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Development of Antigen Detection ELISA for the Diagnosis of Brugian and Bancroftian Filariasis Using Antibodies to Recombinant Filarial Antigens Bm-SXP-1 and Wb-SXP-1

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***Abstract*: Antibodies specific to recombinant filarial antigens Wb-SXP-1 and Bm-SXP-1 have been used to develop a sandwich ELISA for the detection of circulating filarial antigen (CFA) in sera from patients with lymphatic filariasis caused by *Wuchereria bancrofti of Brugia malayi*. In patients with *W. bancrofti* infections, a high proportion of microfilaria (mf) positive (MF) and low proportions of patients with chronic pathology (CP) and endemic normals (EN) showed the presence of CFA. Similarly in patients with brugian infections a high proportion of mf positive individuals contained CFA while none of the patients with chronic pathology or endemic normals showed the presence of CFA. Sera from patients with other parasitic infections (OPI) like *O. volvulus, Loa loa, Ascaris lumbricoides* and from individuals residing in areas non-endemic to filariasis did not exhibit any reactivity. This assay shows promise for the detection of microfilaremic infections in lymphatic filariasis and its usefulness as a diagnostic tool especially in *B. malayi* infections, needs to be further evaluated.**

***Key words*: Filariasis, *Wuchereria bancrofti*, *Brugia malayi*, ELISA, Recombinant antigen**

The lymphatic dwelling filariae *Wuchereria bancrofti* and *Brugia malayi* are estimated to infect approximately 119 million people worldwide (8). Affected individuals may suffer from clinical manifestations such as elephantiasis, hydrocoele, and adenolymphangitis or may have clinically silent manifestations of infection that are associated with subtle abnormalities of the lymphatic system. A diagnostic technique based on direct demonstration of the parasite or parasite products such as circulating filarial antigen (CFA) is the only definite way to detect infection in humans.

Various groups of researchers have developed diagnostic tests that detect and quantify CFA (1, 3−5, 10, 13, 15, 18) in individuals harbouring *Wuchereria bancrofti*. At present, monoclonal antibody based assays in an ELISA (9) and a rapid-format card test (16, 17) are being employed globally for the identification of infection with *W. bancrofti*. In brugian filariasis no test is available to detect CFA and night blood smear to identify microfilaria (mf) positive (MF) individuals is the only reliable method for diagnosis.

Thus, there is a need to develop immunodiagnostic tests that identifies CFA in *B. malayi* infections. In the

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Abbreviations: BmA, soluble extract of adult *Brugia malayi*; Bm-SXP-1, *Brugia malayi* derived SXP; CFA, circulating filarial antigen; CP, patients with chronic pathology; EN, asymptomatic amicrofilaremic individuals; IMAC, immobilized metal affinity chromatography; MF, asymptomatic microfilaremic patients; mf, microfilaria; NEN, individuals from areas non-endemic to filariasis; OPI, other parasitic infections; Wb-SXP-1, *Wuchereria bancrofti* derived SXP.

present study we have developed an ELISA for the detection of CFA in lymphatic filarial infections caused by both *W. bancrofti* or *B. malayi* using antibodies to the recombinant filarial antigens Bm-SXP-1 and Wb-SXP-1 (11).

Materials and Methods

Study population. Serum samples from a variety of patients were used (Table 1). The serum samples from *W. bancrofti* infected individuals were collected from an area endemic for bancroftian filariasis in and around Chennai, India. The microfilarial status of the individuals was assessed by membrane filtration of 1 ml of heparinized venous blood collected between 9.00 PM and 2.00 AM using 3 μm polycarbonate filters (Nucleopore, Pleasanton, Calif., U.S.A.) followed by visualization under a light microscope. Sera were collected after informed consent from 34 microfilaremic patients (MF), 31 with chronic lymphatic disease (CP), and 23 microfilariae negative healthy individuals residing in the endemic areas (endemic normals [EN]). The serum samples from *Brugia malayi* infected individuals were obtained from patients residing in areas endemic to Brugian filariasis such as Kerala, India and in Kelantan, Malaysia (supplied by Dr. N. Rahmah, Universiti Sains Malaysia, Malaysia) consisting of 30 with MF, 13 with CP, and 10 uninfected individuals residing in endemic areas (EN). Non-endemic normal (NEN) sera were obtained from healthy US blood bank donors who had never traveled outside the United States ($n=19$). Five sera from patients with other parasitic infections such as *O. volvulus*, *Loa loa* and *Ascaris lumbricoides* were also included. These patients had no history of *W. bancrofti* or *B. malayi* infections, and the sera were Og4C3 negative.

