

Original papers

The prevalence of *Ehrlichia canis*, *Anaplasma platys* and *Babesia* spp. in dogs in Nueva Ecija, Philippines based on multiplex polymerase chain reaction (mPCR) assay

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ABSTRACT. The aim of the study was to determine the prevalence of *Ehrlichia canis*, *Anaplasma platys* and *Babesia* spp. in dogs. It describes the practice of veterinarians in detecting tick-borne diseases in Nueva Ecija, Philippines. Seventy blood samples were collected and were subjected to multiplex PCR for the detection of *E. canis*, *Babesia* spp. and *A. platys*. The prevalence of babesiosis is the highest in Cabanatuan City (2/10), while a 10% prevalence (1/10) was observed in Science City of Muñoz, Talavera and Sta. Rosa. *E. canis* were only detected in Cabanatuan City. However, no anaplasmosis was detected in any area. The prevalence of babesiosis and ehrlichiosis in Nueva Ecija is 7.14% (5/70) and 2.85% (2/70) respectively. In addition, 70% (7/10) of the Nueva Ecija veterinary practitioners encountered cases of suspected ehrlichiosis in their practice. The diagnosis of ehrlichiosis is based primarily on presented clinical signs and complete blood counts, which include a platelet count. Of the 10 respondents, half utilized test kits while 90% interpreted blood samples. Meanwhile, only 60% of the respondents used an ELISA test kit for ehrlichiosis. For some practitioners, the main reason for not utilizing a kit is the high cost. None of the respondents had previously attended cases of suspected anaplasmosis. Only one respondent diagnosed a case of babesiosis by blood smear microscopy.

Key words: *Anaplasma platys*, *Babesia* spp., *Ehrlichia canis*, mPCR, tick-borne, Philippines

Introduction

Ticks are blood-sucking ectoparasites of great medical and veterinary significance that can transmit bacteria, protozoa, fungi and viruses, and cause a variety of human and animal diseases worldwide [1]. Tick-transmitted diseases are an emerging problem in dogs. In addition to causing serious disease in tropical and semi-tropical regions, they are now increasingly recognized as a cause of disease in dogs in temperate climates and urban environments [2].

Being haematophagous, ticks are well designed to transmit disease agents such as viruses, bacteria and protozoa. Ticks attach securely to their hosts, facilitating not only the effective transmission of infectious agents, but also the spread of both ticks and microorganisms to different geographical habitats through travelling pets.

Infection with multiple tick-transmitted pathogens can occur in individual animals following heavy exposure to ticks. The same tick species can be a vector for several pathogens and co-infection of individual ticks can occur. Infection with tick-borne

pathogens can also be complicated by other arthropod-borne diseases that share the tick biohabitat, such as leishmaniasis. In dogs, co-infection with combinations of *Ehrlichia*, *Bartonella*, *Babesia*, *Hepatozoon*, *Leishmania* and *Rickettsia* species occurs in endemic areas [2,3].

For many years, *Ehrlichia* spp. has been known to cause illness in pets and livestock [4]. Canine monocytic ehrlichiosis (CME), caused by tick-transmitted *Ehrlichia canis*, has been reported in the United States and throughout the world, causing extensive morbidity and mortality [5–7]. The tropical climate of the Philippines favours the development of vector ticks, the brown dog-tick, (*Rhipicephalus sanguineus*), and the transmission of hemoparasites [8,9]. Clinical and hematological abnormalities are often nonspecific during *E. canis* infections [3]; therefore a definitive diagnosis may be difficult to make [10].

Ehrlichiosis, despite being an emerging disease in humans and animals, is not extensively studied in the Philippines [11]. Along with ehrlichiosis, anaplasmosis and babesiosis are underrated tick-borne diseases of dogs in the Philippines. CME in Philippines is routinely diagnosed in veterinary clinics and hospitals using commercially available serological test kits and peripheral blood smear examination methods [12], but not by molecular means [11]. On the other hand, animals suspected of infection with *Babesia* spp. are conventionally diagnosed through examination of blood smears alongside disease manifestations, a common practice in places that do not have the capability of other more specific tests. In chronic cases where there is low parasitemia, the determination of the specific etiologic agent merely based on blood smears tends to be problematic. In the Philippines, studies on animal babesiosis are scarce and are largely based on Giemsa-stained blood smears and/or clinical manifestations and hematological parameters [13].

