CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The neglected tropical diseases (NTD) are considered as the most debilitating among the common chronic infections of the world’s poorest population. It represents a group of chronic parasitic and bacterial infections such as hookworm infection, ascariasis, schistosomiasis, lymphatic filariasis, onchocerciasis, chaga’s disease, leishmaniasis, and trachoma. It also includes selected viral, especially arboviral, fungal and ectoparasitic infestations. Nematodes are abundant and diverse in terms of biology and ecology. In large part, helminthiases are caused by members of the phyla nematoda and platyhelminthes. Species belonging to both phyla occupy numerous niches within their mammalian hosts, ranging from intestinal lumen to intravascular and even intracellular sites (Colley et al 2001). Parasitic species of nematode are the main causative agents of NTDs, which afflict around 2.7 billion people (Hotez et al 2006). Human evolution and parasitic evolution have run hand in hand and has resulted in an amazing number of parasites; about 300 species of helminth worms and more than 70 species of protozoa. Parasitic nematodes infect nearly half the world's human population, resulting in significant morbidity and mortality. Their presence is known for more than a century, and they continue to have a devastating influence on less privileged populations throughout the tropical and subtropical regions of the world. Helminth parasitic diseases, viz., filariasis, ascariasis and hookworm infections, contribute significantly to the problem.
Lymphatic filariasis (LF) is a disease caused by a group of filarial nematodes transmitted by mosquito vectors. They are caused by three species of tissue-dwelling filaroid nematodes, such as Wuchereria bancrofti, Brugia malayi and Brugia timori. Approximately 120 million people (Schwab et al 2006), 2% of the world’s population (Figure 1.1), in over 90 countries are infected.

![World map showing global endemicity of Filariasis](Source: www.biologie.de/biowiki)

**Figure 1.1 World map showing global endemicity of Filariasis**

W. bancrofti is the most common causative agent and accounts for about 90% of cases while B. malayi accounts for 10% of cases and is confined to East and Southeast Asia. B. timori is found only in Timor and nearby islands. Lymphatic filariasis has been identified by the World Health Organisation (WHO) as the second leading cause of permanent and long-term
isability worldwide. LF is a major cause of morbidity, with the loss of 4.6 million DALYs (disability-adjusted life years) (Remme et al. 2002), severely affecting socio-economic development in endemic areas (Ramaiah et al. 2000; Zagaria and Savioli 2002). WHO estimates that 44 million people have overt clinical disease – lymphedema, elephantiasis, hydrocoele, recurrent infections associated with damaged lymphatics, lung disease, chyluria and renal disease. Another 76 million have pre-clinical damage to their renal systems. India alone accounts for 40% of the global burden of LF and at least one-third of the people affected with the disease live in India. There are ~21 million people with symptomatic filariasis and 27 million with asymptomatic microfilaraemia, while a total of 553 million people are at risk of infection (Ramaiah et al. 2000). W. bancrofti is the predominant species, accounting for about 98% of the national burden, widely distributed in 17 states and union territories of the country (Figure 1.2) (Sabesan et al. 2000), with bancroftian infections localized to Kerala and scattered pockets of Orissa and Assam. Recent report showed that an estimated 554.2 million people are at risk of LF infection in 243 implementation units in India (WHO 2008).

WHO initiated the ‘Global Program to Eliminate Lymphatic Filariasis’ (GPELF) by the year 2000 and it has been successfully implemented in China, Malaysia, Korea and in certain islands of the Pacific (Ottesen 2000, Burkot et al. 2002, Molyneux and Zagaria 2002). GPELF mainly focuses on mass drug administration (MDA) using either diethylcarbamazine (DEC) (Gelbrand et al. 1994) or ivermectin (Eberhard 1997, Molyneux et al. 2002) in single- or two-dose regime combined with albendazole once a year to interrupt transmission of LF (Molyneux et al. 2001, Gyapong et al. 2005) and morbidity alleviation. Management of acute and chronic filariasis cases requires treatment of adenolymphangitis (ADL) with antibiotics since majority of acute episodes appear to be of bacterial aetiology.
Rigorous local hygiene measures like washing of legs with or without local antibiotic and antifungal agents to reduce the severity of ADL.

