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Original Article

Canine Heartworm (*Dirofilaria immitis*) Infection and Immunoglobulin G Antibodies Against *Wolbachia* (Rickettsiales: Rickettsiaceae) in Stray Dogs in Bangkok, Thailand

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Abstract

Canine heartworm (*Dirofilaria immitis*) infection in stray dogs was studied by using parasitological (fresh blood smear, thin blood smear, thick blood smear, and modified Knott's test) and serological methods. Blood samples were collected from stray dogs in Bangkok metropolitan area during 2006 and 2008. There were 10% (50/500) of stray dogs infected with *D. immitis*, which was indicated by using parasitological methods. Microfilaria levels were evaluated from 36 infected dogs and the range of microfilarial levels were between 17 and 78,417 microfilariae per milliliter of blood. Fifty serum samples from *D. immitis* infected dogs and 310 serum samples from non-infected dogs were subjected to the study of total immunoglobulin G (IgG) against *Wolbachia* bacteria, the endosymbiont of the parasite, by using Indirect Enzyme-Linked ImmunoSorbent Assay (ELISA). The sensitivity and specificity of ELISA were analyzed by comparing with the parasitological methods. The ELISA has 52% sensitivity and 85% specificity when the cut-off level was 1.73 (mean+SD). The ELISA from this study which detected the total IgG response to *Wolbachia* bacteria infection may not be a useful method for using as a diagnostic tool to distinguish between *D. immitis* infected and non-infected dogs because of its low sensitivity. Further study of the IgG subclass responses against *Wolbachia* would be useful to evaluate the diagnostic potential.

Keywords: *Dirofilaria immitis, Wolbachia*, immune response, stray dogs, Thailand

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บทคัดย่อ

การติดเชื้อหนอนพยาธิหัวใจสุนัขและการตอบสนองทางภูมิคุ้มกันต่อเชื้อแบคทีเรียวูลบาเคียในสุนัขจรจัดในเขตกรุงเทพมหานคร

สนธยา เตียวศิริทรัพย์[1] ธีรัตต์ ธนไพศาลกิจ[2] เอื้อพงศ์ วราทร[2] ทัดดาว อภิชนพงศา[3] นัฐพล บำเพ็ญเกียรติกุล[3]*

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งานวิจัยนี้มีวัตถุประสงค์เพื่อทำการศึกษาการติดเชื้อหนอนพยาธิหัวใจสุนัข (*Dirofilaria immitis*) ในสุนัขจรจัดโดยใช้วิธีทางปรสิตวิทยา ได้แก่ การทำแผ่นฟิล์มเลือดสด การทำแผ่นฟิล์มเลือดบาง การทำแผ่นฟิล์มเลือดหนา และ Modified Knott's test และวิธีทางซีรั่มวิทยา โดยเจาะเลือดจากสุนัขจรจัดในเขตกรุงเทพมหานครจำนวน 500 ตัวอย่าง ในระหว่างปี พ.ศ. 2549 ถึง 2551 พบว่าสุนัขมีการติดเชื้อหนอนพยาธิหัวใจสุนัขจำนวนร้อยละ 10 ซึ่งตรวจด้วยวิธีทางปรสิตวิทยา และได้ทำการศึกษาถึงระดับของตัวอ่อนระยะไมโครฟิลาเรียในกระแสเลือดของสุนัขจำนวน 36 ตัวอย่าง โดยการทำแผ่นฟิล์มเลือดหนาและย้อมด้วยสียิมซ่า พบว่ามีปริมาณของไมโครฟิลาเรียระหว่าง 17 ถึง 78,417 ไมโครฟิลาเรีย ต่อเลือดจำนวน 1 มิลลิลิตร สำหรับการศึกษาทางซีรั่มวิทยาที่เกี่ยวข้องกับการตอบสนองทางภูมิคุ้มกันต่อเชื้อแบคทีเรียวูลบาเคีย โดยแบคทีเรียชนิดนี้สามารถตรวจพบได้ในหนอนพยาธิหัวใจสุนัข การศึกษานี้ใช้ตัวอย่างเลือดที่ตรวจพบตัวอ่อนระยะไมโครฟิลาเรียจำนวน 50 ตัวอย่าง และตัวอย่างเลือดที่ตรวจไม่พบตัวอ่อนระยะไมโครฟิลาเรียจำนวน 310 ตัวอย่าง ตรวจวัดการตอบสนองทางภูมิคุ้มกันชนิด IgG (total IgG) ต่อเชื้อแบคทีเรียโดยวิธีอีไลซ่า (Indirect ELISA) พบว่าวิธีอีไลซ่านี้เมื่อใช้ค่า cut-off level ที่ 1.73 (ค่าเฉลี่ยบวกค่าเบี่ยงเบนมาตรฐาน) เปรียบเทียบกับวิธีทางปรสิตวิทยานั้นมีความไวร้อยละ 52 และความจำเพาะร้อยละ 85 วิธีอีไลซ่าจากการศึกษาครั้งนี้อาจจะไม่สามารถนำมาใช้งานในการตรวจวินิจฉัยได้เนื่องจากมีความไวค่อนข้างต่ำ การศึกษาเพิ่มเติมถึงการตอบสนองทางภูมิคุ้มกันชนิด IgG ในแต่ละกลุ่ม (IgG subclass) อาจจะเพิ่มความไวและความจำเพาะมากขึ้นซึ่งอาจนำมาใช้ได้ต่อไปในอนาคต

