CHAPTER 3

RESULTS AND DISCUSSION

3.0 PART I

An unequivocal method of diagnosis of active filarial infection is still based on the demonstration of mf in the blood of the infected individuals. In the last twenty years there has been considerable output from the scientific community to develop various immunodiagnostic techniques like counterimmunoelectrophoresis (CIEP), indirect hemagglutination test (IHAT), indirect fluorescent antibody test (IFAT) radioimmunometric assay (RIA) and enzyme linked immunosorbent assay (ELISA) for the identification of parasitic infections. Among all these tests ELISA appeared to be more simple and reliable for detecting either circulating antigens or specific antibody for identifying individuals with active infection in tropical countries. Though there is considerable number of publication on the development of antibody based immunoassay, very little work has been carried out in developing antigen detection ELISA using antibodies raised against recombinant filarial proteins or measuring subclass specific antibodies (like IgG4) against the recombinant proteins. In the present study an attempt was made to develop ELISA reagents for diagnosing the individuals having active filarial infection using recombinant DNA technology. The results of the assays have also been confirmed by using commercially available antigen detection kit (Og4C3 antigen assay).

3.1 CIRCULATING FILARIAL ANTIGEN (CFA) DETECTION BY MONOCLONAL ANTIBODY BASED SANDWICH ELISA - Og4C3 ANTIGEN ASSAY

An attempt was made to study the possible application of Og4C3 assay in the detection of active filarial infection. Circulating filarial antigen (CFA) levels in different clinical groups of filariasis were quantitated by Og4C3 mAb based ELISA. The Og4C3 monoclonal antibody was developed against a non phosphocholine antigen of adult Onchocerca gibsoni (More and Copeman, 1990).
The Og4C3 mAb recognises epitopes present in 50-60kDa and 130kDa antigens of adults and microfilariae of *O. gibsoni*. Og4C3 antigen does not cross-react with human sera infected with *O. volvulus*, *B. malayi*, *B. timori*, *Loa loa*, *Mansonella perstans*, *Strongyloides stercoralis*, *Dracunculus medinensis* or *Ascaris lumbricoides*. Various research groups have employed the assay and they have shown promising results that it detects only individuals with active bancroftian infection (McCarthy *et al.*, 1995). Currently a filariasis card test is being developed based on AD12.1 mAb which binds to a repeated epitope on a 200kDa adult worm excretion product present in the sera of *Wuchereria bancrofti* infected patients (Weil *et al.*, 1987b). This is a new, rapid format antigen test, which is specific for *W. bancrofti* infection when evaluated independently with sera from three different regions of filariasis (Weil *et al.*, 1997). The main disadvantage of this card test is that it is not quantitative in terms of antigen detection, however this test can be used as a qualitative test to survey large endemic populations in field conditions.

### 3.1.1 Og4C3 antigen assay using Bancroftian filarial sera

Hence in the present study the possible application of CFA using Og4C3 monoclonal antibody based assay was investigated for the early diagnosis of filariasis and subsequently the results were compared with the CFA assay developed using monospecific antibodies to a recombinant filarial antigen (pRBSXP). Forty microfilaricemic patients who were positive for microfilaria by nucleopore membrane filtration technique and 10 each of chronic pathology, endemic normals and non-endemic normals were included in the study. The patient’s sera exhibiting circulating filarial antigen units (Og4C3) 80 and above were considered to be positive by this assay. All the 40 Bancroftian MF patients were positive with the antigen units ranging from 80-2560 indicating the high sensitivity of the assay and further, its use to identify individuals with active filarial infection (Table 3.1). The CP sera showed negative reactivity by this assay which might be due to the absence of living adult worm or an efficient immune clearance of the parasite antigen by the host. This result differs from that of
<table>
<thead>
<tr>
<th>Clinical category</th>
<th>Positive by ELISA (Ag units range)</th>
<th>Negative by ELISA (Ag units range)</th>
<th>Mean mf count (mf/ml range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic microfilaraemic (MF)</td>
<td>40 (80-2560)</td>
<td>0 (0)</td>
<td>252 (4-1000)</td>
</tr>
<tr>
<td>n=40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic Pathology (CP)</td>
<td>0 (0)</td>
<td>10 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endemic normals (EN)</td>
<td>2 (449,558)</td>
<td>8 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non endemic normals (NEN)</td>
<td>0 (0)</td>
<td>10 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 3.1: Quantitation of circulating filarial antigen (CFA) by Og4C3 assay in different clinical groups of bancroftian filariasis patients

The serum samples exhibiting antigen units 80 and above were considered positive.
More and Copeman (1990) where the presence of Og4C3 (CFA) antigen in 23% of CP patients was reported. This may be due to reinfection or may be they are in the early stages of chronic pathology. Similar work done by Weil et al., (1996) in Egypt using a different mAb AD12.1 based assay showed positive reactivity with all the MF whereas two of the ten CP were antigen positive. But the antigen positive CP individuals were found to be mf positive by night blood smear.

Further in the present study the 2 of the 10 Endemic normals (mf negative by membrane filtration) had detectable Og4C3 antigen levels. (Fig 3.1), suggesting that the test can be used to identify individuals with very low worm load or unisex worm infection. Chanteau et al., (1994) have also reported the antigen positive EN by Og4C3 mAb ELISA and has suggested that the test would help to identify individuals with prepatent infection or those who have young worms that are incapable of producing the larva. Similarly, CFA was detected in some of the EN individuals (Weil et al., 1996) using AD12.1 mAb. Thus filarial antigenemia in endemic normals maybe a marker for prepatent infection. Previous studies done by (Lammie et al., 1994) using Og4C3 assay have shown that in endemic areas filarial antigenemia is more than mf prevalence in all the age group. This can be because of the poor sensitivity of the detection of mf by blood smear or due to the existence of occult filarial infection.

The Og4C3 antigen assay was carried out using brugian samples obtained from B. malayi infected areas of India and Malaysia. The demographic details of the brugian sera are shown in Table 2.2. It is observed that all the brugian MF sera were negative by the Og4C3 assay and 2 of the 13 CP had very low levels of CFA which was less than 80 antigen units and hence taken as negative (Table 3.2). Moreover the CFA levels were negative with individuals having other parasitic infections (OPI) which included sera from people infected with O. volvulus L. loa and Ascaris. This result thus reflects the specificity of the Og4C3 assay.
Fig 3.1: Circulating filarial antigen (Og4C3) level and microfilaria count (mf) in different clinical groups of bancroftian filariasis

The above graph is a representative data of 10 serum samples from MF, CP, and EN group. Og4C3 antigen levels and the mf count were plotted against different clinical group of patients. Samples with OD values greater than standard 2 (80 antigen units) were considered to be positive.
<table>
<thead>
<tr>
<th>Clinical category</th>
<th>Positive by ELISA (Ag units range)</th>
<th>Negative by ELISA (Ag units range)</th>
<th>Mean mf count (by smear)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic microfilaraemic (MF)</td>
<td>0 (0)</td>
<td>30 (0)</td>
<td>8</td>
</tr>
<tr>
<td>n=30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic pathology (CP)</td>
<td>2 (23, 50)</td>
<td>11 (0)</td>
<td>0</td>
</tr>
<tr>
<td>n=13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endemic normals (EN)</td>
<td>0 (0)</td>
<td>10 (0)</td>
<td>0</td>
</tr>
<tr>
<td>n=10</td>
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<td>Non endemic normals (NEN)</td>
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<td>15 (0)</td>
<td>0</td>
</tr>
<tr>
<td>n=15</td>
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<td></td>
<td></td>
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<tr>
<td>Other parasitic infection (OPI)</td>
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</tr>
<tr>
<td>n=5</td>
<td></td>
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</tr>
</tbody>
</table>

Table 3.2: The circulating filarial antigen units (Og4C3) and microfilaria count in different clinical groups of Brugian filariasis patients and other parasitic diseases

The sera exhibiting antigen units 80 and above were considered positive
3.1.2 CFA and microfilarial density in MF patients

To study whether there is any correlation between the circulating antigen levels and microfilarial density, CFA levels were measured in 40 Bancroftian MF individuals. These individuals had microfilaria levels ranging from 1-1000/ml by nucleopore membrane filtration technique. All the MF individuals had high levels of CFA and there was a least correlation ($r^2 = 0.624$) between microfilarial count and antigen levels (Fig3.2). This suggests that the antigen detected by Og4C3 mAb based assay may not be of microfilarial origin alone. These results are in concordant with a previously published report where negative correlation was observed between CFA and mf levels (More and Copeman 1990). Our finding can be further substantiated by another study by Chanteau et al (1994) in which under experimental conditions no detectable level of Og4C3 antigen was identified in the mf culture supernatant.

