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Detection of *Mycoplasma* and *Hepatozoon* spp. in Philippine Dogs

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ABSTRACT

Tick-borne diseases (TBD) in dogs have been an emerging issue worldwide. In the Philippines, most reports are in the northern areas, which include *Babesia*, *Ehrlichia*, *Anaplasma,* and *Hepatozoon* spp. The *Mycoplasma* spp., a suspected TBD pathogen, has not been reported in Philippine dogs while *Hepatozoon* spp. has not been previously documented in the southern Philippines. The present study aimed to evaluate the presence of *Mycoplasma* and *Hepatozoon* spp. in dogs in Cebu, Philippines. A total of 100 dogs from four veterinary clinics and hospitals were tested for *Hepatozoon* and *Mycoplasma* spp. using PCR. The inclusion criteria used were presence or history of ticks, anemia and/or thrombocytopenia. Clinical signs of the dogs were also obtained. There were four and two dogs found positive for *Hepatozoon* and *Mycoplasma* spp., respectively using PCR, and two dogs were found positive for *Hepatozoon* spp. using PBSE. Clinical signs such as lethargy, inappetence, fever, weight loss, and paleness were observed in most of the subjects. This study is the first of *Mycoplasma* spp. detection in Philippine dogs, and the initial report of *Hepatozoon* spp. in the southern Philippines.

Keywords: *Hepatozoon* spp.; *Mycoplasma* spp.; PBSE; PCR; tick-borne diseases

INTRODUCTION

Tick-borne diseases (TBDs) pose a serious threat to the health and welfare of dogs. TBDs are considered to be the most significant subcategory of canine vector-borne infectious diseases worldwide because of the broad geographic spectrum of many tick species, the capability of tick-borne pathogens to induce infections, and the highly zoonotic potential of some of these pathogens (Elsheikha, 2016). The occurrence and detection of these diseases in dogs have a multi-factorial cause, which includes improved animal care, faster and more efficient diagnostic tools, and a wider distribution of the vectors in a favorable environment through population migrations (Chomel, 2011).

Among the TBD pathogens is the *Hepatozoon canis*, which is an apicomplexan protozoa with a worldwide distribution affecting the health of domestic dogs and wild canids (Smith, 1996; Baneth, 2011). It is transmitted differently compared to other arthropod-borne pathogens since the ticks containing mature oocysts have to be ingested and later resulting in infection in leukocytes and parenchymal tissues (Baneth et al., 2001). Its vector is the ubiquitous brown dog tick, *Rhipicephalus sanguineus*. Meanwhile, *Mycoplasma haemocanis* is a pleomorphic bacterium that can be visualized in the peripheral blood smear of the host either singly or in chains appearing like a "violin-bow" form (Lumb, 1961). *Mycoplasma* spp. was already identified as a canine hemoplasma of worldwide distribution (Compton et al., 2012; Hamel et al., 2013; Torkan et al., 2014). Its implicated vector is still the *R. sanguineus*.

In the Philippines, the only recorded tick vector in dogs is the *R. sanguineus* (Ybañez, 2013; Ybañez et al., 2015a; Ybañez et al., 2016; Ybañez et al., 2017). The presence of several TBD pathogens has already been documented in the Philippines, but *Hepatozoon* spp. has only been reported in the northern part of the country (Baticados et al., 2010; Ybañez, 2013; Corales et al., 2014; Ybañez et al., 2017; Adao et al., 2017). On the other hand, the detection of *Mycoplasma* spp. in dogs in the Philippines is yet to be confirmed. *Mycoplasma* spp. in dogs were already detected in other ASEAN countries, including Thailand and Cambodia (Inpankaew et al., 2016; Liu et al., 2016). Hence, the present study aimed to document the presence of *Hepatozoon* spp. in the southern area of the Philippines and confirm the presence of *Mycoplasma* spp. in dogs in the country.

MATERIALS AND METHODS

Ethical considerations

The procedures performed in this study were guided by the principles of animal welfare, Animal Welfare Act of the Philippines (RA 8485) and Administrative Order No. 45 of the Bureau of the Animal Industry of the Philippines. Prior to sampling, this study was approved by the Institutional Animal Care and Use Committee (IACUC) and was given an IACUC Clearance with approval no. 2018-003. Owners of the dogs from where the samples were obtained gave consent.

Blood Sample Collection

A total of 100 dogs were sampled from four veterinary clinics and hospitals in Cebu, Philippines. Three milliliters of blood sample was collected from the cephalic vein using a BD K3 EDTA Vacutainer® tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and were stored at –20 ºC until DNA extraction. Peripheral blood smear examination (PBSE) was performed to evaluate the presence of the selected tick-borne pathogens. Complete blood count (CBC) was also performed.

DNA Extraction and PCR

DNA was extracted from the blood samples using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, U.S.A.), following the protocol recommended by the company. After measuring their concentrations, the DNA samples were stored at -20 ºC until use.

Previously described PCR assay was used to detect the selected tick-borne pathogens. Detection for *Mycoplasma* and *Hepatozoon* spp. were respectively performed using PCR based on 16S rRNA and 18S rRNA genes (Ybañez et al., 2015b; Inokuma et al., 2002). The PCR assay was carried out in a final volume of 25 μl reaction mixture containing 5 μl of each DNA template. Double distilled water (DDW) was used as a negative control. Amplification cycles included initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, and extension at 72 °C for 90 s followed by a final extension at 72 °C for 5 min. The PCR amplicons were visualized using a 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer stained with ethidium bromide under the UV transilluminator.

