Detection and characterisation of *Leptospira* spp. in dogs diagnosed with kidney and/or liver disease

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Abstract

Background

Leptospirosis is a zoonotic bacterial disease that affects both humans and animals. In humans, a wide range of symptoms had been described but in dogs, it is commonly associated with kidney and/or liver disease. In Malaysia, information with regards to the common serovars causing leptospiral infection in dogs remains limited. Therefore, this study investigated the occurrences of leptospiral infection in 124 pet dogs diagnosed with kidney and/or liver disease in Malaysia.

Results

Based on microscopic agglutination test, 42.7% (53/124) of the dogs were seropositive for leptospiral infection. The predominant serovars detected were Bataviae (n = 12), Javanica (n = 10) and Icterohaemorrhagiae (n = 10). The direct detection using polymerase chain reaction showed that 33.9% (42/124) of the whole blood and 31.9% (36/113) of the urine samples were positive to pathogenic *Leptospira* spp. For tissue samples, 9.1% (2/23) of the kidney and 9.1% (2/23) of liver were positive for pathogenic *Leptospira* spp. Addition samples of abdominal effusion from four dogs were positive for pathogenic *Leptospira* spp. The species detected were *L. interrogans*, *L. borgpetersenii*, *L. kirshneri* and *L. kmetyi* by partial 16S rRNA sequencing. In this study, 11 *Leptospira* spp. isolated successfully from the eight dogs were further identified and characterised as Bataviae, Javanica and Australis. Unfortunately, the mortality rate of the infected dogs was high at 34.0% (18/53).

Conclusions

This study allowed for greater understanding of canine leptospirosis through the provision of crucial diagnostic confirmation.

Background

Leptospirosis is a bacterial disease caused by infection of the pathogenic variants of *Leptospira* spp., which can virtually affect all mammals [1]. The disease is recognised as the most widespread zoonosis and has emerged as a major public health issue in many developing countries [2]. Transmission of the disease is strongly driven by environmental factors, such as high rainfall precipitation rates, flooding, natural disasters, uncontrolled urban expansion, and poor sanitation [3, 4, 5]. Exposure to water and soil contaminated by the urine of infected animals is the most common route of transmission to humans and domestic animals [4], and rodents are considered the major source for human infection, a role likely attributed to its synanthropic behaviour and widespread distribution [2].

Case reports of canine leptospirosis had been described worldwide and dogs are considered highly susceptible to the infection because of a marked environmental exposure to leptospires [1, 6]. However, the actual role of dogs in the zoonotic transmission of leptospirosis remains poorly documented, and the overall contribution of dogs to the burden of human leptospirosis has yet to be determined [7]. Alarming, asymptomatic urinary shedding of leptospires among dog populations has been widely reported [8, 9, 10]. Thus, there is a possibility that dogs may contribute to the spread of pathogenic *Leptospira* spp. in the environment too.

Recent reports exhibited re-emergence of clinical illness in dogs and humans [1, 11], highlighting the importance of improving current diagnostic approaches and prevention strategies. Infected dogs may manifest a broad spectrum of clinical symptoms such as vomiting, depression, icterus, dehydration, diarrhoea, and anorexia, varying from hepatic and/or renal failure, often accompanied by haemorrhagic and pulmonary disorders, to mild, self-limiting febrile illness and asymptomatic infections [12, 13, 14]. Clinical and laboratory findings are usually non-specific, and a definitive diagnosis requires additional confirmatory tests for the direct or indirect identification of the pathogen, such as darkfield microscopy, polymerase chain reaction (PCR), bacterial culture and microscopic agglutination test (MAT) [1]. For serodiagnosis of acute leptospiral infection, MAT is still widely employed, despite its limitation and poor ability to predict the infecting serovar and may not distinguish between infection and vaccine-induced titres [15]. Conversely, PCR has been successfully used to confirm leptospiral infection at the early stages of infection [16, 17], and sequencing PCR amplicon has enabled the identification of the different leptospiral species infecting dogs [18].

In Malaysia, the seroprevalence of canine leptospirosis among dogs ranged from 3.1–22.2% [19, 20, 21, 22]. However, there is a limited report in dogs diagnosed with kidney and/or liver disease locally. Only one study reported in dogs with kidney disease by prevalence of 5.3% [23]. Therefore, this present study described the diagnosis of leptospirosis in dogs diagnosed with kidney and /or liver disease using multiple diagnostic strategies, such as serological, molecular, and bacteriological tests, along with the characterisation of the recovered leptospiral strains. The establishment of a panel of leptospiral strains circulating among dog populations remains the major strategy to support the development and commercialization of vaccines incorporating more specific serovar compositions, which would hypothetically increase immunization effectiveness for local canine populations.

