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Immunoblotting analysis of canine *Brugia malayi* microfilarial antigens

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Abstract. *Brugia malayi* is a filarial parasite that causes lymphatic filariasis in human beings. Kerala (India) is endemic for human Brugian filariasis. *B. malayi* microfilariae, similar to that causing filariasis in human beings, have been identified in dogs in Kerala. This is the first report of western blotting analysis of canine *B. malayi* microfilarial proteins. SDS PAGE analysis of *B. malayi* microfilarial protein revealed 5 major protein bands with molecular weights of 125, 80, 64, 54 and 27 kDa. Among these, the prominent bands were those having molecular weights of 64, 54 and 27 kDa. We raised polyclonal antibodies against these somatic proteins of dog microfilariae in a rabbit. The polyclonal antibodies recognized predominantly the 54 kDa and 64 kDa antigens in a Western blot analysis. Based on previous publications with *B. malayi*, these two protein bands appear to be important for diagnosis and for vaccine development against lymphatic filariasis.

INTRODUCTION

B. malayi is a filarial parasite that causes lymphatic filariasis in humans. Kerala (India) is endemic for human Brugian filariasis. Mass drug administration programs advocated by the World Health Organization for the control of lymphatic filariasis are based on the assumption that human beings are the only reservoir of infection (Bockarie *et al*., 2009). Recently *B. malayi* microfilariae, similar to that causing filariasis in the humans, have been identified in dogs (Ambily *et al*., 2011). Detection of zoonotic *B. malayi* in domestic dogs, in a highly endemic area for lymphatic filariasis, poses great threat to the control and eradication of human lymphatic filariasis. Therefore, accurate diagnosis of canine microfilariasis and successful treatment and elimination of microfilariae from canine hosts are essential for the success of disease eradication.

The most common diagnostic procedures for filarial infections in dogs are detection of microfilaremia, detection of circulating antigens or detection of antibodies raised against filarial antigens. Antigen detection is less sensitive where as antibody detection is less specific because of cross reactions. The use of electrophoretical and Western blot techniques in the field of parasitology is useful in immunodiagnosis.

In this study a preliminary analysis of canine *B. malayi* microfilarial antigens was carried out aiming to identify the immunogenic antigens. This is the first report of western blotting analysis of *B. malayi* microfilarial antigens isolated from dogs.

MATERIALS AND METHODS

Dogs presented to the University Veterinary Hospital, Mannuthy, Kerala were screened by wet blood film examination for microfilariae. Thick blood smears prepared from positive animals were subjected to giemsa staining and acid phosphatase staining. For acid

phosphatase activity, blood smears were air dried, fixed in chilled acetone for 1 minute, and stained using the naphthol AS-TR phosphate method of Barka (Chalifoux and Hunt, 1971). Identification of *B. malayi* microfilariae was based on the somatic distribution of the acid phosphatase activity as described by (Kobosa *et al.*, 2004 and Chirayath *et al.*, 2015).

Two clinical cases with severe microfilaremia (more than 5 microfilariae per low power field) identified as belonging to *B. malayi* based on the presence of sheath were selected for these studies.

Isolation of Microfilariae from Blood

Isolation of live microfilariae from fresh blood free of host cells was done by density gradient centrifugation using Ficoll 400, immediately after collection (Dissanayake, 2000).

For isolation of microfilariae, 10 mL of blood was collected from microfilaremic animal in EDTA. The blood sample was diluted with 10 mL of sterile PBS mixed with 2 mM EDTA. Three milliliter of 10% ficoll solution was pipetted in two 15 mL centrifuge tubes. Ten mL of diluted blood were carefully added over the ficoll layer without mixing the phases. Samples were then centrifuged at 400 x g for 30 minutes at room temperature in a swinging bucket rotor without brake. The contents in the centrifuge tubes were separated into three clear layers. Bottom layer consisted of red blood cells with a creamy coloured lining of granulocytes. The next layer was of ficoll with a hazy top margin containing mononuclear cells and the top layer was plasma mixed with PBS. The top layer was carefully aspirated and removed. The ficoll layer from both the centrifuge tubes was carefully transferred into a fresh 15 ml centrifuge tube, without disturbing the bottom layer of erythrocytes and leaving a little portion of ficoll layer just above erythrocytes. One drop of ficoll layer was examined under low power objective (10X) of light microscope to confirm the presence of live microfilariae.

