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DETECTION OF PATHOGENIC LEPTOSPIRES AND ANALYSIS OF FACTORS AND CLINICAL SIGNS ASSOCIATED WITH CANINE LEPTOSPIROSIS

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ABSTRACT

Early diagnosis of canine leptospirosis in the leptospiremic phase is crucial to provide appropriate treatment and better prognosis to affected patients, however, this may be challenging due to varying risk factors, non-specific signs and non- predictive hematological values. In this descriptive, cross-sectional study, 60 blood samples from canine patients were tested for leptospirosis using polymerase chain reaction assay. Of the 60 samples, 11 samples (18%) tested positive for pathogenic leptospires which is statistically significant based on Z – test (α=0.05). In contrast, statistical analysis revealed no significant correlation between positive result upon PCR and several variables (age, sex, breed, type of housing and vaccination history). Majority of the clinical signs observed in dogs which tested positive for pathogenic leptospires were vomiting and diarrhea (7/11) while leukocytopenia (5/11) was the predominant hematologic finding. This study was able to detect leptospiral DNA in the blood of dogs and provided a clinical picture of dogs infected with pathogenic leptospires.

Keywords: dog, clinical signs, DNA, leptospirosis, PCR

INTRODUCTION

Leptospirosis is a widespread and fatal zoonotic bacterial disease affecting a wide range of hosts including humans and dogs, caused by pathogenic spirochetes of the genus *Leptospira*. Rats are considered as a global reservoir and a main source of zoonoses, however, dogs may also play a role as pathogen reservoirs especially in urban areas (Morikawa *et al.,* 2015). There is varied geographic distribution of infecting serovars among dog populations, depending on exposure to infected wild or domestic animal reservoir hosts, however, knowledge on serovars infecting dog population has been limited because published studies usually have not included isolation efforts (Sykes *et al*., 2010).

Previous studies have shown evidence of canine leptospirosis in the Philippines. A preliminary survey on the incidence of leptospirosis in dogs using rapid plate agglutination test was done by Alojipan (1961) with 0.88% positive results at the final dilution of 1:40 and 1:160 against *Leptospira canicola* antigen. Using microscopic agglutination-lysis test, it was found that 41 (12.7%) out of 321 dogs tested were positive, including those from the provinces of Laguna, Batangas, Pampanga and Rizal (Topacio *et al*., 1974). The study confirmed that the following Leptospiral serotypes were present: *L. pyrogenes* (36.5%), *L. canicola* (34.1%), *L. grippothyposa* (12.2%), *L. javanica* (7.3%), *L.manilae* (7.3%) and *L. autumnalis* (2.4%) (Topacio *et al*., 1974). Within that period, these studies were able to prove the presence of canine leptospirosis and the prevalent serotypes in the Philippines.

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Albeit there was a low prevalence ranging from 0.88% to 12.7%, these studies indicate the importance of continuous monitoring and surveillance of prevailing serovars.

Arriving at a definitive diagnosis of suspected leptospirosis cases should be of significance to small animal practitioners for several reasons. Early diagnosis and treatment of leptospirosis may improve the prognosis of infected dogs, and can prevent potential zoonotic infection of attending veterinary staff. This may be difficult because of vague and nonspecific clinical signs of the disease (Goldstein, 2010). Moreover, knowledge of prevailing serovars in a particular location are of interest, because vaccines produced must be serovar specific to be effective.

The most commonly available diagnostic tool in veterinary clinics and hospitals is ELISA-based, however, this may prove to have limitations such as low sensitivity (negative results early in the disease process) and specificity (reacts positively with vaccinal antibodies) when a single test is performed (Goldstein, 2010). Polymerase chain reaction (PCR) may prove to be potentially advantageous, as leptospires present in the blood within a week post-infection, known as the leptospiremic phase, can be detected. Leptospires can also be detected in the urine 7–14 days after infection, at which time leptospires may or may not be detected in the blood (Goldstein, 2010). Polymerase chain reaction (PCR) can be made available for small animal practitioners and is a direct test for the detection of specific leptospiral DNA of canine *Leptospira*.

