

Prevalence of canine ehrlichiosis in Perak State, peninsular Malaysia

Wahab A. Rahman, Chen Hee Ning & Chandrawathani, P.

School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang

*Veterinary Research Institute, Jalan Sultan Azlan Shah, Ipoh, Perak

Email address:

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Abstract. The Indirect Fluorescent Antibody Test (IFAT) and thin blood smears were conducted to establish the prevalence of *Ehrlichia canis* infection in dogs presented for treatment by pet owners at five private and one government veterinary clinic. Results showed that 15% of the dogs were positive for the parasite via IFAT, but none using blood smears. However, infected dogs did not show severe clinical symptoms of canine monocytic ehrlichiosis (CME).

INTRODUCTION

Canine blood parasitic diseases transmitted by parasitic vectors include ehrlichiosis (also known as tropical canine pancytopenia), babesiosis, dirofilariasis, hepatozoonosis, bartonellosis, leishmaniasis, and haemabartonellosis. *Ehrlichia canis* is an obligatory intracellular parasite which causes canine ehrlichiosis. Brown dog tick, *Rhipicephalus sanguineus* is the primary vector of *E. canis* infection (Hagan *et al.*, 1988).

In North America, it was first detected from a dog in Oklahoma, United States in 1962 (Ewing, 1969). According to Ewing (1969), this organism can be found in the blood, bone marrow, and other tissues, particularly in the lungs of dogs. When it is found in blood, it is mostly found in monocytes, lymphocytes and occasionally in neutrophil leukocytes. German Shepherd dogs are predisposed to more severe disease (Miller & Zawistowski, 2004). In Thailand, although positive results were shown Labrador Retrievers, Doberman Pinchers, German Shepherd-Labrador cross-breed dogs, German Shepherd dogs showed more severe symptoms (Nims *et al.*, 1971).

Although *E. canis* host are canines, the first human case was reported in Venezuela (Perez *et al.*, 1996). Recent cases reported involve *E. canis* infection in 6 humans in Venezuela. Simpson (1972) observed *E. canis* in monocytes using electron microscopy. The organism was found to be surrounded by a single membrane that enclosed many elementary bodies, each of which presented a double membrane and with fine fibrils and granules within it. All *Ehrlichia* species are biologically transmitted by ticks (Stich *et al.*, 2008).

Besides dogs, wild canids such as wolves, jackals, coyotes, red and grey foxes are also susceptible to *E. canis* infection (Harvey *et al.*, 1979). In Israel, Fishman *et al.* (2004) reported that 36% of foxes were infected by *E. canis*. According to Hagan & Dorsey (1988), *E. canis* often occurs in dogs infected with *Babesia canis* because both organisms are transmitted by the brown dog tick, *Rhipicephalus sanguineus*.

According to Wilkins *et al.* (1967), British military dogs in Singapore during 1963 were attacked by an acute frequently fatal haemorrhagic disease with signs such as high fever, epistaxis, unusual corneal opacity etc. This disease was then known as tropical

canine pancytopenia (TCP). TCP also occurred in dogs in the Malayan peninsula and Aden (Spence *et al.*, 1967).

Yeoh & Loh (1996) conducted a survey between 1986 to 1995 on the incidence of canine ehrlichiosis in Malaysia. A total of 579 cases were reported with an average of 58 cases per year or 5 cases per month. The number of cases of canine ehrlichiosis was highest in May and December.

The presence of *E. canis* can be detected using a few diagnostic tests. These include blood smears, Indirect Fluorescent Antibody Tests (IFAT), Enzyme-linked Immunosorbent Assay (ELISA), Immunoblotting etc. The Indirect Fluorescent Antibody Test (IFAT) with *E. canis* antigen has been the most widely used serological test for the diagnosis of *E. canis* infection of dogs (Waner *et al.*, 2001).

In the present study, sera were extracted from blood samples collected from a few veterinary clinics located in the State of Perak, Malaysia to detect the presence of *E. canis* using Indirect Fluorescent Antibody Test (IFAT) and also thin blood smears. Questionnaires were distributed to veterinarians to get information on dogs and the possible reasons for *E. canis* infection. *Ehrlichia canis* infection in dogs with different ages, sexes, breeds and tick infestation were compared.

MATERIALS AND METHODS

Collection of blood samples

From 25th November 2008 until 15th December 2008, 80 blood samples were collected from 5 private veterinary clinics (Clinic A, Clinic B, Clinic C, Clinic D and Clinic E), and a government veterinary clinic located in Perak State, Malaysia. Blood samples were collected from dogs of different breeds, sexes and ages. Needles, syringes, ethylene-diamine-tetra-acetic acid (EDTA) tubes, eppendorfs and plain tubes were provided to these veterinarians. Forms were also given to veterinarians to fill up the history of dogs. Blood samples were filled into plain tubes or eppendorfs and EDTA tubes.