3.1.3 CFA levels in paired (day and night) serum samples

In order to establish the utility of the assay using daytime samples, paired serum samples were obtained from ten Bancroftian MF individuals during the day and night. It was observed that the antigen levels in the night or day blood samples were almost similar and CFA levels showed a positive correlation which was statistically significant ($r = 0.732$ $p < 0.05$ Fig 3.3 ). Thus this test can be performed using day blood samples and could replace the existing night blood survey, which poses practical difficulties in an endemic area. Lammie et al (1994) have also demonstrated that the antigen levels measured by Og4C3 assay were equivalent for samples collected either in the day or night but in contrast the daytime mf counts were two logs lower than the night time mf levels. Thus CFA assay can be performed with the daytime samples and serve as an initial screening procedure to detect the individuals active filarial infection in endemic areas. Further only those individuals who are antigen positive need to be tested in the night time when mf data are required to study the filariasis transmission.
Fig 3.2: Correlation between circulating filarial antigen level and microfilaria counts in the serum samples of bancroftian MF patients.

The relationship between the number of microfilariae (No./mL) and levels of circulating antigen in sera (expressed as antigen units using the Og4C3 assay) from 40 MF patients is shown in the graph. The antigen units were plotted against the mf count and a negative correlation was seen between them. Correlation coefficient: \( r^2 = 0.624 \)

Fig 3.3: Comparison of circulating filarial antigen level as measured by Og4C3 assay in the day and night serum samples collected from Bancroftian microfilaraemic patients.

Paired serum samples from 10 bancroftian MF individuals were collected during day and night time and the CFA levels were measured. There was no marked variation in the CFA levels in the day and night blood samples. Pearson Correlation coefficient: \( r = 0.732 \) \( p < 0.05 \)
3.1.4 Og4C3 ELISA using blood collected on filter strips

To make this assay more adaptable for large-scale field trials and participation of large number of individuals in the community based studies, blood samples were collected from 40 Bancroftian MF patients by venipuncture and by finger prick on filter paper strips from the same individuals. The CFA level were quantitated in the serum and filter eluate samples. Necessary calculations have been made to identify the blood volume of both samples. The CFA levels from the filter paper eluate were compared with the corresponding serum antigen levels. The results showed a positive correlation between the CFA levels in the serum and filter eluate of the same patient which was found to be statistically significant \( (r = 0.835 \ p < 0.05) \) Fig 3.4. This result suggests that the Og4C3 assay can be performed with either of them.

Advantages of collecting finger prick blood samples on filter strips:

- Filter strips blood collection method can be used in seroepidemiological surveys where preservation of serum is a problem.
- It can be performed on a finger prick specimen taken at any time of the day.
- It requires minimum facilities under field conditions and is less troublesome than the more invasive venipuncture procedure.
- The samples collected on the filter strips are dry, light, easy for handling and transportation.

Thus measuring the CFA levels appears to be the first step in identifying the patients carrying prepatent infection. Thus it is clear from the above results of Part I that mAb Og4C3 based sandwich ELISA is a reliable, sensitive and reproducible test for the diagnosis of bancroftian filariasis.

However, besides the high cost factor involved in performing the Og4C3 assay and its specificity for bancroftian filariasis, the assay fails to identify individuals with active brugian filarial infection. Brugian filariasis caused by \( B. \ maltai \) infects around 13 million people mainly in certain parts of South east Asia including certain parts in India. So far antigen assays of an acceptable degree of sensitivity and specificity for brugian filariasis have not been developed,
Fig 3 4: Comparison of circulating filarial antigen levels in the serum and the blood absorbed onto the filter strip from the same bancroftian MF individual.

Results are expressed as mean antigen units ± SEM of 40 microfilaraemic patients. 50μl of serum or filter eluate from same individuals sample was used for the assay. The antigen levels in the serum and filter eluate showed a positive correlation. Correlation coefficient (r=0.835, p<0.05)
although it can be identified by night blood smear and the \textit{BmA} based IgG4 assay which has its own limitations. Hence to address this problem there is a need to develop a sensitive immunodiagnostic assay to detect brugian infection. The results of such a study is presented in the next part of the thesis.

3.2 DEVELOPMENT OF A RECOMBINANT FILARIAL ANTIGEN (pRBSXP) BASED ELISA FOR THE DIAGNOSIS OF BANCROFTIAN AND BRUGIAN FILARIASIS

There is no suitable animal model to study the human lymphatic filariasis caused by \textit{W. bancrofti} and also lack of sufficient parasite materials makes it difficult to study the various stages of the disease. But with advent of recombinant DNA strategies it has been possible to get several stages specific antigens, which has been isolated and cloned from the cDNA libraries. These filarial antigens are important in diagnosing patients with the early stage of infection or who appear amicrofilaraemic by other conventional method.

Thus in the present study we have used a recombinant filarial antigen purified from the clone pRBSXP and developed an antigen and antibody based ELISA for the diagnosis of both brugian and bancroftian filariasis. The ELISA has been simplified and made to suit the field condition using finger prick day blood samples. It has also been used to evaluate chemotherapeutic follow up in MF individuals treated with the DEC.

3.2.1 Characterization of recombinant filarial clone pRBSXP

The gene encoding SXP was identified from \textit{\textlambda}gt11 cDNA library of adult \textit{B. malayi} by immunoscreening with MF sera by Dissanayake \textit{et al.}, 1992. This gene was then cloned into pMal vector as a maltose binding protein (MBP) fusion protein. The MBP present along with the recombinant fusion protein exhibited extensive cross reactivity with the human sera. Moreover the proteolytic cleavage of the MBP protein was difficult, leading to difficulty in purifying the protein (Wang \textit{et al.}, 1997). To overcome this problem, the MBP-SXP protein SXP gene alone was recloned to a better expression vector i.e., T7 expression vector,
pRSETB, at the EcoRI site (Rao 1998). The clone was renamed as pRBSXP. The orientation of the clone was confirmed by performing PCR with the insert specific primers and the size of the insert was determined by restriction digest with EcoRI enzyme. The protein size was determined by SDS-PAGE and the specific reactivity tested by western blot analysis.

3.2.1.1 PCR profile

PCR was carried out with the pRBSXP transformants using forward and reverse SXP specific primers (Table 2.4). The amplification profile of the clone is shown in (Fig 3.5). It was found that the SXP gene is 489bp in length and it codes for a protein of 23kDa in size (Table 2.5).

3.2.1.2 Restriction analysis of pRBSXP

The transformants were grown overnight in LB medium and the plasmid DNA was extracted as per the standard protocol (Sambrook et al., 1989). The clone pRBSXP was restricted with the EcoRI restriction enzyme and ran on a 0.8% agarose gel. The restriction profile showed that the insert size of 489bp and 2.9kb as the vector backbone (Fig 3.6).
Fig 3.5 Characterization of *B. malayi* SXP in pRSETB by PCR.

1% agarose gel electrophoresis pattern of PCR products amplified from transformants using SXP specific forward and reverse primers. Lane M: 100 bp ladder, Lanes 1-3 Transformants, Lane 4: +ve control and Lane 5: -ve control for PCR.
Fig 3.6 Restriction analysis of pRBSXP using EcoR I and BamHI enzyme

Approx., 2 μg of pRSETB and the recombinant pRBSXP plasmid DNA were analysed with EcoR I and BamHI and resolved on 1% agarose gel. Lane 1: λ Hind III digest, Lane 2: pRBSXP undigested, Lane 3: BamHI digest of pRBSXP, Lane 4: EcoR I digest of pRBSXP, Lane 5: pRSETB undigested, Lane 6: EcoR I digest of pRSETB, Lane 7: λ Hind III digest, Lane 8: 100bp ladder. The insert from pRBSXP was released upon digestion with EcoR I (Lane 4).
3.2 1.3 SDS-PAGE analysis

After the plasmid was established in a non-expression host, they are transformed into an host bearing the T7 RNA polymerase gene ((DE3 lysogen) for expression of the target protein. The commonly used host is BL-21 (DE3) which has the advantage of being deficient in both lon (an ATP dependant protease) and ompT (an outer membrane protease), which will enhance the expression of intact protein without proteolytic cleavage by proteases. In this host T7 RNA polymerase is under the control of the inducible lacUV promoter, which allows some degree of transcription in the uninduced state. The plasmid was transformed into BL-21(DE3) and the expression of the recombinant clone was studied under uninduced and induced condition using IPTG at a final concentration of 1mM. The protein was expressed even at the basal level due to the leaky expression of T7 RNA polymerase. The size of the protein was found to be 23kDa (Fig 3.7).