MATERIALS AND METHODS

Selection of patients suspected for leptospirosis

Dogs presented to the University of the Philippines' Veterinary Teaching Hospital-Diliman Station during the rainy season of 2014 (June to July) were considered for the current study as infection is said to peak during this season in tropical regions (Bharti *et al*., 2003). Patients exhibiting at least one of the following clinical signs of leptospirosis were chosen: fever, anorexia, lethargy, vomiting, bloody diarrhea, anemia, dehydration, voiding dark yellow or reddish urine or jaundice. These clinical signs were based on the findings of the retrospective studies of canine leptospirosis cases in UP VTH-Los Baños and -Diliman Stations (Ramos, 2007; Malazo, 2010)

The signalment of the dogs (age, sex and breed), vaccination history, exposure/ housing and hematology values from the problem oriented medical records (POMR) of the hospital were recorded. Age was categorized as <1year (puppy), 1-4 years (young), 5-9 years (middle-aged) or >10 years (adult), sex as male or female and breed as pure-breed, native or mixed-breed. Housing was categorized as indoor or outdoor. Vaccination history was categorized as vaccinated ≤12 months, vaccinated >12 months, never vaccinated or with insufficient data. Date of the last vaccination was recorded to ascertain whether the dog had an updated or lapsed vaccination. The patient was considered to have updated vaccination if the last vaccination was within the last 12 months, since the duration of immunity for bivalent vaccines against leptospirosis persists for 1 year. Insufficient vaccination history data was considered for patients which did not have a history of vaccination written in the POMR or was vaccinated according to the owner but did not present a vaccination card during the consultation.

Blood collection

About 1 to 3 ml of blood was collected from the cephalic vein or lateral saphenous vein and stored in a vial with EDTA at 4ºC. Samples were kept in ice during transport to the Veterinary Molecular Biology Laboratory, College of Veterinary Medicine, University of the Philippines Los Baños. Blood samples were stored at – 40ºC until further processed.

DNA Extraction and Polymerase Chain Reaction

DNA from the whole blood samples were extracted using a DNA extraction kit (Promega Wizard® Genomic DNA Purification Kit Madison, Wisconsin, USA) following the manufacturer instructions. Polymerase chain reaction (PCR) was carried out in 30 µL reaction volumes consisting of 12.5 µL of master mix (Promega Gotaq® Colorless Master Mix, Madison, Wisconsin, USA), 5 µL of DNA template, 5 µL each of both forward and reverse primers at 10µM concentration and 2.5 µl of nuclease-free water.

The primers G1 and G2 that were used in this study were derived from the genomic DNA library of *L. interrogans* strain RGA as described previously. The primer set G1 /G2 amplified DNA by PCR from all pathogenic *Leptospira* species, i.e. strains of *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai* and *L. meyeri* strain ICF, with an expected band size of 285 bp. PCR reactions were carried out in thermal cycler (Thermo Scientific® Arktik®, Watham, Massachusetts, USA) programmed as described in the study by Gravekamp *et al*., (1993) with few modifications: initial denaturation step of 15 minutes at 95°C, followed by 35 cycles of 90 seconds at 94°C, 60 seconds at 50°C, and 30 seconds at 72°C. The reaction was complete with a final run at 72°C for 5 mins.

