

During the acute phase of infection, leptospires circulate and multiply rapidly in the blood circulation causing leptospiraemia which last for about a week (Goldstein, 2010; Levett, 2001). It is then followed by an immune phase characterized as leptospiruria and production of antibodies occurs 8 to 10 days after the onset of the symptoms (Levett, 2001; Ooteman *et al*., 2006). Molecular test using PCR assay is able to amplify and detect leptospiral DNA in the blood during the acute phase. A period of 7 to 9 days is required before antibodies can be detected serologically using MAT (Ahmad *et al*., 2005; Dutta & Christopher, 2005; Goldstein, 2010). Therefore, PCR assay is more suitable for detection of leptospires in the blood during acute phase while MAT is suitable for antibodies detection during immune phase. In this study, negative PCR assay result was speculated could be either due to the absence of active leptospiral infection or the blood samples were collected during the immune phase. To further support the result, it was observed that all the dogs appeared clinically healthy with a good body condition during sample collection. This indicated that those MAT positive dogs might be in subclinical stage or act as a carrier which warrants further investigation.

The second factor could be due to the different cut-off point titre adopted for the interpretation of the MAT results. Different cut-off point titer of such as 1:50 and 1:100 were used in other studies by Zwijnenberg (2008) and Cruz-Romero (2013), respectively. In our study, the cut-off point titre used for MAT as recommended and used in our local setting was 1:80. The criteria for the selection of cut-off point titer for MAT are often based on the endemicity of the disease in the selected area. Area with higher endemicity of the disease would requires a higher cut-off point and vice versa in order to prevent overestimation of disease burden (Harkin *et al*., 2003; Ahmad *et al*., 2005). In Malaysia, further investigation to determine or to set an appropriate cut-off point titer is needed as there is still lacking on the documentation of canine leptospirosis epidemic status locally. Besides that, although a paired sera sample demonstrating four-fold or greater increase in titre is the most definitive criteria to confirm leptospirosis, at times it can be difficult to obtain the second sample from the same subject in prevalence study (Dutta & Christopher, 2005). However, single elevated titre is suggestive of acute infection with a compatible history and clinical signs observed (Levett, 2004). For example, a single titre of $\geq$ 1:800 is indicative of leptospirosis with compatible clinical signs such as fever, icterus, disseminated intravascular coagulopathy, and renal failure in endemic area (Faine, 1988). In our study, none of the dogs were observed ill during sample collection. Therefore, further investigation with collection of urine sample subjected to PCR assay and culture could determine if these dogs had leptospiral infection and pose a risk as a source of disease to the rest of the dog population and the personnel in this shelter.

The third factor that leads to differences in results published (Oliveira *et al*., 2012; Cruz-Romero *et al*., 2013) could be due to different samples used for PCR assay (such as blood, urine, aqueous humor, and cerebrospinal fluid) for detection of leptospiral DNA (Dutta & Christopher, 2005). For blood sample, PCR assay enables early detection of the DNA during acute stage of infection prior to antibodies production (Musso & La Scola, 2013). As for urine sample, leptospires can only be detected 10 to14 days after the onset of symptoms, which is during the convalescent stage of infection. According to Oliveira (2012), leptospiruria was detected in 20.0% (n=65) of the shelter dogs examined with PCR assay using urine samples. In this study, all blood samples tested with PCR assay showed negative result which can be explained that either there is an absence of acute early infection or the disease was detected during the convalescent stage where the leptospires has been cleared from the circulation and localized in the kidney. Therefore, subsequent PCR assay on urine sample is recommended.

The three main components of epidemiology triad are the agent, environment, and host. The condition of the dog shelter was identified as the environment

while the hosts were the animals in the shelter such as dogs and rodents. In this study, three out of the 80 dogs from the dog shelter had evidence of infection to *Leptospira bataviae* has been documented to infect both humans and animals resulting in fever, renal and hepatic insufficiency, pulmonary manifestation, and reproductive failure (Adler & Moctezuma, 2010). Seroprevalence of *L. bataviae* was reported in various species of mammals in different countries. According to Meeyam (2006), Bataviae was the most prevalent serovar among dogs in Thailand with seroprevalence of 5.2% (n=210). Similar serovar was also found in both bandicoot (4.2%) and rat (3.1%) in India (Venkataraman & Nedunchelliyan, 1992). In Malaysia, Bataviae was first detected in a dog that was kept in a pig farm with a cut-off titre of 1:100 (Joseph, 1979). In humans, serovar Bataviae was reported in Vietnam with the seroprevalence of 18.8%, Indonesia (18.7%), and Bulgaria (7.0%), respectively (Van *et al*., 1998; Hartskeel, 2002; Taseva *et al*., 2005). Although *L. bataviae* was found in both humans and animals, to our knowledge, there is no documented evidence that indicated a direct disease transmission between humans and animals, especially dogs. Further investigations and studies focusing on public health zoonosis are crucial.

