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Immunological Responses of Dogs Experimentally Infected with *Dirofilaria immitis*

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ABSTRACT

Three dogs were experimentally infected with *Dirofilaria immitis*. All dogs were euthanised at 30, 36 and 37 weeks after inoculation of *D. immitis* for the recovery of adult worms. Three cases accounted to 42.91 % recovery of inoculated worms. Serum samples from dogs experimentally inoculated with *D. immitis* were analyzed by ELISA and immunoblotting methods. Antibody titers of dogs detected by ELISA peaked between 7 and 14 weeks then decreased between weeks 15 to 24 followed by another increase during weeks 25 to 30 and persisted throughout the remainde of the experiment period. Analysis of adult *D. immitis* protein stained with Coomassie brilliant blue R-250 indicated separately more than 10 bands, and the major bands were 22, 40, 46, 56, 70, 72 and 89 kDa. Antigenic identification of extracts antigens of adults *D. immitis* by immunoblotting analysis revealed several bands from pooled sera of patent infection (30 weeks after inoculation). The detected bands were 24, 70, 80 and 110 kDa, 22, 72 and 84 kDa, and 58 and 72 kDa in dogs 1, 2 and 3, respectively. Results of antibody titers reached high levels on the 4th molting stage after inoculation of infective larva (L3), and reinforced previous findings that high molecular weight regions are detected in young animals.

Key words: *Dirofilaria immitis*, experimental infection, ELISA, immunoblotting

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Introduction

Dirofilaria immitis, commonly called canine heartworm, is the cause of a serious parasitic disease of dogs endemic in temperate, subtropical and tropical countries [4]. In epizootiologic surveys of dirofilariasis in dogs, various methods for determining infection status have been used, including microscopic examination of blood smears, blood sample concentration techniques, Knott's test for detecting circulating microfilariae, examination for identification of adult worms at necropsy, and radiographic and angiographic evaluations [2,23,26]. A major problem in making diagnosis is the dirofilariasis without microfilaremia (occult dirofilariasis) which has been shown to occur in 10 % to 67 % of dogs that have been infected naturally. This has been attributed to single-sex infection, presence of immature adults or immune-mediated clearance of microfilariae [21]. These occult infections are very difficult to diagnose by microscopic examination of blood, so that, the veterinary practitioners usually make the diagnosis based on clinical signs, radiographic evidence, and other indirect indication of infection [21].

The occult *D. immitis* infections pose the most serious diagnostic challenge. And, sometimes, even in patent infections, obstacles could be avoided in the diagnosis of dirofilariasis due to the low numbers of microfilariae or the difficulty in distinguishing microfilariae of *D. immitis* from those of *Dipentalonema reconditum* [5]. To overcome these problems, various immunological techniques have recently been developed [3,5,6,8-12,14,15,18,19,28]. Among these methods, enzyme-linked immunosorbent assay (ELISA) and immunoblotting analysis have been recognized as simple, sensitive and particularly suitable for the parasitic diseases like *D. immitis* infection [5,6,9,11]. The present study was performed to elucidate the immunological responses of dogs experimentally infected with infective larvae of *D. immitis* using ELISA and immunoblotting.

Materials and Methods

Experimental animals

Five healthy mongrel juvenile dogs of approximately three months of age were housed in mosquito-proof runs, and treated with piperazine for intestinal parasites prior to experimentation.

Experimental infection

Three dogs were experimentally infected with *D. immitis* by the procedures described by Hayasaki [8]. Briefly, mosquitoes, Aedes togoi, collected during the larval stage from their natural spawning areas were reared under laboratory conditions and inoculated by feeding on a dog with about 200 circulating microfilarial (Mf) counts per 20㎕ of blood (Table 1). Two dogs were used as the negative control. Infective larvae (L3) of *D. immitis* were recovered from the proboscis of the infected mosquitoes 10 to 14 days after blood feeding and suspended in saline during microscopic observation. Three dogs were subcutaneously injected in the inguinal region with each dog receiving 228 (dog No. 1), 278 (dog No. 2) and 248 (dog No. 3) infective larvae (L3), respectively. Experimental dogs were euthanised at 30, 36 and 37 after inoculation for the recovery of adult worms.

