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Diagnostic utility of fractionated urinary filarial antigen

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Abstract. Urinary filarial antigen isolated from urine samples of microfilaraemic patients was analysed for its antigenic activity by immunoblotting and enzyme linked immunosorbent assay techniques. SDS-PAGE fractionation of urinary filarial antigen showed 11 protein bands, of which two showed reactivity with immunoglobulin-G fraction of filarial serum immunoglobulin in immunoblotting. Antigenic analysis of SDS-PAGE fractions of urinary filarial antigen by inhibition enzyme linked immunosorbent assay using filarial serum immunoglobulin-G and *Wuchereria bancrofti* microfilarial excretory-secretory antigen revealed 3 fractions, numbers 5, 6 and 9 with significant activity. In indirect enzyme linked immunosorbent assay using fractions 5 and 6, filarial immunoglobulin-G antibody was detected in about 90% of microfilaraemics, 80% clinical filariasis and 20% of endemic normal individuals. Further, there was no phosphorylcholine epitope in these fractions. Fractions 5 and 6 can be a candidate antigens for the immunodiagnosis of filariasis.

Keywords. Urinary filarial antigen; immunodiagnosis; SDS-PAGE; immunoblot; ELISA.

1. Introduction

Filarial antigen in body fluids as secretions and as surface products of the parasite have immense value in immunoparasitology and it acts as a marker for the diagnosis of active infection. Purification and characterization of filarial antigen from body fluids is a prerequisite for identifying the antigens of immunodiagnostic importance. Several investigators have isolated filarial antigens from body fluids *viz.,* blood (Reddy *et al* 1986), urine (Ramaprasad and Harinath 1987) and hydrocele fluid (Malhotra *et al* 1985; Ramaprasad *et al* 1989) to study the nature and properties of these antigens to use them as diagnostic reagents in the detection of bancroftian filariasis as there are considerable difficulties in obtaining required human parasite material due to lack of suitable animal model. Earlier studies from our laboratory have shown that absorption of urinary albumin improved the sensitivity of urinary filarial antigen ($UFAC_2$) in detection of filarial infection (Ramaprasad and Harinath 1987). The present study was carried out to identify the antigens of diagnostic importance in $UFAC_2$ and to study their potential in diagnosis of human filariasis.

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Abbreviations used: UFA, Urinary filarial antigen; $UFAC_2$, UFA column fraction-2; FSI, filarial serum immunoglobulin; *Wb* mf ES, *Wuchereria bancrofti* microfilarial excretory-secretory; NCP, nitrocellulose paper; ELISA, enzyme linked immunosorbent assay; PC, phosphorylcholine.

2. **Materials and method**

2.1 *Human sera*

Human sera belonging to different groups *viz.,* normal (endemic) and filarial (microfdaraemia and clinical filariasis) were collected from Wardha, an endemic region for *Wuchereria bancrofti* filarial infection. The presence of microfilariae in microfilaraemic cases was confirmed by night blood (wet smear) examination. Clinical filarial samples were from individuals with manifestations such as hydrocele, swelling of limbs and elephantiasis. Endemic normal blood samples were from healthy individuals living in an endemic region for filariasis. Non-endemic normal blood samples were collected from students coming to this Institute from places like Kashmir, Chandigarh, etc., where there is no filariasis.

Sera from patients infected with different filarial and non-filarial parasites *viz., W. bancrofti, Brugia malayi, Loa loa, Mansonella perstans, Onchocerca volvulus, Drancunculus medinensis* and *Strongyloides stercoralis,* provided by World Health Organization during the 1991 multicentre study were also screened in this study.

2.2 *UFA C_2*

UFA was isolated from microfilaraemic urine samples as described by Ramaprasad and Harinath (1987). Briefly, 24 h urine samples were collected from microfilaraemic carriers after treatment with diethylcarbamazine, pooled, concentrated by ultra-filtration (Millipore, USA) and dialized against sodium phosphate buffer (SPB) 0·01 M pH 7·2. Active urinary filarial antigenic fraction $UFAC_2$ was obtained by fractionation of UFA on ultrogel ACA 44 column chromatography.

