Serological Evidence for *Ehrlichia canis* Exposure in Military Dogs and Other Canines in Metropolitan Manila, Philippines

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ABSTRACT

Canine monocytic ehrlichiosis (CME) is a fatal worldwide tick-borne disease of canines. To date, there are no published reports on the serological detection of *Ehrlichia canis* in dogs in Metropolitan Manila, Philippines. In an attempt to establish the serological status of canine ehrlichiosis in the country, a total of 169 canine blood samples were processed for ELISA assay. A high percentage of the blood samples, 95.3% (161/169), were found seropositive for *E. canis* antibodies using Immunocomb® *Ehrlichia canis* test kit. Moreover, out of the total seropositive animals, 59.0% (95/161) exhibited significant antibody titers whereas 41.0% (66/161) displayed low antibody titer levels. Only 4.7% (8/169) of the blood samples showed seronegative results. The study reports seropositive Immunocomb® Canine Ehrlichia Antibody test kit results from randomly sampled canines of Metropolitan Manila, Philippines. Consequently, the significant levels of antibody titers only indicate possible exposure to the pathogen but not necessarily the presence of the aetiologic agent. In summary, the outcome of the study indicates that the aetiologic agent of the fatal disease in canines (CME), *E. canis*, is possibly in existence in the Philippines. The paper describes the first serological evidence for *E. canis* exposure in canines from Metropolitan Manila, Philippines using an ELISA-based diagnostic test kit.

Key words: *E. canis*, ELISA, Metropolitan Manila, Philippines, canines, military dogs

INTRODUCTION

Ehrlichia canis is the etiologic agent of a serious and fatal infection in canines known as canine monocytic ehrlichiosis (CME) or tropical canine pancytopenia. It is an acute, subclinical and chronic disease involving an obligate rickettsia established to be localized in the cytoplasm of monocytes and transmitted during blood meals by the brown dog tick, *Rhipicephalus sanguineus* (1, 2, 3, 4).

The disease is a cosmopolitan tick-borne infection (5) which was first recognized in Algeria in 1935, and has reached other parts of Africa, the Middle East, and the Orient particularly tropical and subtropical countries (6, 7). The emergence of multiple *Ehrlichia* species infection in humans and animals led to the development of more rapid and accurate techniques such as serology and polymerase chain reaction (PCR) for diagnosis of ehrlichiosis (8, 9). Furthermore, the limited sensitivity, difficulty of examination and need for skilled personnel in performing direct microscopical parasite examination of blood (6, 10, 11, 12), were among the primary reasons for the current use of molecular and serological tests for ehrlichial infection.

A proven and tested ELISA-based test kit is readily accessible and presently used in the country. Immunocomb® Canine Ehrlichia Antibody test kit has been widely utilized to detect previous exposure of dogs to *E. canis* (2). This test uses small quantities of purified whole ehrlichial antigen mounted on a nitrocellulose surface, for detection of serum IgG antibodies. It requires only minimum equipment and

is easy and rapid to perform since it involves only a single step dilution (13). Consequently, the intensity of the color reaction is directly influenced by the amount of antigen-antibody-conjugate-enzyme complexes formed.

The diagnosis of ehrlichiosis is largely based on clinical signs and laboratory findings during the acute and chronic stages. Whereas a decreased platelet count is the most consistent hematologic finding, serology is one of the preferred methods for confirming infection by *E. canis*, especially during the subclinical stage of disease when clinical manifestations are absent (14). The ELISA kit can therefore be used efficaciously during all phases of CME (2).

Ehrlichiosis can also be specifically diagnosed by direct examination of morulae in peripheral blood smears as was demonstrated in the Philippines in a previous study (15). It is an important method for confirmatory diagnosis of ehrlichiosis but in most cases unrewarding due to the sparse presence of morulae in monocytes. Morulae are evident albeit in small numbers in acute cases but not in convalescing or carrier animals (16). Upon onset of the subclinical stage, there may be complete disappearance of morulae in the blood.

Since *E. canis* infection is a fatal disease (6, 17, 18, 19) it requires appropriate diagnostic procedures for immediate and accurate treatment to attain good prognosis (17). The detection of *E. canis* antibodies in canines from Metropolitan Manila was therefore carried out using an ELISA-based diagnostic test kit. This paper reports for the first time evidence of *E. canis* exposure in canines from Metropolitan Manila, Philippines using an ELISA-based diagnostic test kit.

