CHAPTER 4
DISCUSSION

Human Lymphatic filariasis is a major debilitating disease. World Health Organization has initiated the Global Program to Eliminate Lymphatic Filariasis (GEPLF) by the year 2020 through mass drug administration and alleviation of disability (Ottesen 2000). A regional programme to combat lymphatic filariasis in the Pacific Islands is showing great promise as it reaches its halfway point. The Pacific Programme to Eliminate Lymphatic Filariasis (PacELF), established in 1999, aims to eliminate the disease from the Pacific by 2010, ten years ahead of the global target. Certainly much emphasis is given to easy, rapid and early diagnosis of filariasis especially in individuals having new exposure to infections. WHO recommends baseline surveys using kits to assess prevalence of infection, elimination activities in countries that show prevalence of 1% by Mass Drug Administration and mosquito control (WHO 2005).

Extensive research has been carried out on diagnostic development and evaluation by several researchers. A simple and sensitive rapid immunodiagnostic method for detection of antigen and antibody for the ‘differential diagnosis’ of filarial infections is an imminent need. An unmistakable method of diagnosis of lymphatic filariasis has been the direct confirmation of microfilariae in blood by Geimsa stained smears by microscopy (Khamboonruang et al 1987, Sabry 1992), knot’s concentration and membrane filtration techniques. However such techniques may become
less sensitive since microfilariae can be present in very low numbers and inaccessible, absent as in prepatent cases or show nocturnal periodicity.

The advent of recombinant DNA technology has paved way for construction of gene libraries or cDNA of parasite DNA. Such libraries were screened with either B. malayi or W. bancrofti infected patient’s sera or specific DNA probes to pick up antigen which are of diagnostic importance. Several important contributions have been made in this field of research and some are herewith highlighted. An initial work of immunoscreening of lg11 cDNA library of B. malayi adult parasite with MF sera resulted in the identification of clone expressing an antigen (SXP-1) which has been used in diagnosing individuals with patent infection (Dissanayake et al 1992). Another recombinant clone derived from Brugia malayi BmM14 fused to GST-tag was developed to measure IgG4 levels in the different clinical groups (Ramzy et al 1995).


Studies have clearly shown that filarial specific antibodies are the direct index of the degree and duration of filarial exposure in endemic populations (Mahanty et al 1994). Maizels et al (1995) found that the level of IgG4 antibody correlates with microfilariae counts. The recombinant antigen based rapid IgG4 antibody ELISA (Rahmah et al 2001) and Brugia rapid dipstick test (Rahmah et al 2003) has been developed for the detection of
antibodies in sera of patients with brugian infection. However, this test showed only 54.5% positive reaction in W. bancrofti-infected individuals.

Antibody assays can be used extensively for diagnosis of clinical cases in travellers and expatriates, primary surveillance in areas of unknown endemicity, detection of early infection in childhood and monitoring of chemotherapeutic programs. However, antibody tests have limitations in being unable to discriminate between past exposure and current infection.

It has been emphasized that rapid and easy tests are essential for early identification of filarial infections or exposure in children, travelers, assessing endemicity, identify new infected areas, monitoring of mass drug administration and surveillance programs for elimination of the disease. The limitation in differentiating between current and past infection is based on effective antigen assays. Significant amount of research effort was focused on parasite antigen detection from the patient’s blood and other body fluids (Dissanayake et al 1982, Hamilton et al 1984). Several investigators demonstrated circulating antigens in filariasis using monoclonal antibodies. Circulating filarial antigen (CFA) has been detected by using a MAb Og4C3 directed against antigen of O. gibsoni in a sandwich ELISA. This antigen was detected only in those patients infected with bancroftian filariasis but not B. malayi, B. timori, O. volvulus or Loa loa and is a biomarker for Wuchereria bancrofti adult worm infection (More and Copeman 1990).

In the present work the gene encoding WbSXP-1 was cloned in baculovirus eukaryotic expression system to enhance the diagnostic efficiency of WbSXP-1 protein, and expressed in Sf21 (Spodoptera frugiperda) insect cells. Monoclonal and polyclonal antibodies were raised against rWbSXP-1 expressed in osmotically inducible, E.coli GJ1158 and was used for the development of Sandwich ELISA based antigen detection assay in lymphatic filarial infection.
4.1 CHARACTERIZATION OF SXP GENE IN BACULOVIRUS SYSTEM

The baculovirus expression vector is a helper-independent, eukaryotic DNA viral vector that infects lepidopteran (butterflies and moths) insects and insect cells. Approximately 500 recombinant genes have been expressed in the BEVS under the transcriptional regulation of single or multiple baculovirus promoters representing genes of immediate early, delayed early, late, and very late promoter classes, the most common being the polyhedrin promoter. An advantage of BEVS over bacterial expression systems is that BEVS expressed proteins are often post-translationally modified similar to mammalian cells, which is an important factor for biological activity and protein function studies. Secretion signal peptides, nuclear localization signals, chaperone proteins, and catalytic folding proteins, missing in the BEVS, can be cloned into the viral genome and co-expressed with the desired recombinant protein, permitting proper folding, transport, and localization.