คำสำคัญ: หนอนพยาธิหัวใจสุนัข แบคทีเรียวูลบาเคีย การตอบสนองทางภูมิคุ้มกัน สุนัขจรจัด ประเทศไทย

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Introduction

Wolbachia (Rickettsiales: Rickettsiaceae), the endosymbiont bacteria in filarial nematodes such as *Brugia spp., Dirofilaria spp., Onchocerca spp.*, and *Wulchereria spp*. have been known for many years (Bandi et al., 1998; Bazzocchi et al., 2000[b]). The importance of these bacteria in the pathogenesis of filarial infection has been studied in both humans and animals (Kramer et al., 2005[a]; Porksakorn et al., 2007; Kramer et al., 2008). *Wolbachia* releasing into the blood stream of patients infected with filarial nematodes normally occurs following the damage or death of the parasites. Host immune response to these bacteria is also initiated and can be detected (Bazzocchi et al., 2000[a]; Bazzocchi et al., 2003; Kramer et al., 2005[b]). Detection of the host's immune response for *Wolbachia* might be a useful diagnostic tool to indicate the filarial infection particularly in the case of occult filariasis (Kramer et al., 2005[b]; Oleaga et al., 2009).

Dirofilaria immitis, the life-threatening filarial nematode causes heartworm disease in dogs and other animal species (Nakagaki et al., 2007; McCall et al., 2008). Dogs become infected with *D. immitis* when they were bitten by infected mosquitoes and the infective stage larvae move into the dogs' skin. Adult nematodes reside in the right ventricle and pulmonary artery. The clinical signs that can be seen in infected dogs are exercise intolerance, coughing, ascites and heart failure (Grauer et al., 1987; Atkins et al., 1988; Paes-de-Almeida et al., 2003). Stray dogs without any heartworm prevention can become

infected easily and can serve as the source of infection for other stray dogs, pet dogs and humans. Infection in humans has been reported; however, there are only skin or lung lesions caused by dead larvae found in the patients (Feld, 1973; Simon et al., 2005; Morchon et al., 2006).

This study was performed for the surveillance of canine heartworm infection in stray dogs in Bangkok, Thailand in 2006 and 2008. Parasitological study for *D. immitis* microfilaria detection and serological study for IgG immune response against *Wolbachia* bacterial infection were carried out and compared to indicate the possibility of using the detection of canine immune response against *Wolbachia* as a diagnostic tool for canine heartworm infection.

Materials and Methods

Blood samples: Five hundred blood samples were collected from stray dogs with the approximate age of at least 6 months in Bangkok metropolitan area during 2006 and 2008. This study was approved by the Chulalongkorn University Institutional Animal Care and Use Committee

Parasitological methods: Fresh blood smear, thin blood smear, thick blood smear and modified Knott's test were performed for each blood sample. For fresh blood smear, one drop of blood was mixed with 0.85% NaCl and smeared onto a glass slide. For thin smear, one drop of blood was smeared onto a glass slide and stained with 10% Giemsa. For modified Knott's test, 1 ml of blood was mixed with 2% formalin and centrifuged. Then, the sediment was examined for the microfilaria. For microfilaria counting, 3-line thick blood smear made of 20 µl of blood on a glass slide, was allowed to air dry, hemolyzed in distilled water, fixed in absolute methanol, and stained with 10% Giemsa. The stained slide was examined, and microfilaria counted under a light microscope.

Detection of Wolbachia antigen: Twenty four *D. immitis* positive serum samples collected in 2006 were tested for *Wolbachia* infection by polymerase chain reaction (PCR).