2.3 *Filarial serum immunoglobulin-G*

Filarial serum immunoglobulins (FSI) were isolated from clinical filarial sera by 33% ammonium sulphate precipitation and the IgG fraction was separated by DEAE-cellulose (DE 52) ion-exchange chromatography (Reddy *et al* 1984).

2.4 *W. bancrofti microfilarial excretory-secretory antigen*

W. bancrofti microfilarial excretory-secretory *(Wb* mf ES) antigen was obtained by *in vitro* maintenance of *W. bancrofti* microfilariae in medium 199 at 28°C as described by Kharat *et al* (1982).

2.5 *SDS-PAGE and immunoblotting*

SDS-PAGE analysis of $UFAC_2$ under reduced conditions was carried out using 10% non-gradient slab gel with a 3·5% stacking gel (Laemmli *et al* 1970). The sample was electrophoresed at 20 mA current. The gel was calibrated with the molecular weight (M_r) markers, carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (67 kDa), Phosphorylase (97 kDa) and β-galactosidase

(116 kDa). Proteins in part of the gel were visualized by silver staining (Biorad 1982). Proteins in the other part of the gel were electrophoretically transblotted to a strip of nitrocellulose paper as described by Towbin *et al* (1979). After electroblotting, the blot was blocked with 3% (w/v) bovine serum albumin (BSA) in PBS/T, washed with PBS/T and cut into 5 mm strips. The strips were incubated with optimally diluted FSIgG-peroxidase conjugate (at 37°C for 3 h). The antigenic bands with bound FSIgG-peroxidase conjugate were visualized with the chromogenic substrate containing 0·003% H_2O_2 and 0·05% 3, 3'-diaminobenzidine in citrate phosphate buffer, pH 5·0.

2.6 *Gel elution*

In another experiment, the unstained portion of SDS-PAGE resolved components were cut at 1 cm intervals into 12 slices and the proteins from each slice were eluted as described earlier (Ramaprasad and Harinath 1989). Briefly, each gel slice was ground separately with 5 ml 0·05 M SPB, pH 7·2 and left over night in the sample buffer at 4°C for complete elution. The supernatant was separated, dialysed and concentrated by lyophilization. The protein content in each fraction ($UFAC_2$-1 to 12) was estimated (Lowry *et al* 1951) and analysed for antigenic activity by enzyme linked immunosorbent assay (ELISA).

2.7 *ELISA*

Anti-human IgG and *Wb* mf ES antigen were conjugated separately with enzyme penicillinase by single step glutaraldehyde method as described by Avrameas (1969). The substrate for penicillinase ELISA consisted of 150 mg soluble starch in 27·5 ml of 0·25 M SPB pH 7·2 and added with 10·64 mg penicillin V and 100 μl of 0·08 M iodine in 3·2 M potassium iodide. The substrate was freshly prepared before use.

Detection of IgG antibody in human sera was carried out by indirect ELISA using stick assay system as described by Parkhe *et al* (1986). Briefly, about 5 μl volumes of optimally diluted *Wb* mf ES antigen (100 pg/stick) or antigen fractions $UFAC_2$-5 and 6 [10 pg/stick in 0·05 M SPB (pH 7·2)] were coated on the cellulose acetate membrane sticks. The unbound sites were saturated with 3% gelatin in the same buffer. The sticks were incubated with 0·5 ml of optimally diluted (1:300) human sera samples in PBS/T at 37°C for 90 min. After washing the sticks, 0·5 ml of optimally diluted anti human IgG-penicillinase conjugate in PBS/T was added and incubated at 37°C for 30 min. After final washing, the immune reaction was observed by incubating the sticks with 0·5 ml of starch-iodine-penicillin substrate. The disappearance of blue colour denoted the positive reaction.

2.8 *Inhibition ELISA*

Inhibition ELISA was carried out to determine the antigenic titres of $UFAC_2$-SDS-PAGE fractions as described by Parkhe *et al* (1990). Optimal concentrations of FSIgG (l00ng/stick), serially diluted $UFAC_2$ SDS-PAGE fractions (starting dilution l0 μg ml^{-1} and ten-fold) and *Wb* mf ES antigen-penicillinase conjugate were used in the assay system. The persistance of blue colour indicated positive reaction.