Development of antibodies to recombinant Bm-SXP-1 and Wb-SXP-1. We have previously reported the cloning and characterization of the SXP protein from *B. malayi* adult worm cDNA library and its orthologue from *Wuchereria bancrofti* L3 cDNA library (11). The full-length Bm-SXP-1 and Wb-SXP-1 cDNAs were cloned into pRSET B and expressed as N-terminal six-histidine-tagged fusion proteins. The recombinant proteins were purified on immobilized metal affinity chromatography (IMAC).

The purified recombinant *Brugia malayi* SXP-1 (Bm-SXP-1) and *Wuchereria bancrofti* SXP-1 (Wb-SXP-1) were used for raising antibodies in mice and rabbits. A group of five outbred male BALB/c mice were immunized intraperitoneally with 2 μg of Bm-SXP-1 or Wb-SXP-1 in complete Freunds adjuvant (CFA) followed by three intramuscular booster doses of similar concentrations in incomplete Freunds adjuvant (IFA) at two-week intervals. Antibodies to Bm-SXP-1 or Wb-SXP-1 were raised in rabbits by immunizing with 5 μg of each protein in CFA followed by three booster doses of similar concentrations in IFA by intramuscular injections at two-week intervals. Sera were collected from mice and rabbits after two weeks of each immunization. The immunoreactivity of these antibodies was tested against the purified Bm-SXP-1 and Wb-SXP-1 by Western blot and the antibody titers were determined by ELISA. The antisera were adsorbed with *E. coli* proteins to remove cross reactivity. The rabbit and mouse monospecific antisera against Bm-SXP-1 reacted only with the recombinant protein of 23 kDa and not with the vector protein. Normal mouse and normal rabbit serum did not show any

Table 1. Details of patients selected in the present study

Category	No. samples	mf+	Og4C3+	Source
W. bancrofti				
MF	34	34	34	Centre for Biotechnology, Anna University, Chennai, India
CP	31	0	0	
EN	33	0	0	
B. malayi				Dr. V. Kumaraswami, Tuberculosis Research Centre, Chennai, India and Dr. N. Rahmah, Universiti Sains Malaysia, Malaysia
MF	30	30	0	
CP	13	0	0	
EN	10	0	0	
Non-endemic normal (NEN)	19	0	0	Dr. T.B. Nutman, Laboratory of Parasitic Diseases, NIH, U.S.A.
Other parasitic infections (OPI)				
Onchocerca volvulus	5	0	0	Dr. T.B. Nutman, Laboratory of Parasitic Diseases, NIH, U.S.A.
Loa loa	5	0	0	
Ascaris lumbricoides	5	0	0	

reactivity with Bm-SXP-1. Similar findings were observed with Wb-SXP-1 using rabbit and mouse antisera.