This particular dog shelter is located near a highway and surrounded by ponds and factories. Approximately 15 to 20 dogs were kept in a kennel which may increase the risk of disease transmission among the dogs through direct contact. The dogs were managed with an ad libitum feeding regime and it was observed that other animals such as rodents and birds have access to the dry food left in the kennel. *L. bataviae* had been reported in rat before (Venkataraman & Nedunchelliyan, 1992). Therefore, there is a possibility that *L. bataviae* may be carried by the rats in the shelter and act as a maintenance host for spreading the disease to the dogs.

The animal shelter has enrichment activities such rotation of dogs of different kennel that would eventually allow all the dogs to roam and play around or in the ponds situated in the dog shelter. These common areas were exposed to rodents and birds too. It was observed that the pond has stagnant water that might be contaminated with leptospires from urine of the infected dogs or rats that may contribute to disease transmission leading to possible occurrence of infection among the dogs in the shelter. In addition, rats were found roaming freely in the shelter with their faeces observed all over the floor i.e. in the kennels, isolation cages and food preparation areas. Presence of free roaming rats may expose the shelter dogs to a higher risk of infection as the rats in this shelter might be the source of leptospirosis by shedding the organism via their urine and contaminating the environment.

The shelter had improved their water system by using underground water for washing the shelter area instead of using the pumped pond water, and covered the water tanks which were not done prior to the suspected outbreak of leptospirosis in the dog shelter. Since then, there were no suspected leptospirosis cases among the dogs. This suggests that the water source could be the source of infection during the December, 2013 outbreak.

The possible hosts of *L. bataviae* in this study could be either the dogs and/or rats which play a role as the incidental host or the maintenance host. In this study, the incidental hosts were the dogs in the shelter while the maintenance hosts could be the dogs and rats. Incidental hosts will develop clinical leptospirosis while maintenance hosts serve as source of infection for the disease (Goldstein *et al*., 2006). Leptospirosis is endemic and the maintenance hosts such as rats harbor leptospires in their kidney without showing clinical symptoms. Therefore, infection could occur through direct contact with infected animals or their urine as they shed leptospires into the environment through their urine (Levett, 2001; Goldstein, 2010). However, there could still be a possibility that the dogs in the shelter were the maintenance host for *L. bataviae* as all the 3 dogs detected with antibodies against serovar Bataviae appeared clinically healthy during the time of sample collection. Similar assumption can also be made on the rats. For further

investigation, collection of urine samples and subjecting them to PCR assay for leptospiral detection in order to confirm the carrier state could determine the role of the dogs in disease transmission. Samples from the rats also should be obtained for the same purpose.

CONCLUSION

In conclusion, the seroprevalence of canine leptospirosis in the shelter was determined to be 3.8% (n=80) with *L. bataviae* identified as the infecting serovar. This is the second reported presence of *L. bataviae* in dogs in Malaysia. The rats in the shelter were believed to be the source of leptospirosis. Further investigations are required to determine the role of dogs in disease transmission between dogs, humans and rats. Blood and urine samples of the 3 positive dogs should be closely examined to determine whether they are carriers. Urine samples and other animal shelters should be included in future prevalence study.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements. The authors would like to acknowledge the contributions of Ms Tan Yi Wei during sample collection. We are grateful to Shelter X and the management committee for their participation in the study.

REFERENCES

Adler, B. & Moctezuma, A. (2010). Leptospira and Leptospirosis. *Veterinary Microbiology* **140**(3-4): 287-96.

Agampodi, S., Peacock, S.J. & Thevanesam, V. (2009). The Potential Emergence of Leptospirosis in Sri Lanka. *Lancet Infectious Disease* **9**: 524-526.

Ahmad, S., Shah, S. & Ahmad, F. (2005). Laboratory Diagnosis of Leptospirosis. *Journal of Postgraduate Medicine* **51**(3): 195-200.

Bahaman, A.R. & Ibrahim, A.L. (1987). A Short Review of Animal Leptospirosis with Special Reference to Malaysia. *Tropical Biomedicine* **4**: 93-99.