However, the anti-filarial drugs are micro-filaricidal drugs which cannot clear the adult worms and there is a need for more macro-filaricidal drugs. The effective control of filariasis lies in the early diagnosis, treatment of the infected individuals, particularly the microfilaremics, and effective follow-up of drug administration. The unequivocal methods of diagnosis of lymphatic filariasis are the microscopic examination of microfilariae (mf) by Giemsa-stained night blood smears, membrane filtration techniques (Schultz 1988, Schuurkamp et al 1990, Sabry 1992). The nocturnal periodicity of mf areas requires night-time blood collection and survey, which is often unpopular with the local population. Furthermore, low numbers of mf are sequestered in inaccessible sites making these methods ineffective in diagnosis. Immunodiagnostic methods have expanded with time and now they remain as one of the most powerful and sensitive tool for the demonstration of parasitic infections either for individual cases or for epidemiological studies. The circulating filaria-specific antibodies are largely explored to develop antibody-based diagnostic methods that conversely detect mf.

For early diagnosis immuno assays using crude antigens or other antigens from animal filarial parasites have been developed. Antibody assays are usually problematic due to extensive cross-reactivity among the antigens of other helminthic parasites. Hence assays developed using homologous (W. bancrofti and B. malayi) antigens were preferred to heterologous antigens for developing specific immunodiagnostic techniques. The expansive development in rDNA technology provides excellent scope for developing filarial specific antigen for diagnosis. Constructs of gene libraries of parasite DNA were expressed and screened either with B. malayi or W. Bancrofti infected patient’s sera or specific DNA probes to pick up antigen genes which

Better understanding of the transmission of LF is crucial for predicting the impact of control programmes and assessing the prospects of elimination (Stolk et al 2004). The combined impact of density dependence in both uptake and development of mf determines the relationship between mf density in the human blood and the number of third infective larval stage (L3) eventually developing in mosquitoes after feeding (Snow and Michael 2002). The rapid flow-through immunodiagnostic kit (Baskar et al 2004) developed in our centre is used in diagnosing the mixed infection of brugian and bancroftian filariasis in India. This is also recommended to WHO by Lammie et al (2004) as a good antigen candidate to diagnose filariasis. The advantage in this device is that it not only picks strong but also the weak infection which is assessed by the intensity of the colour development during assay (Janardhan et al 2007). It can also be used to estimate the rate of infection based upon the intensity of the colour developed and thus can help in interrupting the transmission. Recombinant antigen-based rapid IgG4 antibody ELISA (Rahmah et al 2001) and dipstick test (Rahmah et al 2003) have been developed for the detection of antibodies in sera of patients with brugian infection.
However with the fact remaining that antibody assays are indiscriminate between past exposure and current infection, they could still 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 for monitoring chemotherapeutic programs.

The diagnosis of *W. bancrofti* infection has been much improved with the advent of filarial antigen detection assays. A sensitive and simpler antigen assay for early detection of filariasis has been developed by More and Copeman (1990) which was easier compared to microscopic examinations. Assays could effectively detect antibody in serum or blood samples alike when taken onto filter paper strips Lalitha et al (1998) and Itoh et al (1998). Currently, the MAb-based Og4C3 assay and the ICT card test have been used widely for the early diagnosis of bancroftian filariasis. Although Og4C3 assay is the most sensitive in detecting CFA levels in bancroftian filariasis, it cannot be used for the detection of active filarial infection in brugian filariasis. On the other hand, the ICT card test is only a qualitative test and is found to be specific for bancroftian filariasis (Weil et al 1997).

Previous work carried out in our research group has reported the cloning and characterization of the novel recombinant SXP-1 protein from *B. malayi* adult worm cDNA library (Rao et al 2000) and its orthologue WbSXP-1 from *Wuchereria bancrofti* L3 cDNA library. The antigen rWbSXP-1 has already been reported and well known for its diagnostic potential (Rao et al 2000). The gene encoding WbSXP-1, 573 bp in length (Accn.No AFO98861) was cloned in pRSETB expression vector. The recombinant WbSXP-1 was expressed as 26 kDa His-tag fusion protein. The ELISA developed using polyclonal antibodies to recombinant filarial proteins BmSXP-1 and WbSXP-1 have been successfully used to quantitate circulating filarial antigens in sera from Bancroftian and Brugian infections (Lalitha et al
2002). This has been found useful in endemic areas with mixed infection due to W. bancrofti and B. malayi (Rajendran et al 1997, Srividya et al 2002). The imminent need to develop a simple, sensitive, rapid diagnostic test for the detection of antigen and antibody that can identify both Bancroftian and Brugian infections were investigated (Basker et al 2004).

