Diagnosis of Heartworm (*Dirofilaria immitis*) Infection in Dogs and Cats by Using Western Blot Technique

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ABSTRACT

Heartworm (*Dirofilaria immitis*) infection is the causative parasite of severe disease in dogs and cats. This mosquito-borne parasite is also found in wild animals and occasionally evident in humans. Diagnosis of dirofilariasis in companion animals is mainly performed by modified Knott's technique and commercial serological tests. The objective of this study was to develop an alternative diagnosis of heartworm infections in pet animals. Blood samples of dogs and cats were collected and demonstrated the heartworm infection. Somatic proteins of nematodes such as *D. immitis, Toxocara canis, T. cati, Ancylostoma ceylanicum* and *A. caninum* were used to compare the protein profiles by using polyacrylamide gel electrophoresis (PAGE). The sera of heartworm-infected animals were used in detection of antigens associated with *D. immitis* proteins by Western blot (WB) analysis. WB results revealed that somatic antigens of *D. immitis* contained seven major peptide bands from 25 to 250 kDa recognized by the sera of infected dogs and three bands from 33 to 64 kDa recognized in cats. There were cross-reacted protein bands (50 and 64 kDa) found in all nematodes. The protein at 33 kDa was unique since it was only demonstrated by heartworm infected dog and cat sera. WB results indicated that this technique might be considered as the alternative diagnosis for heartworm infection.
Key words: *Dirofilaria immitis*, dogs and cats, Western blot

INTRODUCTION

D. immitis is a filarial parasite that causes an animal disease commonly known as heartworm. This vector-borne parasite can cause patent infections in companion and wild animals and occasionally can infect humans. Dogs are the most common natural host while cats are not easily infected. However, the infection is becoming more and more often diagnosed in cats and now recognized as a potential cause of serious disease in cats. Epidemiological studies indicate that in locations where the infection is endemic in the dog, cats are at risk (Kramer and Genchi, 2002). Descriptions of clinical manifestations and the pathogenesis of canine heartworm disease are based on both experimental infection and clinical case studies (Rawling, 1986). Unlike dogs, cats with naturally acquired infections usually do not harbor many worms. Clinically, the majority of heartworm infections in cats are asymptomatic. However, a low number of worms can cause a severe condition, including sudden death (Genchi *et al.*, 1992; McCall *et al.*, 1994; Atkin *et al.*, 2000).

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Heartworm disease is present in all continents including Asia. Some attempts to obtain reliable tests for detecting specific antibodies against *D. immitis* using a direct fluorescent antibody test which used killed microfilariae as antigen (Wong and Suter, 1979) and an ELISA-based test, similar to that developed by Grieve *et al*. (1981). However, the results were inconclusive due to poor sensitivity and low specificity. The detection of circulating antibodies against *D. immitis* has long been the alternative for epidemiological studies of feline heartworm infection since the recent development of antibody tests with improved sensitivity and specificity was considered as an important tool in the study of feline heartworm disease and its prevalence among the cat population (Genchi *et al*., 1998; Miller *et al*., 1998; Watkins *et al*., 1998). In Thailand, stray dogs and cats are becoming of public concern since their numbers are seriously rising annually in the Bangkok metropolitan areas. Jittapalapong *et al* (2005) reported the prevalence of heartworm infection of stray dogs and cats in Bangkok areas were 13.9 and 0.3%, respectively. Therefore, under these circumstances stray animals are likely at risk with heartworm infections and might be transmitted to other pets in the surrounding area. The objective of this study was to develop an alternative diagnosis by using the unique protein pattern of *D. immitis* recognized by positive *D. immitis* sera of stray dogs and cats by Western blot analysis.

MATERIALS AND METHODS

1. Animal sera

Blood sample was collected from the cephalic vein of stray dogs and the jugular vein of stray cats in Bangkok area and examined for *D. immitis* microfilariae by using the modified Knott technique. Sera were separated after coagulation of blood cells and were stored at -20°C until used.

2. Parasite antigens preparation

Somatic antigens of adult *D. immitis, T. canis, T. cati, A. ceylanicum* and *A. caninum* were used. The worms obtained by necropsy of infected dogs were washed, homogenized and sonicated in phosphate buffer saline solution (0.15 M, pH 7.2). The homogenate was then centrifuged at 16,000 G for 30 min and the supernatant was dialyzed and measured the protein concentration according to the procedure described by Bradford (1976). Antigens were stored at -20°C until used.