Thin Blood Smear

EDTA tubes with blood were placed on a blood roller mixer to mix the blood. After 1 min, blood was taken out from EDTA tube using a hematocrit capillary tube and a thin blood smear was made. When the blood was dried, it was fixed with methanol. And stained with Giemsa stain for 45 min and examined under the microscope.

Serum extraction

Blood in the plain tubes was stored overnight and centrifuged (Heraeus Labofuge 300) at 2000 rpm for 15 min. Sera (the upper supernatant) were extracted by using micropipette and transferred into eppendorf tubes. Subsequently, the sera were labeled and placed in eppendorf rack. The sera were then stored in a freezer at -20°C.

Indirect Fluorescent Antibody Test (IFAT)

Materials

The detection of antibodies by using IFAT required fluorescent antibody substrate slides containing 12 wells spotted with *E. canis*. The cells were unstained and slides were sealed in moisture-free foil pouches. Positive and negative control sera, serum diluting buffer, anti-immunoglobulin conjugate, rinse buffer and mounting fluid were also used in IFAT.

Procedure

Before removing the slide from foil pouch, the slide was warmed to room temperature. Sera were diluted at 1:20 with serum diluting buffer at pH 7.2 in a serum dilution plate. Next, 10µl diluted sera from each samples were placed on the designated wells. Subsequently, the slide was incubated in humid chamber at 37°C for 30 min. Then, the slide was gently rinsed in a fluorescent antibody rinse buffer with pH 9.0 using a wash bottle and the slide was then soaked in fluorescent antibody rinse buffer with pH 9.0. After 10 min, the slide was drained and flicked to remove excess moisture. After this was done, 10 µl of labeled anti-IgG was placed on the wells. The wells were again incubated in humid chamber at 37°C for 30

min. Then, the wells were rinsed and soaked similarly as the step mentioned before. After 10 min, the slide was drained and the back and edges were dried with a paper towel. The stained surface was not allowed to dry and not rinsed with water. After that, the slide was mounted with mounting fluid which was prepared by mixing glycerol and fluorescent antibody rinse buffer with pH 9.0 in equal proportion. Lastly, the slide was viewed under the fluorescence microscope at 400x magnification and the presence of antibody was indicated by the presence of brightly fluorescent cytoplasmic inclusion bodies (morulae), clustered elementary bodies (initial bodies) or free elementary bodies.

RESULTS

Eighty-two blood samples were collected from 5 private veterinary clinics and one government veterinary clinic. These samples were examined by using thin blood smears and the presence of *E. canis* was not detected. However, other blood parasites such as *Babesia canis* and *Hepatozoan canis* were found.

Thirty-four samples were collected from male dogs and 7 of these samples were found positive for *E. canis*. Thus, 8.75% of the total samples infected were male dogs. On the other hand, 5 female dogs (6.25%) were infected by *E. canis*. There were no significant differences between male and female dogs for *E. canis* ($X^2 = 1.45$, $p = 0.229$).

Fifteen samples were collected from private veterinary Clinic A, 27 from private veterinary Clinic B, 7 from Clinic C, 4 from Clinic D, and 17 samples from veterinary Clinic E. The remaining 10 samples were provided by the government veterinary clinic. For Clinic A, 2 dogs (13.33%) were infected by *E. canis*, 4 dogs (14.81) from Clinic B, 2 dogs from the Clinic C (28.57%) and 2 dogs from Clinic D (50%), while Clinic E contributed 5.88% positive result, that is 1 dog infected. On the other hand, 1 dog (10%) was found to be infected in government veterinary clinic. No significant differences were noted for ehrlichiosis between Clinic

A, Clinic B, Clinic C, Clinic D, Clinic E and government veterinary clinic ($X^2 = 6.19$, $p = 0.288$).

Samples collected were categorized according to the range of age of dogs. Eleven samples were collected from dogs of 1-6 months old, 17 samples from dogs 6-12 months old, 29 samples from 1 to 5-year-old dogs, 18 samples from 5 to 10-year-old dogs while the remaining 5 samples were collected from dogs with 10-16 years old. Two dogs from age group between 10-16 years old showed positive result for *E. canis* infection, contributing the highest infection (40%). From the 17 samples collected from dogs which are 6 to 12 months old, 3 samples (17.65%) showed positive towards *E. canis* infection. Five dogs (17.24%) from the age group 1-5 years old were found to be infected. One sample from the age group 1-6 months old (9.09%) was found to be infected and one sample from the age group of 5-10 years old (5.56%) showed positive result for *E. canis* infection. There were no significant differences for *E. canis* between dogs of different age group ($X^2 = 4.22$, $p = 0.377$).

Among the 37 dogs infested by ticks, 4 dogs (10.81%) were found to be infected by *E. canis*. However, 8 dogs (18.60%) from 43 dogs which were free of tick infestation were positive for *E. canis* infection. There were no significant differences of *E. canis* between dogs with tick infestation and dogs which were free of tick ($X^2 = 0.95$, $p = 0.33$).