Data Analysis

Z–test was used to determine the presence and absence of significance between the PCR result and the occurrence of leptospirosis infection. Chi–square test of independence was used to establish the association between sex and leptospirosis infection. Fisher's exact test was used to determine correlation between leptospiral infection and the following variables: breed, age, type of housing and vaccination history

RESULTS AND DISCUSSION

This study detected pathogenic *Leptospira* species affecting canine patients. Eighteen percent (11/ 60) of the dogs tested were positive for leptospirosis infection upon PCR (Figure 1). This percentage of detection was considered high considering that the leptospira-positive patients included in this study were treated for disorders other than leptospirosis. These were gastrointestinal parasitism (3), bacterial gastroenteritis (2), blood parasitism (2), wound abscess (1), renal failure (1), protozoan infection (1) and parvovirus infection (1). In spite of a different treatment plan, it is possible that leptospira- positive patients were also infected with these diseases, in concurrence with leptospirosis. The proportion of 18% samples showed to be statistically significant using the Z-test ($P<0.05$).

Polymerase chain reaction assay as a diagnostic tool in the detection of pathogenic leptospires is important in the early stage of the disease as demonstrated in the other studies (Gravekamp *et al.*,1993; Bal *et* al., 1994) wherein serum and urine samples from human patients were used. Previously tested primers (Gravekamp *et al*., 1993) were used in this study for the detection of pathogenic leptospires using PCR. The primer set G1 /G2 can amplify DNA of all pathogenic *Leptospira* species, i.e. strains of *L. interrogans, L. borgpetersenii, L. weilii, L. noguchii, L. santarosai* and *L. meyeri* strain ICF. The same primer sets were also used in the study conducted by Cai *et al.* (2002) wherein differentiation of *L. grippotyphosa* and *L. sejroe* from other pathogenic *Leptospira* serovars were demonstrated. The practical value of using PCR is crucial to arrive at a definitive diagnosis because it specifically detects leptospiral DNA; whereas, presently used antibody tests may inaccurately detect antibodies induced by vaccines and/ or exposure to non-pathogenic leptospires (Goldstein, 2010).

Using the chi-square test of independence, it was found that sex was not significantly related to leptospirosis infection. Age, breed, type of housing and vaccination history were also statistically analysed using Fisher's exact test; however, no significance

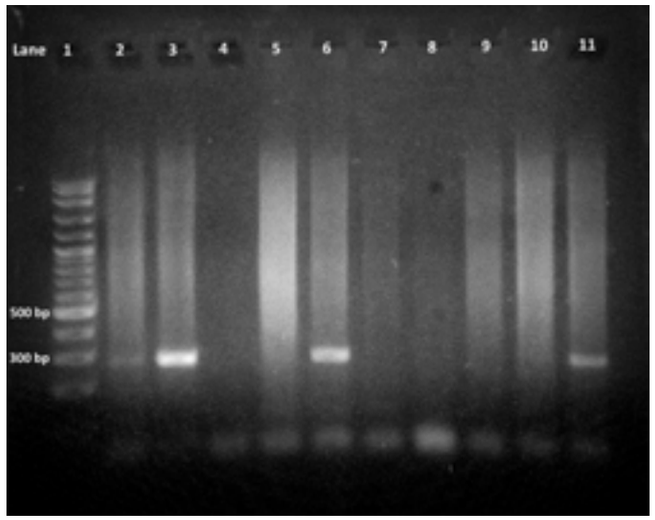

Figure 1. Agarose gel electrophoresis of PCR products amplified from extracted DNA using blood of dogs presented to the UP VTH Diliman from June to July 2014. Lane 1 – DNA ladder. Lanes 4-5 and 7-10 - Clinical blood samples showing no positive results. Lanes 2, 3, 6 and 11 - Clinical blood samples showing positive results at approximately 285 bp.

was found to correlate these variables to leptospirosis infection. Table 1 shows the association of the different variables sex, age, breed, type of housing and vaccination history with the results of polymerase chain reaction assay.

Although other studies (Goldstein *et al*., 2006; Goldstein, 2010; Rentko *et al*., 1992; Ross *et al*., 2011; Sykes *et al*., 2011) have shown the highest percentage of infection in male dogs, as attributed to roaming behaviour, this study showed no correlation in the occurrence of leptospirosis in either male (55%) or female (45%) dogs.

