Antigenic Identification of Excretory-Secretory Products of Adult *Dirofilaria immitis*

Hideto KANEKO, Mineo HAYASAKI, and Isamu OHISHI

Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan

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ABSTRACT. Excretory-secretory (ES) products collected from adult *Dirofilaria immitis* cultured *in vitro* were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. ES products of male (M-ES) and female (F-ES) worms were separated into 16 and 21 bands by SDS-PAGE with Coomassie blue and silver staining, respectively. The antigenic bands were then analyzed by immunoblotting, using pooled sera from dogs that had naturally contracted *D. immitis*. Sera from dogs with microfilaremic infection showed 7 bands in M-ES and 10 bands in F-ES, while those from dogs with occult infection revealed 3 bands in M-ES and 10 bands in F-ES. Among these bands, those of 14, 18, 21, 22, 29, and 32 kilodaltons (Kd) were common to M-ES and F-ES, those of 39 and 44 Kd were specific to M-ES, and those of 20, 38, 43, 53, 63, 90, 110, 125 and 136 Kd were specific to F-ES.—KEY WORDS: *Dirofilaria immitis*, dog, excretory-secretory product, immunoblotting.

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Metabolites released from parasites are apparently recognized by their hosts as major parasitic antigens and cause various immunoreactions in the hosts.

Dirofilaria immitis inhabits the right ventricle and pulmonary arteries of dogs and releases metabolites called excretory-secretory (ES) products into the circulatory system [1, 3, 15–18]. Circulating ES products are essential to the occurrence of pathologic lesions of dirofilariosis as immune complexes, as in the case of nephropathy [3, 4]. Although circulating ES products are present in extremely small amounts, they can be detected by immunological assay using monoclonal antibodies [15, 16, 18]. Thus, detection of circulating antigens should facilitate the diagnosis of dirofilariosis [15]. ES products released from *D. immitis* include substances such as allergens [2] and neutrophil chemotactic factors [12] and are responsible for immune response to the hosts. Humoral response to ES products is qualitatively the same in dogs with microfilaremic (microfilaremic dogs) and occult (occult dogs) infection [13]. However, differences in the components of male and female ES products and immune response to these products in infected dogs are unknown. Analysis of ES products should thus help clarify the host-parasite relationship in immune response and lead to more effective applications of these products to immunological diagnosis.

In the present study, ES products of adult male and female *D. immitis* were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

MATERIALS AND METHODS

Collection of ES products: Adult *D. immitis* recovered from the right ventricle and pulmonary arteries of euthanatized dogs were washed three times with 0.85% sterilized saline and then three times with Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical Co., Ltd., Tokyo). Male and female worms were separately

incubated with Eagle's MEM at 37°C for 1 week in dark. The medium was collected and changed every 24 hr. Following removal of microfilariae from the medium of female worms with filter paper (pore size: 5 μm, Millipore Co., Bedford, Mass), the media of the male and female worms were separately concentrated by ultrafiltration (molecular weight exclusion 10,000, Amicon Co., Lexington, MA) at 4°C, dialyzed at 4°C for 72 hr against distilled water, and finally lyophylized. The lyophylized materials were reconstituted with adequate amounts of distilled water, and their protein concentrations were determined by the method of Lowry *et al.* [9]. The materials were designated as the ES products of male (M-ES) and female (F-ES) worms and stored at −50°C until use.

SDS-PAGE: A minislab gel consisting of 12.5% acrylamide and 0.1% SDS was used as described by Laemmli [8]. The samples were diluted to 500 μg protein/m*l* in the sample buffer (0.0625M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.00125% bromophenol blue) and heated for 5 min in boiled water. Ten micrograms of protein were then loaded per well, followed by electrophoresis at 100 volts for 120 min. The gels were subsequently stained with Coomassie brilliant blue R-250 (Merck, Darmstadt) or a silver stain kit (Daiichi Pure Chemicals Co., Ltd., Tokyo). Approximate molecular weights of the separated bands were estimated using molecular weight markers (Sigma Chemical Co., St. Louis, MO) consisting of myosin (205 kilodaltons (Kd)), β-galactosidase (116 Kd), phosphorylase B (97.4 Kd), bovine serum albumin (66 Kd), ovalbumin (45 Kd), glyceraldehyde-3-phosphate dehydrogenase (36 Kd), carbonic anhydrase (29 Kd), trypsinogen (24 Kd), soybean trypsin inhibitor (20.1 Kd) and α-lactalbumin (14.2 Kd).

