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First Molecular Evidence of *Ehrlichia canis* Infection in Dogs with Probable Disease Relapse in the Philippines

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Ehrlichia canis is a gram-negative intracellular rickettsial organism that can cause infection in dogs referred to as canine monocytic ehrlichiosis (CME) (Perez *et al*., 2006). In the Philippines, routine diagnoses of CME in veterinary clinics and hospitals involve using commercially available serological test kits and peripheral blood smear examinations (PBSE) (Morales and Baticados, 2007). However, both methods have known limitations, including serological cross reactions (Ramos *et al*., 2009; Waner *et al*., 1998) and difficulty in identifying closely related and/or morphologically similar pathogens by visual means (Pusterla *et al*., 1998). In one study, Ehrlichia spp. pathogens were observed in blood smears from dogs in 2 areas in Luzon (upper part of the Philippines) (Morales and Baticados, 2007). In a recent study by Baticados *et al*. (2011) covering more areas and canine samples from Luzon, the use of molecular methods and PBSE failed to detect *E. canis* in dogs that were observed to be infested with ticks or had histories of tick exposure. In contrast, another study performed involving the previously mentioned authors (Baticados *et al*., 2011) reported a high seroprevalence of *E. canis* infection exposure in almost the same sampling areas and number of dogs tested in the Luzon area (Baticados and Baticados, 2011).

Previous studies mentioned above (Baticados *et al*., 2011; Baticados and Baticados, 2011) can apparently present confusing epidemiological information as both studies were reported in the same year but with conflicting results (high seroprevalence but with negative PCR results) in almost the same sampling area and samples. Also, the results in one study (Baticados *et al*., 2011) wasn't mentioned in the other study (Baticados and Baticados, 2011). However, the authors expressed uncertainty about their claim on the complete absence of the pathogen in the area due to the negative PCR results in their study (Baticados *et al*., 2011). Thus, it is essential to clarify the presence of *E. canis* in the Philippines not only by serological means, but also by molecular methods. *E. canis* has already been molecularly detected in ticks in the country (Ybañez *et al*., 2012b). The present study aimed to provide molecular evidence on the presence of *E. canis* infection in dogs in the Philippines.

Two canine cases suspected to have *E. canis* infections were examined in 2011. The cases were presented at the GPY Veterinare Animale Veterinary Clinic, Cebu City, Philippines. EDTA-anticoagulated peripheral blood samples were collected for hematological examination and PBSE at different observation days. Hematological analysis was performed using BC-2800 Veterinary Auto Hematology Analyzer (Mindray, Shenzen Mindray Biomedical Electronics Co., Ltd., Shenzen, China). Blood samples for PCR were obtained only once from each dog on days 1 and 22. Clinical signs were recorded. Blood smears stained with giemsa solutions were prepared from each dog at different observation days. Only 1 dog was serologically tested for *E. canis* antibodies using Immunocomb® (Biogal, Israel), a commercial test kit for *E. canis* detection with a reported sensitivity and specificity

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of 86% and 98%, respectively (Harrus *et al*., 2002; Waner *et al*., 2000).

DNA extraction, elution and storage were performed as previously described (Ybañez *et al*., 2012b). PCR assay to amplify partial 16S rRNA gene fragments were performed as previously described (Ybañez *et al*., 2012a). Briefly, for the partial 16S rRNA gene amplification for the genus Anaplasma and Ehrlichia, primer pairs fD1/Rp2 and EHR16SD/EHR16SR (Weisburg *et al*., 1991; Parola *et al*., 2000) were respectively used for the 1st and 2nd round PCR to amplify a final target of 345 bp. To amplify a longer amplicon (786 bp), additional round of PCR using primer pair fD1/EHR16SR was used. The negative and positive controls used were double distilled water, and A. bovis, respectively. Final amplicons were visualized using 1.5 % agar gel after electrophoresis.

Final amplicons were purified using a Gel Extraction Kit (QIAGEN, Valencia, CA) and were cloned using a TA cloning kit (Invitrogen, USA). DNA sequencing and sequence comparison were performed as previously described (Ybañez *et al*., 2012a).