MATERIALS AND METHODS

Blood sample collection

One hundred sixty nine venous blood samples from military working dogs of the Armed Forces of the Philippines and dogs presented to large veterinary hospitals in Metropolitan, Manila were used. Randomized sampling was utilized and dogs included in the study were either apparently healthy or showing various clinical signs of differing severities. Samples were analyzed using an ELISA test kit within 30 minutes after blood collection. Unstained blood smears were posted to the Veterinary Histopathology Laboratory, University of the Philippines and stained with Giemsa® stain for future use. The rest of the venous blood were kept at -44°C in the BioFreezer at the Veterinary Molecular Biology Laboratory,

A

Locality	No. of samples	%
Malabon	1	0.59
Quezon City	22	13.01
Manila	35	20.71
San Juan	5	2.96
Pasig	3	1.78
Makati	71	42.01
Mandaluyong	6	3.55
Taguig	4	2.37
Parañaque	7	4.14
Muntinlupa	8	4.73
Las Piñas	1	0.59
Caloocan	5	2.96
Rizal	1	0.59

B

Figure 1. Map of sampling areas (A). Number and percentage distribution of samples per locality (B).

College of Veterinary Medicine, University of the Philippines Los Baños for future experimentation. Blood collection was performed with prior permission and approval of the Institutional Animal Care and Use Committee (IACUC) (Protocol No. 2008-031).

Serology

ImmunoComb® Canine Ehrlichia Antibody Test Kit (Biogal Galed Lab., Israel) was performed at room temperature (20°-25°C) and carried out following the manufacturer's instructions. An equivalent intensity of the color reaction in comparison with a positive reference point was used as guide in order to denote the level of antibodies in each sample: intense color reactions as compared to the reference spot were considered positive for antibodies against *E. canis* whereas a colorless or faint gray color reaction indicates either a negative result or undetectable levels of antibodies. The "gold standard" for the detection of antibodies to *E. canis*, is the indirect immunofluorescence antibody (IFA) test. In the clinic or laboratory, ELISA units can be translated to IFA IgG *E. canis* antibody titers by using the color scale provided in the kit. S3 is considered the "cutoff" level of IgG antibody, which is roughly equivalent to a positive immune response at a titer of 1:80 by IFA assay. Antibody titers for the different "S" levels (ImmunoComb® scores (IC)) were as follows; S1 and S2 (1:20-1:40), S3 and S4 (1:80-1:160) S5 and S6 (1:320-1:1280).

Table 1. Tabulated serological profile of canine breeds represented in the study.

No. per breed	ImmunoComb® Score (IC)							Canine Breed		ImmunoComb® Score (IC)							No. per breed
	S0	S1	S2	S3	S4	S5	S6			S0	S1	S2	S3	S4	S5	S6	
20	1	2	6	6	4	1	0	Mongrel	Lhasa Apso	0	0	1	1	1	0	0	3
16	0	1	8	6	1	0	0	Shih Tzu	French Bulldog	0	1	0	1	1	0	0	3
15	2	1	4	6	1	1	0	Labrador Retriever	Yorkshire Terrier	0	0	2	0	0	0	0	2
13	0	3	2	5	1	2	0	German Shepherd	Sharpei	0	0	0	2	0	0	0	2
10	0	0	3	4	3	0	0	Japanese Spitz	Great Dane	0	0	1	1	0	0	0	2
9	0	0	2	0	3	2	2	Belgian Malinois	Dutch Shepherd	0	0	0	0	0	1	1	2
8	0	0	3	3	1	1	0	Cocker Spaniel	Bull Terrier	0	0	0	0	1	0	0	1
8	1	0	4	2	1	0	0	Chihuahua	Boston Terrier	0	0	1	0	0	0	0	1
7	1	1	2	1	2	0	0	Mini Pinscher	Bichon	0	0	1	0	0	0	0	1
5	0	1	2	1	1	0	0	Pug	Whippet	0	0	1	0	0	0	0	1
5	1	1	1	1	1	0	0	Dalmatian	Irish Setter	0	0	0	1	0	0	0	1
5	0	0	0	5	0	0	0	Toy Poodle	Ridgeback	0	0	0	0	1	0	0	1
5	1	1	1	1	1	0	0	Beagle	Chow Chow	0	0	0	1	0	0	0	1
4	0	2	1	1	0	0	0	Dachshund	Maltese	0	0	1	0	0	0	0	1
4	0	0	1	2	1	0	0	Rottweiler	Basset Hound	0	0	0	0	1	0	0	1
3	1	0	1	1	0	0	0	Golden Retriever	Belgian Shepherd	0	0	0	0	1	0	0	1
3	0	0	3	0	0	0	0	Pomeranian	Siberian Husky	0	0	0	1	0	0	0	1
3	0	0	0	3	0	0	0	Pekingese	Dobermann	0	0	0	1	0	0	0	1
143	8	13	44	48	21	7	2	= Sub-total =		0	1	8	9	6	1	1	26

Summary								
IC	S0	S1	S2	S3	S4	S5	S6	TOTAL
Dogs (%)	8(4.7)	14(8.3)	52(30.8)	57(33.7)	27(16.0)	8(4.7)	3(1.8)	169(100)

RESULTS

A total of 169 dog blood samples from various breeds of dogs and different areas of Metropolitan, Manila were obtained (Figure 1A). The bulk of the samples were collected from the municipality of Makati (42.0%) while the least number of samples came from Malabon (0.6%), Las Piñas (0.6%) and Rizal (0.6%) respectively (Figure 1B). The most represented breeds belonged to mongrel dogs known locally as Aspin or Askal *colloq*. (11.8%), Shih Tzu (9.5%), Labrador Retriever (8.9%) and German Shepherd (7.7%) (Table 1).