Immunoblotting: Following SDS-PAGE, the protein bands in polyacrylamide gel were transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuel, Dassel) at 80 volts for 120 min, at 4°C, in transfer buffer (0.025M Tris, 0.192M glycine, 20% methanol, 0.1% SDS) [14]. The nitrocellulose membrane was blocked overnight in Tris-buffered saline (TBS) (0.02M Tris-HCl, pH 7.5, 0.5M NaCl) containing 3% gelatin, as a blocking reagent. The blocked membrane was treated at room temperature for 2 hr with canine sera diluted 1:500 in TBS containing 1% gelatin. Pooled sera from microfilaremic or occult dogs were used as sources of the above canine sera. The membrane was then treated at room temperature for 1 hr with peroxidase-conjugated goat anti-dog IgG (Cappel Laboratories Inc., Malvern, PA) diluted 1:1,000 in TBS containing 1% gelatin. Antigenic bands on the nitrocellulose membrane were developed with a substrate solution (0.5 mg/m*l* 4-chloro-1-naphthol, 0.015% H_2O_2 in TBS).

RESULTS

Collection of ES products: The protein concentrations of M-ES and F-ES collected from 13 male and 13 female worms were 711.8 μg (7.8 μg/worm/day) and 680.9 μg (7.5 μg/worm/day), respectively. Both male and female worms showed active motility, and female worms released microfilariae during culture.

Analysis of ES products by SDS-PAGE: M-ES and F-ES were separated into 8 and 12 bands by analysis with Coomassie blue staining; 8 bands in M-ES and 9 bands in F-ES were additionally detected by silver staining, thus giving a total of 16 bands in M-ES and 21 bands in F-ES (Fig. 1, Table 1).

Analysis of ES products by immunoblotting: Antigenicity of the bands in both M-ES and F-ES was assessed by immunoblotting.

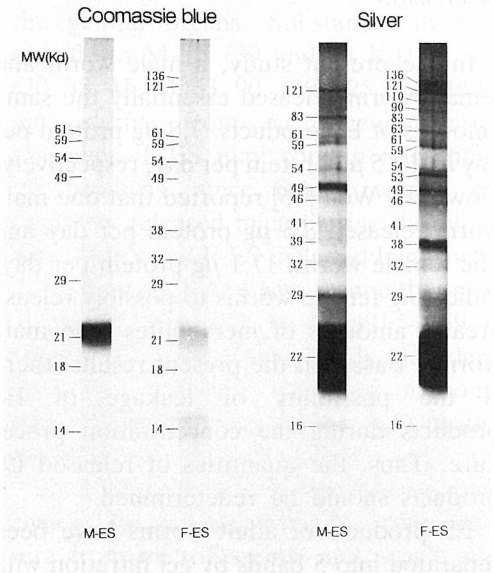

Fig. 1. Patterns of excretory-secretory products of male (M-ES) and female (F-ES) *D. immitis* as detected by Coomassie blue and silver staining. Approximate molecular weights of detected bands were determined using molecular weight markers.

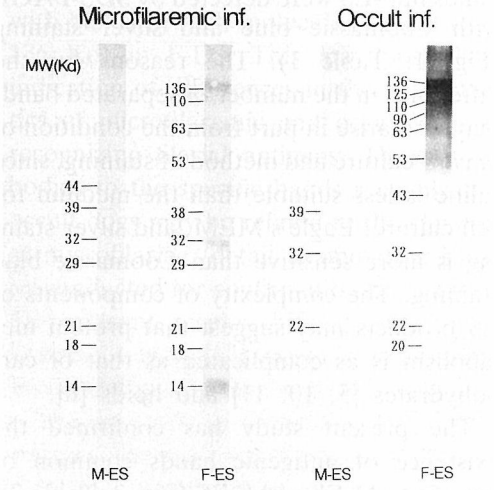

Fig. 2. Patterns of excretory-secretory products of male (M-ES) and female (F-ES) *D. immitis* detected by sera from dogs with microfilaremic and occult infections. Approximate molecular weights of detected bands were determined using molecular weight markers.