3.2.1.4 Western blotting

In order to study the immunoreactivity of the recombinant SXP protein, western blotting was carried out using various clinical groups of filarial patients' sera. Protein extracted from the recombinant clone pRBSXP was ran on a SDS-PAGE with suitable molecular weight markers and then electro transferred onto an NCP membrane. The membrane was probed with different clinical group of filarial sera. The 23kDa protein distinctively reacted only with the MF sera of *W. bancrofti* or *B. malayi* infection (Fig.3.8).

The earlier studies done by Dissanayake *et al.* (1992) have shown that when the SXP gene is expressed as maltose binding protein (MBP) fusion protein (pMal vector), it detected MF individuals who were infected only with bancroftian filariasis. But in the present study when the SXP gene was expressed in pRSETB which has only six histidine tag as the fusion partner, it reacted with MF individuals infected with brugian and bancroftian filariasis. Hence it appears that the removal of MBP fusion protein and subsequent expression of the SXP gene in the pRSETB vector is advantageous over pMal vector since it is able to detect filarial infection caused by both *B. malayi* and *W. bancrofti*. Moreover it is easy
Fig 3.7. SDS-PAGE analysis of pRBSXP protein

Total protein extracts from recombinant pRBSXP and control pRSET B were solubilised in 1 X SSB, separated on 12% SDS-PAGE gel and stained with CBB dye. 75 μg of protein was loaded in each of the respective lanes. Abbreviations: UI=Uninduced, I= Induced, M: Molecular weight marker. The recombinant protein expressed from the clone pRBSXP is approx. 23kDa in size. The recombinant protein is shown as marked by a arrow.
Fig 3.8 Western blot analysis of pRBSXP

Induced pRBSXP protein was separated on SDS-PAGE, transferred to nitrocellulose membrane and probed with 1:2000 diluted pooled (A pool of 10) *W. bancrofti* MF (*Wb MF*), *B. malayi* MF (*Bm MF*), CP, EN and NEN sera. The 23 kDa recombinant protein is distinctly detected by bancroftian and brugian MF sera. Lane M=Molecular weight marker is shown on the left side. The recombinant protein is shown as marked by a arrow.
to purify the recombinant antigens from pRSET by metal affinity column since it is expressed as the fusion protein with polyhistidine tag.

3.3 PURIFICATION OF pRBSXP PROTEIN BY IMMOBILIZED METAL AFFINITY COLUMN (IMAC)

The recombinant protein was expressed in *E. coli* host BL21. Earlier studies have shown that when foreign genes that are highly expressed under the T7 promoter system form inclusion bodies (Marston 1986). Moreover depending on the nature of the expressed protein, the rate of their expression, and the level of expression exert a profound influence on the formation of inclusion bodies. High expression rates allow insufficient time for the nascent polypeptide chain to fold into the native conformation. This combined with increased and localized concentration of the protein in the cytoplasm, lead to non-specific precipitation and thus results in the formation of inclusion bodies.

3.3.1 Solubility of inclusion bodies

The outer membrane of *E. coli* is composed of lipoproteins, lipopolysaccharides and proteins whereas phospholipids and proteins are the two components of the inner membrane. In addition, the periplasmic space contains peptidoglycans, all of which contribute to the rigidity of the cell wall. Inclusion bodies formed within the cytoplasm cannot be satisfactorily isolated by mechanical methods for cell disruption, which include high-pressure homogenization and the French press. These methods release nucleic acids that lead to viscosity associated problems and produces complex mixtures of contaminants and partially damaged products (Kula and Schute 1987). Non mechanical techniques like, osmotic shock and enzymatic lysis are generally preferred. But the disadvantage is that the osmotic shock leads to lower yield of the protein and enzymatic lysis with lysozyme is good but for economic reasons it is available only at laboratory scale and not for large scale preparation. Thus solubilization of the inclusion bodies can be achieved by using chemical release methods using chaotropic agents or detergents. The common chaotropic agents are Guanidine hydrochloride (GuHCl)
and urea. Thus in our study we have used 8M urea solution in phosphate buffer (0.1M pH 8.0) to solubilise the proteins. The solubility of the protein was analyzed by SDS-PAGE. It was observed that by the end of 8 hours 50% of the protein was solubilised and the remaining protein was present in the pellet (Fig 3.9). The solubilised protein solution was passed through the IMAC column to purify the proteins. The purification was performed under denaturing conditions.

### 3.3.2 Studies on the efficiency of the column

The solubilised protein was allowed to bind to the matrix for 2 to 4 hours. The column was washed with starting buffer and the flow-through fractions were collected until the A$_{280}$ reached the baseline. The coupling efficiency of the column was 75-80% as observed by the significant difference in the A$_{280}$ values of the protein before and after allowing it to bind to the column.

### 3.3.3 Elution of the protein

Preliminary experiments were carried out to standardize the elution procedure. The pH gradient, competitive ligand, imidazole or a complexing agent, like EDTA was used for desorbing the target protein from the affinity matrix. The percentage recovery of target protein was high (20%) with 100mM imidazole elution in comparison to pH and EDTA elution, which was around only 1-2% (Table 3.3).
Fig 3.9 SDS-PAGE analysis of solubilised recombinant protein using 8M urea at different time point

The total protein extract from the recombinant protein (pRBSXP) was solubilised with 8M urea at different time points and the level of solubility was analyzed by loading about 50μg of the solubilised protein on a 12% SDS-PAGE and CBB stained.

Lane M Molecular weight marker
Lane 1 pRBSXP protein solubilized for four hours
Lane 2 pRBSXP protein solubilized for eight hours
Lane 3 pRBSXP protein present in the cell pellet after eight hours of solubilization
Lane 4 pRBSXP protein solubilized for an hour
Lane 5 pRBSXP protein solubilized for two hours

The recombinant protein is shown as marked by an arrow.
<table>
<thead>
<tr>
<th>Type of elution</th>
<th>Concentration of Total protein/ml matrix</th>
<th>Amount of protein eluted (µg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH = 4.5</td>
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<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>0.1M EDTA</td>
<td>2mg/mL</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>0.1M Imidazole</td>
<td>2mg/mL</td>
<td>200</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 3.3: Recovery of purified pRBSXP antigen from IMAC column using different elution technique.
The possible reason could be that imidazole is a strong unidentate ligand displacer and it is less prone to scavenge metal ions from the gel than EDTA or by buffers of low pH. Most of the protein was eluted in the first few fractions, which was read spectrophotometrically at 280nm and the chromatogram is shown in the (Fig3.10).

The wash and eluted fractions were analyzed on a 12% SDS-PAGE (Fig3.11). The recovery of the protein in the eluted fraction was found to be 20%. The purified protein was dialyzed against stepwise decreasing gradient of urea ranging from 2M to 0.5M to facilitate proper folding of the protein and prevent precipitation of the protein during dialysis.

The purified protein was used:

- To develop an recombinant antigen based IgG4 assay
- To raise antibodies in mice and rabbits
- To develop an antigen detection system using the monospecific antibodies for the diagnosis of filariasis.

### 3.3.4 Recombinant antigen (pRBSXP) specific IgG4 antibody levels in the blood samples collected on filter paper eluate from bancroftian filariasis

The purified recombinant filarial antigen (pRBSXP) was used to develop a IgG4 specific antibody assay. Previous studies in our laboratory by Rao (1998) have shown that pRBSXP specific IgG4 levels were preferentially high in the MF and TPE patient’s sera. Moreover the pRBSXP specific IgG4 assay was nonreactive with the nonendemic sera as well as sera from patients with other helminthic infection like *O. volvulus L. loa and Ascaris lumbricoides*, and hence suggesting its potential use for immunodiagnostic purposes.

In order to study the feasibility of recombinant protein specific IgG4 ELISA for field study, the same blood samples collected from 40 bancroftian MF individuals which was used for studying the CFA levels were used. The pRBSXP specific IgG4 ELISA was performed and the results compared with the
Fig 3.10 Chromatogram of pRBSXP protein purification profile using IMAC column

The flow through and the eluted fractions were collected and the absorbance measured at 280nm.