2.9 *Assay for phosphorylcholine epitopes*

Albumin conjugated to phosphorylcholine (PC) and anti-PC IgM monoclonal antibody (Vector, USA) were kindly supplied by Dr Mario Philipp, Primate Research Centre, Tulane University, Covigton, LA, USA. Phosphorylcholine epitope assay was carried out using PVC microtitre plates (Dynatech). The wells were sensitized with 100μl volumes of serially diluted $UFAC_2$ and $UFAC_2$- 5 and 6 (1 μg/ml, l00ng/ml, l0ng/ml) in carbonate buffer ppH 9·6, at 4°C overnight. The unbound sites were blocked with 200 μl of PBS containing 0·2% Tween-20. After washing the plates, the wells were incubated with 100 μl of optimally diluted anti-PC IgM monoclonal antibody at 37° C for 2 h. The bound antibody was detected by incubation at 37°C for 2 h with anti-mouse IgM-peroxidase conjugate followed by the addition of O-phenylene-diamine (Sigma, USA) substrate. After the development of colour, the reaction was stopped by additon 50 μl of 5 NHCl and read at 490¯ nm in an ELISA reader. The results were expressed as the antigen dilution that resulted in an absorbance greater than 0·1.

3. Results

$UFAC_2$ when analysed by SDS-PAGE followed by silver staining revealed 11 polypeptide bands within the r range of 12 to ≥ 165 kDa. In immunoblotting technique, FSIgG isolated from clinical filarial sera samples identified 2 polypeptide bands of approximate M_r 49 kDa and 42 kDa in $UFAC_2$ (figure 1). Analysis of 12 SDS-PAGE fractions of $UFAC_2$ by inhibition ELISA using *Wb* mf ES antigen and FSIgG showed antigenic activity in fractions $UFAC_2$-4,5,6,7 and 9 (table 1). The fraction $UFAC_2$-5 showed high inhibitory antigen concentration of 0·1 ng whereas the fractions $UFAC_2$-6 and 9 inhibited the binding of *Wb* mf ES antigen to FSIgG with the inhibition antigen concentration of 1 ng.

The diagnostic use of pooled antigen fractions $UFAC_2$-5 and 6 for the detection of filarial antibody in human sera samples was studied by indirect ELISA. A total of 40 sera samples collected from both filarial endemic and non-endemic area were screened and the results are compared with the results of *Wb* mf ES antigen. Among 30 filarial endemic sera, 9 out of 10 microfilaraemic (90%), 8 out of 10 clinical filarial (80%) and 2 out of 10 endemic normal sera (20%) showed positive reaction for filarial antibody. None of the 10 non-endemic normal sera were positive for filarial antibody in this assay system. The test shows the sensitivity of 90% and 80% for microfilaraemia and clinical filariasis respectively and a specificity of 90%.

The results of analysis of filarial and non filarial sera provided by WHO/TDR Sera Bank for filarial IgG antibodies using $UFAC_2$-5 and 6 fraction and *Wb* mf ES antigen are shown in table 2. While all the 11 microfilaraemic sera of *W. bancrofti* infection were positive for filarial IgG antibodies using $UFAC_2$-5 and 6 antigen, only 10 of them were positive when *Wb* mf ES antigen was used. Among, the *B, malayi* microfilaraemic sera, all the 5 were positive against $UFAC_2$-5 and 6 where as 4 of them were positive against *Wb* mf ES antigen. Out of the six onchocercal sera 2 and 4 were positive for filarial IgG antibodies with $UFAC_2$-5 and 6 and *Wb* mf ES antigen respectively