3. Polyacrylamide gel electrophoresis (PAGE)

One dimensional polyacrylamide gel electrophoresis (PAGE) was carried out in 12% gels with an acrylamide/bis ratio of 37.5:1 in the presence of 10% sodium dodecyl sulphate (SDS) supplemented (Bio-Rad, USA) and ammonium persulphate in Tris-HCl buffer pH 8.8, according to Laemmli (1970). The antigen was diluted in a Tris (pH 6.8) sample buffer and then loaded in a gel with a concentration of 4-8 μg protein/lane. The electrophoresis was monitored using 0.5% bromophenol blue and the current was set at 200 V for 40 minutes. Kaleidoscope prestained (BioRad, USA) was used as protein markers.

4. Western blot

After running for electrophoresis, these gels with *D. immitis*, *T. canis*, *T. cati*, *A. ceylanicum* and *A. caninum* antigen were electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane according to the procedure described by Jittapalapong (1999). The transfer was performed in a mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA) for 2 hours in a constant current of 100 V in a transfer buffer (Tris-glycine-methanol). The PVDF membranes were blocked by a blocking buffer (Tris-gelatin-Tween-20) and incubated overnight. Then, membranes were washed in washing buffer for 3 times and incubated for 3 hours at room temperature with diluted (1:100) primary antibody (sera of infected

dogs and cats) on a shaker. Subsequently, membranes were washed again. Specifically bound antibodies were detected by anti-dog and anti-cat Horseradish Peroxidase conjugate (ICN, USA) diluted to 1:1000 for 60 minutes. After rinsing three times in washing buffer, the blots were incubated at room temperature for 10 minutes in enzymatic substrate (3,3'-Diaminobenzidine tetrahydrochloride) (DAKO,USA) to develop color.

RESULTS

Western blot analysis revealed that somatic antigens of adult *D. immitis* contained seven peptide bands at 250, 108, 71, 50, 46, 33 and 25 kDa recognized by the sera of infected dogs (Figure 1), while four bands at 64, 50, 46 and 33 kDa were recognized by positive heartworm cats (Figure 2). However, the unique signals were found at 108, 71, 46 and 33 kDa in dogs and 46

and 33 kDa in cats. There was cross-reacted protein bands (50 and 64 kDa) found in all nematode. The protein at 33 kDa was unique since they were only demonstrated by heartworm infected-dog and -cat sera. The WB result of protein patterns was summarized in Table 1.