Bahaman, A.R. & Ibrahim, A.L. (1988). A Review of Leptospirosis in Malaysia. *Veterinary Research Communications* **12**: 179-189.

Brown, C.A., Roberts, A.W., Miller, M.A., *et al.* (1996). Leptospira interrogans Serovar Grippotyphosa Infection in Dogs. *Journal of the American Veterinary Medical Association* **209**: 1265-1267.

Cruz-Romero, A., Romero-Salas, D., Aguirre, C.A., Aguilar, M. & Bautista-Piña, C. (2013). Frequency of Canine Leptospirosis in Dog Shelters inVeracruz, Mexico. *African Journal of Microbiology Research* **7**(16): 1518-1521.

Dutta, T.K. & Christopher, M. (2005). Leptospirosis-An Overview. *The Journal of The Association of Physicians of India* **53**: 545-551.

Everard, C.O.R., Jones, C.J., Inniss, V.A., Carrington, D.G. & Vaughan. A.W. (1987). Leptospirosis in Dogs in Barbados. *Israel Journal of Veterinary Medicine* **43**: 288-295.

Faine, S. (1988). Leptospirosis. Balows, A., Hausler, W.J., Ohashi, M., Turano, A., Eds. Laboratory Diagnosis of Infectious Diseases: Principles and Practice. New York: Springer-Verlag, 344-352.

Faine, S., Adler, B., Bolin, C. & Perolat, P. (1999). Clinical Leptospirosis in Humans. Leptospira and Leptospirosis, 2nd Eds. Medical Science, Melbourne; 1999.

Feigin, R.D., Lobes, L.A., Anderson, D. & Pickering, L. (1973). Human Leptospirosis from Immunized Dogs. *Annals of Internal Medicine* **79**: 777-785.

Fletcher, W. (1928). Recent Work on Leptospirosis, Tsutsugamushi Disease and Tropical Typhus in the Federated Malay States. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **21**: 265-88.

Fraser, D.W., Glosser, J.W., Francis, D.P., Phillips, C.J., Feeley, J.C. & Sulzer, C.R. (1973). Leptospirosis Caused by Serotype Fort-Bragg – A Suburban Outbreak. *Annals of Internal Medicine* **79**: 786-789.

Gautam, R., Wu, C.C., Guptill, L.F., Potter, A. & Moore, G.E. (2010). Detection of Antibodies Against Leptospira Serovars via Microscopic Agglutination Tests in Dogs in The United States, 2000-2007. *Journal of The American Veterinary Medical Association* **237**(3): 293-298.

Goldstein, R.E., Lin, R.C., Langston, C.E., Scrivani, P.V., Erb, H.N. & Barr, S.C. (2006). Influence of Infecting Serogroup on Clinical Features of Leptospirosis in Dogs. *Journal of Veterinary Internal Medicine* **20**: 489-494.

Goldstein, R.E. (2010). Canine Leptospirosis. *The Veterinary Clinics of North America. Small Animal Practice* **40**(6): 1091-1101.

Harkin, K.R., Roshto, Y.M. & Sullivan, J.T. (2003). Clinical Application of a Polymerase Chain Reaction Assay for Diagnosis of Leptospirosis in Dogs. *Journal of The American Veterinary Medical Association* **222**: 1224-1229.

Hartskeel, R.A. (2002). Report of the leptospirosis outbreak in Indonesia, WHO/FAO/OIE/RIVM Leptospirosis Reference Centre, KIT Biomedical Research, Amsterdam. Report submitted to WHO South East Regional Office.

Hartskeel, R.A., Collares-Pereira, M. & Ellis, W.A. (2011). Emergence, Control and Re-Emerging Leptospirosis: Dynamics of Infection in The Changing World. *Clinical Microbiology and Infection* **17**(4): 494-501.

Jimenez-Coello, M., Vado-Solis, I., Cárdenas-Marrufo, M.F., Rodríguez-Buenfil, J.C. & Ortega-Pacheco, A. (2008). Serological Survey of Canine Leptospirosis in the Tropics of Yucatan Mexico Using Two Different Tests. *Acta Tropica* **106**: 22-26.

Joseph, P.G. (1979). Leptospirosis in Animals in West Malaysia. *Malaysian Journal of Pathology* **2**: 15-21.

Kikuti, M., Langoni, H., Nobrega, D., Corrêa, A. & Ullmann, L. (2012). Occurrence and Risk Factors Associated with Canine Leptospirosis. *The Journal of Venomous Animals and Toxins including Tropical Diseases* **18**(1): 124-127.