The transferred ficoll layer was diluted with equal volume of sterile PBS and centrifuged at 400 x g for 15 minutes at room temperature. The microfilariae were sedimented at the bottom. The supernatant was aspirated and discarded. The procedure of dilution with PBS and centrifugation was repeated three times to remove the contamination of host serum and cells. Finally the microfilarial pellet was suspended in 3 mL of sterile PBS and stored at 4°C till the extraction of microfilarial protein within 48 hours. The microfilariae remained live in sterile PBS at 4°C for one week.

Extraction of Microfilarial Protein

The extraction of microfilarial protein was done by sonication (Maizels *et al.*, 1991). Three millilitres of live microfilarial suspension were used for extraction of proteins. An aliquot of 0.05 mL of cocktail protease inhibitor (Sigma catalogue no. p 2714 – 1BTL) was added into the centrifuge tube. The mixture was sonicated for 4 minutes on ice at 400 W in pulsed mode using Branson 450 Sonifier. The suspension was allowed to stand on crushed ice for 60 min, agitating occasionally. The disrupted parasite suspension was centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was collected and stored at -20°C. Protein concentration of the extract was measured by Lowry's method (Lowry *et al.*, 1951).

Analysis of Microfilarial Protein by Poly Acrylamide Gel Electrophoresis in the presence of Sodium Dodecyl Sulfate (SDS-PAGE)

The profile of *B. malayi* microfilarial proteins was analysed by one – dimensional SDS-PAGE (5% stacking gel and 12% resolving gel) (Laemmli, 1970) in a vertical electrophoresis apparatus (Axygen). In brief, 40 µl of each of the sample solubilized in 2X gel loading buffer were incubated at 100° C for 5 minutes and spun for 30 seconds. The samples were then run through the gel along with the standard protein medium

range molecular weight marker (Termo Scientific). The stacking gel was then snipped off and the resolving gel was subjected to the Coomassie brilliant blue G staining solution (2%) for one hour. Destaining was carried out for six hours with four changes of destaining solution containing glacial acetic acid (10%) and methanol (30%), till the background became clear. The gel was then transferred to distilled water and viewed in white light and photographed. Molecular weights of unknown proteins were determined by plotting a graph using the relative migration distance and the logarithm of molecular weights of the standard medium range protein ladder (Thermo Scientific, USA) and then interpolating the relative migration distances of unknown proteins to the graph (*Bulletin 3133 US/EG Rev Bio-Rad*).

Raising Hyper Immune Serum against Microfilarial Protein in Rabbit

One New Zealand White male rabbit weighing approximately 2kg was obtained from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy. Five hundred microgram of protein corresponding to 1 mL of microfilarial protein suspension (concentration- 0.05 mg/mL) in one mL of Freund's complete adjuvant (FCA) were injected intramuscularly. Two booster doses of microfilarial antigen were given in Freund's incomplete adjuvant (FIA) at weekly intervals. Third booster dose was given intravenously one week after the second booster. The rabbit was bled by cardiac puncture one week after the last injection. Whole blood was allowed to clot at room temperature for 1 hour and centrifuged at 1000 x g for 5 minutes to separate serum. Serum was collected and stored at -20°C until used.

Western Blot Analysis

The proteins fractionated in the SDS-PAGE gel were transferred onto a nitrocellulose membrane using semi- dry western blotting apparatus following the standard protocol (Towbin *et al*., 1979). The membrane was then incubated in blocking buffer (100mM Tris-HCl buffer with 0.1% Tween 20 and 5% bovine serum albumin) for two hours at 37°C, followed by washing twice in PBS with 0.1% Tween 20 (PBS-T). The membrane was incubated with primary antibody (*i.e*., the hyperimmune serum raised against microfilarial protein diluted in blocking buffer at 1:100 dilution) for one hour at 37°C with constant agitation. The membrane was washed four times by agitation with sufficient PBS – T, 15 min each followed by incubation at 37°C in suitably diluted goat anti- rabbit immunoglobulin G- horse raddish peroxidase conjugated [Millipore, Cat. No. 12-348] and diluted in blocking buffer (1:2500), for one hour with constant agitation. The membrane was washed four times with PBS-T as described earlier and the blots were developed in the chromogenic visualization solution (100 mM Tris Chloride (pH 7.5)-5mL, Diaminobenzidine stock solution (40 mg/mL)-100 µl, Nickel Chloride stock (80 mg / ml)-25 µl and 3% H_2O_2-15 µl) at room temperature, with mild rocking, until colour developed. The reaction was terminated by washing the membrane with distilled water. Membrane was air-dried and photographed.