Case 1: A 23-month old female mixed-breed dog. During first presentation (day 1), a history of tick infestation, fever, weight loss, lethargy and coughing were noted. Hematological examination revealed anemia and thrombocytopenia (Table 1). Serological testing for *E. canis* revealed strong positive reaction (S5). The dog was treated and prescribed with doxycycline (10 mg/kg, once a day) for 28 days and prednisone (1 mg/kg, in 2 divided doses per day) for 14 days. Hematological values were monitored on day 8 and day 38, which revealed good recovery as the total WBC count, erythrocyte count, packed cell volume (PCV) and platelet count appeared to be normalizing (Table 1). However, on day 55, the dog was again presented with signs of fever (39.6 ℃) and pale mucous membranes. Hematologic examination revealed thrombocytopenia and a macrocytic hypochromic anemia (Table 1). Although it is not completely understood, the observed anemia may be a result of an initial subclinical infection. The dog was again treated and prescribed with doxycycline and corticosteroids for 14 days. Hematologic monitoring on days 62 and 92 revealed good recovery as its WBC count, PCV and platelet counts appeared to be normalizing. The dog was also observed to be apparently healthy during the last presentation. All examined blood smears at different observation days were found to be negative of any morulae.

Case 2: An 11-month-old male golden retriever. During initial examination (day 1), tick infestation, nasal discharge, panting, coughing, and gagging were observed. The owner also reported inappetence and lethargy. The dog was treated with 2 ml of a vitamin B complex supplement Biodyl (Merial, Georgia, USA), and prescribed with 14 days medication of generic ascorbic acid (500 mg/tab) at 1/2 tab, 2 x a day, and multivitamins LC-Vit Forte Syrup (Apt Vet Link, Inc, Quezon City, Philippines) at 5 ml, once a day, and 7 days with an local oral herbal medicine that is indicated for the treatment of respiratory infections in small animals, Broncure (Vetmate Farma Corporation, Quezon City, Philippines) at 5 ml, 2x a day. On day 6, the owner reported that coughing was minimal, but pinpoint hemorrhages or ecchymoses were observed on the body. On day 8, the dog was treated with Fipronil and Heartgard® (Merial, Georgia, USA), and was prescribed with multi-vitamin supplements. On day 22, lethargy, inappetence and gagging were still observed. Hematological examination revealed thrombocytopenia, leukopenia, and microcytic hypochromic anemia (Table 1). The dog was prescribed with 14 days treatment of doxycycline (5 mg/kg, 2 x a day), and prednisone (1 mg/kg, 2 divided doses) for 14 days. On day 51, the dog appeared to be more active and was apparently healthy as the previously observed clinical signs were not seen. Weight gain was also observed. At approximately 8 months post-recovery (or day 276), the dog was presented again with signs of fever and coughing. Hematological examination revealed marked pancytopenia (Table 1). The dog was treated with an injectable NSAID Tolfine (Tolfenamic acid; Vetoquinol, France), erythropoietin (Epoietin Alfa; Renogen, Shandong Kexing Bioproducts Co. Ltd., Shandong, China), and injectable doxycycline (10 mg/kg) for the said day. Medication was continued with a prescription of doxycycline (same dose) and vitamin B complex for 14 days. After 14 days, the owner reported that the dog had recovered and was apparently healthy. Similar to the first case, no morulae were seen on all examined blood smears at different observation days.

In this study, two suspected canine ehrlichiosis cases were presented. In both of the cases, anemia,