Sera were obtained from dogs of different breeds of dogs and these dogs were categorized into 3 main groups: local breeds, cross bred and pure bred. Forty-two dogs were purebreds, 27 were local dogs and 11 were crossbreds. Seven pure breeds (8.75%), 3 local dogs (3.75%), and 2 cross breeds (2.5%) were found to be infected with ehrlichiosis. There were no significant differences of ehrlichiosis between dogs of different breeds ($X^2 = 0.50$, $p = 0.779$).

DISCUSSION

From this study, there were no morulae of *E. canis* found in blood smears though positive results were shown in Indirect

Fluorescent Antibody Test (IFAT). Thus, it showed that *E. canis* morulae are hardly observed in stained blood smears. Therefore, indirect fluorescent antibody test (IFAT) was used to determine the prevalence of *E. canis* in Ipoh, Perak.

Both *Babesia* sp. and *H. canis* are also transmitted by *R. sanguineus* (Service & Ashford, 2001). Concurrent infections of canine babesiosis which caused by *B. canis* were reported in Nairobi and Thailand (Price *et al.*, 1987; Assarasakorn & Niwetpathomwat, 2007). Thus, before conducting IFAT, *E. canis* were still considered to be present in these samples. One of the samples was positive for *E. canis* and *H. canis* infection. From the questionnaire, it was found that this was a local dog with tick infestation. Hence, this dog probably was bitten by ticks carrying *B. canis* and *E. canis*. There was another case where there were two dogs, infected by *E. canis* and *B. canis*, respectively. These dogs belong to the same owner and the dog with *E. canis* infection was infested by ticks. One of these dogs might be newly brought into this house, and therefore, ticks transmit neither of these parasites to another dog.

A total of 15% dogs were found positive for ehrlichiosis. The results showed that 6.25% were infected. For male dogs, 8.75% were infected. The percentage of male and female dogs infected by *E. canis* does not show much difference. In this study, there was no significant difference between female and male dogs for *E. canis*. In Yabsley *et al.* (2008) study, they also found that there was no significant difference between the sex of dogs and *E. canis* infection. The probability of tick infestation between female and male dogs were similar, thus they have equal chance to be infected.

Among the age group, older dogs (10-16 years old) have highest percentage of infection. Two out of 5 dogs from this group were positive for ehrlichiosis, meaning 40% of dogs from this group were infected. Previous study was done by Rembeck *et al.* (2007), showing that old dogs have higher percentage of infection. In their study, 60% of dogs older than 5 years old, 33.6% of dogs younger than 2 years old and 52.7% of dogs

were positive for *E. canis* infection. As dogs get older, their probability of being exposed to *E. canis* also increased (Watanabe *et al.*, 2004).

In this survey, dogs without *R. sanguineus* infestation have higher positive results compared with dogs with *R. sanguineus* infestation. However, there was no correlation between presence of tick with ehrlichiosis (M'Ghirbi *et al.*, 2009). Veterinarians checked for tick infestation when dogs were sent into their clinics. Some dogs were sent to clinics after a bath or treated with acaricide. Consequently, they were temporarily free of tick but this did not mean that they were not infested by tick before they were sent to veterinary clinics.

In this study, there is no significant difference between dog breeds and ehrlichiosis. Previous study also showed that there was no significant difference between dog breeds and the positive results (M'Ghirbi *et al.*, 2009). From the data obtained, purebred which gave positive results were Doberman, Golden Retriever, Poodle, Shih Tzu and Siberian Husky. These dogs have medium length to long fur which provides good hiding side for ticks. Thus, it is suggested that dog having longer fur have higher chance to get infected. On the other hand, 3 local breeds were infected. These infected dogs might include stray dogs which were not under proper care. Stray dogs environment are not as good as pet dogs and this increases their chance to be exposed to tick vectors. According to Sainz *et al.* (1996), habitat of dogs can affect the prevalence of canine ehrlichiosis. Besides, stray dogs are not bathed and sprayed with acaricides to remove ticks.

In conclusion, no positive results for *E. canis* were found in the blood smears. The prevalence of *E. canis* infection in Perak was determined by indirect IFAT, which is also the standard approach to diagnose *E. canis*. By using IFAT, 12 of 80 dogs in were positive for *E. canis* infection (15%). Besides, there was 1 positive case for each of the *B. canis* infection and *H. canis* infection. Since *E. canis* is distributed in the State, veterinarians and dog owners should be aware so that the prevalence of ehrlichiosis will not increase.

Control on *R. sanguineus* is important to avoid transmission of ehrlichiosis, babesiosis, hepatozoonosis and other tick-borne diseases among dogs. Furthermore, elevation of tick vector can also reduce the risk of *E. canis* transmission to human. Thus, acaricides should be treated on dogs and their kennels from time to time to get rid of *R. sanguineus*.

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