The development of immunodiagnostic method using rWbSXP-1 protein, evaluation and testing of kits were commenced in the year 2000 and carried out under a MoU with M/s SPAN diagnostics Ltd., Surat. The technological knowhow was transferred in 2007.

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. The baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) has become a popular vehicle for the cloning and expression of recombinant proteins in insect cells (Smith et al 1983, O’Reilly 1992). 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.

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 caused by both W. bancrofti or B. malayi. The results showed the potential of the rWbSXP-1 for the development of antigen
assay for detection of active filarial infection. Monoclonal antibodies were used to develop dipstick method for rapid detection of antigen.

1.2 OVERVIEW OF THE THESIS

*Wuchereria bancrofti* SXP-1 orthologue of *Brugia malayi*, was isolated from L3 stage cDNA library due to its high reactivity with microfilaremic positive sera of individuals living in the endemic area. The results of previous experiments carried out in our lab established the fact that recombinant *WbSXP-1* has the potential to diagnose both *Brugia* and *Bancroftian* filariasis (Rao et al 2000, Lalitha et al 2002, Basker et al 2004). Recombinant *WbSXP-1* based diagnosis was developed into a rapid flow through kit format (Basker 2004). These developments made the r*WbSXP-1* a diagnostically important protein. In previous study optimization of media and cultivation conditions for expression of r*WbSXP-1* in osmotically inducible, *E. coli* GJ1158 was designed and established.

In the present study the gene encoding *WbSXP-1* was cloned in baculovirus eukaryotic expression system to enhance the diagnostic efficiency of SXP-1 protein detection, and expressed in Sf21 (*Spodoptera frugiperda*) insect cells. *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 characterized with filarial patient serum from different clinical groups collected from various endemic regions.

In the present work Sandwich ELISA based antigen detection assay in lymphatic filarial infection caused by both W. bancrofti or B. malayi was developed using monoclonal and polyclonal antibody raised against rWbSXP-1 expressed in osmotically inducible strain, E. coli GJ1158. Assay was standardized using recombinant (rWbSXP-1 and rWbBAC-SXP-1) as well W. bancrofti and B. malayi mf antigen with different combination of antibodies as capture and detection antibody. Monoclonal antibodies were used to develop dipstick method for rapid detection of antigen. The response of sandwich ELISA was studied with W. bancrofti MF positive cases while EN and CP groups were used as control. Development of rapid dipstick diagnostic assay for filarial antigen detection was attempted using monoclonal antibodies raised against rWbSXP-1. The device was tested with clinical samples (MF and EN patient blood samples) to develop field-mode-rapid diagnostic prototype.

1.3 OBJECTIVES

The overall objective of the thesis is the development of efficient antibody and antigen detection assay methods.

- Cloning, expression, purification and characterization of WbSXP-1 in Baculovirus expression system to enhance the diagnostic efficiency of antigen for antibody detection assay.

- Expression and purification of rWbSXP-1 in E. coli towards development and characterization of monoclonal antibodies for the development of prototype antigen based immuno-diagnostics for Human Lymphatic Filariasis.
1.4 REVIEW OF LITERATURE

1.4.1 Filarial Parasites

Humans and animals are infected by nematode parasites called “Filariae” which include several hundred species of worms that are slender, elongated and parasitic in tissues of various vertebrate hosts. Wuchereria and Brugia are mainly responsible for human lymphatic filariasis in India and neighbouring countries. W. bancrofti belongs to Kingdom – Animalia, Phylum – Nematoda, Class – Secernentea, Order – Spirurida, Sub-Order – Spirurina, Family – Filarioidea and Genus – Wuchereria. There are two species of Wuchereria: (i) W. bancrofti (Cobbold 1877) and (ii) W. Kalimantani (Palmieri et al 1980); and there are nine species of Brugia: (i) B. malayi (Brug 1927), (ii) B. pahangi (Buckley and Edeson 1956), (iii) B. patei (Buckley et al 1958), (iv) B. brugiella buckleyi (Dissanaike and Paramanathan 1961), (v) B. ceylonensis (Jayewardene 1962), (vi) B. guyanensis (Orihel 1964), (vii) B. beaveri (Ash and Little 1964), (viii) B. tupaiiae (Orihel 1964) and (ix) B. timori (Partono et al 1977). The B. malayi and W. Bancrofti adult worms reside in the lymphatics and cause brugian and bancroftian filariasis, respectively.