DNA extraction: DNA was extracted from each dog serum sample using QIamp DNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's recommendation with slight modification. Briefly, 100 µl of serum was mixed with 20 µl of protease and 200 µl of lysis buffer and incubated at 56°C for 10 min. Two hundred ml of absolute ethanol were added and transferred into the DNeasy Mini spin column. The column was then centrifuged and washed twice with washing buffer. One hundred µl of elution buffer were added into the column to elute the DNA and DNA samples were kept at -20°C until tested.

Polymerase chain reaction: PCR was performed to amplify a fragment of the 16s ribosomal DNA (rDNA) gene of *Wolbachia* (Werren et al. 1995; Werren and Windsor 2000). The sequence of forward primer was 5′-CAT ACC TAT TCG AAG GGA TAG-3′ and the sequence of reverse primer was 5′-AGC TTC GAG TGA AAC CAA TTC-3′. PCR cycling conditions were 95°C for 15min followed by 38 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 90 sec and 72°C for 10 min for the final extension. PCR was performed in 25 µl-reaction using Hot Star Taq DNA polymerase (QIAGEN, Valencia, CA). The reaction is composed of 2.5 µl of 10x buffer (Tris-Cl, KCl, (NH4)2SO4, 15mM MgCl2, pH 8.7), 1 unit of Taq DNA polymerase, 100 µM dNTP (Deoxynucleotide Solution Mix, New England Biolabs Inc., Ipswich, MA), 0.2 µM of forward primer, 0.2 µM of reverse primer, 17.3 µl of ultra pure water (Invitrogen Corp., Carlsbad, CA), and 2 µl of DNA template. PCR product was analysed in 1.2% agarose gel (Ultrapure™ Agarose, Invitrogen Corp., Carlsbad, CA) and stained with SYBR safe™ DNA gel staining (Invitrogen Corp., Carlsbad, CA). The 16s rDNA *Wolbachia* PCR product had 438 base pairs.

Preparation of Wolbachia surface protein (WSP): Cloning and expression of WSP: The entire coding sequence of the WSP gene excluding the predicted N-terminal signal sequence was cloned from *Brugia malayi* genomic DNA by PCR into pET100/D-TOPO expression vector (Invitrogen, Carlsbad, CA). The recombinant WSP was expressed as a fusion protein containing N-terminal 6xHis tag, and a specific-enterokinase cleavage site. *B. malayi* genomic DNA was isolated from a pool of adult worms through standard phenol-chloroform procedures. The forward primer, 5′-CACC ATG GAT CCT GTT GGT CCA ATA GC-3′, had 4 additional bases at the 5′ end to enable directional cloning into the pET TOPO vector. The sequence of reverse primer was 5′- TTA GAA ATT AAA CGC TAT TCC AGC-3′. The PCR reaction was performed in 50-µl volumes under the following final conditions: 2X Pfx amplification buffer, 1 mM $MgSO_4$, 0.3 mM of each dNTP, 0.3 µM each of forward and reverse primers, and 1.25 unit of Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The PCR amplification was performed by 35 cycles of 94°C for 15 sec, 50°C for 30 sec, and 68°C for 60 sec. The PCR products were cloned in the pET100/D-TOPO expression vector and transformed into one-shot TOP10 cells. Plasmids containing inserts were selected by growth on Luria-Bertani plates containing ampicillin, and sequenced to confirm that the WSP gene represented correct orientation. For expression, the plasmids containing the WSP gene were extracted from TOP10 cells and transformed into *E. coli* BL21 (DE3) pLys (Invitrogen, Carlsbad, CA). A recombinant WSP (rWSP) fusion protein was then induced to express by adding 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Invitrogen, Carlsbad, CA) into the cultivated clone at O.D.600 about 0.5-0.8. The cultivated clone was harvested at 1 hr after the induction. Cultures were centrifuged at 10,000 g at 4°C for 10 min. Cell pellet was lysed by sonication. Soluble and insoluble parts were then separated by centrifugation at 14,000 RPM at 4°C for 5 min. Expression profile was analyzed by SDS-PAGE, and

Western blot analysis with anti-WSP antibodies and HisProbe-HRP (Pierce, Rockford, IL).