4.1.1 Cloning of WbSXP-1 Gene with SXP Signal Sequence in Baculovirus Expression System

An orthologue of BmSXP-1 was identified from W. bancrofti L3 cDNA library using BmSXP-1 specific DNA primers as a probe (Rao et al 2000). The identified WbSXP-1 gene (Accession no. AF098861) was cloned in EcoRI site of pRSETB vector and was expressed in E.coli BL21 to derive the diagnostic antigen rWbSXP-1 (Rao et al 2000). The SXP gene from pRSETBSXP-1 was amplified from SXP forward and reverse primer with BamH1 and EcoR1 restriction sites and subcloned in pFastBac1 donor vector. The SXP-1 protein contains signal peptide at amino-terminal region with possible cleavage site. The SXP gene with signal sequence was cloned to Baculovirus expression system for extracellular secretion of protein.
Recombinant baculoviruses are commonly used for the expression of foreign gene products in cultured lepidopteran insect cells (Luckow and Summers 1988a, Miller 1988, Webb and Summers 1990, Luckow 1991; O’Reilly et al 1992). Two major advantages of the baculovirus/insect cell system are that the virus provides unusually high levels of foreign gene expression, and the host cells provide most of the protein processing pathways found in higher eukaryotes (Jarvis and Summers 1992, O’Reilly et al 1992). Thus, this system is well suited for the overexpression of complex secretory or membrane-bound glycoproteins, which must be co- and post-translationally, processed in various ways. However, these products are generally not as well expressed as cytoplasmic or nuclear proteins in the baculovirus system. One possible reason for this is that heterologous signal peptides might be inefficiently recognized by the insect cell protein translocation machinery. This could limit the amount of a newly synthesized foreign protein which can enter the secretory pathway, thereby limiting the amount of processed end product which can be obtained from this system. However the effect of different signal peptide and signal peptide prosequence combinations on baculovirus-mediated expression and secretion on human tissue plasminogen activator (TPA) was studied and result showed that insect-derived signal insect-derived signal peptides and/or prosequences cannot always enhance the expression and/or secretion of foreign secretory pathway proteins in the baculovirus system. They also suggested that the inability of insect cells to recognize the processing signals in human t-PA efficiently is probably not the major factor preventing its high level production in this system (Donald L. Jarvis. et al 1993).

4.1.2 Expression of rBAC-WbSXP-1 from Insect Cells Infected with Recombinant Baculovirus

Expression of rBAC-WbSXP-1 was studied in insect cells infected with recombinant baculovirus with 5 multiplicity of infection (MOI) in serum
free TC100 medium. Serum free medium was used to evade the interference of serum protein during IMAC purification. Viable cell density of infected cells remains nearly constant during the initial culture period (0-2 day post infection). On 3rd day post infection viable cell density rapidly decreased. This may indicate that almost all the cells were synchronously infected with initial added recombinant baculovirus. Recombinant BAC-WbSXP-1 expression was observed on day 2 post infection and concentration reached plateau on day 3 post infection. There was slight increase in expression on day 4 post infection. Expressed rBAC-WbSXP-1 showed streak in western blot in concentrated infected insect cell supernatant and multiple bands after IMAC purification. This may indicate discrete glycosylation of expressed protein in insect cells, which was confirmed with PAS staining of IMAC purified rBAC-WbSXP-1 and deglycosylation of pure protein with Peptide N Glycosidase F (PNGase F) treatment.

4.1.3 Reactivity of rBAC-WbSXP-1 with Patient’s Sera

The rWbSXP-1 antigen based simple and rapid format for antibody flow through assay of human lymphatic filariasis was developed successfully developed by Basker et al (2004). The kit underwent extensive evaluation in India (Basker et al 2004) and global WHO surveillance (Lammie et al 2004). The kit showed with 91% sensitivity and 100% specificity in both these evaluation. The rapid kit was suitable to finger prick blood samples collected on filter paper (Basker et al 2004). However, Lammie et al (2004) have also emphasized on making antibody flow through kits to detect even low levels of antibody reactivity. Thus, the potential of the protein made it imminent to investigate the strategies to suit industrial or commercial production.

The baculovirus expression vector system (BEVS) offers significant advantages over prokaryotic and other eukaryotic systems for the production of many proteins. Heterologous genes placed under the
transcriptional control of the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcNPV) are often abundantly expressed during the late stages of infection. In most cases, the recombinant proteins are processed, modified, and targeted to their appropriate cellular locations, where they are functionally similar to their authentic counterparts.