Table 1 summarizes the serological profile of each canine breed included in the study. The study illustrated that the majority of the breeds had significant antibody titers with IC scores varying between S3 to S6 (n= 95; titer=1:80-1:1280)

denoting possible exposure to *E. canis*. In contrast, less than 5% of the animals failed to exhibit demonstrable level of antibodies reactive to *E. canis* against the parasite.

S3 Immunocomb® scores were consistently observed in groups of greater than 5 dogs per breed except for Belgian Malinois. However, among the breeds which had more than five dogs represented, only the Belgian Malinois exhibited S6 Immunocomb® scores. Additionally, for the following serological groups S2, S3 and S4, equal distribution of one dog per breed was observed. Furthermore, S2 and S3 scores were both seen in house pets (Bichon, Maltese and Chow Chow), S3 and S4 scores were observed with terriers (Boston Terrier and Bull Terrier) while S4 and S2 scores were detected for gun dogs (Ridgeback and Irish Setter respectively) (Table 1).

In general, the results of ImmunoComb® *E. canis* test showed that 95.3% (161/169) of the blood samples were found seropositive for *E. canis* antibodies. Out of the total seropositive animals, 41.0% (66/161) exhibited very low antibody titer levels while 59.0% (95/161) animals displayed significant antibody titers confirming the widespread exposure to *E. canis*. Only 4.7% (8/169) of the blood samples exhibited seronegative results. In addition, based on history and physical examination, all dogs from different municipalities were determined infested with the brown dog tick, *Rhipicephalus sanguineus*.

DISCUSSION

Several techniques have been routinely utilized in the diagnosis of ehrlichiosis in the Philippines including microscopy, hematology and serology. Molecular studies using PCR are carried out as well but only in referral centers and research laboratories. A study in 2007 in the Philippines of 45 Belgian Malinois military working dogs showed that 36.0% of the examined blood smears showed *Ehrlichia* morulae in peripheral blood leukocytes. Furthermore, thrombocytopenia was seen in 84.0% of the canine blood smears with identifiable morulae (15). These findings along with the known existence of the tick vector host (*Rhipicephalus sanguineus*) in Asia (4), including the Philippines, coupled with 95.3% seropositive results in the examined animals of the current study, are strongly indicative of the presence of *E. canis* in Metropolitan Manila, Philippines. The serological study was undertaken in order to gain more evidence of the presence of *E. canis* in the Philippines.

Serology is the most frequently used diagnostic modality for the confirmation of *E. canis* infection in the dog. Indirect fluorescent antibody (IFA) testing is considered the "gold standard" for the detection and titration of antibodies, the test however is generally available only in selected laboratories and requires trained personnel. A comparative study was done using IFA and enzyme-linked immunosorbent assay (ELISA) (2). Results showed that both were equally sensitive for the early detection of IgG antibodies against *E. canis*. Furthermore, the results correlated well with the appearance of clinical signs. The study therefore proposed the application of in-clinic ELISA test to aid in the diagnosis of CME (2). Based on these suggestions we have set out to use this kit for the demonstration of *E. canis* antibodies in dogs in Metropolitan Manila region.

The majority of dogs (33.7%) had *E. canis* titers of S3, which is equivalent to an IFA titer of about 1:80. Titers of equal to and greater than 1:80 (S3) are considered as evidence of exposure to *E. canis*. This is supported by other studies (19).

The observed serological evidence of the extensive exposure to *E. canis* of dogs in the Philippines in both clinically ill and apparently healthy pet dogs indicates that a sizable proportion of the canine population may have been exposed to the pathogen. These dogs may have been in one of the stages of ehrlichiosis, most frequently in the subclinical phase, or they may have been successfully treated but still demonstrating a titer. Accordingly, 16.0%, 4.7% and 1.8% of the samples demonstrated high anti-*E.canis* IgG at 1:160 (S4), 1:320 (S5) and 1:1280 (S6) antibody titers, respectively.