Results

A total of 124 dogs were recruited in this study. Out of 124 dogs, 68 dogs were diagnosed with both kidney and liver diseases, the remaining 34 dogs had kidney disease (BUN and/or creatinine elevated) and 22 dogs had liver disease (ALT and/or ALP elevated). The demographic data of the dogs were tabulated as shown in Table 1. The most common clinical signs observed in the dogs were inappetence followed by vomiting, lethargy, jaundice, and diarrhoea. All dogs presented were observed to have at least two clinical signs (refer Fig. 1).

In total, 124 sera, 124 whole blood and 113 urine samples were collected. During the period of study, 30 out of 124 dogs did not survive despite treatment and supportive care. Only 23 kidneys and 23 livers were harvested after the post-mortem examination. Abdominal effusion samples were collected from four dogs. Most of the dogs diagnosed with kidney and/or liver samples were of senior, male, non-vaccinated, outdoor, and medium-breed dogs as shown in Table 1. All vaccinated dogs were vaccinated with a commercial tetravalent vaccine which included serovars *Icterohaemorrhagiae*, *Canicola*, *Pomona* and *Grippotyphosa*.

Table 1
Demographic data of the dogs diagnosed with kidney and/or liver disease (N = 124)

Characteristic	No. of dogs (%)	Characteristic	No. of dogs (%)
Age	13 (10.5%)	Breed	28 (22.6%)
Young (≤ 1 year)	48 (38.7%)	Small	65 (52.4%)
Adult (< 6 years)	63 (50.8%)	Medium	31 (25.0%)
Senior (≥ 6 years)		Large	
Sex	76 (61.3%)	Type of household	68 (54.8%)
Male	48 (38.7%)	Single	56 (45.2%)
Female		Multiple	
Vaccination status	52 (41.9%)	Rat exposure	78 (62.9%)
Vaccinated	72 (58.1%)	Exposed	46 (37.1%)
Non-vaccinated		Not exposed	
Management	51 (41.1%)	Clinical illness	102 (82.3%)
Indoor	73 (58.9%)	Acute (≤ 7 days)	22 (17.7%)
Outdoor		Chronic (> 7 days)	

Serological detection

Out of 124 sera tested using MAT, 53 (42.7%; 95% CI: 34.0% – 51.4%) dogs were seropositive with titre ranged between 1:100 to 1:800 (refer Fig. 2). The three most frequent serovars detected were *Bataviae* (n = 12), *Javanica* (n = 10) and *Icterohaemorrhagiae* (n = 10) (refer Fig. 3). However, all the tested sera were negative serologically for serovars *Grippotyphosa*, *Tarrasovi*, *Djasiman*, *Hebdomadis* and *Patoc* as shown in Fig. 3. In addition, among 52 vaccinated dogs, 22 dogs were seropositive towards *Icterohaemorrhagiae* (n = 5), *Javanica* (n = 3), *Bataviae* (n = 2), *Hardjobovis* (n = 2), *Pomona* (n = 2), *Malaysia* (n = 2), *Australis* (n = 1), *Autumnalis* (n = 1), *Canicola* (n = 1), *Copenhageni* (n = 1), *Cynopteri* (n = 1) and *Celledoni* (n = 1).

Molecular detection and partial 16S rRNA sequencing

Total molecular detection of leptospiral infection by PCR was 42.7% (53/124; 95% CI: 34.0% – 51.4%). The positive samples were obtained from 42 whole blood, 36 urine, tissue samples of two kidneys and two livers and four abdominal effusions. All of them were positive for pathogenic *Leptospira* sp. and four *Leptospira* species had been detected using partial 16S rRNA sequencing. *L. interrogans* (n = 62) was the most detected species followed by *L. borgpetersenii* (n = 17) and *L. kirschneri* (n = 6). Only one blood sample was detected as *L. kmetyi* as shown in Fig. 4. Out of 53 positive dogs, 18 dogs had died, therefore the mortality rate was 34.0% (95% CI: 21.2% – 46.7%).

Isolation and characterisation of *Leptospira* spp.

The *Leptospira* spp. were successfully isolated from eight out of 124 dogs (6.5%; 95%CI: 2.1% – 10.8%), where three dogs were vaccinated, and five dogs were not vaccinated. From the eight dogs diagnosed with kidney and/or liver disease, 11 isolates were obtained from three blood, seven urine and one abdominal effusion samples. Serological characterisation using MAT showed eight of the isolates were Bataviae, two were Australis and one was Javanica. Molecular characterisation using PCR revealed all the isolates were pathogenic. Further analysis of the partial 16S rRNA sequencing using BLAST (www.ncbi.nlm.nih.gov/blast) confirmed 10 isolates had 95% identical towards *L. interrogans* and one isolate had 95% identical towards *L. borgpetersenii*. The sequences were submitted to the GenBank and accession number was obtained (refer Table 2).