Data Processing and Analysis

Gathered data were manually encoded in Microsoft Excel, and imported to statistical software. Descriptive statistics were employed.

RESULTS AND DISCUSSION

PCR analysis revealed that four and two dogs were positive for *Hepatozoon* spp. and *Mycoplasma* spp., respectively. The low detection rate of *Hepatozoon* spp. in the area is similar to other reports in the northern Philippines (Baticados et al., 2010; Adao et al., 2017), but it is lower than those reported by nearby countries like Thailand (Liu et al., 2016) and Cambodia (Inpankaew et al., 2016). PBSE revealed two of the four *Hepatozoon* spp.-positive PCR to have inclusion bodies, while none was observed for the *Mycoplasma* spp.-positive PCR samples. The negative detection using PBSE may have been affected by the low level of parasitemia during the time of sample collection or maybe due to the chronic stage of the disease (Rani et al., 2011). High parasitemia level is usually seen in acute stages (Sakuma et al., 2009), which can be evident in the blood smears. However, PBSE is considered to be less reliable as PCR-positive animals may have negative PBSE results (Ybañez, 2013).

Due to the low detection rates reported in this study, performing statistical analysis on profile, clinical signs, complete blood count and PCR positivity was not ideal. Instead, summarized information on the positive cases is hereby presented.

Upon presentation, clinical signs such as lethargy, inappetence, fever, weight loss, and paleness were observed in most of the subjects. However, not all dogs had high neutrophil counts and not all were anemic and thrombocytopenic (3/4) as shown in their complete blood count findings (data not shown). These results indicate that the dogs, upon the time of presentation, were not in the acute stage of the disease and the similar clinical signs and blood values may have been caused by other pathogens present in the animal (Rojas et al., 2014). *Hepatozoon* spp.-infected dogs exhibited lethargy, paleness of mucous membranes, anemia, and thrombocytopenia. The observed lethargy and inappetence may be due to the inflammatory reactions caused by *Hepatozoon* spp. in the organs such as the bone marrow, lymph nodes, spleen, and liver. Since *Hepatozoon* spp. infects the bone marrow, liver, and spleen, paleness of the mucous membranes can be an expected clinical finding, since these organs are involved in the production of red blood cells (Duncan and Prasse, 1977). Anemia was observed in most of the positive dogs (3/4) *w*hich was similar to previous

studies (Elias and Hommans, 1988; Sakuma et al., 2009; Rojas et al., 2014). Anemia can be due to the necrosis of the spleen and liver in *Hepatozoon* spp infection (Tsachev et al., 2008). *Hepatozoon* spp. can also cause dysmegakaryopoiesis and dysgranulopoiesis in the bone marrow of affected dogs which may lead to the development of anemia in *Hepatozoon* spp. infections (De Tommasi et al., 2014). It should be taken into account that merogony of *Hepatozoon* spp. occurs in the bone marrow (Baneth et al., 2007).

Fever, which would have been an expected finding, was only observed in one patient. One out of the four positive dogs had no clinical signs seen during presentation proving that *Hepatozoon* spp. infection can also be subclinical, and clinical manifestations can only be found when dogs are immunocompromised. Thrombocytopenia in this study can be most likely due to co-infection with other diseases causing thrombocytopenia such as ehrlichiosis and babesiosis, as the same vector, *R. sanguineus*, carries these diseases (Yabsley et al., 2008; De Tommasi et al., 2013). *Mycoplasma* spp.-infected dogs exhibited inappetence, weight loss, and pale mucous membrane. The pathogen seldom causes anemia unless the dogs are immunocompromised or splenectomized. Another factor is co-infection with other TBDs such as babesiosis and ehrlichiosis which could be the cause of the manifestation of pallor or anemia and other clinical signs (Sasanelli et al., 2009; Rojas et al., 2014). Thrombocytopenia was also observed.

An increased neutrophil count was detected in half of the dogs in this study which is attributed to the secondary necrosis of spleen, liver, lymph nodes (Tsachev et al., 2008). Increased neutrophil count was also reported in the studies of Voyvoda et al. (2004) and Kaur et al. (2012). Basophilia was also witnessed in most dogs which might be an indication of chronic infection in TBDs. Other blood values in this study were found to be within the normal range.

The present study reports the presence of *Hepatozoon* spp. in dogs in the southern Philippines and the first detection of *Mycoplasma* spp. in Philippine dogs. Infection with the pathogens mentioned above must be considered as among the differential diagnoses by local practitioners, especially when presenting clinical signs resemble the disease. The results of this study is a valuable addition to the TBD pathogen diversity in the Philippines. Moreover, further studies at the genetic analysis level will have to be endeavoured in the future for species confirmation and for analysis of their relatedness to pathogens isolated in dogs from other countries.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that the work described has not been published previously and that it is not under consideration for publication elsewhere. All the authors approve the publication of this manuscript and that, if accepted, it will not be published elsewhere in the same form, in English or any other language, including electronically without the written consent of the copyright holder.

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