The ages of the infected animals ranged from < 3 months to 7 years, with the highest percentage at 1-4 years of age (64%). This finding agrees with other studies wherein young dogs were reported to be more clinically affected than older dogs (Claus *et al*., 2008; Greene *et al*., 2008; Rentko *et al*., 1992). The breed of the dogs were categorized as pure, mixed and native. The highest percentage of infected dogs were seen in pure bred dogs (73%), while one dog was of native breed (9%) and two dogs were mixed breeds (18%). There was also no relation found between breed and leptospirosis infection although it was mentioned in some studies (Claus *et al*., 2008; Sykes *et al*., 2011) that medium to large breed working dogs are predisposed to the disease. Rather, most of the patients in this study were mainly kept as pets in urban areas. Similarly, outdoor dogs have been shown to be at risk of contracting leptospirosis (Sykes, 2011), however, in this study, type of housing did not show any statistically significant correlation with leptospirosis infection. Leptospira-positive patients were kept mostly kept indoors (10/11) which contradicts claims that outdoor dogs are more prone to the disease. It is possible that these indoor dogs infected with leptospires acquired the infection through contact with contaminated environment such as exposure to rat urine, contaminated feeding troughs, waterers, or contact with contaminated inanimate objects.

There was no significant correlation between vaccination and leptospiral infection. Majority (64%) of the infected animals either had lapsed vaccination or did not have a vaccination history against DHLPPi based on the POMR, while 4 dogs (36%) were vaccinated within the last 12 months. It was assumed that the vaccine given included the bacterin for *Leptospira* spp. as part of DHLPPi (5 in 1) vaccine since it was the vaccine routinely used in most clinics. Locally available commercial bivalent vaccine only protects dogs against two *Leptospira* serovars: *L. canicola* and *L. icterohaemorrhagiae*. Lack of antibodies produced despite vaccination may have caused infection, since immunity to leptospires is serovar- specific and vaccines are not cross-protective against other serovars

Table 1. Sex, age, breed, type of housing and vaccination history of patients tested for pathogenic leptospires and results of polymerase chain reaction assay.

Variables		Total Number of Samples	PCR	
			Positive (n=11)	Negative (n=49)
Sex	Male	32	6	26
	Female	28	5	23
Age	<1 year	15	2	13
	1-4 years	34	7	27
	5-9 years	9	2	7
	>10 years	2	0	2
Breed	Pure	49	8	41
	Native	2	1	1
	Mixed	9	2	7
Type of Housing	Indoor	53	10	43
	Outdoor	6	0	6
	Indoor/Outdoor	1	1	0
Vaccination History	Vaccinated ≤ 12 months	16	4	12
	Vaccinated >12 months	6	0	6
	Never Vaccinated	8	2	6
	Insufficient Data	30	5	25

(Greene *et al*., 1998). Furthermore, vaccination only protects the animal against acute signs but may not prevent infection if the dog is exposed to a high infectious challenge or to a highly invasive strain (Andre-Fontaine, 2006).

In this study, blood samples were collected from dogs exhibiting at least one clinical signs of leptospirosis (Table 2). The dogs that were positive for leptospirosis, exhibited (in decreasing order) vomiting, bloody diarrhea, hematuria, anorexia, fever and lethargy. Other studies have reported that vomiting, lethargy, anorexia are most common findings in cases of leptospirosis (Goldstein *et al*., 2006; Greene *et al*., 1998; Rentko *et al*., 1992).

In this study, none of the icteric dogs (3/60) tested positive for leptospirosis upon PCR. Signs related to hepatic disease in the dog have been most commonly associated with serovars *icterohaemorrhagiae* and *grippotyphosa* and icterus has been reported to be a common in subacute canine leptospirosis (Rentko *et al*., 1992). However, since leptospiremia occurs only for about a week (Levett, 2001), this may be the reason why no leptospiral DNA was detected. It should also be noted that overt icterus on initial presentation is considered an uncommon clinical sign and therefore it should not be considered as a reliable indicator to which a dog should be tested for the disease (Goldstein, 2010). Moreover, jaundice is also associated with chronic hepatitis and babesiosis (DebMandal *et al*., 2011).