Serum samples

The blood was collected from the cephalic vein once a week, and serum separated and stored at -80℃ until analysis.

Circulating Mf counts

Dogs were screened for Mf by concentration method, and microfilarial density in 20㎕of blood was measured by counting Mf on methylene blue stained smears [20].

Preparation of *D. immitis* antigen

The crude extracts of *D. immitis* were prepared as previously described [7]. Briefly, the antigens used in this study were extracted from adult worms of *D. immitis* by phosphate buffered saline (PBS, pH7.2, 0.1M). These worms were homogenized and sonicated by tissue homogenizer (15min, 4℃) and ultrasonicator (50 watt, 15min, 4℃), respectively, and then allowed to incubate overnight at 4℃. After centrifugation at 18,000g, the supernatant as antigen was collected and kept at -80℃. The protein concentration of the antigen was determined using the methods of Lowry et al.[17].

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed by methods of Grieve et al.[6]. *D. immitis* antigen was diluted to 10㎍/㎖ in PBS. 100㎕ of antigen was dispensed into each well and incubated for 30 minutes at room temperature. 200㎕ of 1% bovine serum albumin(BSA) solution in PBS was added and the plates were incubated at 4℃ overnight. The wells were washed three times with 200㎕ of 0.1% Tween 20 in PBS (TPBS) in 3 minutes for each wash. 100㎕ of the serum from the test subject was diluted to 1:100 in PBS and added to each well. The microplates were incubated at 37℃ for 1 hour, washed with TPBS, and 100㎕ of peroxidase-conjugated goat anti-dog IgG (ICN Pharmaceuticals, Inc., USA) diluted to 1:200 in PBS, was added to each well and incubated at 37℃ for 1 hour, then washed with TPBS three times (3 minutes for each wash). A fresh preparation of substrate working solution was made from 1% o-phenylenediamine in methanol, 0.1M citrate buffer (pH4) and 3% hydrogen peroxide, and 100㎕ of the solution was added to each well and left at room temperature for 30 minutes. The enzyme reaction was stopped by adding 50㎕ 4 N sulfuric acid and values of optical density (OD) were read at 498㎚ (Sanko Junyaku co. Japan). The specificity of the assay was evaluated by cross-checking with the DiroCHEK®kit (Synbiotics co., San Diego, USA) using five of the known positive and ten known negative serum samples.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A minislab gel consisting of 12.5% acrylamide and 0.1% SDS was used as described by Laemmli [16]. The serum samples were diluted to 500㎍ protein/㎖ in the sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.00125% bromophenol blue) and heated for 5 minutes in boiling water. 10㎎ of protein was

Table 1. Worm burden of dogs experimentally inoculated with *D. immitis*

Dog No.	No. of L3 injected	Duration of infection (weeks)	No. of adults worm recovered (F/M)	Recovery rate
1	248	37	151(95/96)	60.88
2	278	31	31(16/15)	11.15
3	228	38	127(66/61)	56.70
Mean(%)				42.91

L3: Infective larvae
F/M: Female/Male

then loaded in each well, followed by electrophoresis on 20㎃ currents at 4℃ for 120 minutes. The gels were subsequently stained with Coomassie brilliant blue R-250 (Katayama chemical, Japan). Approximate molecular weight of separated bands were estimated using molecular weight markers (BIO-RAD, USA).

Immunoblotting

Following SDS-PAGE, the protein bands in the polyacrylamide gel were transferred electrophoretically to a nitrocellulose membrane sheets (pore size 0.45㎛, BIO-RAD, USA) at 80 volts at 4℃ for 120 minutes, with a Tris-glycine electro-transfer buffer (0.025 M Tris, 0.192 M glycine, 20% methanol and 0.1% SDS). The nitrocellulose sheets were blocked for overnight in Tris-buffered saline (TBS) (0.02M Tris-HCl, pH 7.5, 0.5M NaCl) containing 3% gelatin (BIO-RAD, USA). The nitrocellulose sheets were cut into strips and placed into plastic trays. Strips were separately reacted to test samples that were diluted 1:500 in TBS containing 1% gelatin, at room temperature for 2 hrs, peroxidase-conjugated goat anti dog IgG (ICN Pharmaceuticals Inc, USA) serum diluted to 1:500 in TBS containing 1% gelatin. Strips were then washed with TBS. Substrate solution consisting of 15㎎ of 4-chloro-1-naphtol dissolved in 5㎖ methanol, 15㎕ hydrogen peroxide, was added to each well and allowed to develop at room temperature for 20 minutes. The reactions were stopped by replacing the substrate with TBS.