Peak 1: represents the amount of protein sample loaded on to the column
Peak 2: represents the amount of protein eluted from the column
Sup: represents the solubilized protein supernatant loaded on to the column
W1-W5: represents the wash fractions from the column
E1-E4: represents the eluted fractions from the column
Fig 3.11 SDS-PAGE analysis of the different fractions of the pRBSXP protein obtained from IMAC column.

The urea solubilized pRBSXP protein was passed through a nickel affinity column (IMAC) and the various wash and eluted fractions were collected. Approximately 10µg of the protein from different fraction was ran on a 12% SDS-PAGE.

Lane C: Crude cell lysate containing the pRBSXP protein (+ control);
Lane S: Urea solubilized protein;
Lane W1&W5: Wash fractions;
Lane E1&E2: Eluted protein fractions and
Lane M: Molecular weight marker
The recombinant protein is marked by an arrow.
commercially available Og4C3 antigen assay. It is seen that 93% of the bancroftian MF patients were positive by this assay in comparison with the Og4C3 assay (Table 3.4). Hence this assay can be used as an alternate for the night blood survey for screening mass population during daytime. The possible reason for the failure of the assay for not detecting 7% of parasitologically confirmed cases of MF could be that the most of the antibody might be complexed with the antigen leading to low levels of free antibody.

Earlier studies by Kwam-lim et al., (1990) and Lammie et al. (1998) have shown the importance of IgG4 assay in relation to the infection status in children. They have observed that IgG4 levels increased significantly among children who acquired infection by four years and were antigen positive (Og4C3) than among children who remained antigen negative. Therefore monitoring the IgG4 levels in children is important parameter to prevent the development of the disease. But monitoring the BmA specific IgG4 levels in children becomes difficult since it requires serum samples. Earlier work carried out in French Polynesia where bancroftian filariasis is prevalent, have reported that BmA specific IgG4 assay could not be performed in the filter eluate since the dilution of sample was high and hence samples with low levels of IgG4 were negative (Chanteau et al., 1991). This problem has been obviated in our study by using recombinant antigen (pRBSXP) in the IgG4 assay, since it can be performed using small volume of sample (finger prick blood) rather than serum samples in young children.

Moreover the recombinant antigens can be obtained in large quantities in shake flasks at laboratory conditions at anytime and thus avoid the need of obtaining crude filarial adult antigens from the peritoneal cavity of the animals which is difficult at times. Further recombinant antigens have advantage over the crude or fractionated antigens in that they do not exhibit any cross reactivity among other nematode infections. Earlier studies have shown that much of the cross reactivity is due to the nonprotein determinants (e.g., carbohydrates and phosphorylcholine) which are present in many components of the crude antigen extracts prepared from adult worm (Lai and Ottesen 1989).
Table 3.4: Detection of circulating filarial antigen (Og4C3) and pRBSXP specific IgG4 antibody levels from blood collected on filter strips from bancroftian MF individuals

<table>
<thead>
<tr>
<th>Number of bancroftian MF samples</th>
<th>Positive by Og4C3 assay (Range- Antigen units)</th>
<th>Positive by pRBSXP antibody assay (Range- OD 405nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF 38/40 N=40 (80 - 261)</td>
<td></td>
<td>34/40 (0.15 - 1.5)</td>
</tr>
</tbody>
</table>

For Og4C3 assay, the sera exhibiting antigen units 80 and above were considered positive.

For pRBSXP specific IgG4 assay, the cut off value was taken as mean OD +SD of NEN sera (0.1 + 0.09) which was used as the negative control.
3.3.5 Comparison of pRBSXP specific IgG4 assay with BmA and BmMF specific IgG4 assays for the diagnosis of Brugian filariasis

The study was carried out to design an the pRBSXP specific IgG4 ELISA to diagnose patients harboring B. malayi infection. This study is more important for our country were dual infection caused by W. bancrofti and B. malayi is prevalent.

The brugian serum samples were obtained from two places (i) Shertalli taluk of Alleppay, Kerala, India, which consists of 22 MF and 8 CP sera and (ii) Malaysia consisting of 8 MF, 5 CP, 10 EN and 15 NEN sera. The demographic details of the brugian filariasis patient are given in Table 2.2. The IgG4 assays were carried out using crude Brugia malayi adult worms(BmA) and microfilariae (BmMF) and the purified recombinant protein (pRBSXP) antigens and the results compared. The serum samples were considered positive for the respective assays if it had optical density (OD) greater than the mean OD of the NEN samples plus three SD (mean NEN OD+3SD).

The results are shown in Table 3.5. The pRBSXP specific IgG4 assay was able to detect the highest number of brugian MF patients 26/30 (86.66%) when compared with the other IgG4 assays performed using crude antigens BmA 22/30 (73.33%) and BmMF 21/30 (70%). Two of the ten endemic normals were positive for the recombinant SXP based IgG4 antibody ELISA whereas only one of the individual was positive by the BmA and BmMF IgG4-ELISA. The specificity of the assays was found to be 100% for pRBSXP specific IgG4 whereas it was 86.66% when IgG4 assays was performed using crude antigens.

The above results demonstrates that the recombinant antigen specific IgG4 assay is preferable over the conventional assay using the saline extracts of crude antigens identifying individuals with active of brugian filariasis. Moreover the recombinant antigen based ELISA can be used to detect patent and/or cryptic infection in areas where brugian filariasis is prevalent. There is no reliable test
<table>
<thead>
<tr>
<th>Clinical category</th>
<th>pRBSXP specific IgG4 (% positive)</th>
<th>BmA specific IgG4 (% positive)</th>
<th>BmMF specific IgG4 (% positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic</td>
<td>26/30 (86.66)</td>
<td>22/30 (73.33)</td>
<td>21/30 (70)</td>
</tr>
<tr>
<td>microfilaraemic (MF)</td>
<td>n=30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic pathology</td>
<td>2/13 (15)</td>
<td>4/13 (30)</td>
<td>2/13 (15)</td>
</tr>
<tr>
<td>(CP)</td>
<td>n=13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endemic normals</td>
<td>2/10 (20)</td>
<td>1/10 (10)</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td>(EN)</td>
<td>n=10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non endemic normals</td>
<td>0/15 (0)</td>
<td>2/15 (13.33)</td>
<td>2/15 (13.33)</td>
</tr>
<tr>
<td>(NEN)</td>
<td>n=15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5: Comparison of pRBSXP specific IgG4 assay with the existing IgG4 assay using different crude filarial antigens in brugian filariasis

The brugian serum samples were obtained from two places (i) Shertalli taluk of Alleppay, Kerala, India, which consists of 22 MF and 8 CP sera and (ii) Malaysia consisting of 8 MF, 5 CP,10 EN and 15 NEN sera
now available for the diagnosis of Brugian filariasis. The Giemsa-stained thin blood smear is used to differentiate *W. bancrofti* microfilariae, which have no nuclei in the tip of the tail from those of *B. malayi*, which have two prominent nuclei at the tip of the tail. Rahmah *et al.*, (1994) has using crude *BmA* antigen have shown the use of IgG2 in the diagnosis of CP and IgG4 to detect the MF in brugian filariasis. Currently a new PCR-ELISA has been developed for the detection of *B. malayi* infection using fingerprick blood Rahmah *et al* (1998) but it is not cost effective.

### 3.3.6 Comparison of pRBSXP specific IgG4 assay with Og4C3 antigen assay for brugian filariasis

In India we have dual type of lymphatic filariasis caused by *B. malayi* or *W. bancrofti*. Thus it becomes difficult to identify brugian filariasis by the current antigen assay (Og4C3) or the ICT filariasis card test which is specific only for bancroftian filariasis.

Therefore this study was carried out to study the advantage of pRBSXP specific IgG4 over mAb based Og4C3 antigen assay for detecting active brugian filariasis. The assays were performed with the brugian sera obtained from India and Malaysia. It was observed that all the brugian MF patients had negative CFA levels by Og4C3 assay whereas they were positive by the recombinant antigen based IgG4 assay (Fig 3.12A&B). Thus pRBSXP specific IgG4 assay is specific in identifying active brugian filariasis, which can otherwise go unnoticed by the currently available immunological assays.
Fig 3.12: Circulating filarial antigen level (Og4C3) and pRBSXP specific IgG4 levels in brugian filariasis.

A: The CFA level were quantitated in different clinical groups of brugian filariasis patients using Og4C3 assay. The CFA level were plotted against the different clinical group of patients. It was observed that all the MF patients were negative by this assay.