1.4.2 Important Vectors Transmitting Filarial Parasites

The three lymphatic-dwelling parasites W. bancrofti, B. malayi and B. timori are vectored by haematophagus arthropods. The three important arthropods with the blood-sucking habits of ectoparasites are mosquitoes, bedbugs and fleas. LF is transmitted by intermediate hosts which are always blood-sucking arthropods of the order Diptera, belonging to the four families of arthropods. Some of the known vectors of human filariae are given in Table 1.1.
Table 1.1 Human filaria-transmitting vectors of the family Diptera

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diptera</td>
<td>Culicidae – mosquitoes</td>
<td>Culex quinquefasciatus, Mansonia sp. and Anopheles barbirostris</td>
</tr>
<tr>
<td></td>
<td>Simulidae – black flies</td>
<td>Simulium damnosum</td>
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<tr>
<td></td>
<td>Certopogonidae – biting midges</td>
<td>Culicoides furens</td>
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<tr>
<td></td>
<td>Tabanidae – horse flies</td>
<td>Chrysopsis dimidiate</td>
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Mosquitoes are the important vectors in filariasis, which is a major public health problem next to malaria. Filarial parasites are transmitted by many mosquito species, whereas in malaria, Plasmodium is transmitted by only one mosquito species. Culex quinquefasciatus is a ubiquitous species and abundant in tropical and subtropical countries. C. quinquefasciatus is the principal vector of bancroftian filariasis throughout the mainland of India. Anopheles vectors are important in the transmission of periodic W. Bancrofti in Africa, Southern Asia and the island of New Guinea. It is also a significant vector of periodic brugian infection in Southern Asia. New transmission and distribution records include A. gambiae from the island of Grande Comore and A. flavirostris from Sabah, while A. philippinensis has very limited distribution in the northeastern part of India.

Aedes species such as Ae. Polynesiensis and Ae. samoanus mainly transmit filariasis in Samoa and French Polynesia. Ae. poecilius transmits in Philippines. Transmission by Aedes is limited only to Andaman and Nicobar islands of India. Mansonioides include the important vectors M. annulifera, M. uniformis and M. indiana of B. malayi infection in Southern and south-
eastern Asia. Mansonia vectors are mainly from India, Indonesia, Malaysia and Thailand.

1.4.3 Life Cycle of the Parasite

Lymphatic filariae have biphasic life cycle where larval development takes place in the mosquito (intermediate) host, and larval and adult development takes place in the human (definitive) host (Figure 1.2).

![Figure 1.2 Life cycle of W. bancrofti parasite](Source: www.dpd.cdc.gov/dpd)

These parasites have no free-living forms. The infection is initiated by the deposition of the L3 onto the skin of the human host by an infected mosquito during its blood meal. The larvae penetrate into the human host through the bite wound and undergo additional moult to the fourth larval stage (L4). They mature and develop into lymph-dwelling adult male and female
worms. Adults are dioecious and undergo ovoviviparous reproduction and produce first stage larvae (L1 or mf), which are sheathed and have nocturnal periodicity, except the South Pacific mf which have the absence of marked periodicity. The mf migrates into lymph and blood channels moving actively through lymph and blood. A mosquito ingests the mf during a blood meal. After ingestion, the mf lose their sheaths and some of them work their way through the wall of the proventriculus and cardiac portion of the mosquito's midgut and reach the thoracic muscles and moult to the second stage larvae (L2) and subsequently moult into L3 after few days. The L3s migrate through the haemocoel to the mosquito's proboscis and can infect another human when the mosquito takes a blood meal.

