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

Sonthaya Tiawsirisup1*  Teerat Thanapaisarnkit2  Euapong Varatorn2  Taddao Apichonpongsa3  Nattapon Bumpenkiattikun3  Sirirat Rattanapuchpong3  Sudchit Chungpiwat1  Vivornpun Sanprasert4  Surang Nuchprayoon4

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

1Veterinary Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330 Thailand
2Senior Veterinary Student Year 2006, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330 Thailand.
3Senior Veterinary Student Year 2008, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330 Thailand.
4Lymphatic Filariasis Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330 Thailand.

Corresponding author E-mail: sonthaya.t@chula.ac.th

Original Article

บทคัดย่อ
การติดเชื้อหนอนพยาธิหัวใจสุนัขและการตอบสนองทางภูมิคุ้มกันต่อเชื้อแบคทีเรียวูลบาเคียในสุนัขจรจัดในเขตกรุงเทพมหานคร

สมชาย เดชวิทยาภูมิ รศ. อนิชิต อนโพสกิจ เล็กพงศ์ วรารัฐ ทัดดาว อภิชนพงศา นัฐพล บุญเพ็ญเกียรติกุล

ศิริวัฒนา รัตนคุณศักดิ์ สุธิจักร จุฬาลงกรณ์ วิวัฒน์ คาร์ลมาร์ส ซุปแรก บุญประยูร

งานวิจัยนี้มีวัตถุประสงค์เพื่อทำ การศึกษาการติดเชื้อหนอนพยาธิหัวใจสุนัข (Dirofilaria immitis) ในสุนัขจรจัดโดยใช้วิธีทางปรสิตวิทยาได้แก่ การทำแผ่นฟิล์มเลือดสด การทำแผ่นฟิล์มเลือดบาง การทำแผ่นฟิล์มเลือดหนา และ Modified Knott’s test และวิธีทางซีรั่มวิทยาโดยเจาะเลือดจากสุนัขจรจัดในเขตกรุงเทพมหานครจำนวน 500 ตัวอย่าง ในระหว่างปี พ.ศ. 2549 ถึง 2551 พบว่าสุนัขมีการติดเชื้อหนอนพยาธิหัวใจสุนัขจำนวนร้อยละ 10 และได้ทำการศึกษาถึงระดับของตัวอ่อนระยะไมโครฟิลาเรียในกระแสเลือดของสุนัขจำนวน 36 ตัวอย่าง โดยการทำแผ่นฟิล์มเลือดหนาและย้อมด้วยสียิมซ่า พบว่ามีปริมาณไมโครฟิลาเรียระหว่าง 17 ถึง 78,417 ไมโครฟิลาเรียต่อเลือดจำนวน 1 มิลลิลิตร สำหรับการศึกษาทางซีรั่มวิทยาที่เกี่ยวข้องกับการตอบสนองทางภูมิคุ้มกัน พบว่ามีการติดเชื้อแบคทีเรียวูลบาเคียในหนอนพยาธิหัวใจสุนัข ร้อยละ 10 และได้ทำการศึกษาการตอบสนองทางภูมิคุ้มกันชนิด IgG (total IgG) โดยวิธีอีไลซ่า (Indirect ELISA) พบว่ามีการตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจพบตัวอ่อนไมโครฟิลาเรียจำนวน 50 ตัวอย่าง และตรวจพบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG ในตัวอย่างที่ตรวจไม่พบการตอบสนองทางภูมิคุ้มกันชนิด IgG

คำสำคัญ: หนอนพยาธิหัวใจสุนัข, แบคทีเรียวูลบาเคีย, การตอบสนองทางภูมิคุ้มกัน, สุนัขจรจัด, ประเทศไทย

*ผู้รับผิดชอบบทความ E-mail: sonthaya.tg@chula.ac.th

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., 2000b). The importance of these bacteria in the pathogenesis of filarial infection has been studied in both humans and animals (Kramer et al., 2005a; 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., 2000b; Bazzocchi et al., 2003; Kramer et al., 2005a). 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., 2005b; 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, 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 QIAamp 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 µl 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* (Warren et al. 1995; Warren and Windsor 2000). The sequence of forward primer was 5'-CAT ACC TAT TCG AGG 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 1X 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-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 TCG AAG GGA TAG-3'. The PCR reaction was performed in 50-µl volumes under the following final conditions: 2X Pfx amplification buffer, 1 mM MgSO4, 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 SDSPAGE, 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 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 extraction reagent. Affinity-purified rWSP was solubilized in denaturing (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 bichinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

**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 H2SO4 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.

<table>
<thead>
<tr>
<th>Microfilaremia</th>
<th>ELISA Test</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (50)</td>
<td>True positive (A)</td>
<td>False positive (B)</td>
</tr>
<tr>
<td>Negative (310)</td>
<td>True negative (C)</td>
<td>False negative (D)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>[A/(A+C)] x 100</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>[D/(B+D)] x 100</td>
<td></td>
</tr>
<tr>
<td>Accuracy</td>
<td>[(A+D)/(A+B+C+D)] x 100</td>
<td></td>
</tr>
<tr>
<td>Predictive value positive test</td>
<td>[A/(A+B)] x 100</td>
<td></td>
</tr>
<tr>
<td>Predictive value negative test</td>
<td>[D/(C+D)] x 100</td>
<td></td>
</tr>
</tbody>
</table>

**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

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

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., 2005b). 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., 2005b).

Acknowledgement

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