In the present study the gene encoding WbSXP-1 was cloned in baculovirus eukaryotic expression system to enhance the diagnostic efficiency of WbSXP-1 protein and expressed in Sf21 (Spodoptera frugiperda) insect cells. The reactivity of pure WbSXP-1 expressed in baculovirus expression system was tested in ELISA with filarial patient serum where non endemic normal sera used as control. Protein (rBAC-WbSXP) purified from baculovirus expression system showed 100% sensitivity and specificity with filarial clinical samples.

4.1.4 Isotype ELISA for rBAC-WbSXP-1 with Patient Sera

The results of previous studies demonstrated that the antibody response to WbSXP-1 in W.broncrofti infected individuals was restricted to the IgG4 subclass (Rao et al, 2000). Sera from MF positive patient had significantly elevated level anti-WbSXP-1 IgG4 and IgM levels compared to other clinical group. It was interesting to note that the IgM levels against SXP-1 were significantly higher specifically in individuals with circulating mf. This could be used in design of novel improved method of antibody based diagnosis to improve specificity. This could be used in design of novel improved method of antibody based diagnosis to improve specificity.

4.1.5 Growth and Expression Profile of Sf21 Cells in Shake Flask

Insect cells have been widely cultured in basal cell media supplemented with vertebrate serum, in particular with a concentration of 5 to
20% fetal bovine serum (FBS), which has been shown to support cell growth, baculovirus infection and recombinant protein production (Wu et al 1989). The use of serum, however, has a number of disadvantages, including high cost, variable lot-to-lot performance, difficulty in downstream processing and purification of expressed protein, and biohazard arising from pathogenic contaminants such as mycoplasma, viruses, and proteinaceous infectious particles (prions). Most commercially available serum-free insect media are essentially simple variations of IPL-41 basal medium supplemented with undefined protein hydrolysates and a lipid/surfactant emulsion (Inlow et al 1989) Second-generation serum free formulations such as Sf-900 II SFM and EXPRESS-FIVE SFM are specifically designed for large-scale production of recombinant proteins.

In the present study, growth of Sf21 cells were optimized in suspension with basal medium supplemented with and without serum supplementation. In serum supplemented medium the maximum cell density achieved was 4.5 million/ml on 5th day. Medium without serum supplement did not support cell growth. Suspension culture techniques generate mechanical shear forces.

Although serum concentrations between 5% and 20% in medium appear to provide some protection from shear forces most insect cell lines require shear force protection during suspension cell culture. Pluronic F-68 was supplement with medium to protect the cells from mechanical shear forces.

4.1.6 Comparison of Reactivity of rBAC-WbSXP-1 and rWbSXP-1 with Patient Sera

E. coli is the most preferred host for expressing recombinant proteins, though it has certain limitation includes formation of inclusion
bodies, express more in insoluble form and improper folding. Recombinant baculoviruses have become widely used as vectors to express heterologous genes in cultured insect cells and insect larvae. Heterologous genes placed under the transcriptional control of the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcNPV) are often abundantly expressed during the late stages of infection. In most cases, the recombinant proteins are processed, modified, and targeted to their appropriate cellular locations, where they are functionally similar to their authentic counterparts. The recombinant BAC-SXP-1 was purified and its diagnostic efficiency was compared with the SXP-1 expressed in E.coli. with filarial patient serum from different clinical groups collected from various endemic regions. SXP-1 protein expressed in eukaryotic baculovirus expression system found to be post-translationally modified processed and showed higher reactivity towards MF positive clinical samples compared to SXP-1 expressed in E.coli.

4.2 SELECTION OF MAb FOR ANTIGEN DETECTION AND ITS CHARACTERIZATION

It is vital to detect circulating filarial antigen in the blood to identify active filarial infection in endemic populations and hence an attempt was made to develop monoclonal antibodies for the microfilaria specific antigen, rWbSXP-1. Two of the monoclonal antibodies, namely 1F6H3 and 2E12E3 showed better reactivity with recombinant and native mf antigen, higher affinity and avidity to recombinant antigen which were used for validating capture assay as individual antibody and in combination. The assay showed higher sensitivity when MAbs were used in combination. The two MAbs recognize two different epitopes in SXP-1 protein, leading to synergistic binding that allows a more stable antigen-antibody interaction and
shows higher sensitivity in combination. The other clones showed low affinity towards the recombinant and native mf antigen.