1.4.4 Morphology of Filarial Parasites

The ultra-structural morphology of cuticular surface membranes of adult B. malayi has been studied by freeze fracture electron microscopy (Smith et al 1996). Localization and isolation of W. bancrofti adult worms by ultrasonography has facilitated the ultra-structural studies of the surface by scanning electron microscopy (Araujo et al 1995). Adults are found primarily in lymphatic vessels, less commonly in blood vessels. Adult W. bancrofti and B. malayi are minute, thread like and have a smooth cuticle. Adult males measure 40 mm in length and 0.1 mm in diameter, whereas females measure 80–100 mm in length and 0.24–0.3 mm in diameter (Nanduri and Kazura 1989). The mf of both W. bancrofti and B. malayi are sheathed and measure 244–296 μm and 177–230 μm in length, respectively (Figure 1.3). B. malayi mf has two terminal nuclei distinctly separated from each other and the last terminal nucleus is small and is present at the tip of the tail, whereas the body nuclei of W. bancrofti mf do not extend to the tip of the tail.
1.4.5 Clinical Groups

The clinical symptoms of filariasis vary from one endemic area to the other and depend upon the species involved in the infection. In Africa, hydrocoele is common compared to lymphedema and elephantiasis. But in other countries such as India, hydrocoele and lymphedema are distributed in equal frequency. Tropical pulmonary eosinophilia is restricted to certain parts of the world such as South Asia, South East Asia and Brazil. The clinical groups are classified as below.

1.4.5.1 Asymptomatic Amicrofilaraemics (Endemic Normals, EN)

‘Endemic normal’ is the terms given to those individuals living in endemic region and who inspite of constant exposure to filariasis do not exhibit any clinical evidence of the disease. They are alternatively called putatively immune individuals. Immune responses to filarial antigens are higher in these individuals compared to other clinical groups (Ottesen et al 1977).
1.4.5.2 **Asymptomatic Microfilaraemics (MF)**

The most common clinical manifestation observed among the endemic population is asymptomatic microfilaraemia. Individuals in this clinical group do not exhibit disease symptoms but harbour large number of circulating mf in the peripheral blood and are termed as ‘asymptomatic microfilaraemics’. These individuals have no inkling that their blood contains large numbers of mf. This situation may persist for decades without any progression to overt clinical disease (Ottesen 1992). Filarial antigen-specific hyporesponsiveness is the characteristic of these MF individuals (Mahanty et al 1997, Ravichandran et al 1997).

1.4.5.3 **Acute Manifestations**

The most common acute manifestation of filariasis is identified by recurrent attacks of fever associated with inflammation in lymphatics termed as adenolymphangitis (ADL), which is characterized by intense lymphangitis, lymphadenitis with retrograde extension from the affected node and reddening of the overlying skin. These attacks are usually accompanied by chills and fever (filarial or ‘elephantoid’ fevers). In endemic areas two types of ADL occur: ADL caused by bacteria or fungus and by the parasite itself. Repeated occurrence of ADL is associated with progression of the disease. The progression of lymphedema from one grade to the next in bancroftian filariasis is associated with increased frequency of ADL attacks (Pani and Srividya 1995).

1.4.5.4 **Chronic Manifestations (CP)**

Individuals belonging to this group are clinically affected and the manifestations usually develop after 10–15 years from the onset of the first acute attack. They suffer from either acute attacks of lymphatic inflammation
such as lymphadenitis, lymphangitis or from chronic obstructive form of the disease characterized by elephantiasis of the legs, hydrocoele or chyluria and lymphurias. When people from non-endemic areas move to endemic areas, they become more susceptible than the inhabitants of endemic area (Partono 1984). These individuals show hyper-responsiveness to filaria-specific antigens than MF individuals (Nutman et al 1987, Ravichandran et al 1997).

1.4.5.5 Tropical Pulmonary Eosinophilia (TPE)

Tropical pulmonary eosinophilia (TPE) is the least common manifestation of filariosis and it is a form of occult filariosis. It is manifested by recurrent asthmatic attacks, transitory lung field mottling, low-grade fever and marked leukocytosis and eosinophilia. There is a severe hypersensitivity response with marked eosinophilia, extreme levels of serum IgE and high titres of antifilarial IgG and IgE antibodies. TPE is most likely caused by the remnants of mf, and chronic TPE can lead to pulmonary fibrosis. Other extra lymphatic signs include chronic microscopic haematuria and proteinuria, and mild polyarthritis, all presumed to result from immune complex deposition. Clinical condition of TPE can be treated with DEC, and treatment leads to reduction of symptoms as well as eosinophil numbers.