Results

Recovery of worms at necropsy

Dogs were euthanised on week 30, 36 and 37 post inoculation for the recovery of adult worms. At necropsy, 151 adult worms (95 females and 56 males) in Dog No.1, 31 adult worms (16 females and 15 males) in Dog No.2 and 127 adult worms (66 females and 61 males) in Dog No.3. were recovered from the right ventricle and pulmonary arteries. These represent approximately 42.91% recovery of inoculated worms.

ELISA of sera from experimentally infected dogs

In the Dog No.1, No.2 and No.3, antibody titers in the sera of inoculated dogs were significantly detected 7 weeks post inoculation and persisted until 14 weeks. Antibody titers of dogs detected by ELISA peaked between 7 and 14 weeks then decreased between weeks 15 to 24 followed by another increase during weeks 25 to 30 and persisted throughout the remainde of the experiment period.

Analysis of adult *D. immitis* protein by Coomassie brilliant blue R-250 staining

D. immitis proteins were separated into more than 10 bands stained with Coomassie brilliant blue R-250 (CBB) staining, the major bands were 22, 40, 46, 56, 70, 72 and 89 kDa.

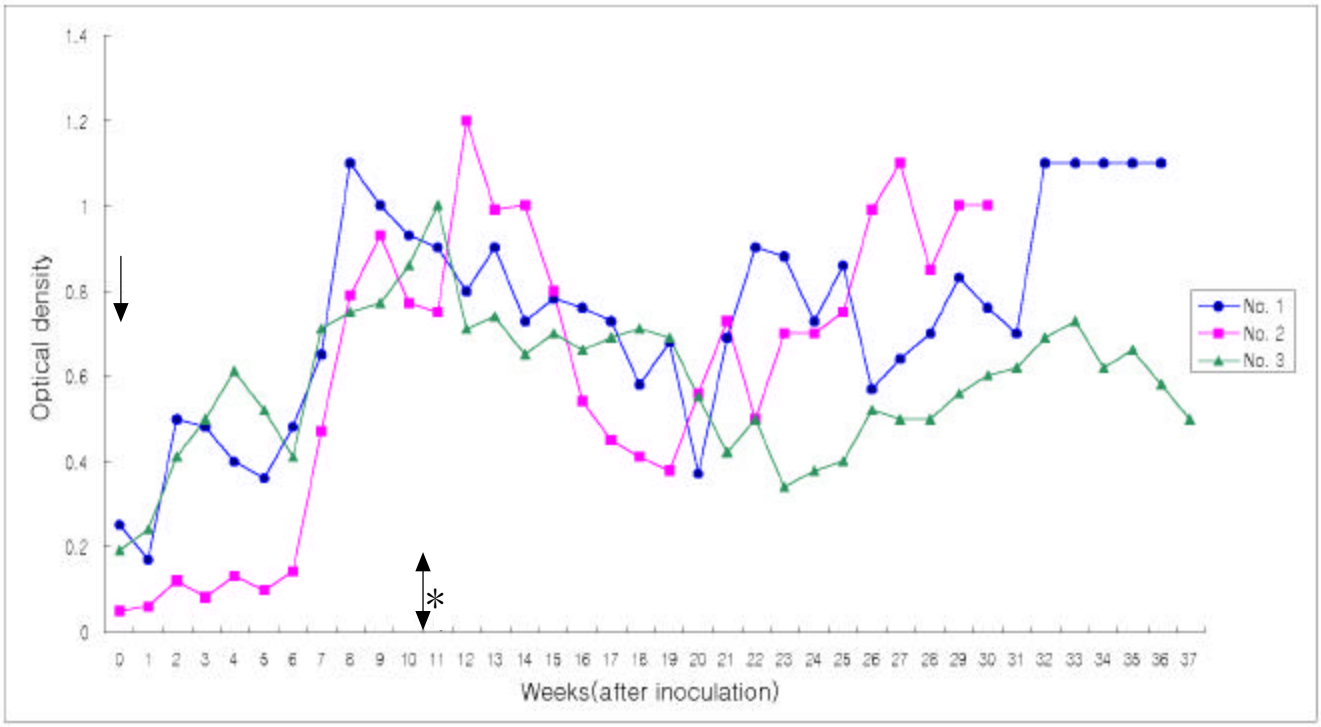

Fig 1. Antibody responses of dogs to experimental *D. immitis* infection by ELISA using *D. immitis* antigens. *The range of optical density of control group. The arrow indicates the day of inoculation.

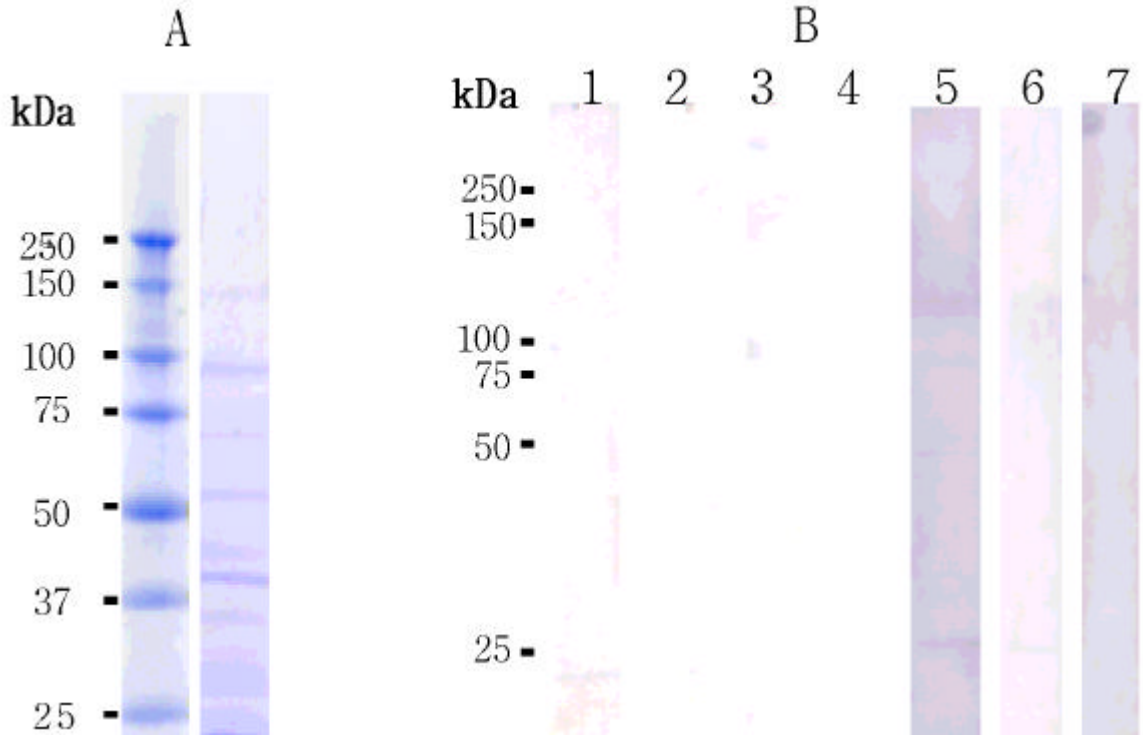

fig. 2. Protein fractions of adult *D. immitis* were separated by SDS-PAGE stained with Coomassie brilliant blue R-250(A), and *D. immitis* antibody responses with *D. immitis* antigen by immunoblotting analysis(B). Lane 1:standard marker, lane 2-4: negative control, lane 5-7: patent infection (lane 2,5: No.1, lane 3,6: No.2, lane 4,7: No.3).

Antigenic identification of crude extract antigens of adult *D. immitis* by immunoblotting

Pooled sera of patent infection (30 weeks after inoculation) from Dog No.1 revealed four antigenic bands with 24, 70, 80 and 110 kDa. Dog No.2 showed three antigenic bands of 22, 72 and 84 kDa, while two antigenic bands were detected in Dog No. 3 with 58 and 72 kDa.