4.3 DEVELOPMENT OF PROTOTYPE ANTIGEN BASED IMMUNO-DIAGNOSTICS FOR HUMAN LYMPHATIC FILARIASIS

Wuchereria bancrofti accounts for more than 90% of the total filarial infection in India. It is one of the major public health problems in South-East Asia. It predominantly afflicts poor people in both urban and rural areas as well as the marginalized and neglected populations. Rapid diagnostic procedures for human lymphatic filariasis are essential and emphasized by the WHO for early detection, proper surveillance and eradication of the disease. The microscopy based parasitological examination of blood smear is the simplest Mf detection method which is most widely used in the field till date. But it poses problems like night blood collection, labour-intensive technique, low sensitivity and variations based on blood volume and parasite load.

Immunological assays like detection of parasite specific antibodies and circulating parasite antigens have been developed to overcome these limitations. The antibody assays based on recombinant proteins have shown improved specificity than those based on native filarial antigens (Weil et al 1997). Antibody assays using recombinant antigens Bm14, WbSXP and BmR1 were evaluated in a multi-centre trial. However, the specificity of the tests has been reported to be a great concern since they cannot distinguish current infection from past infection or exposure to the parasite and because there is some degree of cross-reactivity with other helminth infections. Although Wb14 showed antibody reactivity with both W. bancrofti and B. malayi infections, the considerable cross-reactivity with L. loa and O. volvulus infections limits its utility (Lammie et al 2004). Also, the antibody titers are
not correlated with infection intensity, i.e. the number of worms in the patient (Weil et al 1997).

Recombinant W. bancrofti SXP-1 (rWbSXP-1), an orthologue of BmSXP-1 was identified and used to develop an antibody based immunoassay for diagnosis. The assay could detect SXP specific circulating antibodies in both bancroftian and brugian infections but also shows recognition of sera from L. loa infection (Rao et al.2000; Lalitha et al 2002). An improved rWbSXP-1 based immunoassay to detect the circulating antibodies was found to be more specific and had rapid field-application formats for diagnosis, surveillance and elimination programmes (Basker et al 2004) although it showed cross reactivity in loaiasis.

Currently, Og4C3 ELISA (More & Copeman, 1990; Weil et al 1997) and an immunochromatographic card test (ICT) (Weil et al 1997) are available to detect circulating antigens of W. bancrofti in serum or whole blood. Although, the ICT test showed positive for highly infected MF individuals, the kit nevertheless showed lower sensitivity (71.2%) and a large proportion (25%) of mf-negative individuals were positive as it detects CFA of adult parasite as well as mf. This was reported to be due to the detection of antigen in individuals harboring low levels of mf (not detectable by night blood smear), growing worms or non-fecund adults or adults of a single sex may be antigen positive. Further, a significant proportion of microfilaraemics, especially those with low-level parasitaemia detected by the membrane filtration technique, were ICT negative. Similarly, the sensitivity of the Og4C3 test was lower than that of the ICT test (Pani et al 2000). The major drawback however is that, both are not effective in detecting B. malayi antigens.

Hence, there is a need to develop effective diagnostics for detection of active infections by both W. bancrofti and B. malayi (Pani et al 2004). In
the present study, monoclonal antibodies against the filarial mf specific antigen, WbSXP-1 have been used to develop a sandwich ELISA to detect circulating antigen in microfilaria positive individuals.

Capture assay was developed with rWbSXP-1 monoclonal as capture antibody and rabbit anti-SXP polyclonal as detection antibody and validated against recombinant as well as native mf antigen. The assay showed 100% sensitivity with MF positive samples, whereas 16.6% of EN samples were shown positive. This could be explained as some of the EN individuals who are microfilaria negative by microscopic examination may carry antigens without any detectable mf in blood since they are exposed to infective bites of mosquitoes and may be in their early stages of harboring very low levels of parasite. However the blood samples from CP and NEN groups were negative for SXP-1.

Lymphoproliferative and cytokine response studies on various clinical categories of filarial patients have shown that MF individual have low Th1 response to filarial specific antigens compared to CP and EN individuals, wherein enhanced Th1 activity is observed (Ravichandran et al; 1997, Suba et al., 2000). Thus MF individuals were not able to clear the antigen and were positive in antigen assay. Since CP and EN show elevated levels of gamma interferon, there is increased macrophage activation and the elimination of the antigen.

Rapid dipstick diagnostic assay for the rapid detection of filarial antigen was optimized using 2E12E3 and 1F6H3 monoclonal antibodies as capture and detection antibody and pure recombinant SXP-1 protein was used as standard test antigen. Clinical samples (MF and EN patient samples) were tested as a means to develop field-mode-rapid diagnostic prototype which showed the promising sensitivity and specificity. The samples tested in this fashion showed a moderate sensitivity (41.7%). An extensive on-the-field
trials (with samples procured and tested immediately) in the future could remedy and enhance sensitivity if fresh samples are used. Moreover, the reduced sensitivity may be directly related to the MF load which may in turn result in low circulating antigens. The development of high efficiency MAb’s against eukaryotic expressed protein could enhance the sensitivity of rapid diagnostic assay.