Table 2

The *Leptospira* sp. isolates obtained from sample with their respective characterisation (n = 8)

Dog ID	Samples	Characterisation		
		Serovar (MAT titre)	PCR	GenBank Submission (GenBank Accession No.)
D2	Urine	Bataviae (1:3200)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain UVH huseli (MF589180.1)
D19	Blood	Bataviae (1:3200)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain UVH khor_samei3 (MH745144.1)
	Urine	Bataviae (1:3200)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain UVH khor_samei1 (MH745142.1)
	Abdominal Effusion	Bataviae (1:6400)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain UVH khor_samei2 (MH745143.1)
D27	Urine	Bataviae (1:3200)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain UVH khor_saron (MH799846.1)
D41	Urine	Bataviae (1:3200)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain PV huseli (MH799845.1)
D52	Urine	Bataviae (1:3200)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain UVH khor_savi (MH799847.1)
D63	Blood	Javanica (1:3200)	Pathogenic <i>Leptospira</i> sp.	<i>L. borgpetersenii</i> strain khorsayo (MN226648.1)
D82	Urine	Australis (1:3200)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain UVH khor_sapy1 (MN698724.1)
	Blood	Australis (1:3200)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain UVH khor_sapy3 (MN698723.1)

Dog ID	Samples	Characterisation		
		Serovar (MAT titre)	PCR	GenBank Submission (GenBank Accession No.)
D85	Urine	Bataviae (1:6400)	Pathogenic <i>Leptospira</i> sp.	<i>L. interrogans</i> strain khorgoh_jacky (MN226649.1)

The phylogenetic tree showed three distinct clades related to pathogenic, intermediate, and saprophytic species. All the isolates obtained in this study were placed within the pathogenic group. Ten of the isolates were placed within species of *L. interrogans* while one isolate was placed within *L. borgpetersenii* as shown in Fig. 5. Three isolates namely *L. interrogans* strain UVH khor_sapy1, *L. interrogans* strain UVH khor_sapy3 and *L. borgpetersenii* strain khorsayo were found to diverge from the respective species. Condensed tree was conducted with 50.0% cut-off point from original tree to further investigate the significance of the divergence and showed no significant divergence for all the isolates as shown in Fig. 6. From condensed tree, all 10 isolates were 61.0% closely related with all the representative species of *L. interrogans*. In addition, *L. interrogans* strain UVH khor_sapy1 and *L. interrogans* strain UVH khor_sapy3 were 99.0% related because those two isolates were isolated from one dog. The other isolate was 68.0% closely related to all the representative species of *L. borgpetersenii*.

Discussion

Leptospira spp. can be found worldwide regardless of climate and has been presumed to be the most widespread zoonoses [24]. Despite it being common, the diagnosis of canine leptospirosis is not often made unless the dog was presented with clinical manifestations such as fever, jaundice, renal and/or liver failure. Clinical diagnosis remains a challenge but with the aid of confirmatory laboratory tests, a diagnosis can be derived to allow immediate administration of therapeutic regime [25] especially for dogs diagnosed with leptospirosis. This study demonstrated the utility of direct detection using serological and molecular methods followed by bacterial isolation in dogs with kidney and/or liver disease and found that dogs can shed *Leptospira* spp., further contaminating the environment and poses a risk of infection to their owners.

Microscopic agglutination test is a sensitive assay, but because of the antigenic heterogeneity of *Leptospira* spp., the test requires many serovars as antigens [26]. The overall serological detection of leptospiral infection in dogs with kidney and/or liver disease was 42.7%, much higher compared to the previous studies locally. The reason could likely be due to the specific selection of recruited dogs and serum tested against 20 leptospiral serovars selected. In comparison, previous studies investigated a larger population of apparent healthy shelter and working dogs [19], some investigated healthy dogs from a single location [21, 22] and one study was carried out among the pet dogs [23]. All these studies only

aimed to determine seropositive among apparently healthy dogs using a panel of 10 leptospiral serovars, unlike in this study.

The MAT titre frequently observed were at 1:100 (n = 21), and perhaps could be used as a potential cut-off titre for diagnosis of leptospirosis as these dogs were clinically sick with the supportive evidence of elevated kidney and/or liver profiles. Alarming, three dogs with a titre level of 1:100 did not survive despite being treated and serovar Bataviae was detected. These three dogs showed clinical signs such as inappetence, diarrhoea, vomiting and jaundice within three days with history of post-exposure to rats. Another four dogs died (titre of 1:200), in which serovars Australis (n = 2) and Icterohaemorrhagiae (n = 2) were detected. The highest MAT serological titre obtained were at 1:800 (n = 8) that could suggest a severe condition in these infected dogs. Only two dogs died with a titre of 1:800 (Javanica), whilst six other infected dogs survived post-treatment (2-Javanica; 1-Hardjobovis; 1-Pomona; 1-Copenhageni; 1-Malaysia, respectively). This shows that different level of titre detected does not correlate with the risk of mortality and prognosis. On the other hand, six dogs with high infection titres did survive and therefore perhaps the dogs' immunity level, infecting serovar and treatment initiated might all have played important roles in the survivability of infected dogs.