1.4.6 Importance of Immune Response in Filariasis

Acquired immune responses are classified as humoral and cellular immune responses. Humoral immune responses are antibody mediated and are effective against extracellular infectious agents. Filariasis elicits strong humoral immune response in human host (Maizels et al 1991, Ottesen 1994). The most striking feature of the humoral responses in filariasis is the elevation of total as well as filarial antigen specific IgG4 in the MF. IgG4 is a monovalent antibody and is not capable of fixing complement by the classical pathway and hence it may lower complement-mediated parasite clearance in
microfilaremics (Ottesen et al 1985). Studies by Hussain and Ottesen (1987) and Maizels et al (1995), showed the role of IgG4 and IgE antibody levels leading to hypersensitivity in Brugia malayi infections. MF is characterized by high levels of anti-filarial antibodies whereas CP generally has higher anti-filarial IgG2, IgG3 and IgE levels (Simonsen et al 2002). Several investigators have adopted the IgG4 assay to diagnose the disease status in filariasis. Thus, these studies have to be rightly considered while developing diagnostics for human lymphatic filariasis.

1.4.7 Diagnosis of Filariasis

The lymph dwelling filariae infects million of people who may suffer from clinical manifestations such as elephantiasis, hydrocoele and ADL or may have clinically silent manifestations of infection associated with abnormalities in the lymphatic system. The infection goes unnoticed for years in some cases until they develop clinical signs of the disease. It is therefore essential to develop diagnostics that can detect the infection earlier. MDA programme under GPELF is in progress to minimize the rate of LF transmission. So it is essential also to realize the impact of these MDA programs which will help in deriving appropriate chemotherapy measures. Also there is a need to improve the available diagnostic tests and surveillance tools, especially for lymphatic filariasis (Weil and Ramzy 2007).

1.4.7.1 Parasitological Diagnosis

Traditionally, diagnosis of lymphatic filariasis depended on the detection of mf by microscopic examination of the blood. It is the simplest and conventional technique to detect the presence of the parasite by conventional night blood smear stained with Giemsa stain (Schultz 1988, Schuurkamp et al 1990, Sabry 1992). Membrane filtration technique is widely used when large volumes of samples need to be processed. About 1–10 mL of
diluted blood is passed through a filter with polycarbonate membrane which traps the mf. The membrane is removed and the mf are stained and counted (Bell 1967, Chulerek and Desowitz 1970, Moula-Pelat et al 1992). Though it had implication in isolating mf from large volume samples, it cannot be applied in the field as it is a time-consuming method and requires large volumes of blood for examination. Also community opposition to venipuncture is a major drawback (Nanduri and Kazura 1989).

1.4.7.2 General Imaging Techniques for Detection of Filariasis

A close study on dilated lymphatics and rapid lymph flow proved to be very important in diagnosing asymptomatic cases. Lymphoscintigraphy is a special type of nuclear medicine imaging that provides pictures called scintigrams, by which the lymphatic abnormalities in both asymptomatic MF and clinical cases can be detected (Freedman et al 1994). Although it is a safe and non-invasive technique, it is costly and not suitable for field evaluations. Another method called lymphangiography is used to study alterations in lymphatic function of filarial patients (Sen and Ellapan 1968). Ultrasound is a non-invasive, highly sensitive and widely available tool to identify adult worms in parasitized lymph vessels. It detects structures with a diameter of 2.8 mm. In a small study, Amaral et al (1994) showed tubular juxtatesticular structures suggestive of dilated lymphatic vessels and demonstrated a peculiar pattern of movement called the ‘filarial dance sign’ in microfilaraemic but asymptomatic individuals. Later, using high-phased transducers 3SMHz and 7SMHz, they detected 85 % of adult W. Bancrofti worms showing this dance sign in microfilaraemic but clinically asymptomatic individuals (Noroes et al 1996). Finally, there is evidence that ultrasound could be even more sensitive than antigen-detection assays (Rocha et al 1996, Dreyer et al 1999). Ultrasound could also be particularly valuable to evaluate macrofilaricidal effects of antifilarial drugs (Dreyer et al 1995). In spite of these advantages, it
is laborious, impracticable for mass screening, time consuming, invasive and uses oil-based contrast material for imaging (Miller et al 1990), which can induce local morbidity and aggravate the secondary infections.