Purification of WSP: *E. coli* BL21 (DE3) pLys clone containing the expression vector was harvested at 1 h after induction with IPTG by centrifugation at 10,000 g at 4°C for 10 min. Cells were lysed by B-PER bacteria protein extraction reagent (Pierce) supplemented with 1% protease inhibitor cocktails. The inclusion bodies were then isolated from the crude cell lysate by centrifugation at 10,000 g at 4°C for 15 min. The rWSP was purified by treatment of lysozyme and washed with B-PER bacteria protein extraction reagent. Affinity-purified rWSP was performed by chromatography with Ni-NTA resin (QIAGEN, Valencia, CA) under denaturing condition. The insoluble protein was solubilized in denaturing binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 1 mM β-mercaptoethanol, 6M Urea, pH 7.9), and incubated on ice for 1 hr. The solution was clarified by filtration through a 0.45-µm nylon membrane (Millipore, Billerica, MA). The purification column was prepared by washing with 5-column volume of the binding buffer. Then, the sample was loaded onto the column and wash with 10-column volume of the binding buffer. The column was washed with another 10-column volume of denaturing wash buffer (10 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 1 mM β-mercaptoethanol, 6M Urea, pH 7.9). The rWSP protein was eluted with denaturing elution buffer (1M imidazole, 500 mM NaCl, 20 mM Tris-HCl, 2 mM β-mercaptoethanol, 6M Urea, pH 7.9). The purified rWSP protein was dialyzed against 20 mM Tris-HCl (pH 8.5) containing 0.1 mM DTT and finally 20 mM Tris-HCl (pH 8.5). Protein concentration of purified rWSP protein was determined by a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Ill).

Indirect Enzyme-Linked ImmunoSorbent Assay (ELISA): One hundred µl of 1 µg/ml of WSP in 0.05 M carbonate buffer (pH 9.6) were coated onto each well of microtiter plates and incubated at 37°C for 2 hrs and at 4°C overnight. Coated plates were then kept at -80°C until used. Before using, frozen ELISA plates were warmed up at room temperature and washed 3 times for 3 min each with 200 µl of PBST (0.01 M PBS (pH 7.4)/0.05% Tween 20). Each well was then blocked with 200 µl of 5% non-fat dried milk in PBST at 37°C for 3 hrs and washed 3 times for 3 min each with 200 µl of PBST. One hundred microliters of each serum sample were added to each well at 1:20 dilution in 5% non-fat dried milk in PBST. After incubating at 4°C overnight, the ELISA plate was washed 5 times for 5 min each with 200 µl of PBST and 50 µl of anti-dog IgG-horseradish peroxidase conjugates diluted in PBS/T20 (1:5,000) were added to each well and incubated at 37°C for 1 hr. The ELISA plate was then washed 5 times for 5 min each with 200 µl of PBST, and 100 µl of TMB substrate were added to each well and incubated at room temperature for 10 min. The reaction was ceased by adding 100 µl of 2 M H_2SO_4 and the optical density was read at 450 nm. The cut-off level for ELISA, sensitivity and specificity of ELISA analyzed by comparing with the parasitological methods were calculated using the following formulas.

Microfilaremia	ELISA Test		Total
	Positive	Negative	
Positive (50)	True positive (A)	False positive (B)	A+B
Negative (310)	False negative (C)	True Negative (D)	C+D
Total	A+C	B+D	A+B+C+D

Sensitivity = [A/(A+C)] x 100

Specificity = [D/(B+D)] x 100

Accuracy = [(A+D)/(A+B+C+D)] x 100

Predictive value positive test = [A/(A+B)] x 100

Predictive value negative test = [D/(C+D)] x 100

Results

Parasitological study: Five hundred blood samples were collected from stray dogs in Bangkok metropolitan area during 2006 and 2008. There were 10% (50/500) of stray dogs infected with *D. immitis*, which was indicated through parasitological methods. Microfilaremia levels were evaluated from 36 infected dogs and the range was between 17 and 78,417 microfilariae per ml of blood. For other parasitic infections, there was one dog infected with *Brugia pahangi* and 4 dogs infected with *Hepatozoon canis*.

Detection of Wolbachia antigen: Twenty four *D. immitis* positive serum samples were tested for *Wolbachia* infection by Polymerase Chain Reaction (PCR), and all of them were negative.

IgG response against Wolbachia bacteria infection: Through parasitological methods, 50 serum samples from *D. immitis* infected dogs and 310 serum samples from non-infected dogs were subjected to the study. Total IgG response against *Wolbachia* infection was studied by using Indirect Enzyme-Linked ImmunoSorbent Assay (ELISA). Mean±SD of the optical density for *D. immitis* infected dogs and non-infected dogs were 1.70±0.64 and 1.33±0.40, respectively.

The cut-off level for ELISA, sensitivity and specificity of ELISA analyzed by comparing with the parasitological methods were shown in Table 1. The ELISA has 52% sensitivity and 85% specificity when the cut-off level was 1.73 (mean+SD). The ELISA specificity increased to 94% and 99% when the cut-off levels were 2.009 (mean+2SD) and 2.528 (mean+3SD), respectively. The sensitivity, however, decreased to 34% and 10%. There was no correlation between microfilaria level and optical density from ELISA (Fig. 1).