B: The pRBSXP specific IgG4 levels were monitored in different clinical groups of brugian filariasis patients. The optical density measured at 405nm was plotted against the different clinical groups of patients. The serum samples were used at a dilution of 1:100. The cut off value was taken as the mean OD +3 SD of 15 NEN sera {0.1 +3(0.03)}. The MF group showed the highest antibody reactivity 86.66% (26/30) whereas the CP and the EN group had least antibody reactivity 15% (2/15) and 20% (2/10) respectively.
3.4 APPLICATION OF IMMUNOASSAYS FOR CHEMOTHERAPEUTIC FOLLOW UP STUDIES IN BANCROFTIAN MF INDIVIDUALS TREATED WITH DEC

Earlier studies have shown that the most commonly used drug for treatment of filariasis was diethylcarbamazine (DEC), which has got microfilaricidal effect and partial macrofilaricidal effect (Duke, 1980). But an specific end point of DEC treatment has not been established still, since most of the study are based on clearance of microfilaria in the blood, because of the difficulty in quantitating and identifying the adult worm which resides in the human lymphatics. But nowadays, ultrasonography has been used directly to assess the in vivo effect of antifilarial drugs on the adult worms (Dreyer et al., 1995). But the potential disadvantage of this technique is the lack of the equipment in all the areas where the disease is endemic and also a shortage of skilled personals in ultrasonography in developing countries. Recently Nutman et al (1996) have used a PCR based assay to assess the infection status Onchocerca volvulus patients treated with amocarzine, to predict the likelihood of having recurrence of microfilaramia after treatment.

Therefore the present study was designed to examine the utility of CFA levels as quantitated by (Og4C3) and a recombinant antigen (pRBSXP) specific IgG4 assay as a tool for studying chemotherapeutic follow up studies bancroftian MF individuals when treated with DEC.

A pilot experiment was carried out in our laboratory by Lalitha et al (1998) to study the use of Og4C3 assay in immunomonitoring the bancroftian MF patients treated with DEC. It was observed that the CFA antigen levels were remarkably stable during the course of treatment with DEC and upto a period of one month, inspite of the mf density as measured by membrane filtration technique being also zero by the end of one month. Thus suggesting that this assay is a better marker for studying the infection status than rather than looking at the mf by the night blood smear. Since the initial results of using CFA to study the state of ongoing infection were promising this prompted us to study the changes in CFA by Og4C3
and pRBSXP specific IgG4 levels for a longer period (5 months) in two different endemic areas in Chennai India where different DEC drug regimens were given. Samples were collected on filter strips before and after treatment over a period of five months and ELISA was performed instead of measuring the mf counts by the conventional method.

3.4.1 Application of CFA assay in chemotherapeutic follow up of bancroftian MF patients treated with DEC

Two groups of bancroftian MF individuals, who were positive by night blood smear and living in two different endemic areas (Guduvancherry and Manimangalam) in Chennai, India were chosen for this study. This study was performed with the help of Department of Public Health, Chennai India. The demographic data of the MF individuals are given in Table 2.3. The MF individuals in the first group were administrated the standard dose of DEC for 12 days (6-mg/kg-body weight) and the second group was given a single dose (6mg/kg-body weight) of DEC. The treatment was given by qualified Medical Officer. Before therapy night blood samples was collected by finger prick on filter strips to quantify the CFA level and blood smear was done simultaneously to assess the mf count. After therapy blood samples were collected on filter strips at the end of 7, 14 and 30 days then every month up to a period of five months and the assay was carried out as mentioned in the earlier section. The side effects due to DEC treatment were qualitatively similar in both the groups. Fever, headache, lethargy and myalgia was observed between 12-24 hours after the medication and subsiding in essentially all the patients within few days. The side effects were probably due to the host reaction to antigens released by the mf that were killed following treatment with DEC (Hawking, 1979).

The pretreatment mf counts ranged between 2-25 by night blood smear ( 20µl blood) in both the groups (Table 2.3). The overall changes in the CFA levels and pRBSXP specific IgG4 levels following the two different dose of DEC treatment were summarized in the Table 3.6 as mean ± SEM. The CFA levels were measured using the commercial Og4C3 mAb based ELISA kit, since the
pRBSXP antigen assay was not standardized for samples collected on filter strips. The antifilarial IgG4 antibody levels were studied using the purified recombinant filarial antigen (pRBSXP).

Figure 3.13 represents the mean changes in the CFA profile in both the groups of MF individuals before and after treatment with DEC. The change in the CFA levels after treatment follow the same trend irrespective of the different DEC regimes. The pre treatment levels of CFA were not the same in both the groups of patients. There was no correlation between the mf count and the antigen units before treatment thus confirming our earlier findings that Og4C3 is an indicator of adult worm burden. Following DEC therapy, there was a initial decrease in the levels of CFA by day 7, which further dropped on day 14. The decrease in the levels of CFA on the 14-day was statistically significant in both the groups when compared to the pre treatment CFA levels (p<0.05). This is followed by an increase in CFA levels by day 30 which remains high upto an period of two months. The possible explanation for the initial decrease in the CFA following treatment could be that upon treatment with DEC some of the adult worms might be killed or immobilized and thereby lowering the release of specific circulating antigens which is recognized by the Og4C3 assay. But by day 30, DEC is cleared from the host and the immobilized adult worms regain its activity, which is indicated by an increase in the CFA levels.

Our results are in parallel with the findings of Weil et al., (1988) who have observed a drop in CFA levels as quantitated by AD12.1 mAb based assay following DEC treatment, suggesting that the decrease maybe due to the slow clearance of antigens from degenerating dead worms. By third month the CFA levels decrease and the decrease was statistically significant when compared with pretreatment levels (p<0.05). An enhanced level of CFA followed this by fourth month, which then remains high for the entire observation period (five months). Similar results have been observed by Zheng et al (1991a&b) who have studied the immunological and parasitological changes that take place when treated with Ivermectin following DEC therapy. They have observed that irrespective of the treatment all the individuals were amicrofilaraemic by day one.
<table>
<thead>
<tr>
<th>Endemic areas</th>
<th>Guduvancherry</th>
<th>Manimangalam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time intervals</td>
<td>Mean Og4C3 Ag units (±SEM)</td>
<td>Mean OD pRBSXP-IgG4 levels (±SEM)</td>
</tr>
<tr>
<td>Pre treatment</td>
<td>13590.59 (+1045.64)</td>
<td>0.263 (+0.046)</td>
</tr>
<tr>
<td>7th day</td>
<td>7923.10 (+1249.49)</td>
<td>0.405 (+0.077)</td>
</tr>
<tr>
<td>14th day</td>
<td>8841.19 (+938.902)</td>
<td>0.780 (+0.078)</td>
</tr>
<tr>
<td>1st month</td>
<td>22635.88 (+1445.46)</td>
<td>0.642 (+0.081)</td>
</tr>
<tr>
<td>2nd month</td>
<td>23673.14 (+1734.34)</td>
<td>0.661 (+0.080)</td>
</tr>
<tr>
<td>3rd month</td>
<td>14635.18 (+1505.48)</td>
<td>0.590 (+0.105)</td>
</tr>
<tr>
<td>4th month</td>
<td>17256.48 (+1345.80)</td>
<td>0.567 (+0.089)</td>
</tr>
<tr>
<td>5th month</td>
<td>17681.78 (+1342.34)</td>
<td>0.5589 (+0.096)</td>
</tr>
</tbody>
</table>

Table 3.6 Mean ± SEM Og4C3 antigen levels and pRBSXP specific IgG4 levels in bancroftian MF patients from two different endemic areas.
Fig 3.13: Circulating filarial antigen level (Og4C3) in filter eluate collected from MF patients before and after treatment with two different doses of DEC

The results were expressed as mean antigen units ± SEM and plotted against different time points. The change in the CFA level was similar in both the groups.

In X-axis 0 = Pretreatment CFA level and 0.5, 1, 2, 3, 4 and 5 represents the post treatment CFA level by the end of 14 days, 1, 2, 3, 4 and 5 months respectively. The Y-axis represents the mean antigen units.

**Guduvancherry group:** This group of MF patients were given the standard 12 days dose of DEC (6mg/kg body weight) and the CFA levels were monitored for a period of five months.

**Manimangalam group:** This group of MF patients was given DEC as a single annual dose (6mg/kg-body weight) and the CFA levels was monitored for a period of five months.
They have measured the circulating antigen levels using HC11, which is suppose
to be an indicator of the adult worm burden. They have observed an initial
increase followed by a decrease in the antigen levels by third month and followed
by an increase up to an year.