1.4.7.3 Detecting Lymphatic Filariasis Using Microwaves

The microwave method for detecting LF is based upon the changes in the dielectric properties. This measurement technique is simple and suggests a novel, alternative, in vitro method of diagnosing LF using microwaves and is completely independent of time. The initial characterization of normal blood samples and infected blood samples is done using cavity perturbation technique. This method is adopted because it requires very low volume of sample and hence applicable to samples such as blood. An appreciable change in the dielectric properties of filarial blood sample is observed when compared with the normal healthy samples. These measurements were in good agreement with clinical analysis (Lonappan et al 2007). Although simple, it needs sophisticated laboratories to perform the tests and skill to interpret the results. Moreover, it cannot differentiate between other parasitic co-infections and filariasis. Although it cannot be used in the field, it is encouraging to see the advancement in diagnosing LF.

1.4.7.4 PCR-Based Diagnosis of Filariasis

DNA sequences allow the identification of even single adult worm or mf in the definitive host or a developing larva in the intermediate host. Polymerase chain reaction is a powerful tool in the study of parasitic infections. Species-specific DNA probes have been developed for B. malayi, W. bancrofti, O. volvulus, and Loa loa (Nutman et al 1994). Such DNA-based diagnostic methods have been recently developed for detecting parasitic DNA in human blood samples and in vector population. The genome-based survey
was helpful in differentiating the species of infection. B. malayi Hha I DNA repeat sequence was used to develop PCR assays for filariasis (Lizotte et al 1994). A rapid PCR based on W. bancrofti 185 bp repeat sequence SspI was developed and standardized for the detection of W. bancrofti in mosquitoes (Williams et al 1996, Hoti et al 2001). Earlier, this genus-specific repeat sequence from W. bancrofti was shown to detect L3 in mosquitoes (Zhong et al 1996). Another 969 bp repeat sequence pWb12 that could detect W. bancrofti DNA in human blood samples, hydrocoele fluid and in mosquito vector was developed by Siridewa et al (1996).

There are other modern approaches such as multiplex-PCR where primers specific for brugian and bancroftian species are used together to detect both W. bancrofti and B. malayi in single step (Mishra et al 2005). The primers designed were novel and have been tested with the parasite DNA. In a single reaction, they amplify a 188 bp fragment specific for B. malayi and ~129 bp fragment specific for W. bancrofti. The test is highly sensitive for both B. malayi and W. bancrofti as it detects parasitaemia up to the level of one mf per reaction. PCR is found to be more efficient in comparison to microscopy, as it can detect 8 % and 5 % more filarial parasites in field-collected blood and mosquito samples, respectively.

Thus, several of these DNA-based assays are promising for the diagnosis of filarial infection and are currently in the research phase, and its large-scale field applicability in the endemic areas will require further validation. The PCR-based techniques have their own disadvantage such as requirement of skilled personnel with technical capabilities and good laboratory infrastructure to carry out the assay and are hence not appropriate for large field conditions.
1.4.8 Immunodiagnosis of Filariasis

For the diagnosis of lymphatic filariasis two different techniques, namely circulating antibody and antigen detection assays, have been developed.

1.4.8.1 Antibody Detection Assay

The conventional immunoassay or methods were based on skin test or serological determination using complement fixation, diffusion in gel, latex agglutination and indirect haemagglutination (IHAT) to evaluate antibodies generated by the host. *W. bancrofti* mf antigens were used to develop antibody-based immunofluorescent antibody test (IFAT), IHAT and enzyme linked immunosorbent assay (ELISA) methods for the diagnosis of filariasis, and the efficiency of such tests was compared (Kaliraj et al 1981a). It was found that ELISA technique was simple and sensitive over the other detection methods. A method was developed for measurement of antibodies in filarial patients, using filarial antigens obtained by rearing *B. malayi* and *B. timori* worms intraperitonially in jirds. Using such antigens, the conventional antibody assay was replaced with labelled reagent assays such as IFAT, immunoradiometric assays (IRMA) and ELISA (Kaliraj et al 1981b).