The panel of leptospiral serovars selected in this study was based on the important serovars circulating in Malaysia for human, rats and dogs as found in the previous studies [19, 20, 21, 22, 23, 27]. It was observed that the three most frequent leptospiral serovars detected in diseased dogs were Bataviae (n = 12), Javanica (n = 10) and Icterohaemorrhagiae (n = 10), then followed by Ballum (n = 3), Australis (n = 3), Hardjobovis (n = 3), Malaysia (n = 3) and Pomona (n = 2). The least frequent leptospiral serovars observed in this study were Canicola, Lai, Pyrogenes, Copenhageni, Celledoni, Cynopteri and Autumnalis. Therefore, incorporating known local leptospiral serovars is highly recommended in the diagnostic workout to improve detection rate as these serovars had shown to cause disease among dogs. Locally, both serovars Bataviae and Javanica had been reported in dogs [19, 21] and high serological detection of serovars Bataviae and Javanica (32 out of 53 seropositive dogs) was directly linked to direct contact with rats. This is not surprising as Benacer *et al.*, states that Bataviae and Javanica are two leptospiral serovars circulating among the urban rats' population in Peninsular Malaysia [28]. Besides that, Icterohaemorrhagiae, Australis, Pomona and Canicola had been detected in rats within Kuala Lumpur [29] which further supports the findings in the current study. In contrast, serovars Hardjobovis and Copenhageni were commonly reported in working dogs from livestock farms in New Zealand [30]. Thus, the dogs there were at greater risk of exposure to those serovars and detection was governed by local endemicity which was similarly observed in this study.

Direct PCR on specimens enables rapid and direct diagnosis, in both early and convalescent stages of infection. In this study, the overall molecular detection of leptospiral infection in dogs diagnosed with kidney and/or liver disease was 42.7%. Results were consistent with study by Miotto *et al.*, at 42.4% (14/33; 95%CI: 25.6% – 59.3%) [31]. Despite having a smaller sample size in that study, the target population was similar. In contrast, Santanna *et al.*, and Latosinski *et al.*, reported a lower molecular detection rate at 19.8% (26/131; 95%CI: 13.0% – 26.7%) and 1.0% (1/106; 95%CI: 0.0% – 2.8%),

respectively [32,33]. Despite the similarity in large sample size with this study, the target population recruited were apparently healthy dogs which could explain the lower detection rate. The PCR can detect leptospiral DNA in the whole blood, down to extremely small amounts equivalent to the DNA content of about 10 leptospires or less [26]. In this study, 42 pathogenic *Leptospira* spp. (33.9%; 42/124; 95%CI: 25.5% – 42.2%) were detected in the whole blood and suggestive of leptospiraemia phase. Out of 42 dogs diagnosed with leptospirosis, 21 dogs were presented at the acute stage as *Leptospira* spp. with positive detection only from the blood samples. Sixteen dogs were positive for both PCR and MAT, which suggested that the dogs were in convalescent phase, where antibodies might have started to react with the antigens, and that could reduce the amount of leptospires circulating in the body, but still detectable using the molecular method.

The optimum time of *Leptospira* spp. evident in the urine of infected dogs were reported at seven or more days of clinical illness [26, 34]. Positive detection of 36 pathogenic *Leptospira* sp. from urine samples suggested a leptospiruria phase in these diseased dogs. Twenty-three out of 36 dogs were presented at the convalescent phase. Twenty-five dogs detected positive for pathogenic *Leptospira* sp. in both whole blood and urine samples and 14 of the diseased dogs were presented with chronic stage, either in the period of active infection and/or actively shedding. Using the conventional PCR method, blood samples allowed higher detection compared to urine samples. This could be related to samples obtained at different phases of infection or the biological feature of urine itself. There are limitations to PCR with regards to urine samples as urea (act as PCR inhibitor) may lead to polymerase degradation affecting the sensitivity of the assay or even leads to false-negative results [35], which is a challenging issue with direct detection from the clinical sample obtained.

Only two kidneys and two livers were positive for pathogenic *Leptospira* spp. Low detection from tissue samples could be due to the presence of PCR inhibitors such as haemoglobin and hormones [35]. However, to our knowledge, this study could be the first to demonstrate all four abdominal effusion samples obtained from four different dogs were tested positive for pathogenic *Leptospira* spp. and perhaps abdominal effusion can be the preferred sample for molecular diagnostic investigation. Cerebrospinal fluid (CSF) was not collected in this study, but leptospiral DNA has been expressed in CSF fluids from both, human [36, 37] and animal [38] studies. None of the dogs in this study showed neurological signs,