The filarial antigen $UFAC_2$ and $UFAC_2$-5 and 6 were tested for phosphoryl-

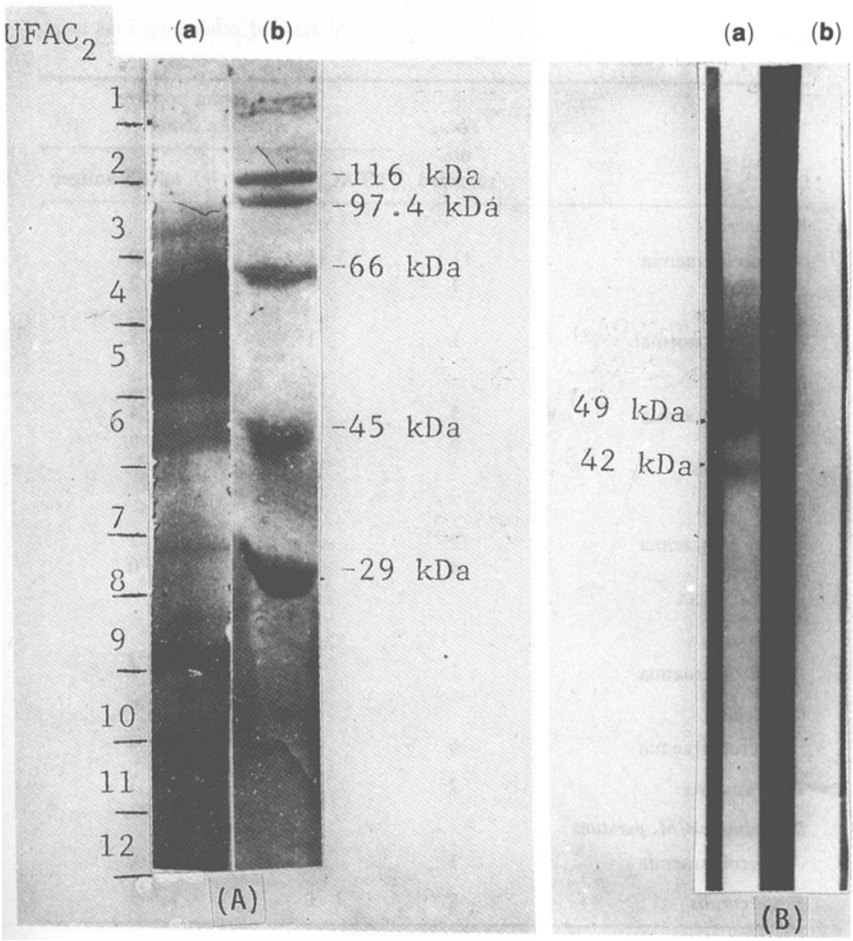

Figure 1. (A) SDS-PAGE pattern of $UFAC_2$ **(a)** and molecular weight markers **(b). (B)** Recognition of $UFAC_2$ by FSIgG in immunoblotting. Blots incubated with peroxidase conjugates of **(a)** FSIgG and **(b)** normal human serum IgG.

Table 1. Concentration of inhibitory antigen in SDS-PAGE fractions of $UFAC_2$ analysed by inhibition ELISA.

$UFAC_2$ fraction number	Approximate M_r range (kDa)	Concentration of inhibitory antigen (ng)
1	≥160	0
2	95–160	0
3	68–95	100
4	56–68	10
5	45–56	0·1
6	37–45	1
7	31–37	100
8	25–31	0
9	20–25	1
10	17–20	0
11	14–17	0
12	12–14	0

Table 2. Detection of filarial IgG antibodies in filaria and other infections using $UFAC_2$-5 and 6 and *Wb* mf ES antigen.

Sera	Total no. screened	No. showing positive reaction using	
		$UFAC_2$-5 and 6	*Wb* mf ES antigen
W. bancrofti			
Microfilaraemia	11	11	10
Clinical filariasis	3	3	2
Endemic normal	1	1	1
B. malayi			
Microfilaraemia	5	5	4
Clinical filariasis	6	6	6
L. loa			
Microfilaraemia	2	2	2
Clinical filariasis	4	0	0
M. perstans			
Microfilaraemia	1	0	1
O. volvulus			
Microfilaraemia	6	2	4
D. medinensis	2	1	1
D. medinensis/M. perstans			
Microfilaraemia	1	1	0
S. stercoralis	2	0	1

choline epitope, using anti-PC antibodies and PC-conjugated BSA as positive control. Both the antigen fractions showed minimum reactivity (optical density values of 0·028, 0·036 for $UFAC_2$ and $UFAC_2$-5 and 6 respectively) compared to their reactivity with the PC conjugated BSA (absorbance 0·426).