Serologic cross-reactions between ehrlichial species may pose a substantial downside in the interpretation of serological results; therefore it is crucial that the diagnostician take into account the range of cross-reactivities that may also confound the diagnosis. *E. canis* and *E. chaffeensis* share many common antigens and serological cross reactions are known to occur. The immunodominant proteins of *E. canis* cross-react with *E. chaffeensis* major antigens in the 30 kDa range. These *E. canis* and *E. chaffeensis* cross-reacting proteins were demonstrated to be major outer membrane proteins (OMP) encoded by a polymorphic multigene family (13). In areas endemic to other *Ehrlichia* species, cross-reactivity between *E. canis* and *E. ewingii*, *E. risticii* or *E. equi* should be taken into consideration. Cross-reactions

between *E. canis* and *Anaplasma phagocytophilia* after artificial infection with the Israel strain of *E. canis* have been documented (20). In addition, experimentally infected dogs developed antibodies, which reacted with both *E. canis* and *C. ruminantium*, with very similar IFA titers against the two antigens.

Furthermore, antibodies against *E. canis* are also known to cross-react with antigens of *Neorickettsia helminthoeca* and *N. risticii*, and *E. sennetsu* (22). On the other hand, there is no serological cross reactivity between *E. canis* and the etiological agent of canine thrombocytotropic anaplasmosis, *Anaplasma platys* (21) and seldom if any, cross-reactions were observed between *E. canis* and *Rickettsia rickettsii*, the etiologic agent of Rocky Mountain Spotted Fever (RMSF).

It is possible that the dogs in Metropolitan, Manila could have been infected by an organism closely related to *E. canis* as seen with several (41.0%) of the animals having low titers, that may have been associated with cross reactivity (15).

The possibilities of exposure to *E. canis* without disease, and/or the spontaneous recovery from ehrlichiosis should also be considered (23). Differences in the virulence of various strains have been proposed for *E. canis*. The existence of a less virulent strain of *E. canis* in Africa has been hypothesized and may explain why seroconversion to antibodies reactive with *E. canis* in Zimbabwe was found to be 42.0% even among apparently healthy dogs and how 50.0% of previously seronegative French military dogs in Tunisia, Senegal and Chad developed titers while in Africa without showing clinical signs during a 15-day observation period (22).

The populations studied in published serosurveys for *E. canis* differ considerably, thus making comparisons between the studies difficult. The prevalence of antibodies against *E. canis* in 371 pound dogs in California was zero. Eleven percent of 2077 apparently healthy USA military working dogs situated around the world were seropositive, with a higher prevalence in tropical and temperate zones than in colder zones. Fifty-two percent of 105 dogs, ill with a variety of conditions, which were observed at a veterinary hospital in Harare, Zimbabwe, had antibodies reactive with *E. canis* (22). In addition, overall seroprevalence in Spain is 3.0% to 67.0% (24) and in Turkey 65.0% (25). The geographical distribution and seroprevalence of *E. canis* in the Middle East are as follows: Israel 30.0% (26) and Egypt 33.0% (27). Results of the studies in Israel, demonstrated significantly higher seroprevalence in adult dogs (1 to 8 years) as well as those coming from animal control facilities as compared with young dogs (<1 year) and dogs visiting veterinary clinics. Furthermore, the highest incidence was observed in northern Israel (71.4%) and the lowest in the central hills (Jerusalem) (10.3%). These findings indicate that *E. canis* infection is endemic in the Middle East (26).

Most of the cities in Metropolitan Manila, Philippines are lowland terrains except for Rizal, which is generally a hilly and mountainous region, and Malabon, which lies in the Guadalupe plateau. All are residential/industrial areas that had become increasingly commercialized over the years. These lands have been altered substantially by human intervention since the American colonial times. Some of the natural variations in topography have been evened out due to the urbanization of the city. The cities of Rizal, Malabon and Las Pinas had similar seropositive results at 0.59% regardless of varying topography thus, based on these results no differences in seropositivity were seen in terms of the physical geography of the sampled municipalities.

Although antibody titers, in general are not absolutely correlated with either the duration of infection, the current carrier status, or the presence of severity of clinical disease (19), results of the study demonstrated that majority of the animals were possibly exposed to *E. canis* as evidenced by their obtained level of anti-*E. canis* titers (2, 19, 28). The possibility of cross-reactions still remains to be resolved.

These results reinforce the applicability of in-clinic ELISA for the determination of disease exposure as well as the possible diagnosis of canine ehrlichiosis. Consequently, the study advocates the use of ELISA as part of the routine diagnostic procedures in the Philippines.

In summary, the study established for the first time the possibility that the etiological agent of CME, *E. canis*, is endemic in the Metropolitan Manila region. Further isolation and genetic studies will be necessary to confirm this conclusion taking into account the possibility of cross-reactions with other related ehrlichial organisms.

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