Multiple tick-transmitted pathogen infection can occur in animals. The same tick species can be a vector for several pathogens and co-infection of individual ticks can occur. Detection of these tick-transmitted pathogens can be difficult. Microscopic detection is more fruitful in acute than sub-clinical or chronic phases because of the tendency for chronic and/or sub-clinical disease to be associated with cryptic infection, which reduces the sensitivity of microscopic examination and does not allow intra-species differentiation. Serological assays that

detect antibodies, such as indirect immunofluorescent antibody and dot-ELISA tests, are very sensitive in detecting the prevalence of exposure to parasites or cross reactivity with other species, but can be misleading, especially in endemic areas of these diseases. However, they are not useful for determining current infection status or assessing clearance of parasites after antibiotic treatment. Hence, there is a need for a specific, simple and more sensitive method to directly detect the organisms, which has led to the development of multiplex PCR, a one-step PCR, to detect *E. canis*, *Anaplasma platys* and *Babesia* spp. which can be useful for the selection of specific treatment to each disease or co-infection and assessing clearance of parasites after treatment [14–16].

Materials and Methods

Collection of blood samples. The samples were collected from the cities and municipalities of Nueva Ecija densely populated by dogs: Cabanatuan City, San Jose City, Science City of Muñoz, Talavera, Gapan City, Guimba and Sta. Rosa. From each selected city or municipality, 10 dogs were chosen for sampling, making a total of 70 samples. Dogs that were previously or currently infested with ticks were selected. Blood samples were collected through the cephalic vein using disposable syringe with a 1-inch 23-gauge needle. About 3 ml of blood was collected from each dog. Blood samples were then stored in EDTA-coated tubes. After collection, the blood samples were stored in an ice box and later stored at -20°C prior to processing. Information such as age, breed, sex, source of puppy, and movement of each animal was recorded, as well as the client's name, address and contact number.

Questionnaires were given to veterinarians of existing veterinary clinics in Nueva Ecija. A questionnaire was also given to the owners of the dogs involved in the study.

DNA extraction. A total of 500 μl of whole blood was mixed with 600 μl of cell lysis buffer in a microcentrifuge tube. The mixture was centrifuged at 14000 rpm for 1 min and the supernatant was then discarded. The precipitates were mixed with 700 μl of cell lysis buffer. The mixture was vortexed well and was centrifuged at 14000 rpm for another minute. The supernatant was then discarded. Using the microcentrifuge rack, the bottom of the microcentrifuge tube was then tapped

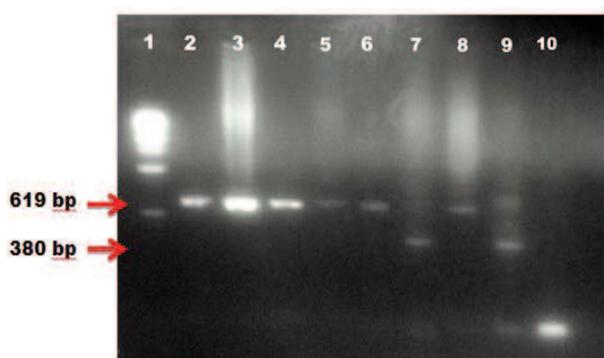


Fig. 1. Agarose gel-electrophoresis of amplicons of *E. canis*, *Babesia* spp. and *A. platys* genes. DNA extracted from blood samples or parasite-positive blood samples were analyzed by multiplex PCR using specific primers for *E. canis*, *Babesia* spp. and *A. platys*. Lane 1: 1 kb ladder; lane 2-6: *Babesia* spp.; lane 7: *E. canis* positive samples; lane 8: *Babesia* spp. (+) control; lane 9: *E. canis* and *Babesia* spp. positive samples; lane 10: (-) control.

14000 rpm for 10 seconds. A 300 μ l volume of nuclei lysis buffer and 100 μ l of protein precipitate was mixed to the flash centrifuged pellets. These were vortexed for 15–30 seconds and were centrifuged at 14000 rpm for 10 min. A 400 μ l volume of the supernatant was transferred to a new microcentrifuge tube and 500 μ l of propanol was added to each. These were mixed well but not vortexed. The mixture was centrifuged at 14000 rpm for 1 min then the supernatant was discarded. A total of 500 μ l of 70% ethyl alcohol solution was added and the mixture was centrifuged at 14000 rpm for 1 min. The supernatant was removed and the extracted DNA was confirmed by the presence of white pellets at the bottom of the microcentrifuge tubes. The DNA pellets were air-dried for an hour. These were rehydrated with 100 μ l of DNA rehydrate

solution. The primers used were based on Kledmance et al. [14] and Ybanez et al. [11] as shown in Table 1.