DISCUSSION

Cross reaction to other nematode parasites occurred with peptide bands of 50 and 64 kDa (Table 1) in all parasitic nematodes (*T. canis*, *T. cati*, *A. caninum* and *A. ceylanicum*) in dogs while cats only 50 kDa were cross reaction with *T. canis* and *T. cati*. No cross-reaction appeared with *A. caninum* and *A. ceylanicum* in cats. Based on these findings, this might be concluded that *D. immitis* shared common antigens with other nematodes. This cross reactivity may depend on many antigenic epitopes existing in somatic components of these parasites since the
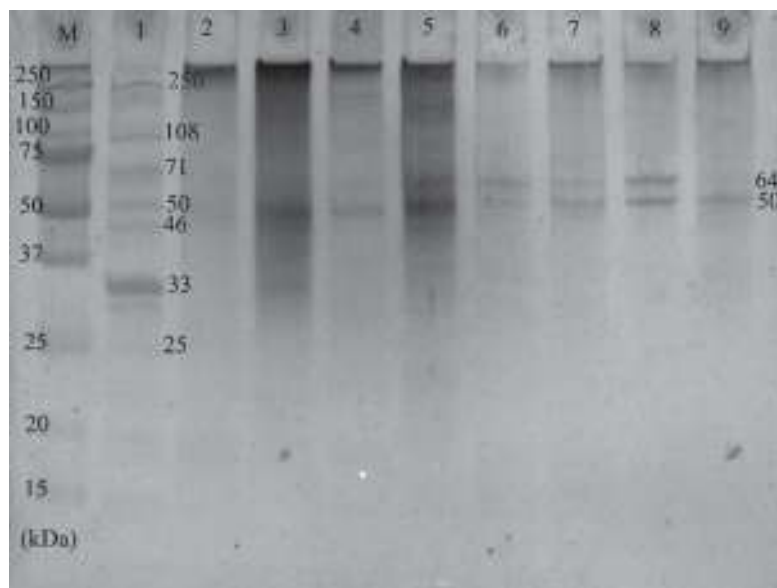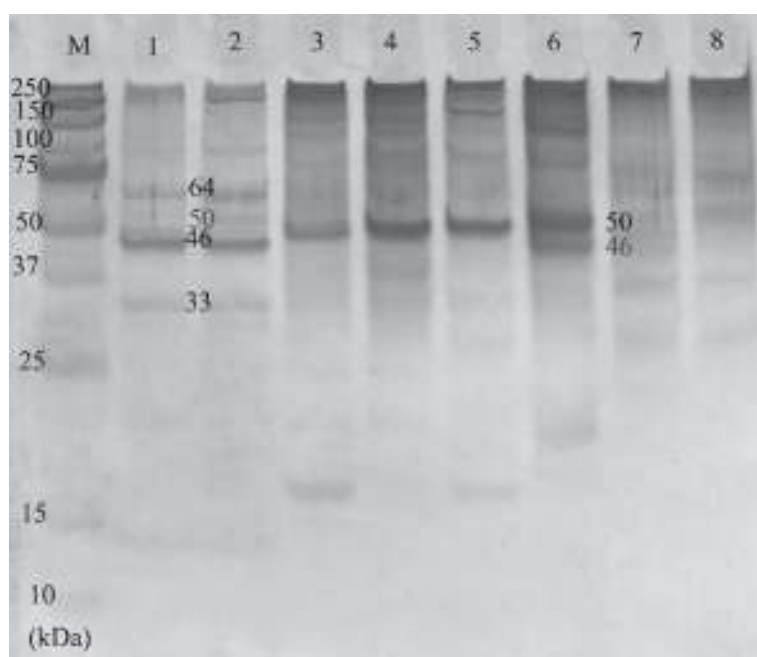

Figure 1   Immunoblot patterns of adult *D. immitis* infection in dogs, lane M, molecular weight markers; lane 1, *D. immitis* male; lane 2, *T. canis* female; lane 3, *T. canis* female; lane 4, *T. cati* male; lane 5, *T. cati* female; lane 6, *A. caninum* male; lane 7, *A. caninum* female; lane 8, *A. ceylanicum* male; lane 9, *A. ceylanicum* female, respectively.

previous report indicated that *D. immitis* was consisted of various protein components and their antigenicities were complicated. These parasites also originated from the same progenitor and still possess partially similar antigenicity (Song *et al.*, 2002). Therefore, certain interspecies antigenic epitopes may be isolated from one species which maybe useful in eliciting a highly specific immune response in a host toward cross-reactive epitopes in different species (Hayasaki *et al.*, 1994).

From our results, we suggested that this technique might be an alternative technique for heartworm detection especially in cats. However, screening with dog and cat population will be needed to get information about sensitivity and specificity of this technique.

Figure 2 Immunoblot patterns of adult *D. immitis* infection in cats, lane M, molecular weight markers (kDa); lane 1, *D. immitis* male; lane 2, *D. immitis* female; lane 3, *T. canis* male; lane 4, *T. canis* female; lane 5, *T. cati* male; lane 6, *T. cati* male; lane 7, *A. caninum* male; lane 8, *A. ceylanicum* male, respectively.

Table 1 Immunoblot patterns of peptide bands recognized by sera of *D. immitis* infected-dogs and cats.

Parasite nematodes	Dogs (kDa)								Cats (kDa)			
	25	33	46	50	64	71	108	250	33	46	50	64
Dirofilaria immitis	+	+	+	+		+	+	+	+	+	+	+
Toxocara canis			+	+						+		
Toxocara cati			+	+						+	+	
Ancylostoma caninum			+	+								
Ancylostoma ceylanicum				+	+							

kDa, Kilo Dalton

CONCLUSION

The results showed that specific peptide bands for the detection of *D. immitis* in dogs sera were 71, 46, 33, 25 kDa while in cats were 46 and 33 kDa. Further study will be needed to compare the unique protein pattern of *D. immitis* from both adult and microfilarial antigens and also to elucidate the immunological implications antigenic cross reactivity between *D. immitis* and other filarial nematodes such as *Brugia malayi*.

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