Table 1. Hematologic findings in dogs infected with *E. canis*

Hematologic Parameters	Reference Values*	Case 1						Case 2	
		Observation Days							
		1	8	38	55	62	92	1	274
WBC count (x10^3/µl)	6-17	8.5	21.6	13.2	6.8	23.6	16.5	4.4	1.2
PCV (%)	37-55	31.8	33	49.2	40.1	34.1	46	23.5	6
Hemoglobin (Hgb) (g/l)	120-780	96	104	141	110	96	153	66	18
RBC count (x10^6/µl)	5.5-8.5	4.7	4.7	7	5.6	4.6	5	4	0.9
MCV (fl)	66-77	67.7	70.2	70.3	71.6	74.1	92.0	58.8	66.7
MCH (pg)	21.0-26.2	20.4	22.1	20.1	19.6	20.9	30.6	16.5	20
MCHC (g/dl)	32.0-36.3	30.2	31.5	28.7	27.4	28.2	33.3	28.1	30.0
Platelet count (x10^3/µl)	200-500	50	321	256	56	219	203	54	19
Differential Count									
Basophils (%)	0-2	0	0	0	0	0	0	0	0
Eosinophils (%)	2-10	0	0	0	0	0	3	0	0
Monocytes (%)	3-10	7.6	7.5	8	7.6	7.9	4	11.5	3.4
Lymphocytes (%)	12-30	56.1	53.6	59.6	38.8	35.1	24	34.1	21.7
Neutrophils (%)	60-70	36.3	38.9	32.4	53.6	57	69	54.4	74

*(Duncan and Prasse, 1986)

thrombocytopenia, coughing, lethargy, fever and probable disease relapse after 14-28 days of treatment were seen. PCR and sequencing results revealed positive infection for *E. canis*. The shorter partial 16S rRNA nucleotide sequences (345 bp) obtained from 2 dogs was found 100% identical to each other and to several *E. canis* sequences registered in Genbank. Since the shorter sequences were also found 100% identical to several other Ehrlichia spp. sequences, longer partial sequences (786 bp) were sought for further clarification. The obtained longer partial sequences were still found 100% identical to each other, and to registered *E. canis* sequences from Taiwan and USA. Interestingly, the obtained sequences were found only 99.5-99.6% identical to previous sequences obtained from ticks in the Philippines (JN121379-80) (Ybañez *et al*., 2012b). The partial longer sequences obtained in the present study were registered at GenBank with the accession numbers JX893522-3.

In contrast to the results of Baticados *et al*. (2011) and Baticados and Baticados (2011), this study reports the first successful molecular detection of *E. canis* in a dog that was found serologically positive in the Philippines. Baticados *et al*. (2011) explained the negative PCR results they obtained, due to subdetectable quantities of pathogens in the blood due to localization of the pathogen in other organs of the body, stages of chronic and asymptomatic carriers of infection, timing of collection, ongoing or past treatments and presence of other diseases. In addition, this difference may be due to strain variations or low sensitivity of the PCR conditions and/or method previously used by Baticados *et al*. (2011). Results have been shown to vary when different PCR methods were used in the same set of samples (Fernández *et al*., 2009).

Although PCR testing was only performed once prior to the doxycycline treatment, it is possible that the *E. canis* rickettsia was not cleared after the initial antibiotic treatment for CME (Harrus *et al*., 1998), leading to the probable relapse of the disease. However, re-infection was also possible as the dogs returned to their natural environment and were not monitored daily. Moreover, tick prevention medications were not administered regularly. Thus, tick re-exposure might have occurred leading to reinfection. Using the platelet count to assess recovery from the disease (Harrus *et al*., 1998), it can

be observed in the first case that the dog had good recovery at day 8 and 38, but the disease recurred at day 55 as evidenced by the low platelet count. While the platelet count of the 2nd case was not assessed periodically, the disease was again observed at 8-months post-treatment in which thrombocytopenia was seen. It is also possible that this dog has entered the chronic phase of CME judged by the pancytopenia observed (Mylonakis *et al*., 2004). In one study, *E. canis* was still molecularly detected from an experimentally infected dog at 6 weeks post-treatment (Harrus *et al*., 1998). Even with appropriate treatment, *E. canis* infection may also relapse (Iqbal and Rikihisa, 1994). However, it is also possible that the dogs were re-infected with the pathogen (Hess *et al*., 2006), or co-infected with another pathogen, *Anaplasma platys*, which may not have been detected during the PCR testing due to low-level bacteremia (Sekeyova *et al*., 2011). In the Philippines, A. platys has been detected in Rhipicephalus sanguineus tick (Ybañez *et al*., 2012b), which is believed to be its vector (Gaunt *et al*., 2010). This pathogen also causes non-specific signs, but thrombocytopenia is commonly observed. More severe clinical signs may be seen in dogs co-infected with *E. canis* and A. platys (Gaunt *et al*., 2010). Therefore, if chronic CME is suspected, doxycycline treatment may be administered in more than 28 days to increase the chances of clearing the pathogen/s. It is noteworthy that a 6-week doxycycline treatment was previously shown to be insufficient in clearing the *E. canis* pathogen (Harrus *et al*., 1998). However, for acute CME, doxycycline treatments can still be given until 28 days as it has been shown to clear the pathogen in experimentally infected dogs (Eddlestone *et al*., 2007).