Table 1. Protein bands in excretory-secretory products of adult *D. immitis* as detected by Coomassie blue or silver staining

M.W.	M-ES[a]		F-ES[b]	
(Kd)	Coomassie blue	Silver	Coomassie blue	Silver
136			+[c]	+
121		+	+	+
110				+
90				+
83		+		+
63				+
61	+	+	+	+
59	+	+	+	+
54	+	+	+	+
53				+
49	+	+	+	+
46		+		+
41		+		+
39		+		
38			+	+
32		+	+	+
29	+	+	+	+
22		+		+
21	+		+	
18	+		+	
16		+		+
14	+		+	

a) Excretory-secretory products of male worms.
b) Excretory-secretory products of female worms.
c) Detected.

Eight antigenic bands in M-ES and 15 antigenic bands in F-ES were recognized by the pooled sera of microfilaremic and occult dogs (Fig. 2). Among them, bands of 14, 18, 21, 22, 29 and 32 Kd were common to M-ES and F-ES, those of 39 and 44 Kd were specific to M-ES, and those of 20, 38, 43, 53, 63, 90, 110, 125 and 136 Kd were specific to F-ES (Table 2). Pooled sera from microfilaremic dogs specifically recognized the bands of 14, 18, 21, 29 and 44 Kd in M-ES and those of 14, 18, 21, 29 and 38 Kd in F-ES, while pooled sera from occult dogs specifically recognized those of 22 Kd in M-ES and those of 20, 22, 43, 90 and 125 Kd in F-ES. Both groups of sera from infected dogs commonly recognized the bands of 32 and 39 Kd in M-ES and those of 32, 53, 63, 110 and 136 Kd in F-ES (Table 3).

Table 2. Antigenic bands in excretory-secretory products of male (M-ES) and female (F-ES) worms as detected by sera from dogs infected with *D. immitis*

M.W. (Kd)	Common bands[a]	Specific bands[b]	
		M-ES	F-ES
136			+[c]
125			+
110			+
90			+
63			+
53			+
44		+	
43			+
39		+	
38			+
32	+		
29	+		
22	+		
21	+		
20			+
18	+		
14	+		

a) Common bands to M-ES and F-ES.
b) Specific bands to M-ES or F-ES.
c) Detected.

Table 3. Antigenic bands in excretory-secretory products of adult *D. immitis* as detected by sera from dogs with microfilaremic and occult infections

M.W. (Kd)	Common bands[a]	Specific bands[b]	
		Microfilaremic	Occult
136	+[c]		
125			+
110	+		
90			+
63	+		
53	+		
44		+	
43			+
39	+		
38		+	
32	+		
29		+	
22			+
21		+	
20			+
18		+	
14		+	

a) Common bands detected by sera from dogs with microfilaremic and occult infections.
b) Specific bands detected by sera from dogs with two types of infection.
c) Detected.

DISCUSSION

In the present study, a male worm and female worm released essentially the same amounts of ES products: 7.8 μg protein per day and 7.5 μg protein per day, respectively. However, Weil [16] reported that one male worm released 8.3 μg protein per day and one female worm, 17.1 μg protein per day, indicating female worms to possibly release greater amounts of metabolites than male worms. Based on the present results, there is the possibility of leakage of ES products during the concentration procedure. Thus, the quantities of released ES products should be redetermined.