Moreover the change in the CFA levels are almost same in both groups by
the end of five months thus speculating that the efficacy of the two regimes
appears to be similar (Fig 3.13). The efficacy of single dose of DEC has been
studied by Cartel et al (1990) by monitoring the microfilarial density by membrane
filtration technique. They have indicated that a single dose was sufficient to
reduce the mf density by 95% by the end of five months. Using ultrasound
imaging Noroes et al., (1997), have demonstrated that single doses of 6mg/kg
weight of DEC had macrofilaricidal effect against the adult worm and increasing
the DEC dose did not increase the macrofilaricidal activity. Our findings are in
parallel with these results.

3.4.2 Application of pRBSXP specific IgG4 assay in chemotherapeutic
follow up of bancroftian MF patients treated with DEC

The antifilarial antibodies were monitored using the recombinant antigen.
pRBSXP in the same two group of bancroftian MF patients where CFA levels
were monitored by Og4C3 assay using filter eluate. The demographic details of
the patients are shown in Table 2.3.

Unlike the CFA levels the pretreatment levels of pRBSXP specific IgG4
were similar in both the groups of patients as shown in the Fig 3.14. Upon DEC
treatment there was an increase in the pRBSXP specific IgG4 levels which peaks
by day 14. The increase in the antibody is three folds more than the pre treatment
levels and is statistically significant (p<0.05). The increase in the IgG4 levels
following treatment maybe due to the unmasking of the microfilaria by DEC and
exposing it to the host immune system and also because of the antigens released by
the killed microfilariae and adult worms following by therapy. This is followed by
slight decrease in the antifilarial antibody level by one month and thereafter
remains significantly high for the entire observation period of five months when
Fig 3.14 Recombinant antigen pRBSXP specific IgG4 levels in filter eluate collected from bancroftian MF patients before and after treatment with two different doses of DEC

The results were expressed as mean optical density (OD) at 405nm ± SEM and plotted against different time points.

In X-axis 0 = Pretreatment pRBSXP specific IgG4 level and 0.5, 1, 2, 3, 4 and 5 are the post treatment pRBSXP specific IgG4 level by the end of 14 days, 1, 2, 3, 4 and 5 months respectively. The Y-axis represents the mean OD 405nm.

**Guduvancherry group:** This group of MF patients were given the standard 12 days DEC dose (6mg/kg body weight) and the IgG4 levels were monitored for five months.

**Manimangalam group:** This group of MF patients was given a single annual dose of DEC (6mg/kg-body weight) and the IgG4 levels were monitored for five months.
compared to pretreatment levels in both the groups [Fig 3.14 (p<0.05)]. Our results are in accordance with the IgG4 kinetics reported by various workers in other endemic areas where they have used saline extract of antigens. For example, Weil et al (1988) have demonstrated an initial increase in antifilarial antibody levels by one month as measured using BmA or BmMF antigens following DEC treatment. Similarly Wamae et al (1992) have used Brugia phangi crude antigens and have observed an increase in the antifilarial IgG4 levels by day 30 of treatment followed by a significant decrease in IgG4 by day 180. McCarthy et al (1995) have shown an elevated antifilarial antibody response to Brugia malayi antigens after one month of treatment and declines thereafter by a period of six months and eventually reach baseline by 18 months.

The following facts can be inferred from the above study

- CFA (Og4C3 antigen) or recombinant antigen (pRBSXP) based antibody assay is more advantageous over the conventional methods, like routine night blood smear or membrane filtration in studying chemotherapeutic follow studies since it can performed with finger prick blood at daytime.
- Further this study has showed that the single annual dose is as effective as the standard dose of 12 days treatment and may be a suitable regimen for mass chemotherapy programs for the control of filariasis in the future.
- CFA detection represents a valuable tool for monitoring the efficacy of antifilarial drug therapy which will lead to improved use of existing drugs and aid in the evaluation of new drugs for filariasis.

PART II

In this part of the thesis development of a novel antigen-detection sandwich ELISA system has been discussed. The ELISA was developed with antibodies raised to a recombinant antigen (pRBSXP) in rabbits and mice for diagnosing active cases of brugian and bancroftian filariasis. Further the results were compared with the commercially available Og4C3 ELISA.
3.5 DEVELOPMENT OF SANDWICH ELISA (ANTIGEN DETECTION) USING ANTIBODIES RAISED TO RECOMBINANT FILARIAL ANTIGEN (pRBSXP)

3.5.1 Production of monospecific antibodies

The purified recombinant protein (pRBSXP) was used to immunize mice and rabbits. The primary and booster dose of antigen for each animal was given according to the standard protocol. The antiserum was collected 2 weeks after the last immunizing dose. The pre and post immune sera were adsorbed with E. coli proteins to prevent any cross reactivity due to the host protein. The specific immunoreactivity and the antibody titer of the hyperimmune sera were determined by western blotting and ELISA respectively. Western blot analysis was carried out with the pRBSXP antigen, which was probed with mice and rabbit pre and post immune pRBSXP sera. The vector protein served as the control. The anti pRBSXP sera of mice and rabbit were monospecific since it reacted only with the pRBSXP protein (23kDa in size) and were non reactive to the vector protein (Fig 3.15).

3.5.2 Determination of the titers of anti pRBSXP antibodies

ELISA plates (96 wells) were coated with different concentrations (1µg or 10ng /well) of purified antigen (pRBSXP) and control antigen (E. coli) and direct ELISA was performed. The pre and post immune sera were serially diluted starting from 1:1000 - 1:256000. Both mouse and rabbit anti pRBSXP sera showed the same level of reactivity to either 1µg or 10ng /well of the purified recombinant antigen (pRBSXP). The mouse anti pRBSXP sera reacted at a dilution of 1 in 32,000 while the rabbit anti pRBSXP sera reacted at 1 in 16,000 dilution. Both pre and post-immune sera showed no reactivity to the E. coli protein (Fig 3.16A&B).
Fig 3.15: Western blot analysis of pRBSXP protein using anti-pRBSXP antibodies raised in mice and rabbits

Induced samples of the clone pRBSXP and vector pRSETB were separated on SDS-PAGE, transferred to nitrocellulose membrane and probed with 1:1000 diluted mice and rabbit pre and post immune anti-pRBSXP sera.

Lane V : Induced pRSETB protein probed with post immune pRBSXP sera
Lane Pre : Induced pRBSXP protein probed with preimmune pRBSXP sera
Lane Post: Induced pRBSXP protein probed with post immune pRBSXP sera
Lane M : Molecular weight markers

The 23kDa recombinant protein is distinctly detected by both mice and rabbit SXP antisera. The recombinant protein is marked by an arrow.
Fig 3.16: Determination of anti pRBSXP antibody titers using ELISA

The ELISA plates were coated with 100ng/well of either the purified protein (pRBSXP) or the *E. coli* protein and the assay was performed with different dilution of either mice or rabbit pre or post immune pRBSXP sera. The antisera was diluted to various concentration starting from 1 : 1000 to 1:10,000. The optical density was measured at 405nm and the end point of the antibody titer calculated as the last dilution at which the OD for the post immune sera was greater than the mean OD plus two standard deviation for the preimmune sera.

A: OD 405nm was plotted against different dilution of the mice anti pRBSXP sera on a log scale. It was seen that the antisera is highly reactive only with the recombinant protein and the reactivity to the *E. coli* was also zero. The end point of the titer was taken as 1 : 32,000

B: OD 405nm was plotted against different dilution of the rabbit anti pRBSXP sera on a log scale. It was seen that the antisera is highly reactive only with the recombinant protein and the reactivity to the *E. coli* was also zero. The end point of the titer was taken as 1 : 16,000
3.5.3 Optimization of various parameters for the development of sandwich ELISA using antibodies raised to pRBSXP antigen

Criss cross serial dilution analysis was carried out to determine optimal reagent concentration to be used in the ELISA. All the three reactants in this ELISA namely –a primary solid phase coating reagent, a secondary reagent (pRBSXP antigen) that binds to the primary reagent and the second antibody that binds to the secondary reagent were serially diluted and analyzed by criss cross matrix. Thus using different permutation and combinations, it was seen that when mice anti pRBSXP sera was used as the primary solid phase coating reagent (dilution: 1:10,000) it was able to capture the antigen better than the rabbit anti pRBSXP sera used at the same dilution. Hence the sandwich ELISA was performed as follows

Primary (solid phase) coating reagent (mice) anti pRBSXP sera → Patient’s serum/pRBSXP antigen → Second antibody (rabbit) anti pRBSXP sera → Goat anti -rabbit Biotin → Streptavidin conjugate (ALP) → pNPP as substrate.