The molecular confirmation of *E. canis* infection in dogs that is presented in this study clarifies the presence of the pathogen in the Philippines. Since PCR testing may not be readily available in most areas, local veterinary practitioners (LVPs) should maximize the use of PBSE, hematological examination and commercial ELISA-based test kits. However, LVPs should be reminded of the potential limitations of the different detection methods, and that dogs can be infected even when PBSE and serological tests are negative. Peripheral blood smear examination (PBSE) can directly show evidence of an infected cell, but is known to be very insensitive (Sekeyova *et al*., 2011). Serological testing gives evidence of exposure to the pathogen, but dogs can be exposed without developing clinical signs (Harrus *et al*., 1998, Mylonakis *et al*., 2004). This testing may not also delineate active infection from recovery phase. PCR can also be insensitive if samples from blood are used, than if samples from spleen will be tested. However, obtaining samples from spleen may be difficult and invasive. On the other hand, the characteristic probable disease relapse observed in the present study should prompt LVPs to monitor patients even after presumed recovery.

Tick prophyaxis of the dogs and the environment should also be practiced due to the potential re-infection when these preventive measures are not performed. For economical reasons, another daunting task that LVPs face in the Philippines is the unwillingness of the pet owner to commit to recommended diagnostic procedures. LVPs should strive to educate pet owners on the importance of each diagnostic procedure in relation to appropriate disease diagnosis. Because of the high seroprevalence of CME in the Philippines and the non-specificity of the clinical signs produced by the pathogen, LVPs and pet owners should heighten their familiarity with the disease pathogenesis, diagnosis and treatment.

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References

Baticados, A.M., Baticados, W.N., Villarba, L.A., Carlos, E.T., Carlos, S., Fajardo, P.V., 2011. PCR assay and microscopy for examination of mixed *Ehrlichia canis* and Babesia spp. infection in bomb sniffing dogs and other canines in the National Capital Region, Philippines. Eurasian Journal of Veterinary Sciences 27, 111-115.

Baticados, A.M., Baticados, W.N., 2011. Serological evidence of *Ehrlichia canis* exposure in military dogs and other canines in Metropolitan Manila, Philippines. Is. J. Vet. Med. 66, 151-156.

Duncan, J.R., Prasse, K.W., 1986. Veterinary Laboratory Medicine: Clinical Pathology, 2nd ed., Iowa State University Press, AMES.

Eddlestone, S.M., Diniz, P.P., Neer, T.M., Gaunt, S.D., Corstvet, R., Cho, D., Hosgood, G., Hegarty, B., Breitschwerdt, E.B., 2007. Doxycycline clearance of experimentally induced chronic *Ehrlichia canis* infection in dogs. Journal of Veterinary Internal Medicine 21, 1237-1242.

Fernández, D., González-Baradat, B., Eleizalde, M., González-Marcano, E., Perrone, T., Mendoza, M., 2009. Trypanosoma evansi: A comparison of PCR and parasitological diagnostic tests in experimentally infected mice. Experimental Parasitology 121, 1-7.

Gaunt, S.D., Beall, M.J., Stillman, B.A., Lorentzen, L., Diniz, P.P.V.P, Chandrashekar, R., Breitschwerdt, E.B., 2010. Experimental infection and co-infection of dogs with *Anaplasma platys* and *Ehrlichia canis*: hematologic, serologic and molecular findings. Parasit Vectors 3, 33.

Harrus, S., Alleman, A.R., Bark, H., Mahan, S.M., Waner, T., 2002. Comparison of three enzyme-linked immunosorbant assays with the indirect immunofluorescent antibody test for the diagnosis of canine infection with *Ehrlichia canis*. Veterinary Microbiology 86, 361-368.