ES products of adult worms have been separated into 5 bands by gel filtration with DEAE-Sephadex A-50 after saline culture [2] and 2 major bands by SDS-PAGE with Coomassie blue staining [13]. Thus possibly these products may be comprised of simple substances. However, the present study has demonstrated the complexity of ES products. Indeed, 16 bands in M-ES and 21 bands in F-ES were detected by SDS-PAGE with Coomassie blue and silver staining (Fig. 1, Table 1). The reasons for the difference in the number of separated bands may also arise in part from the condition of *in vitro* culture and method of staining, since saline is less suitable than the medium for cell culture, Eagle's MEM, and silver staining is more sensitive than Coomassie blue staining. The complexity of components of ES products may suggest that protein metabolism is as complicated as that of carbohydrates [5, 10, 11] and lipids [6].

The present study has confirmed the existence of antigenic bands common or specific to M-ES and F-ES (Fig. 2, Table 2). Filarial ES products are produced by excretion, secretion and cuticle turnover [7]. Substances in bands common to M-ES and F-ES (14, 18, 21, 22, 29 and 32 Kd) may derive from the digestive system, worm

surface and metabolic systems unrelated to the genital organs. Substances in bands specific to M-ES (39 and 44 Kd) or F-ES (20, 38, 43, 53, 63, 90, 110, 125 and 136 Kd) may derive from each genital organ. However, F-ES may contain microfilarial ES products, since female worms continuously released microfilariae during culture. M-ES and F-ES included bands which were not detected by sera from either group of infected dogs (16, 41, 46, 49, 54, 59, 61, 83 and 121 Kd). These bands may derive from low antigenic materials or nonantigenic materials regarded as host proteins and substances unreleased *in vivo*.

Tamashiro *et al.* [13] reported no difference in the number of bands recognized by sera from microfilaremic and occult dogs. However, in the present study, some differences were detected in the patterns of immunoblotting between sera from both groups of infected dogs (Fig. 2, Table 3). Pooled sera from microfilaremic dogs reacted primarily with the bands of low molecular weight (14 to 32 Kd) in M-ES and F-ES while those from occult dogs reacted with bands of high molecular weight (53 to 136 Kd) in F-ES. This may possibly be indication of differences in the characteristics of microfilaremic and occult dogs for recognizing filarial antigens. Though antibodies to the specific bands recognized by occult dogs may be related to the clearance of microfilariae, detail examination should be conducted for confirmation of this point. The present results differ from those of Tamashiro *et al.* [13], possibly due to differences in the sources of pooled sera.

The presence of circulating ES antigens indicates the presence of parasites; thus, the detection of circulating antigens is a very reliable means for diagnosing *D. immitis* infection [15]. Understanding the characteristics of male and female ES products may lead to more effective applications of these products to immunological diagnosis. Further study on adult ES products as well as microfilarial ES products should be made for greater clarification of immune response in dogs infected with *D. immitis*.

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要　約

犬糸状虫成虫排泄・分泌抗原の抗原性分画の検出：金子秀人・早崎峯夫・大石　勇(東京農工大学農学部家畜内科学教室)——犬糸状虫(*Dirofilaria immitis*)成虫を *in vitro* 培養し，回収した排泄・分泌(excretory-secretory, ES)抗原の免疫生化学的性状をSDS-ポリアクリルアミドゲル電気泳動法(SDS-PAGE)とImmunoblotting法により分析した．SDS-PAGE終了後，Coomassie blue染色と銀染色によりES抗原の蛋白分画について検討した結果，雄成虫ES抗原(M-ES)では16種類，雌成虫ES抗原(F-ES)では21種類の蛋白分画が検出された．次に，Immunoblotting法により抗原性を有する分画について検討した結果，ミクロフィラリア陽性犬プール血清はM-ES中の7分画とF-ES中の10分画を検出し，M-ES及びF-ESの分子量14Kdから32Kdの比較的低い分子量の分画と強く反応した．また，occult感染犬プール血清は，M-ES中の3分画とF-ES中の10分画を検出し，F-ESの53Kdから136Kdの比較的高い分子量の分画と強く反応した．これらの両血清にて検出された分画のうち分子量14，18，21，22，29及び32KdのM-ESとF-ESに共通して見られた．39及び44KdのM-ESのみに見られた．20，38，43，53，63，90，110，125及び136KdのF-ESのみに見られた．