3.5.4 Sensitivity of the pRBSXP antigen ELISA using purified antigen

ELISA and dot blot analysis were carried out to find out the minimum detectable concentration of purified pRBSXP antigen. A known amount of the purified antigen starting from 5000ng to 0.125ng was added to normal sera. The serum was added to the microtitre plate, which was coated with mice anti pRBSXP antibody and the assay performed as mentioned above. The *E. coli* antigen was used as control. It was found that the minimum amount of antigen that could be detected was 40ng/ml by ELISA and no reactivity to *E. coli* antigen was observed even at a higher concentration (Fig3.17).

Dot blot analysis was done where 2.0 to 0.0165µg/well of the purified antigen was dotted on the NCP membrane and probed with mice and rabbit anti pRBSXP antibody. It was observed that mice anti pRBSXP antibody was able to detect upto 0.125µg of the protein whereas the rabbit anti pRBSXP antibody would detect only upto 0.25µg of the protein (Fig3.18). These experiments suggests that anti SXP antibodies are specific.
Fig 3.17: Sensitivity of pRBSXP antigen assay by ELISA

Optical density (OD) at 405nm with different concentration of the purified pRBSXP antigen and E.coli. The plate was coated with mouse anti pRBSXP sera at a dilution of 1 in 5000. The known amount of purified pRBSXP antigen and E.coli (concentration ranging from 5000ng to 0.125ng) was added to the normal sera and aliquot of 100μl was added to well. The second antibody used was rabbit anti pRBSXP sera at a dilution of 1 in 5000. The sensitivity of the assay was 40ng/ml.
Fig 3.18 Sensitivity of pRBSXP antigen assay by dot blot assay

Different concentrations (2μg to 0.0165μg) of the purified pRBSXP antigen and the vector protein (pRSETB) were prepared in 1X PBS (pH 7.4) by serially diluting the stock protein solution. 100μl of each dilution was dotted onto nitrocellulose membrane and the membrane probed with mouse and rabbit anti-pRBSXP antibodies. The sensitivity of the assay was qualitatively by observing the intensity of the dot as compared with the control protein at a given concentration. Hence it was 0.125μg and 0.25μg with mouse and rabbit anti-SXP antibody respectively.
3.5.5 Standardization of releasing the antigen from the immune complex

In order to study the levels of circulating antigens in filariasis it is necessary to pre treat the sera either with certain reagents or by boiling to release the antigens that may be trapped in the immune complex (IC). In the present study different method to release the antigens from the immune complex was used. The different clinical groups of bancroftian filarial patient’s sera were pre treated with either 0.5M EDTA and boiled as described by Weil et al (1987) or with 0.15M glycine (pH 2.0)/0.5MTris (pH 9.5) as described by (Kestens et al., 1991) with some modification.

Using the untreated and pre treated filarial patient's sera the pRBSXP sandwich ELISA was performed to examine the presence of free pRBSXP antigens. It was observed that untreated and EDTA treated serum samples of all the clinical group of filariasis showed very low or no reactivity while serum samples treated with glycine/Tris showed high reactivity by the ELISA as observed by an increase in the optical density at 405nm (Fig 3.19).

Thus pretreatment of the sera with glycine/Tris effectively dissociates the immune complex without compromising the reactivity of the antigen itself. This result indicate that the level of free pRBSXP specific antigens might be very less in the patient’s sera. Our findings are similar to that observed by Lutsch et al (1988), who have demonstrated the absence of free antigens in the W. bancrofti infected individual sera by two site immunoradiometric assay performed using mAb raised against B. malayi microfilariae, whereas these infected patients had detectable levels of antigen in their urine samples. Thus suggesting that the poor detection of antigen in the sera might be related to the presence of circulating immune complexes. Moreover the filarial antigen detection in patient's sample not only depend on the sensitivity of the assay but is also effected by the formation of immune complexes in vivo.
Fig 3.19 Standardization of a method to release the antigen from the immune complex

The bancroftian filarial serum samples namely 4 of MF, 2 of CP, 2 of EN and 2 of NEN samples were treated with different reagent to release the antigens and a 1:100 of the treated samples were used in the pRBSXP antigen-capture ELISA. It was observed that the maximum levels of the pRBSXP specific antigen were present when the sera were treated with glycine/Tris than those that treated with EDTA or the untreated serum.
3.5.6 Comparison of pRBSXP specific antigen assay with the Og4C3 mAb based assay for detecting circulating filarial antigens in Bancroftian and Brugian filariasis

In order to validate the utility of pRBSXP specific antigen assay in early diagnosis of filariasis, a sandwich ELISA was performed with different clinical groups of *W. bancrofti* and *B. malayi* infected filarial sera with appropriate controls. Each serum sample was tested in duplicate.

**Figures 3.20 & 3.21** shows the pRBSXP specific antigen levels in the different clinical groups of bancroftian and brugian filariasis. The results were expressed as mean optical densities at 405nm for each group ± standard error. Sera were considered positive for the assay when the optical density was higher than the mean OD + 3SD value of 19 NEN control sera. The cutoff (OD) values for the bancroftian and the brugian filariasis were 0.35 [0.3026 + 3(0.028)] and 0.25 [0.22 + 3(0.012)] respectively. The cut off line is indicated in the Fig 3.20 & 3.21 as a parallel line drawn to the X-axis. Based on this criterion it was observed that most of the bancroftian and brugian MF individuals had higher OD values above the cut off value compared with other clinical groups.

In bancroftian filariasis, the MF group exhibited the highest optical density (OD) mean OD = 0.60 whereas the CP, EN and NEN showed a mean value of OD=0.302, 0.30 and 0.138 respectively (Fig 3.20). It was interesting to find that MF group had significantly higher OD values than that of control group (Mann-Whitney U test; p < 0.05) whereas the OD values of CP group had no significantly difference than control group.

The MF group had the highest percentage of antigen positive reactivity 30/34 (88.33%) while CP and EN group had the least antigen reactivity 4/24 (16.66%) (Table 3.7). The performance of the pRBSXP antigen ELISA was compared with existing mAb based Og4C3 assay. The results were comparable (Table 3.8). Further the sensitivity and specificity of the pRBSXP antigen assay was 88.33% and 100% respectively.
Mean optical densities ± SEM obtained at 405nm for the pRBSXP specific antigen is plotted against various clinical groups of filariasis patients. The patient’s sera were used at a dilution of 1 : 100 for the assay. The number of patients that comprises the groups were as follows: MF (n=34), CP (n=34), EN (n=28) and NEN (n=19).

Sera were considered positive for the assay when the optical density (OD) was higher than the mean OD + 3SD value of NEN control sera. The mean optical density (OD) value for the 19 NEN sera was 0.3026 OD. The cut off OD = 0.35 \( \{0.3026 + 3(0.018)\} \). The cut off OD value is indicated as a parallel line drawn to the X-axis in the figure. The MF group had the highest antigen positivity 30/34 (88.23%) compared to other clinical groups.
<table>
<thead>
<tr>
<th>Clinical categories</th>
<th>Serum with no reactivity (NR) OD ≤ 0.35</th>
<th>Serum with moderate reactivity (MR) OD = 0.5</th>
<th>Serum with high reactivity (HR) OD ≥ 0.51</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF (n=34)</td>
<td>4</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>CP (n=34)</td>
<td>24</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>EN (n=28)</td>
<td>24</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>NEN (n=19)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.7: pRBSXP antigen levels in different clinical groups of Bancroftian filariasis

<table>
<thead>
<tr>
<th>Clinical categories</th>
<th>Serum with pRBSXP antigen reactivity (% positive)</th>
<th>Serum with Og4C3 antigen reactivity (% positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF (n=34)</td>
<td>30/34 (88.23%)</td>
<td>34/34 (100%)</td>
</tr>
<tr>
<td>CP(n=34)</td>
<td>10/34 (29.41%)</td>
<td>0/34 (0%)</td>
</tr>
<tr>
<td>EN (n=28)</td>
<td>4/28 (14%)</td>
<td>0/28 (0%)</td>
</tr>
<tr>
<td>NEN (n=19)</td>
<td>0/19 (0%)</td>
<td>0/19 (0%)</td>
</tr>
</tbody>
</table>

Table 3.8: Comparison of circulating pRBSXP and Og4C3 antigen levels in different clinical groups of bancroftian filariasis
**Brugian** filariasis is generally diagnosed either by night blood smear or measuring antifilarial IgG4 levels using crude antigens. Currently there is no circulating antigen detection assay available for the diagnosis of brugian filariasis. Hence in our study the pRBSXP antigen assay was carried out for diagnosing brugian filariasis. The microfilaremics group exhibited the highest optical density (OD) the mean OD = 0.42 whereas the CP, EN and NEN showed a mean value of OD=0.152, 0.148 and 0.133 respectively (Fig 3.21). Moreover the MF group had significantly higher OD values than that of control group (Mann-Whitney U test; p < 0.05)

As in bancroftian filariasis, the brugian MF group had the highest percentage of antigen positive reactivity 25/30 (83.33%) while 1/13 (7.5%) of the CP and 2/10(20%) of the EN group had the least antigen reactivity (Table 3.9). The pRBSXP antigen ELISA was compared with existing mAb based Og4C3 assay. All the brugian MF sera were negative by Og4C3 assay (Table 3.10).