Detection of circulating filarial antigen by ELISA using antibodies against recombinant Bm-SXP-1 and Wb-SXP-1. The optimal dilutions of mouse and rabbit antisera, patient's serum, and goat anti-rabbit IgG alkaline phosphatase conjugates were determined by chequer board titration. For the Bm-SXP-1 based antigen assay, flat bottom 96-well microtiter plates (Immunolon 4, Dynatech Laboratories, Inc., Alexandria, Va., U.S.A.) were coated with 100 μl of mouse anti-Bm-SXP-1 antibody diluted in carbonate buffer pH 9.6 (1:1,500) and incubated overnight at 4 C. The plates were washed in phosphate buffered saline (PBS) containing 0.05% Tween 20 (Sigma) and blocked with PBS containing 3% bovine serum albumin (BSA) for 2 hr at 37 C. After further washes, sera samples (1:100) treated with glycine (0.15 M, pH 2.0) and neutralized with equal volume of Tris (0.1 M, pH 9.0) was added (6) to the wells in duplicates and the plates were incubated at 37 C for 2 hr. The plates were washed as before and incubated at 37 C for 1 hr with rabbit anti-Bm-SXP-1 antibody (1:1,000). After washing the plates, biotin labeled goat anti-rabbit IgG was added and incubated at 37 C for 1 hr. The plates were further washed and incubated with Streptavidin conjugated with alkaline phosphatase (1:10,000) for 1 hr at 37 C. After washing as before, substrate solution was added (1 mg/ml *p*-nitro phenyl phosphate in substrate buffer) (Sigma Chemicals, U.S.A.). After 10−15 min the reaction was stopped by addition of 3 M NaOH. Absorbance was measured at 405 nm in a micro plate ELISA reader (BIO-TEK EL311sx, Winooski, Vermont, U.S.A.) and means were taken of the duplicate readings.

The Wb-SXP-1 based antigen assay was performed as described above with the following dilutions: mouse anti-Wb-SXP-1 1:2,000, patient's serum 1:4 and goat anti-rabbit IgG alkaline phosphatase conjugate 1:1,000. The sensitivity of the ELISA was determined using normal human serum spiked with different concentrations of purified Bm-SXP-1/Wb-SXP-1 antigen, and a standard graph was constructed for quantitating the antigen concentration (ng) in the filarial sera.

Circulating filarial antigen detection using monoclonal antibody based Og4C3 commercial kit. Circulating filarial antigen was also quantitated in the sera using a commercially available mAb based Og4C3 ELISA kit (Tropical Biotechnology, Townsville, Australia). The antigen units were extrapolated from a standard curve prepared from standards provided in the kit. Samples with OD values greater than 80 antigen units were considered to be positive as recommended by the manufacturer.

Statistical analysis. Data were analysed by Kruskal-Wallis one-way analysis of variance. Mann-Whitney *U* test was employed to identify the higher reactivity group. *P*-values were adjusted for multiple comparisons by using Bonferroni's correction. For paired values, Sign test was used. In the present study $P<0.05$ was considered statistically significant.

Results

Development of an ELISA with Antibodies to Bm-SXP-1 and Wb-SXP-1 to Quantitate Circulating Filarial Antigens (CFA)

The rabbit and mouse antiserum against Bm-SXP-1 reacted only with the recombinant protein of 23 kDa and not with the vector protein. Normal mouse and normal rabbit serum did not show any reactivity with Bm-SXP-1. Similar findings were observed with Wb-SXP-1 using rabbit and mouse antisera. The specificity of the rabbit and mouse antiserum against Bm-SXP-1 or Wb-SXP-1 were tested for the reactivity with different filarial recombinant proteins by Western blot and the results were negative. Preliminary experiments were carried out with mouse and rabbit anti-SXP-1 antibodies to determine the choice of antisera to be used as the capture antibody and the secondary antibody for the standardization of ELISA. It was observed that the best results were obtained with mouse anti-Bm-SXP-1/Wb-SXP-1 antisera as the capture antibody and the rabbit antisera as the secondary antibody.

Detection of CFA in Bancroftian Filarial Sera

The results of ELISA with anti-Bm-SXP-1 antibodies using sera from patients with *W. bancrofti* infection is given in Fig. 1A. Kruskal-Wallis one-way ANOVA showed that CFA among the groups were highly significant ($P<0.001$). Individual comparisons were made between the groups to identify the higher mean Bm-SXP-1 category using Mann-Whitney *U* test. MF group had significantly higher antigen units ($P<0.001$). Similarly MF group exhibited higher antigen units ($P<0.001$) when Wb-SXP-1 antibodies were used (Fig. 2A).