The partial 16S rRNA sequencing performed after direct PCR revealed that the most common species detected were *L. interrogans* (n = 62) followed by *L. borgpetersenii* (n = 17), *L. kirschneri* (n = 6), and *L. kmetyi* (n=1). *Leptospira interrogans* were detected in all type of samples (blood, urine, abdominal effusion, kidney, and liver). Comparatively similar, *L. kirshneri* was detected in all sample types except in liver. The detection of *L. interrogans* and *L. kirshneri* were expected in this study and both species had been commonly associated with canine leptospirosis [1, 31, 39, 40]. However, the detection of *L. borgpetersenii* was linked to contacts with rats as this bacterium is commonly shed by rats [28, 41, 42]. In this study, nine out of 17 samples from a total of six dogs had history of in contact with rats. Two out of the six dogs did not survive despite aggressive treatment therapy. Previous study in Germany reported

three out of 200 healthy dogs shed leptospires of the species *L. interrogans* (n = 2) and *L. borgpetersenii* (n = 1) [8]. Even though *L. borgpetersenii* is not common in dogs but this species remains a contributing concern to canine leptospirosis with high mortality. *Leptospira kmetyi* was detected from a dog's blood sample and to our knowledge, could be the first report of *L. kmetyi* identified in an animal. This could be associated with environmental exposure because *L. kmetyi* had been isolated from the environment in Malaysia [43, 44].

Isolation and identification of leptospires are critical to confirm the specific leptospiral serovar circulating in this group of dogs diagnosed with kidney and/or liver disease. However, culturing leptospires is challenging due to frequent contamination and the fastidious growth of the pathogen [1]. Leptospires are slow-growing bacteria in comparison to other bacteria. Even though the semisolid EMJH is the selective medium containing 5-FU to improve leptospiral isolation, however high number of contaminants greater leptospires in the samples may likely suppress the growth of the selected bacteria. Despite the 12 weeks challenges of continuous checking and sub-culturing, contaminated samples may alter the possibility of a result. Besides that, recovering leptospires from suspected dogs were limited due to early antibiotic therapy intervention, which is usually required after the disease is suspected [45]. Nonetheless, culturing leptospires still stands as the gold standard reference test for confirmation of leptospiral infection, and only serological characterisation of the isolated strains may provide reliable information regarding serovar identity [1].

Despite the challenges faced throughout the study, 11 *Leptospira* sp. isolates were recovered from whole blood (n = 3), urine (n = 7) and abdominal effusion (n = 1) samples. Comparing with previous studies, leptospires were successfully recovered only from urine sample of the diseased dogs [46], shelter and stray dogs [47] and farm dogs [20]. This supports that urine samples are superior samples for leptospiral isolation in dogs regardless of the target population. Leptospires can persist in the kidneys and colonize the renal proximal tubules, causing live bacteria excretion in the urine [48]. Leptospiruric dogs remain as potential shedders and therefore, may increase the risk of infection to their owners or from dog-to-dog within the same household. The previous study found that prolonged dog handler-dog contact time increases the risk of seropositivity, and the unknowing handling of infected dogs puts the dog handlers at risk from the leptospires shedding [19]. Sometimes, persistence leptospiruria due to inadequate antibiotic usage of failing to penetrate kidneys may ineffectively eradicate leptospires [49]. Therefore, the use of a sensitive molecular technique is recommended to investigate as to whether an infected treated dog is still leptospiruric and/or the post-antibiotics therapy is effective. Besides urine, leptospires were commonly isolated from whole blood in human [50, 51, 52]. According to Gompf, other body fluids (besides blood and urine) might contain leptospires, but the opportunity to isolate them is slim [53]. Surprisingly, this study successfully cultured *Leptospira* sp. from abdominal effusion.

Further serological characterisation of the 11 isolates showed reaction towards three hyperimmune sera namely: Bataviae (n = 8), Javanica (n = 1) and Australis (n = 2). The molecular characterisation revealed that all isolates were pathogenic and further confirmed species of *L. interrogans* (n = 10) and *L. borgpetersenii* (n = 1) by partial 16S rRNA sequencing. These results strengthen the serological and

molecular detection as discussed earlier. Phylogenetic tree analysis revealed that all isolates were within pathogenic clade as follows; eight isolates closely related to reference isolate of *L. interrogans* serovar Bataviae, two isolates closely related to reference isolate of *L. interrogans* serovar Australis, and one isolate closely related to reference isolate of *L. borgpetersenii* serovar Javanica. Phylogenetic analysis concurred with the findings of previous reports that the pathogenic, intermediate, and saprophytic species each formed one clade [54, 55].