Discussion

Experimental *D. immitis* infections in dogs have been performed by various investigators [6, 8, 27, 29]. The recovery rate of adult worms from dogs experimentally inoculated with the infective larvae of *D. immitis* were 45%, Hayasaki [8], 66%, Thrall et al.[27] and 50%, Wong et al.[29], respectively. In this study, three cases accounted to 42.91 % recovery of inoculated worms. Although this study did not confirm the correlation between antibody titer and the number of adult worm, Grieve et al.[6] reported that there was no relationship between them.

The ELISA used in conjunction with a Knott's test, exposure history, clinical signs, laboratory results, and radiographic changes are useful for studying seroepizootiologic pattern and risk factors of heartworm infection. ELISA testing has been shown to be capable of identifying prepatent infections [6]. Diagnosis of prepatent infection or asymptomatic occult infection is important to the practitioner who may be preparing to initiate a heartworm preventive program based on negative results of a Knott`s test. In addition, experimental studies of beagles have shown abnormalities of pulmonary arteriograms 6 months after inoculation with infective *D. immitis* larvae, at which time the dogs were still amicrofilaremic and results of indirect fluorescent antibody testing for antimicrofilarial antibodies were negative [22]. ELISA testing for the detection of *D. immitis* provide a useful and improved assay for serological characterization and detection of infection. Grieve et al.[6] reported that ELISA titers were not significantly increased until 11 or 16 weeks, and remained at maximum levels for the duration of the observation period. However, the present study revealed that antibody levels were detected as early as 7 weeks post inoculation, and developed continuously to 14 weeks, and then diminished to 15 to 24 weeks, then climbed again to 25 to 30 weeks, and high titer levels persisted throughout later experiment time. One explanation of these different results may be the different responses of individual dogs to the variable extent and duration of parasitic exposure [13]. Another explanation may be that different antibodies are being detected by different protocols used [11].

Konno et al.[15] reported that protein bands in extracted

antigen of *D. immitis* was detected by Coomassie brilliant blue R-250 staining were 44 bands (14 to 230 kDa). The present study revealed that *D. immitis* protein was separated into more than 10 bands (22 to 89 kDa) by Coomassie brilliant blue R-250 staining, the major bands detected were 22, 40, 46, 56, 70, 72 and 89 kDa. Although the number of protein bands was less than those of Konno et al.[15], similar protein bands were detected by Coomassie brilliant blue R-250 staining. Immunoblotting in the *D. immitis* infection helps to clarify the relation of antigen- antibody in immune response, a more effective application of immunologic diagnosis [9,12,24]. Tamashiro et al.[24] and Boto et al.[1] reported that low molecular weight reactivity as more prevalent in older and /or more heavily infected dogs in experimental *D. immitis* infection by means of immunoblotting. In natural *D. immitis* infection, Tanaka et al.[25] reported the trends in the distribution of immunoblotting patterns with either young animals and/or mild infection tended to reveal antibodies with reactivity to antigen in the higher molecular weight regions of immunoblotting (more than 80 kDa), he also reported that the majority of antibodies with reactivity to antigens in the low molecular weight regions (less than 37 kDa), occurred more frequently in older dogs and/or heavier infections. The results of Tamashiro et al.[24], Tanaka et al.[25] and Boto et al.[1] suggested that immunoblotting will produce similar results in both experimental and natural infections.

In the present study, antigenic identification of crude extract antigens of adults *D. immitis* by immunoblotting analysis revealed several bands from the pooled sera of patent infection (30 weeks after inoculation). The detected bands were 24, 70, 80 and 110 kDa in Dog No.1, 22, 72 and 84 kDa in Dog No.2, and 58 and 72 kDa in Dog No.3. The results presented here are similar to those of Tamashiro et al.[24] and Boto et al.[1], in which the higher molecular weight regions were detected by immunoblotting in young animals (more than 70 kDa).

In summary, antibody titers by ELISA reach high levels at the 4th molting stage after inoculation of L3, and the higher molecular weight regions are mainly detected by immunoblotting in young animals. Further studies by other techniques are needed to elucidate immunological responses in *D. immitis*-infected dogs.

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