Antigen concentration of 10 ng and above in the samples were considered positive for Bm-SXP-1 and Wb-SXP-1 assay. Eighty-eight percent (30/34) MF, 22% (7/31) CP and 26% (6/23) EN were positive for Bm-SXP-1 CFA (Fig. 1A) and 95% (20/21) MF, 10% (2/20) CP and 3% (1/33) EN were positive for Wb-SXP-1 CFA (Fig. 2A).

Using the above criteria for the positivity, paired comparison with Wb-SXP-1 antigen and Bm-SXP-1 antigen of the same MF ($n=21$) did not show any significant dif-

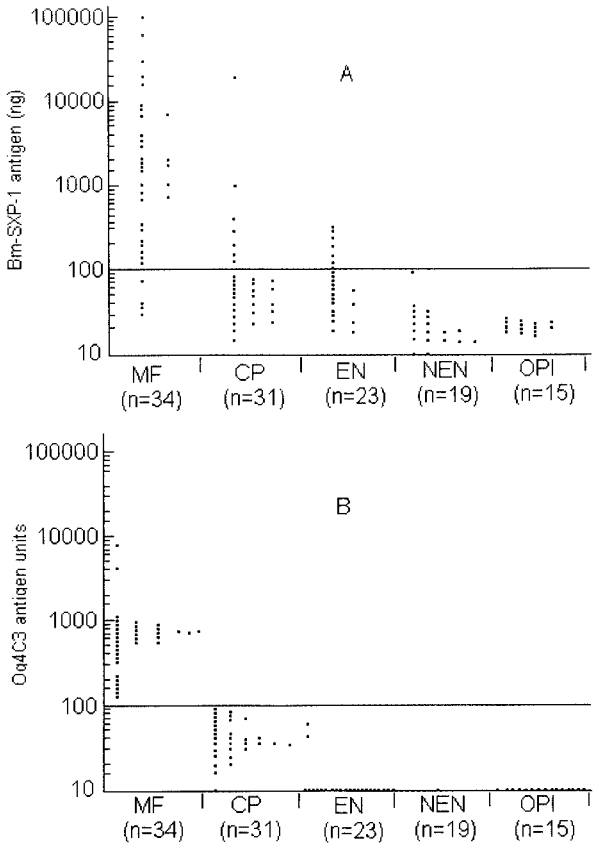

Fig. 1. Circulating filarial antigen levels in individual sera of patients with bancroftian filariasis as determined by Bm-SXP-1 (A) and Og4C3 assay (B). The horizontal line represents the mean antigen concentration (ng) or antigen units+3SD of a group of NEN individuals. Each dot represents corresponding antigen concentration in ng or antigen units of individual patients. MF, microfilaremics; CP, individuals with chronic pathology; EN, endemic normals; NEN, non-endemic normals; OPI, other parasitic infections.