4. Discussion

The scarcity of sufficient parasite material is one of the constrictions for progress in filarial immunodiagnostic research. Sensitive, specific and reproducible diagnosis of filariasis is possible only by using purified and well characterized antigens. Antigens from body fluids have been identified and isolated in bancroftian filariasis, to use as diagnostic reagents (Reddy *et al* 1984; Ramaprasad and Harinath, 1987; Malhotra *et al* 1985). Comparatively, urine is an easily available body fluid and it has been shown that parasite antigen excreted in urine of infected individual is an excellent source for the isolation of antigens of immunodiagnostic importance. Absorption of albumin from $UFAC_2$ increased its sensitivity by 50000-fold (Ramaprasad and Harinath 1987).

SDS-PAGE fractionation delineates the polypeptide pattern of $UFAC_2$. Albumin was detected in $UFAC_2$-3 and 4 fractions. The fractions $UFAC_2$-5 and 6 approximate M_r range of 45 to 56 and 37 to 45 kDa respectively were free of albumin, reacted with FSIgG and inhibited the binding of *Wb* mf ES antigen to FSIgG. In immunoblotting studies FSIgG identified two polypeptides of approximate M_r of 49 and 42 kDa with their corresponding positions in 5th and 6th fractions respectively. These studies showed that these are the major active antigenic proteins of $UFAC_2$. Though $UFAC_2$-9 fraction showed high inhibitory activity, no protein band was detected in the region corresponding to this fraction. The total amount of protein recovered from this fraction was also quite low and it showed non-specific reactivity with endemic normal sera (data not shown). Hence in further studies, only the $UFAC_2$-5 and 6 antigen fraction was evaluated for its diagnostic use.

The analysis of different groups of sera for filarial IgG antibody using $UFAC_2$-5 and 6 fractions gave comparable results with those obtained by using *Wb* mf ES antigen. Further, the comparative analysis of. the other filarial and non filarial sera using $UFAC_2$-5 and 6 and *Wb* mf ES antigen for filarial IgG antibodies (table 2) showed that $UFAC_2$-5 and 6 fraction is marginally sensitive and specific compared to *Wb* mf ES antigen.

In the earlier studies from our laboratory (Harinath *et al* 1984) *Wb* mf ES antigen was shown to be highly useful for detection of filarial antibodies with sensitivity and specificity values of 98% and 86% respectively. This assay system was also found to be helpful for effective monitoring of filarial cases after treatment with diethylcarbamazine (Ramaprasad *et al* 1988). When $UFAC_2$ antigen fraction was used as such for detection of filarial IgG antibodies only 70% of microfilaraemic cases were positive (Ramaprasad and Harinath 1987). Using another *in vivo* released filarial antigen isolated from hydrocele fluid of clinical filarial cases (Ramaprasad and Harinath 1989) detected filarial IgG antibodies in around 80% of microfilaraemic cases. Katiyar *et al* (1985) evaluated the diagnostic utility of *B. malayi* infective larval (L_3) and adult worm antigen by filarial skin test and reported that about 90% of the individuals residing in endemic area are reacted to *B. malayi* antigen. *B. malayi* adult somatic antigen was explored in dot ELISA by Tandon *et al* (1988), who reported it to be useful in discriminating asymptomatic cases from symptomatic filarial cases.

PC epitope is common in all nematode parasite antigens (Maizels *et al* 1987, Wenger *et al* 1989). Though the PC bearing antigen or antibody to it showed diagnostic potential in filariasis (Forsyth *et al* 1985; Lal *et al* 1987; Maizels *et al* 1987) it is safe to have a non-PC antigen to avoid cross reactivity with other parasite infections (Lai and Ottesen 1989). $UFAC_2$-5 and 6 fractions were found to be free of PC epitope and this is added advantage for using these fractions for the immunodiagnosis.

In the absence of a suitable laboratory animal model for bancroftian filariasis for isolation of antigen from filarial worms this study does show that antigen isolated from the easily available urine of microfilaraemic patients may be useful as a candidate antigen for the diagnosis of filarial infection. Large scale isolation of $UFAC_2$-5 and 6 antigenic fractions or mass production of these antigenic epitopes by molecular biological approach may help in overcoming the limitations of getting sufficient antigen material for immunodiagnosis of bancroftian filariasis.

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