Table 1 The cut-off level for the ELISA (total IgG immune response against *Wolbachia* bacteria), sensitivity and specificity of ELISA analyzed by comparing with parasitological methods

Cut-off value	Sensitivity	Specificity	Accuracy	Predictive value positive test	Predictive value negative test
Mean+1/2SD	58%	65%	64%	21%	91%
Mean+1SD	52%	85%	81%	36%	92%
Mean+2SD	34%	94%	86%	47%	90%
Mean+3SD	10%	99%	87%	71%	87%

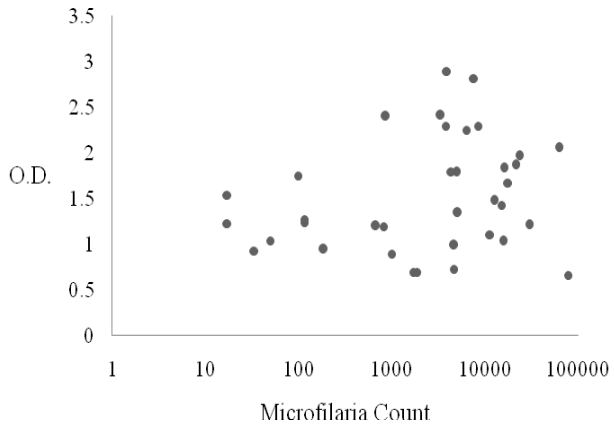

Figure 1. Microfilaria count and optical density (O.D.) from the ELISA (IgG immune response against *Wolbachia* bacteria).

Discussion

Blood samples were collected from stray dogs with the approximate age of at least 6 months in Bangkok metropolitan area because the development of the infective larva stage of canine heartworm; *D. immitis* to the adult stage in dog is approximately 6 months. There was no clinical sign of infection in dogs while collecting the blood samples. This study showed that 10% (50/500) of stray dogs were infected with *D. immitis*. We also found 0.2% (1/500) and 0.8% (4/500) of these dogs were infected with *Brugia pahangi* and *Hepatozoon canis*, respectively. Nevertheless, this study may underestimate the actual infection of stray dogs with *D. immitis* because we only used parasitological methods. In this study, we also sampled 30 sera which had the negative results from parasitological methods and tested by using canine heartworm antigen-ELISA commercial test kit, and 7% (2/30) of them were found infected. Therefore, the actual infection of the stray dogs with canine heartworm in Bangkok during 2006 and 2008 was approximately 17%.

Nithiuthai (2003) also reported that 10.2% and 29.2% of pet dogs which were requested for blood test were positive for *D. immitis* when tested by parasitological methods only and by parasitological methods and antigen-ELISA test kit, respectively. Using other diagnostic tools would be helpful for the diagnosis of occult dirofilariasis or the absence of microfilaremia. In addition, another point that needs to be taken into account in relation to filarial diagnosis in Thailand is not only *D. immitis* but also *B. pahangi*, as indicated in the results, are filarial nematodes that can be found in dogs. Both *D. immitis* and *B. pahangi* are mosquito-borne filariasis. Potential vectors for these filarial nematodes were indicated in several mosquito species for examples, *Aedes aegypti, Aedes albopictus* and *Culex quinquefasciatus* (Tiawsirisup et al., 2005; Tiawsirisup and Nithiuthai, 2006; Tiawsirisup and Kaewthammasorn, 2007).

In this study, we also aimed to evaluate the ELISA that was developed in our laboratory to detect the total IgG response against *Wolbachia* bacteria infection in dogs. It was reported that *D. immitis* infected dogs are also infected with *Wolbachia* bacteria (Simoncini et al., 2001; Kramer et al., 2005[b]). In our study, however, we could not detect any *Wolbachia* in the infected dog sera by regular polymerase reaction (PCR). Nevertheless, in our preliminary study, we could detect these bacteria in the serum by using real time PCR or *Wolbachia* in the serum which needs to be concentrated before tested by regular PCR (unpublished data).

It is noteworthy that the ELISA may not be a useful method to use as a diagnostic tool to distinguish between *D. immitis* infected and non-infected dogs because of its low sensitivity. Another preliminary study in our laboratory on total IgG response against *Wolbachia* bacteria in human filariasis patients in Thailand also revealed similar results with low sensitivity of the test (unpublished data). Further study of the IgG subclass responses against *Wolbachia* in dogs would be useful to evaluate the diagnostic potential (Marcos-Atxutegi et al., 2003; Kramer et al., 2005[b]).

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