The isolation of serovars Bataviae, Javanica and Australis could be alarming due to their absence in commercial vaccines. In Malaysia, bivalent (Canicola and Icterohaemorrhagiae) and tetravalent vaccines (Canicola, Icterohaemorrhagiae, Pomona and Grippotyphosa) were adopted based on the availability of imported vaccine and WSAVA guidelines [56]. Referring to the history obtained from pet owners, three diseased dogs were vaccinated with tetravalent vaccine and five were not vaccinated. Dogs vaccinated annually remained at risk as vaccination does not provide cross-protection towards non-vaccinal serovars. In Australia, commercial vaccines for dogs containing serovars of Icterohaemorrhagiae and Australis have been marketed for use [56]. Meanwhile, trivalent (Canicola, Icterohaemorrhagiae, and Grippotyphosa) and tetravalent vaccines (Canicola, Icterohaemorrhagiae, Grippotyphosa, and Australis) have been licensed in European countries. The decision of in-cooperation incorporating serovars for optimal protection was made due to the observed shift in serovar prevalence in Europe and Australia with emerging serovar Australis [57].

Conclusions

Leptospirosis is not always diagnosed or being considered as a differential diagnosis in small animal practice, mostly due to the disease variable non-specific clinical presentation. This study had shown that, one in three dogs presented with kidney and/or liver disease have the potential to be infected with leptospirosis and those patients with a history of in direct contact with rats have an increased risk. These results would help veterinarians to diagnose or even to consider leptospirosis as a differential diagnosis. The concurrent use of serological, molecular and isolation methods in this study allowed better disease understanding thus improving the diagnosis of leptospirosis especially in pet dogs diagnosed with kidney and/or liver disease. Proper clinical and laboratory diagnosis might increase the survivability of infected dogs as appropriate treatment can be administered immediately. The findings in this study may indirectly increase the awareness of zoonotic risk for dog owners. The potential of zoonotic transmission of the leptospiral infection from dogs to their owners does exist due to the renal shedding of the bacteria and the close contact between humans and their pets, but the true extent or significance has still not been accurately investigated. To prevent the disease in pets and their owners, simple preventive measures can be applied focusing on reducing the chances of infection, including vaccination in dogs, good hygiene practices and avoid the exposure of their pet dogs to other infected animals and reservoir hosts. The detection of non-vaccinal serovars such as Bataviae and Javanica is a reminder of the limitation in vaccination. Proper characterisation of leptospiral isolates remain a crucial bottleneck to access the role of particular serovars or strains in the epidemiology of canine leptospirosis and may provide evidence-

based knowledge to support the development and commercialisation of multivalent vaccines containing serovars that are circulating among local populations.

Methods

Sample collection and inclusion criteria

All the dogs diagnosed with kidney and/or liver disease either presented to the University Veterinary Hospital of Faculty of Veterinary Medicine, Universiti Putra Malaysia or from private veterinary clinics were recruited. Dog owner's permission was obtained prior to sample collection. Ethical approval was obtained from the Institutional Animal Care and Use Committee (IACUC Ref No: UPM/IACUC/AUP-R084/2016).

The selection criteria of the recruited dogs were: (i) dogs with presenting clinical sign of kidney and/liver disease with, (ii) elevation of either kidney and/or liver parameters based on serum biochemistry profile [Elevated parameters were as follows; blood urea nitrogen (> 7.5 mmol/L), creatinine (> 176 μ mol/L), alanine aminotransferase (ALT) (> 90 U/L) and alkaline phosphatase (ALP) (> 100 U/L)]. The signalment of each dog recruited was recorded. Age, breed, and clinical illness characteristics were categorised according to published guidelines [58, 59, 60]. The recruited pet dogs were manually restraint for venipuncture (serum and whole blood) and urine samples were collected either via ultrasound guide cystocentesis or spontaneous micturition by experienced veterinarians. Blood samples collected using plain blood tube (BD Vacutainer®, USA) were allowed to clot at 4 °C then centrifuged for 10 minutes at 4000 rpm (80 – 2 Electronic Laboratory Medical Centrifuge, China). The serum was collected and stored at -20 °C for serological detection. Whole blood was collected in the blood tube containing anticoagulant ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer®, USA) and urine samples were collected in sterile universal containers. Whole blood and urine samples were kept in the chiller at 4 °C until further analyses. After sample collection, the dog was monitored closely by the owner or veterinarian (if warded) and treated appropriately. If the dog was euthanised (procedure done by veterinarian using pentobarbitone intravenously) or found dead due to the sequelae of the disease, a necropsy was performed with permission. The kidney and liver were grossly examined and harvested. A section of the kidney and liver was cut into smaller pieces and homogenised with liquid Ellinghausen and McCullough modified by Johnson and Harris (EMJH) medium for bacterial isolation. If there was abdominal effusion, the fluids were collected and stored appropriately.

Serological detection using microscopic agglutination test (MAT)

Using the serum that was obtained, MAT was performed to detect anti-leptospiral antibodies against a panel of 20 leptospiral serovars as shown in Table 3. The leptospiral serovars comprised of 19 pathogenic strains and one non-pathogenic strain (Patoc I). The endpoint titres were determined using

serial two-fold dilutions until the last well showing 50.0% agglutination was recorded. The cut-off for a positive MAT reaction was defined as a titre $\geq 1:100$ [61, 62].