Multiplex PCR amplification. Multiplex PCR was carried out in a solution containing 5 μ l of extracted DNA and 3 μ l of the following mixture: 0.5 μ l of each primer, 0.5 μ l of each dNTP, 0.2 μ l of *Taq* DNA Polymerase, 1xPCR buffer (20 mMTris–HCl pH 8.4, 50 mMKCl), 0.75 μ l MgCl₂ and 1.5 μ l sterile water. Amplification was performed in a thermocycler. Thermocycling consisted of one step of 5 min at 94°C followed by 30 cycles of 45 sec at 94°C, 45 sec at 65°C, and 90 sec at 72°C with a final extension step of 2 min at 72°C. The amplicons were separated by electrophoresis in 1.5% agarose gel in 40 mMTris-acetic acetate pH 8.4, 1 mM EDTA, stained with ethidium bromide (0.5 μ g/ml) and visualized under UV light.

Results and Discussion

Canine blood samples were collected randomly in San Jose City, Science City of Muñoz, Talavera, Guimba, Cabanatuan City, Sta. Rosa and Gapan City. Ten samples were collected from each area. The blood samples were processed using mPCR to determine the presence of tick-borne diseases caused by *E. canis*, *A. platys* and *Babesia* spp. The results of the multiplex PCR is shown in Fig. 1.

Fig. 2 shows the distribution of detected cases in Nueva Ecija. No cases for these tick-borne diseases were detected in San Jose City, Guimba, and Gapan City. The prevalence of babesiosis was the highest in Cabanatuan City with 20% (2/10). A 10% prevalence (1/10) was observed in Science City of Muñoz, Talavera and Sta. Rosa. Though more cases were observed in Cabanatuan, the prevalence was comparable among towns with reported cases.

Table 1. Oligonucleotide primers that were used in Multiplex PCR [11,14]

Pathogen	Primer	Sequence	Length(Bases)	Size of Product
<i>Ehrlichia canis</i>	Ehr1401F	CCATAAGCATAGCTGATAACCCTGTTACAA	30	380bp
	Ehr1780R	TGGATAATAAAACCGTACTATGTATGCTAG	30	
<i>Babesia</i> spp.	Ba103F	CCAATCCTGACACAGGGAGGTAGTGACA	28	619bp
	Ba721R	CCCCAGAACCCAAAGACTTTGATTTCTCTCAAG	33	
<i>Anaplasma platys</i>	PLATYS	AGAGTTTGATCCTGGCTCAG	23	426bp
	Ehr16sR	ACGGCTACCTTGTTACGACTT	20	

Table 2. Prevalence of *Babesia* spp. and *E. canis* infection among dogs in Nueva Ecija from September 2013 to January 2014

Area	Samples (N)	<i>Babesia</i> spp. (+)	Prevalence (%)	95% CI*	<i>E. canis</i> (+)	Prevalence (%)	95% CI*
San Jose City	10	0	0		0	0	
Science City of Muñoz	10	1	10	0.25-44.50	0	0	
Talavera	10	1	10	0.25-44.50	0	0	
Guimba	10	0	0		0	0	
Sta. Rosa	10	1	10	0.25-44.50	0	0	
Cabanatuan City	10	2	20	2.52-55.60	2	2.85	0.34-9.94
Gapan City	10	0	0		0	0	
Total	70	5	7.14	2.35-15.89	5	2.85	0.34-9.94

Throughout Nueva Ecija, the prevalence of babesiosis was found to be 7.14% (5/70). No *A. platys* infection was detected from any samples in any area. *E. canis* was found in Cabanatuan City, where two samples were confirmed to be positive at

a prevalence of 2.85% (2/70) (Table 2).