Different group of researchers have used a variety of techniques to study the circulating antigens in filariasis. Some have raised polyclonal and monoclonal antibodies to fractionated antigens, ES antigens, animal filarial antigens or recombinant antigens and have developed circulating antigen assays. Earlier studies by Kaliraj et al (1981c) had detected circulating antigen in filarial sera by counter immunoelectrophoresis (CIEP) using the purified human filarial serum immunoglobulin (FSI). They have observed that 23 out of 30 MF sera and 1 of 30 CP sera were positive by CIEP. Similarly CIEP has been used in canine Dirofilariasis to detect the circulating parasite antigens(Weil et al., 1984).

Using microfilarial ES antigen of *Brugia pahangi* monoclonal antibodies were produced to the repetitive epitope of ES and has been used in detecting circulating antigens in the serum of patients infected with lymphatic filariasis Maizels et al., (1985). Similarly Mustafa et al., (1996) have shown that the antibodies raised against the excretory-secretory products released by the cattle filarial parasites (*Seteria cervi*) can be used to detect circulating antigens in filarial patients. Monoclonal antibodies K3AE7 and K3BDS raised against excretory-secretory (ES) antigens of *S. digitata* were shown to be promising in the
Fig 3.21: pRBSXP specific antigen levels in different clinical group of Brugian filariasis.

Mean optical densities ± SEM obtained at 405nm for the pRBSXP specific antigen is plotted against various clinical groups of filariasis patients. The patient’s sera were used at a dilution of 1:100 for the ELISA. The number of patients that comprises the groups were as follows: MF (n=30), CP (n=13), EN (n=10) and NEN (n=15).

Sera were considered positive for the assay when the optical density (OD) was higher than the mean OD + 3SD value of NEN control sera. The mean optical density (OD) values for the 15 NEN sera was 0.22 OD. The cut off OD = 0.25 [0.22 + 3(0.012)]. The cut off OD value is indicated as a parallel line drawn to the X-axis in the figure. The MF group had the highest antigen positivity 25/30 (83.33%) compared to other clinical groups.
### Table 3.9: pRBSXP antigen levels in different clinical groups of Brugian filariasis

<table>
<thead>
<tr>
<th>Clinical categories</th>
<th>Serum with no reactivity (NR) OD ≤ 0.25</th>
<th>Serum with moderate reactivity (MR) OD = 0.4</th>
<th>Serum with high reactivity (HR) OD ≥ 0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF (n=30)</td>
<td>5</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>CP (n=13)</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EN (n=10)</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NEN (n=15)</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 3.10: Comparison of circulating pRBSXP and Og4C3 antigen levels in different clinical groups of brugian filariasis

<table>
<thead>
<tr>
<th>Clinical categories</th>
<th>Serum with pRBSXP antigen reactivity (% positive)</th>
<th>Serum with Og4C3 antigen reactivity (% positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF (n=30)</td>
<td>25/30 (83.33%)</td>
<td>0/30 (0%)</td>
</tr>
<tr>
<td>CP (n=13)</td>
<td>0/13 (0%)</td>
<td>2/13 (6.66%)</td>
</tr>
<tr>
<td>EN (n=10)</td>
<td>2/10 (20%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>NEN (n=15)</td>
<td>0/15 (0%)</td>
<td>0/15 (0%)</td>
</tr>
</tbody>
</table>

Table 3.9: pRBSXP antigen levels in different clinical groups of Brugian filariasis

Table 3.10: Comparison of circulating pRBSXP and Og4C3 antigen levels in different clinical groups of brugian filariasis
diagnosis of *W. bancrofti* infection (Dhas and Raj 1995). Chenthamarakshan *et al.*, (1996) have used fractionated microfilarial ES antigen (BmE DE1) to develop an ELISA and found it to be more superior in detecting IgG levels in microfilaremics than the whole *B. malayi* ES extracts.

Polyclonal antibodies were raised in mouse ascitic fluid against of *B. malayi* adult worms and an sandwich ELISA developed (Cheirmaraj *et al.*, 1991). They have shown that 90% of MF sera, 30% of sub and acute filarial sera and 20% of the CP sera were positive for circulating filarial antigens by this assay.

Nowadays recombinant antigens have been widely used for the development of diagnostic reagents for parasitic and infectious disease. Polyclonal antibodies raised to a 15kDa protein of *S. digitata* and monoclonal antibodies derived from a recombinant protein (pGT7) were used in a sandwich ELISA for the detection of circulating parasite antigens (Theodore *et al.*, 1996). They have shown that all the 16 MF were positive and the other clinical group of patients were negative by this assay. Recombinant filarial antigen BmM5 and BmM15 have been successfully used in diagnosing brugian and bancroftian filariasis in 90% of the Indian and Egyptian patient's samples (Chandrasekhar *et al.*, 1994). Recently a new indirect ELISA using antibodies to recombinant PDi33 (*Dirofilaria immitis*), which was an analogue of aspartyl protease inhibitor was highly sensitive in diagnosing prepatent infection in cats infected with *D. immitis* (Frank *et al.*, 1998).

In our study the development of antigen assay using monospecific antibodies raised to recombinant antigen (pRBSXP) has been evaluated and the initial results are encouraging. Similar work has been carried out by Hassan *et al.*, (1996) using polyclonal antibodies raised to *Dirofilaria immitis* worm homogenate and a monoclonal antibody (AD12) in a sandwich ELISA. It has been reported that using polyclonal antibodies, microfilaraemic group had the highest percentage of antigen positivity 7/12 (58.3%) among all groups while symptomatic amicrofilaraemic group had the least antigen positivity 2/16 (12.5%). This result were in consistent with our finding i.e., by pRBSXP antigen assay the
MF group has the highest antigen reactivity compared with the other clinical group of filariasis.

The present study suggests that the detection of circulating pRBSXP antigen can be a useful tool to diagnose filarial infection particularly brugian and bancroftian filariasis. However this study was based on a small number of serum samples. Hence this test requires further validation with larger number of samples. In addition, by enhancing the amplification technique at the detection level, the sensitivity of the ELISA can be improved.

4.0 CONCLUSION

♦ Og4C3 antigen ELISA using blood collected on filter paper is a sensitive, specific, and practical method for detection of bancroftian microfilaraemics in endemic areas. This technique has been optimized for sero epidemiological studies where in blood samples were collected onto filter strips during the daytime hence, this method of diagnosis appears to be the first step in the control of spread of filarial infection alternative to fingerprick night blood smear.

♦ The recombinant filarial protein (pRBSXP) was overexpressed and purified using IMAC. The purified recombinant protein was used to develop an IgG4 assay and was found to be specific in recognizing bancroftian and brugian MF individuals with active infection.

♦ The Og4C3 antigen assay and the pRBSXP specific IgG4 assay were used as a tool for studying the efficacy of different regimens of DEC therapy and for the chemotherapeutic follow up of in bancroftian MF individuals. It was observed that the single dose of DEC had the same effect as the 12 day dose. Further monitoring antigenemia gives a better insight about the ongoing infection in the individuals.

♦ The purified pRBSXP antigen was used to raise monospecific antibodies in mice and rabbit. An pRBSXP antigen capture ELISA was developed using the monospecific antibodies. The bancroftian and brugian MF individuals
exhibited the highest percentage of reactivity of 88.33% and 83.33% by this assay respectively. This assay is advantageous over the Og4C3 assay which is specific for bancroftian filariasis whereas the pRBSXP antigen ELISA is able to detect individuals with active infection either with bancroftian or brugian filariasis.