Table 3
Panel of leptospiral serovars used in MAT for anti-leptospiral antibody detection

Species	Serovar	Strain
<i>L. borgpetersenii</i>	Hardjobovis	117123
<i>L. Interrogans</i>	Hebdomadis	Hebdomadis
<i>L. weilii</i>	Celledoni	Celledoni
<i>L. kmetyi</i>	Malaysia	Bejo-IS09
<i>L. interrogans</i>	Pomona	Pomona
<i>L. borgpetersenii</i>	Tarassovi	Perepelitsin
<i>L. interrogans</i>	Pyrogenes	Salinem
<i>L. interrogans</i>	Australis	Ballico
<i>L. interrogans</i>	Grippothyposa	Moskva V
<i>L. kirschneri</i>	Cynopteri	3522C
<i>L. interrogans</i>	Canicola	Hond Utrecht IV
<i>L. interrogans</i>	Lai	Lai
<i>L. interrogans</i>	Icterohaemorrhagiae	RGA
<i>L. interrogans</i>	Bataviae	Swart
<i>L. borgpetersenii</i>	Javanica	Veldrat Bataviae 46
<i>L. interrogans</i>	Autumnalis	Akiyami A
<i>L. borgpetersenii</i>	Ballum	Mus 127
<i>L. interrogans</i>	Djasiman	Djasiman
<i>L. biflexa</i>	Patoc	Patoc I
<i>L. interrogans</i>	Copenhageni	M20

Molecular detection using polymerase chain reaction (PCR) and partial 16S rRNA sequencing

The whole blood, urine, abdominal effusion (if available) and tissue sample of kidney and liver were used to detect leptospiral DNA. DNA extraction from the samples and positive control of *Leptospira interrogans* serovar Canicola strain Hond Utrecht IV were performed using DNeasy® Blood & Tissue Kit (QIAGEN,

Germany), as describe on the manufacturer's protocol. The end products (DNA template) were inspected using 1.5% agarose gel for purity.

With reference to previous studies [63, 64], two sets of primers were selected and targeted the 16S rRNA and LipL32 genes (as shown in Table 4). Both genes were present in pathogenic *Leptospira* sp., but only 16S rRNA gene were present in the non-pathogenic *Leptospira* sp. [65].

Table 4
Primers for amplification of *Leptospira* spp.

Primer	Sequence 5' – 3'	Length (bp)	Amplicon size (bp)
16S rRNA (forward)	CATGCAAGTCAAGCGGAGTA	20	541
16S rRNA (reverse)	AGTTGAGCCCGCAGTTTTC	19	541
LipL32 (forward)	GTCGACATGAAAAAAGTTTCGATTTTG	27	756
LipL32 (reverse)	CTGCAGTTACTTAGTCGCGTCAGAAGC	27	756

A total of 25.0 µl reaction volume was optimised as follows: 12.5 µl 2x MyTaq™ Red Mix (BIOLINE, UK), 2.5 µl for primer (forward and reverse) and 10.0 µl DNA template. Amplification was optimised and performed in a Mastercycler Pro S (Eppendorf, Germany) with initial denaturation of 94 °C for 5 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, primer annealing at 58 °C for 45 seconds, and DNA extension at 72 °C for 30 seconds before the final extension step at 72 °C for 6 minutes to complete the synthesis of all strands.

The amplicons were analysed in tris-borate-EDTA (TBE) buffer at 80 volts for 1.5 hours by using 1.5% gel electrophoresis. The gel was pre-stained with SYBR® Safe DNA gel stain (Invitrogen™, North America) and examined using Gel Documentation (AlphaImager™, USA). The amplicons were identified by their band sizes. Figure 7 shows two bands at 541 bp and 756 bp were observed using the positive control of serovar Canicola.

After PCR analysis, the amplicons were subjected to a commercial facility for DNA sequencing together with a forward primer sequence. The sequencing data were compared to reference sequences deposited in GenBank using the BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Isolation and characterisation of *Leptospira* spp.

Isolation of *Leptospira* spp.

The isolation of *Leptospira* spp. in this study was based on the protocol described by World Organization for Animal Health (OIE) [66]. Two drops of whole blood, urine, abdominal effusion and approximately

1 ml of homogenised tissue (kidney and liver) samples were inoculated into semisolid EMJH medium. The medium contained 200 µg/ml 5-fluorouracil (5-FU). The inoculation of all the samples was performed within two hours after collection. Primary cultures were kept in the incubator at 30.0 °C for 12 weeks. The cultures were checked every fortnightly to observe for any presence of leptospires under the darkfield microscopy. The cultured medium was checked and sub-cultured into new semisolid EMJH medium for a few times to reduce contamination. If leptospires were observed within the 12 weeks, the positive cultures were transferred into liquid EMJH medium to enhance their growth and filtered by 0.45 µm (Millex®, Ireland) until pure isolates were obtained. The *Leptospira* spp. isolates were maintained in liquid EMJH medium and further characterised through serological and molecular characterisation. If negative for leptospires within the 12 weeks, the cultures were discarded after final careful examination.