RESULTS

Identification of B. malayi microfilariae

Acid phosphatase staining of sheathed microfilariae was similar to that of *B. malayi* in which, four points in the microfilariae have taken bright red colour, indicating the acid phosphatase activity in these four areas (Figure 1). The four regions were amphids, excretory pore, anal pore and phasmid.

Protein Profile of B. malayi isolated from dogs

The protein concentration of the microfilarial protein extract was 0.5 mg/mL. SDS-PAGE analysis of *B. malayi* microfilarial protein revealed 5 protein bands of molecular weights 125, 80, 64, 54 and 27 kDa. Among them prominent bands were 64, 54 and 27 kDa (Figure 2A).

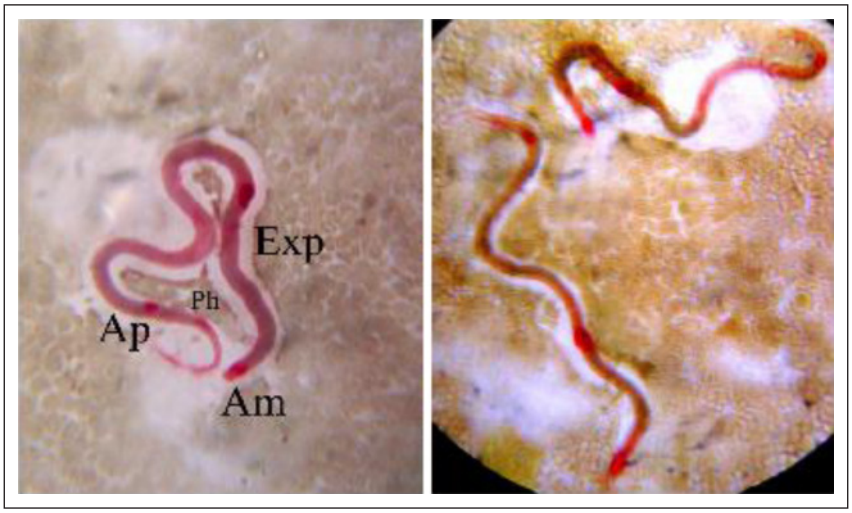


Figure 1. *Brugia malayi* microfilariae showing acid phosphatase staining at amphid (Am), excretory pore (EP), anal pore (AP) and phasmid (Ph) [4 point staining pattern].

Identification of Immunogenic Proteins of B. malayi

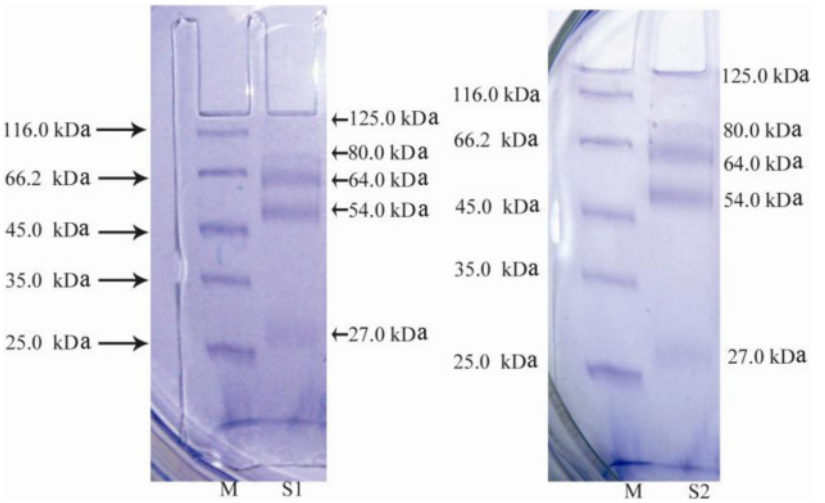
Western blotting analysis of microfilarial extract using antiserum from a rabbit immunized against this protein fraction revealed only two bands, corresponding to 64 kDa and 54 kDa. Out of these two, 64 kDa was more prominent. The results suggest that the most immunogenic proteins of *B. malayi* microfilariae have molecular weights of 64 and 54 kDa (Figure 2B).