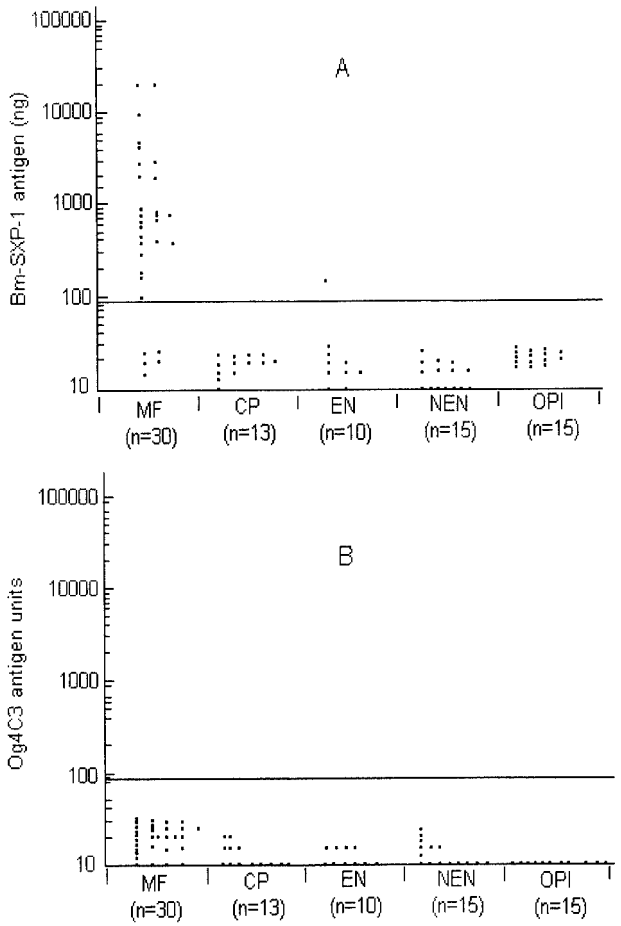

Fig. 2. Circulating filarial antigen levels in individual sera of patients with brugian filariasis as determined by Bm-SXP-1 (A) and Og4C3 assay (B). The horizontal line represents the mean antigen concentration (ng) or antigen units+3SD of a group of NEN individuals. Each dot represents corresponding antigen concentration in ng or antigen units of individual patients. MF, microfilaremics; CP, individuals with chronic pathology; EN, endemic normals; NEN, non-endemic normals; OPI, other parasitic infections.

ference ($P > 0.05$). All the 21 MF samples showed positivity for Bm-SXP-1 CFA and only one did not show positivity for Wb-SXP-1 CFA. All the bancroftian MF sera tested (34/34) were positive for circulating filarial antigen by Og4C3 ELISA while none of the CP, EN and NEN reacted in the assay (Fig. 1B).

Detection of CFA in Brugian Filarial Sera

Circulating antigen levels in patients with brugian filariasis using Bm-SXP-1 antibodies were quantitated by ELISA as described above. MF patients exhibited highest levels of the circulating antigens 25/30 (83.3%) compared to CP [0% (0/13)] and EN [10% (1/10)] (Fig. 3A). Similar results were obtained using antibodies to Wb-SXP-1 {MF [12/15 (80%)], CP [0/10 (0%)] and EN [0/10 (0%)]} (Fig. 2B). All the brugian sera tested were negative for CFA by Og4C3 ELISA (Fig. 3B). None of the sera from NEN and patients with other parasitic infections were positive in either of the assays (Figs. 1, 2 and 3).

Discussion

Antibodies raised against purified recombinant filarial proteins Wb-SXP-1 and Bm-SXP-1 have been used to develop a sandwich ELISA to diagnose microfilaria positive individuals. When compared to ELISA developed using Bm-SXP-1 antibody, the ELISA with Wb-SXP-1 antibody identified apparently a high proportion of MF's with bancroftian filariasis.

Monoclonal antibody based ELISA (Og4C3) has been used by our laboratory and in those of others to diagnose filarial infecton (2) by quantitating circulating filarial