Some hematological values of the dogs were also recorded which consisted of the PCV (%), RBC ($X10^6$/µl), WBC (cells/µl) and platelet count (cells/µl) (Table 3) Hematological findings in this study showed anemia in 4 out of 11 positive animals as indicated by the low

Table 2. Summary of the presenting signs of dogs tested for pathogenic leptospires using PCR.

Presenting Signs	Total	PCR	
		Positive (n= 11)	Negative (n=49)
Fever	5	1	4
Anorexia	11	1	10
Lethargy	12	1	11
Vomiting	14	3	11
Vomiting + Fever	2	0	2
Vomiting + Anorexia	1	0	1
Vomiting + Diarrhea	6	2	4
Blood Tinged diarrhea	5	2	3
Anemia	1	0	1
Bloody Urine	2	1	1
Icteric	3	0	3

packed cell volume and erythrocyte values of the *Leptospira* positive dogs. Many serovars of *Leptospira* are reported to cause anemia in dogs, however the mechanism for the anemia is not well understood (Riegel and Stockham, 2010). Leukocytopenia was seen in 5 out of 11 (45%) *Leptospira*-positive dogs. In the early stages of the disease, transient leukocytopenia may be seen, lasting from 1 to 2 days, which would be then followed by an expected leukocytosis (Andre-Fontaine, 2006; Greene *et al*., 1998).

Another common hematological finding in leptospirosis cases is thrombocytopenia as demonstrated in several studies (Goldstein *et al*., 2006; Rentko *et al*., 1992; Schreiber *et al*., 2005), however, thrombocytopenia was only seen in 3 out of 11 (27%) dogs and the majority (72%) of the dogs had normal platelet count. Thrombocytopenia may occur due to vasculitis associated with leptospiremia and in response to acute systemic infection, wherein leptospiremic phase would seem to be the most likely cause (Rentko *et al*., 1992). In later stages of the leptospirosis, thrombocytopenia may also be seen, accompanied by evidence of acute kidney damage with or without hepatic injury (Sykes *et al*., 2011). Normal hematological findings in this study can be attributed to the time period of infection and development of the disease. Platelets are known to survive for about a week in circulation, and depletion of platelets occurs gradually over a period of 1-3 weeks. In chronic leptospirosis, hematological parameters are known to normalize (Andre-Fontaine, 2006), however, in this study, since pathogenic leptospires were detected in blood, the acute phase of the disease was more likely.

This study has shown that pathogenic leptospires were detected in a significant number of canine patients (18%) with nonspecific clinical signs. It was also shown that pets in urban areas may be susceptible to infection despite the presence or absence of known predisposing factors. Common clinical signs of dogs infected with leptospirosis were analyzed and showed that these can be helpful in screening, diagnosing and treating leptospirosis. Furthermore, the molecular detection of *Leptospira* can be used as preliminary data for further characterization of the infecting serovars of *Leptospira* in dog and may contribute to vaccine efficacy studies and future vaccine development.

Table 3. Summary of the interpretation of hematological values of dogs tested for pathogenic leptospires upon PCR.

Parameters	Total	PCR Results for leptospires	
		Positive (n=11)	Negative (n=49)
Low PCV	27	4	23
Normal PCV	31	6	25
High PCV	2	1	1
Erythrocytopenia	29	3	26
Normal EØ count	29	7	22
Erythrocytosis	2	1	1
Leukocytopenia	17	5	12
Normal LØ count	21	4	17
Leukocytosis	22	2	20
Thrombocytopenia	19	3	16
Normal platelet count	35	6	29
Thrombocytosis	6	2	4

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