Koizumi, N., Gamage, C.D., Muto, M., *et al.* (2009). Serological and Genetic Analysis of Leptospirosis in Patients with Acute Febrile Illness in Kandy, Sri Lanka. *Japanese Journal of Infectious Disease* **62**: 474-475.

Lavinsky, M.O., Said, R.A., Strenzel, G.M.R. & Langoni, H. (2012). Seroprevalence of Anti-Leptospira Spp. Antibodies in Dogs in Bahia, Brazil. *Preventive Veterinary Medicine* **106**(1): 79-84.

Levett, P.N. (2001). Leptospirosis. *Clinical Microbiology*, **14**(2): 296-326.

Levett, P.N. (2004). Leptospirosis: A Forgotten Zoonosis? *Clinical and Applied Immunology Reviews* **4**(6): 435-448.

Low, K.N. (2014). Seroprevalence and Molecular Detection of Canine Leptospirosis in Selected Areas of Klang Valley, Malaysia. Unpublished degree's thesis, University Putra Malaysia, Selangor.

Lau, S.F., Low, K.N., Khor, K.H., Roslan, M.A., Bejo, S.K., Radzi, R. & Bahaman, A.R. (2016). Prevalance of leptospirosis in healthy dogs and dogs with kidney disease in Klang Valley, Malaysia. *Tropical Biomedicine* **33**(2): Accepted for publication.

Mccurry, J. (2009). Philippines Struggles to Recover from Typhoons. *Lancet* **374**(9700): 1489.

Meeyam, T., Tablerk, P., Petchanok, B. & Pichpol, D. (2006). Seroprevalence and Risk Factors Associated With Leptospirosis in Dogs. *Southeast Asian Journal of Tropical Medicine and Public Health* **37**(1): 148-153.

Miller, D.A., Wilson, M.A. & Beran, G.W. (1991). Relationships between Prevalence of Leptospira interrogans in Cattle, and Regional, Climatic, and Seasonal Factors. *American Journal of Veterinary Research* **52**: 1766-1768.

Musso, D. & La Scola, B. (2013). Laboratory Diagnosis of Leptospirosis: A Challenge. *Journal of Microbiology, Immunology and Infection* **46**(4): 245-252.

Oliveira, S.T., Messick, J.B., Biondo, A.W., Pires, A., Stedile, R. & Dalmolin, M.L. (2012). Exposure to Leptospira Spp. in Sick Dogs, Shelter Dogs and Dogs from an Endemic Area/: Points to Consider. *Acta Scientiae Veterinariae* **40**(3): 1-7.

Ooteman, M.C., Vago, A.R. & Koury, M.C. (2006). Evaluation of MAT, Igm ELISA and PCR Methods for the Diagnosis of Human Leptospirosis. *Journal of Microbiology Methods* **65**: 247-257.

Roqueplo, C., Marié, J., André-Fontaine, G., Kodjo, A. & Bernard, D. (2014). Serological Survey of Canine Leptospirosis in Three Countries of Tropical Africa: Sudan, Gabon and Ivory Coast. *Comparative Immunology, Microbiology and Infectious Diseases* **38**: 57-61.

Taseva, E., Christova, I. & Gladnishka, T. (2005). Epidemiological, clinical and serological features of human leptospirosis in Bulgaria in 2005. 17th European Congress of Clinical Microbiology and Infectious Disease, Munich, Germany, March 31–April 4, 2007.

Van, C.T.B., Thuy, N.T.T., San, N.H., Hien, T.T., Baranton, G. & Perolat, P. (1998). Human leptospirosis in the Mekong delta, Viet Nam. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **96**(6): 628-628.

Venkataraman, K.S. & Nedunchelliyan, S. (1992). Epidemiology of an Outbreak of Leptospirosis in Man and Dog. *Comparative Immunology, Microbiology and Infectious Diseases* **15**(4): 243-247.

Weekes, C.C., Everard, C.O.R. & Levett, P.N. (1997). Seroepidemiology of Canine Leptospirosis on the Island of Barbados. *Veterinary Microbiology* **57**: 215-222.

Zwijnenberg, R.J.G., Smythe, L.D., Symonds, M.L., Dohnt, M.F. & Toribio, J.A. (2008). Cross-Sectional Study of Canine Leptospirosis in Animal Shelter Populations in Mainland Australia. *Australian Veterinary Journal* **86**(8): 317-323.