Harrus, S., Waner, T., Aizenberg, I., Bark, H., 1998. Therapeutic effect of doxycycline in experimental subclinical canine monocytic ehrlichiosis: evaluation of a 6-week course. Journal of Clinical Microbiology 36, 2140-2142.

Hess, P.R., English, R.V., Hegarty, B.C., Brown, G.D., Breitschwerdt, E.B., 2006. Experimental *Ehrlichia canis* infection in the dog does not cause immunosuppression. Veterinary Immunology and Immunopathology 109, 117-125.

Iqbal, Z., Rikihisa, Y., 1994. Reisolation of *Ehrlichia canis* from blood and tissue of dogs after doxycycline treatment. Journal of Clinical Microbiology 32, 1644–1649.

Morales, A.B., Baticados, W.N., 2007. Hematology and cytopathology of Ehrlichia spp. Infection in bomb sniffing Belgian Mallinois dogs in the Philippines. Philippine Journal of Veterinary Medicine 44, 76-84.

Mylonakis, M.E., Koutinas, A.F., Breitschwerdt, E.B., Hegarty, B.C., Billinis, C.D., Leontides, LS, Kontos, V.S., 2004. Chronic canine ehrlichiosis (*Ehrlichia canis*): a retrospective study of 19 natural cases. Journal of the American Animal Hospital Association 40, 174-184.

Parola, P., Roux, V., Camicas, J.L., Baradji, I., Brouqui, P., Raoult, D., 2000. Detection of ehrlichiae in African ticks by polymerase chain reaction. Transactions of the Royal Society of Tropical Medicine and Hygiene 94, 707–708.

Perez, M., Bodor, M., Zhang, C., Xiong, Q., Rikihisa, Y., 2006. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. Annals of the New York Academy of Sciences 1078: 110-117.

Pusterla, N., Huder, J.B., Feige, K., Lutz, H., 1998. Identification of a granulocytic Ehrlichia strain isolated from a horse in Switzerland and comparison with other Rickettsiae of the *Ehrlichia phagocytophila* Genogroup. Journal of Clinical Microbiology 36, 2035–2037.

Ramos, C.A., Ramos, R.A., Araújo, F.R., Guedes, D.S. Jr., Souza, I.I., Ono, T.M., Vieira, A.S., Pimentel, D.S., Rosas, E.O., Faustino, M.A., Alves, L.C., 2009. Comparison of nested-PCR with blood smear examination in detection of *Ehrlichia canis* and *Anaplasma platys* in dogs. Brazilian Journal of Veterinary Parasitology 1, 58-62.

Sekeyova, Z., Subramanian, G., Mediannikov, O., Diaz, M.Q., Nyitray, A., Blaskovicova, H. and Raoult, D., 2011. Evaluation of clinical specimens for Rickettsia, Bartonella, Borrelia, Coxiella, Anaplasma, Franciscella and Diplorickettsia positivity using serological and molecular biology methods. FEMS Immunology and Medical Microbiology 64, 82–91.

Waner, T., Strenger, C., Keysary, A., 2000. Comparison of a clinic-based ELISA test kit with the immunofluorescence test for the assay of *Ehrlichia canis* antibodies in dogs. Journal of Veterinary Diagnostic Investigation 12, 240-244.

Waner, T., Strenger C., Keysary, A., Harrus, S., 1998. Kinetics of serologic cross-reactions between *Ehrlichia canis* and the *Ehrlichia phagocytophila* genogroups in experimental *E. canis* infection in dogs. Veterinary Immunology and Immunopathology 66, 237-243.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.L., 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173, 697–703.

Ybañez, A.P., Matsumoto, K., Kishimoto, T., Inokuma, H., 2012a. Molecular analyses of a potentially novel Anaplasma species closely related to *Anaplasma phagocytophilum* detected in sika deer (*Cervus nippon yesoensis*) in Japan. Veterinary Microbiology 157, 232-236.

Ybañez, A.P., Perez, Z.O., Gabotero, S.R., Yandug, R.T., Matsumoto, K., Inokuma, H., 2012b. First molecular detection of *Ehrlichia canis* and *Anaplasma platys* in ticks from Dogs in Cebu, Philippines. Ticks Tick Borne Diseases 3, 288-293.