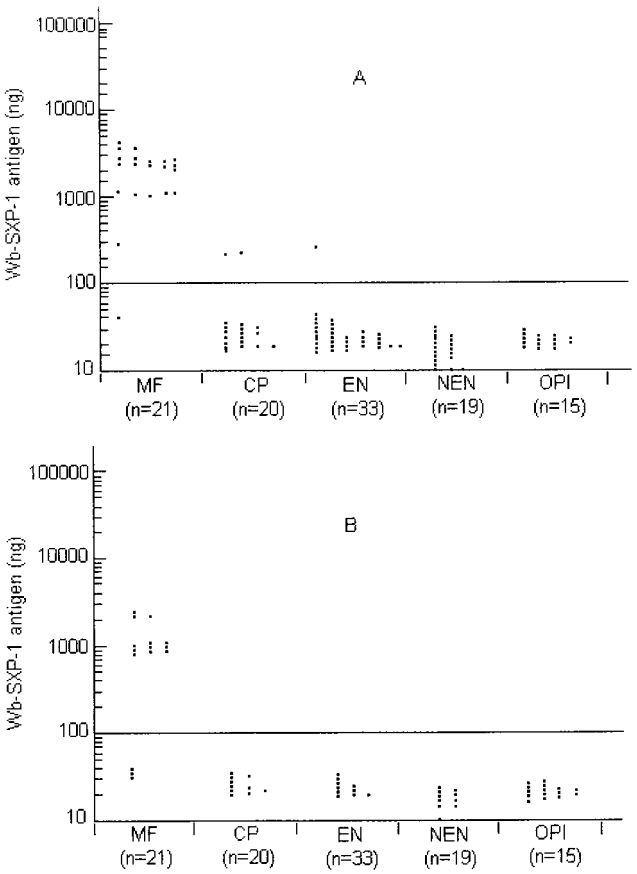

Fig. 3. Circulating filarial antigen levels in individual sera of patients with bancroftian filariasis (A) and brugian filariasis (B) as determined by Wb-SXP-1 assay. The horizontal line represents the mean antigen concentration (ng) or antigen units+3SD of a group of NEN individuals. Each dot represents corresponding antigen concentration in ng or antigen units of individual patients. MF, microfilaremics; CP, individuals with chronic pathology; EN, endemic normals; NEN, non-endemic normals; OPI, other parasitic infections.

antigen. We have shown that all MFs were positive by Og4C3 test while CP, EN and NEN were negative (7). The results of the present study using antibody to Wb-SXP-1 supports the findings of Og4C3 assay though the sample size used for the evaluation was rather small.

Lymphoproliferative and cytokine response studies on various clinical categories of filarial patients have shown that MF individuals have low Th1 response to filarial specific antigens compared to CP and EN individuals where enhanced Th1 activity is observed (12, 14). Thus MF individuals were not able to clear the antigen and hence were positive in the antigen assay. Since CP and EN have high levels of cell mediated immune response due to increased T cell activation and elevated levels of gamma interferon, there is increased macrophage activation and the elimination of the antigen. In spite of this, some of the individuals in the CP and EN group tested positive for SXP-1.

The possible explanations for the above findings could be (a) SXP-1 is a somatic antigen and released by multiple stages of filarial worms, (b) Og4C3 is a monoclonal antibody based ELISA while in the present study polyclonal antibodies have been employed. These antibodies may bind to cross reactive antigens present in the serum samples of CP and EN and may be false positive. (c) It is possible that some of the CP and EN individuals could be infected at the time of sample collection though it is difficult to demonstrate microfilaria positivity in them. (d) Since the CP and EN individuals are in an endemic area they may be exposed to infective bites of mosquitoes and thus may trigger a positive response for SXP-1. The assay developed in the present study appears specific since sera from patients with other parasitic infections (OPI) and sera from non-endemic normals (NEN) were negative for circulating SXP-1. NEN as known were not exposed to infective mosquito bites and hence do not carry the infection. It is possible that patients with OPI have SXP-1 but present in a concentration range not detectable by the ELISA developed in this study. Experiments are in progress to evaluate this assay in normal individuals in areas endemic to bancroftian filariasis to examine the question of false positivity.

In the present study, all the MF patients with *B. malayi* infection were microfilaria positive (mf) (Table 1). Sera from MF patients with brugian filariasis were all negative by Og4C3 assay while a high percentage of them were circulating Bm-SXP-1 and Wb-SXP-1 antigen positive. None of the CP and EN from endemic area of brugian infection contained the circulating antigen. The negative response in Og4C3 assay indicates that the MF patients in question are harbouring only *B. malayi* and not *W. bancrofti* in fection. Thus the test appears to be specific and identifies a high percentage (80%) of microfilariae positive individuals harbouring *B. malayi* infection. The remaining 20% were not positive by this assay. The possible explanation for this could be that the levels of SXP antigen present in these samples could be very low and hence not detectable by this assay. Though there are certain limitations, this assay might be very useful for the epidemiological survey of filarial infections.

In summary, an ELISA developed with antibodies to recombinant filarial proteins Bm-SXP-1 and Wb-SXP-1 has been successfully used to quantitate circulating filarial antigens in sera from bancroftian and brugian infections. This will be helpful for the sero-epidemiological studies in endemic areas of *Brugia malayi* and mixed infections.

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