Results showed that 70% (7/10) of the veterinarians interviewed encountered cases of ehrlichiosis in their practice. The diagnosis of ehrlichiosis was primarily based on clinical signs and



Fig. 2. Map representing the cities and municipalities with *Babesia* spp. and *E. canis* positive samples

complete blood counts with particulars to platelet count. Fig. 3 presents the distribution of respondents according to how cases of ehrlichiosis are diagnosed. Half of the respondents use test kits while 90% evaluate the hemogram interpretation of their patients. Meanwhile, only 60% of the respondents have used an ELISA test kit for ehrlichiosis. The main reason for not using a kit in the diagnostic process is the resistance of dog owners, which may be linked to the high cost of the kit.

Among those who had previously attended to ehrlichiosis cases, the majority (85.71%) treat 1 to 10 suspected *E. canis* patients monthly, however one respondent reported attending at least 11 to 20 suspected ehrlichiosis infections per month. These suspected ehrlichiosis patients are found to be tick-infested. Nobody reported attending cases of anaplasmosis. Meanwhile, one respondent diagnosed a case of babesiosis through a blood smear .

The results of the study supported those of Ybanez et al. [11] regarding the presence of *E. canis* in the Philippines. They report the presence of *E. canis*- and *A. platys*-positive tick samples by polymerase chain reaction in Cebu, Philippines. Likewise, Baticados et al. [17] detected the presence of *E. canis* by serology in Metro Manila and Cavite, Philippines. This study confirms the presence of *E. canis*, and *Babesia* spp. in Nueva Ecija, Philippines.

The emergence of ehrlichiosis and babesiosis in the province may be associated with the import of dogs from other countries or from other provinces. Though it may not be specifically ruled out, it could be a possibility. Likewise, the local spread may be linked to the movement of dogs between provinces as part of dog trading. Trading has been made more efficient through social media and other online networks. Currently, the sole requirement of shipping dogs is a certification from a veterinarian indicating that the animal is vaccinated against rabies. Consequently, the continuous spread of the disease remains unregulated.

As the emerging ehrlichiosis and babesiosis in Nueva Ecija may be linked to the unregulated movement of dogs related to trading, there is the possibility that more new diseases will be introduced to the country unless stricter regulations are put into place. The implementation of the ASEAN Economic Community may possibly serve as an additional avenue in the introduction of new diseases in the country. For instance, foreign veterinarians may be using canine blood for

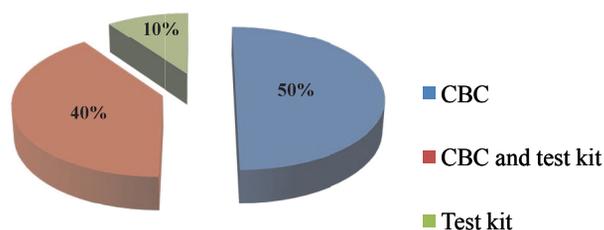


Fig. 3. Diagnostic approach of respondents to evaluate the presence of tick-borne diseases in their patients

transfusion to their patients in the Philippines.

One possible regulation is the adoption of the policy practiced in the livestock industry. For instance, the government will not import pigs, cattle, or buffalo from countries where Foot and Mouth Disease is endemic. Currently, the only regulation observed in the transit of dogs is that it is vaccinated against rabies. Therefore, a policy should be implemented requiring proof that dogs are free from ehrlichiosis, babesiosis, and other non-endemic canine disease in the Philippines be presented prior to import.

Confirming the presence of babesiosis in dogs is another challenge for the small animal practitioner. As there is no test kit available in the market, it may be misdiagnosed as a case of ehrlichiosis. The interview with the veterinarians in this study revealed that one veterinary practitioner does not include babesiosis in his differential diagnoses. Hence, there is the possibility that the wrong treatment will be given, putting the patient's life at risk.

With the current absence of a strong policy to regulate the spread of tick-borne diseases, it is the responsibility of the veterinarian to protect his patients from ticks. One practitioner described encouraging his clients to engage in regular tick protection for their pet by comparing ehrlichiosis with dengue. Both diseases compromise the platelet level of their victims. Such an approach results in a better understanding by the clients of the necessity to prevent ticks from biting their dogs. Another way is to encourage clients to have their dogs tested for ehrlichiosis when the practitioner observes the presence of ticks, despite the absence of any clinical signs. The objective is to address this health concern prior to clinical manifestation, as ehrlichiosis has a subclinical form. This way, the expenses of the client will be much lower as the disease will be addressed earlier.

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