Serological characterisation of *Leptospira* spp. isolates

The serovar of the isolates was determined by MAT using a panel of 18 hyperimmune sera as shown in Table 5. The isolates belonged to a particular leptospiral serovar when they reacted serologically to hyperimmune serum with specific titre. The cut-off titre was based upon the reference value provided by Forensic and Scientific Services, Department of Health, Leptospirosis Reference Laboratory, Queensland, Australia.

Table 5
Panel of reference hyperimmune sera with respective titre

Species	Serovar	Strain	Titre
<i>L. borgpetersenii</i>	Hardjobovis	117123	1:6400
<i>L. Interrogans</i>	Hebdomadis	Hebdomadis	1:6400
<i>L. weilii</i>	Celledoni	Celledoni	1:6400
<i>L. kmetyi</i>	Malaysia	Bejo-ISO9	1:6400
<i>L. interrogans</i>	Pomona	Pomona	1:12800
<i>L. borgpetersenii</i>	Tarassovi	Perepelitsin	1:3200
<i>L. interrogans</i>	Pyrogenes	Salinem	1:12800
<i>L. kirschneri</i>	Cynopteri	3522C	1:6400
<i>L. interrogans</i>	Lai	Lai	1:1600
<i>L. interrogans</i>	Icterohaemorrhagiae	RGA	1:800
<i>L. interrogans</i>	Bataviae	Swart	1:6400
<i>L. borgpetersenii</i>	Javanica	Veldrat Bataviae 46	1:12800
<i>L. interrogans</i>	Autumnalis	Akiyami A	1:6400
<i>L. borgpetersenii</i>	Ballum	Mus 127	1:1600
<i>L. interrogans</i>	Djasiman	Djasiman	1:6400
<i>L. biflexa</i>	Patoc	Patoc I	1:1600
<i>L. interrogans</i>	Copenhageni	M20	1:6400
<i>L. interrogans</i>	Australis	Ballico	1:6400

Molecular characterisation of *Leptospira* spp. isolates

The isolates were identified as either pathogenic or non-pathogenic *Leptospira* sp. The PCR was performed using 16S rRNA and LipL32 primers as mentioned earlier. The partial 16S rRNA sequencing was performed using forward and reverse primers for all isolates. The sequencing data obtained were compared with the GenBank database using nucleotide basic local alignment search tool (BLAST) from National Centre for Biotechnology Information (NCBI). Sequences generated from the isolates, along with the representative sequences from genus *Leptospira* (pathogenic, intermediate, and saprophytic) and *Leptonema illini* strain Habaki (as an outgroup) deposited to the GenBank were aligned with CLUSTAL OMEGA Version 1.2.2 (CLUSTAL, Ireland). All the sequencing data were then subjected to single and multiple pairwise alignments using CLUSTALW Version 2.1 (CLUSTAL, Ireland). All the aligned sequencing data were analysed by using phylogenetic tree analysis. The phylogenetic tree was generated

using Molecular Evolutionary Genetics Analysis Version 7.0 (MEGA7) and was inferred by using the Neighbour-Joining method with 1000 bootstrap based on General Time Reversible model [67].

Statistical analysis

Serological detection, molecular detection, and isolation of *Leptospira* spp. were represented using descriptive statistics with 95% confidence interval (CI) using IBM® SPSS® Statistics Version 23 (IBM®, USA). Mortality rate was calculated based on molecular detection and 95% CI was also applied.

Abbreviations

MAT

Microscopic agglutination test

PCR

Polymerase chain reaction

Declarations

Ethics approval and consent to participate

This study was approved by the Universiti Putra Malaysia ethics board.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All datasets are presented in the paper or additional files supporting the manuscript.

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Authors' contributions

KKH conceived this project and designed the experiments. MSAR performed the experiments, analysed, and interpreted the data. MSAR drafted the manuscript. KKH, SKB, LSF, MM, MAR and GSH analysed and interpreted the data and reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Frequency of clinical signs observed in the dogs diagnosed with kidney and/or liver disease (N=124)

Figure 2

Frequency of seropositive samples by MAT titre in dogs diagnosed with kidney and/or liver disease (n=53)

Figure 3

Frequency of leptospiral serovars detected by MAT in dogs diagnosed with kidney and/or liver disease (n=53)

Figure 4

Frequency of *Leptospira* species by partial 16S rRNA sequencing from different types of samples (n=53)

Figure 5

Original tree with 1000 bootstrap. Evolutionary relationships of taxa based on partial 16S rRNA gene

Figure 6

Condensed tree generated from the original tree with 50.0% cut-off point

Figure 7

Gel image for 16S rRNA and LipL32 with their respective base pair (bp) 68. (M) 100bp DNA ladder; (1) negative control; (2-5) positive control serovar Canicola

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