DISCUSSION

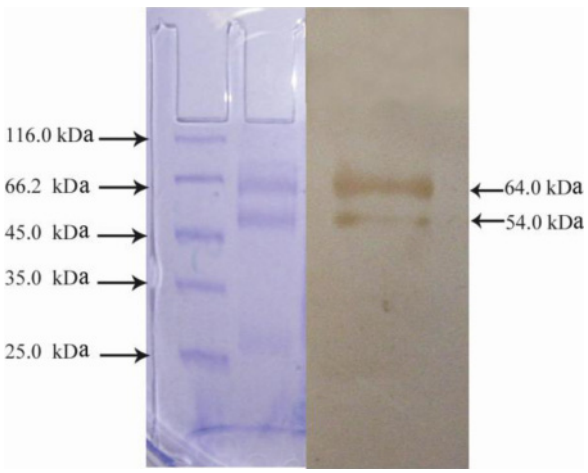
Results presented in this study demonstrate the presence of zoonotic *B. malayi* infections in the dog. Polyclonal antibodies developed against the somatic antigens of the dog derived *B. malayi* microfilariae predominantly recognized two major antigens of *B. malayi* at 54 kDa and 64 kDa. Further studies are needed to characterize the two protein bands by mass spectrometry and determine the usefulness of these two protein bands in the diagnosis and/or development of vaccine against *B. malayi* infections in the dogs.

SDS-PAGE analysis of *B. malayi* microfilarial proteins revealed five bands with molecular weights of 125, 80, 64, 54 and 27 kDa. Among these bands only those with 64, 54 and 27 kDa were prominent. Similar protein bands were observed (Parab *et al.*, 1988) on SDS-PAGE gels of human *B. malayi* microfilarial extract. The protein profile of *Wuchereria bancrofti* microfilariae revealed an array of antigens with apparent molecular weights of 14, 35, 42, 63, 88, 97 and 200 kDa (Saverimuttu *et al.*, 2000). SDS-PAGE electrophoresis of somatic extracts of adult *D. immitis* revealed many protein bands ranging from 6.5 kDa to 205 kDa (Oge *et al.*, 2005).

In the present study western blotting analysis indicated that the most immunogenic protein of *B. malayi* microfilariae have molecular weights of 64 kDa and 54 kDa. Among these, the 64 kDa band was prominent. This finding agrees with the earlier studies on human *B. malayi* microfilarial protein (Parab *et al.*, 1998 and Sahoo *et al.*, 2009). Immune sera developed using different fractions of human *B. malayi* microfilarial extract recognized antigens 76 kDa, 66 kDa and 62 kDa in common (Parab *et al.*, 1998). F6 fraction (54–68 kDa fraction) of *B. malayi* adult worm extract was identified as immunogenic and was suggested as vaccine candidate. The present study also identified 54 and 64 kDa proteins which might correspond to the F6 fraction



A. SDS-PAGE of microfilarial proteins.
M – Molecular weight marker, S1 – sample 1, S2 – Sample 2.



B. Western blot analysis of microfilarial proteins showing bands 64.0 & 54.0 kDa.

Figure 2. SDS-PAGE and Western blot analysis of microfilarial proteins.

previously described, as immunogenic and as putative vaccine candidate. According to Rajasekariah *et al.* (1988) immunogenic proteins of *B. pahangi* microfilariae were of 150, 75, 42 and 25 kDa. Among these the protein with 25 kDa was highly immunogenic and was capable of clearing circulating microfiariae in mice after immunization. Immunogenic proteins of human *W. bancrofti* microfilarial extract have 14 and 42 kDa, out of which 42 kDa antigen was shared by two developmental stages of *W. bancrofti* namely L3 and microfilariae (Saverimuttu *et al.*, 2000). Studies on the sera of dogs affected with *D. immitis* by western blotting analysis observed that specific protein bands for heartworm infection have 85, 66, 42, 20, 16.2 and 14.5 kDa (Oge *et al.*, 2005). Another study

(Saini *et al*., 2011) investigated the utility of biodegradable polymeric lamellar substrate particles of poly (l-lactide) (PLSP) as adjuvant for filarial antigen preparations and compared the immunogenicity of human *B. malayi* adult worm extract (BmA) and its SDS-PAGE resolved the same 54–68 kDa fraction. The results revealed that F6 is more immunogenic than whole worm extract. However the results of studies of Dash *et al.* 2011 on human *B. malayi* abundant larval transcript-2 (BmALT-2) protein (molecular weight around 25 kDa) produced by L3 larvae of *B. malayi* suggest the protein as a potential vaccine candidate.

As in the case of human *B. malayi* infection, immunogenic proteins of *B. malayi* microfilariae isolated from dogs in the present study seem to correspond to the F6 fraction described in human microfilariae, in the 54–68 kDa range. The results show that proteins corresponding to the F6 fraction in canine *B. malayi* microfilariae extracts are immunogenic, and future research can be directed in its usefulness in developing diagnostic tools for filariasis in dogs.

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