1.4.8.2 Assays Based on IgG4 Antibodies

The examination of subclass antibody levels by Hussain et al (1987) have shown that the most significant differences were noticed in the levels of IgG4 in MF patients which was many times higher than those observed in CP. In another serodiagnostic approach, an ELISA was developed using IgG4 antibody to measure the soluble *B. malayi* antigens (Lal and Ottesen 1988). The active filarial infection appears to promote IgG4 and show unusually high levels of filarial specific IgG4 in infected individuals.
(Haarbrink et al 1999). Studies have shown in MF cases, high levels of IgG4 are associated with circulating filarial antigen. IgG4 based assays have been developed for the diagnosis of active filarial infection either using the crude MF antigens or recombinant antigens. Rahmah et al (1994) has shown, that by using soluble B. malayi antigen based antifilarial IgG4-ELISA, the detection was 4.6 times more microfilaric positive cases than the microscopic detection done by night blood smear.

### 1.4.8.3 Excretory Secretory Antigen Based Assay

Excretory secretory (ES) products or antigens of parasites released by the adult worm may induce higher antibody titres than the antigen extracts of the worms. The release of macromolecules by parasites has been reported in human lymphatic filariasis (Kaushal et al 1982). ES antigens have been promoted as potentially useful for the detection of antibody since they appear to be more species specific than crude antigen extracts (Kaushal et al 1984). ES have been studied with respect to function, vaccination potential, pathogenicity, and ability to serve as antigen targets for diagnostic tests. Due to the non-availability of sufficient parasite material from *W. bancrofti*, heterologous ES antigen from *B. malayi* has been used in the diagnosis of bancroftian filariasis. Malhotra and Harinath (1984) have shown that the antibodies to L3-ES antigen was more pronounced in TPE individuals possibly because these might be the first antigens that the immune system is exposed to. In contrast the MF individuals had high levels of antibodies to the mf-ES antigen suggesting that once the microfilaremia state is established the immune response to mf ES becomes stronger. In another work, surface antigens of the bovine filarial parasite *Setaria digitata* were extracted by using EDTA and purified by affinity chromatography using antibodies obtained from chronic human filarial sera. It was observed that the purified antigen showed sensitive and specific reactions in ELISA, for the detection of
antibodies in filarial sera and showed least cross reactivity with other parasitic infections compared with the crude antigens (Theodore and Kaliraj 1990).

1.4.8.4 Circulating Immune Complex for Diagnosis

Immune complexes are formed in circulation system or in tissues due to interactions between the exogenous or endogenous antigens and their corresponding antibodies. Such complexes are common in filariosis patient's sera although the antigens they contain are largely undefined (Au et al 1981). The study of circulating immune complex (CIC) becomes important since they are capable of immunomodulating the immune responses (Barnett 1986). Earlier studies have demonstrated the presence of immune complex antigens in filariosis patients using specific polyclonal antibodies raised against adult Setaria digitata worm (Dissanayake et al 1982). The results demonstrated that, specific immune complexes (IC) were found to occur in nearly 40 % of the clinical filarial patients and only in about 5 % of the microfilaremic carriers. Studies done by Prasad and Harinath (1988) have shown certain fraction of the antigens from IC viz., (IC-9), which was similar to the mf ES antigen in its antigenic determinant and showed higher IgG response in chronic pathology (CP) patients than in MF. Lunde et al (1988) have shown the presence of IC in filariosis patients from all clinical group of filariosis. They report involvement of a 200 kDa glycoprotein when detected with rabbit anti BmA antibodies.

1.4.8.5 Recombinant Antigen Based Assays

Recombinant DNA technology has provided excellent scope for expression and downstream processing for purifying considerable quantities of specific parasite antigens. This is a prerequisite for any strategy for both identifying diagnostic antigens and also for over-expression of targets. Thus it has paved way for a new generation of immunodiagnostic tests with
individual antigenic epitopes to be used as targets, although many tests have or currently employing ‘cocktail’ of such epitopes to cover wide variations in antibody type and response among human populations.

Genomic library of Brugia malayi was constructed by Arasu et al (1987) in bacteriophage vector lgt11 and was screened for species specific clones. Werner et al (1989) derived recombinant clones from genomic expression library of B. malayi, which were recognized by two distinct immunoglobulin classes. The first clone contained a part of the myosin tail region was recognized at IgG level whereas another collagen like clone was recognized at IgE levels.

In a differential immuno-screening of B. malayi cDNA library by Dissanayake et al (1992) a novel parasite antigen SXP was identified. It was experimentally observed that about 78 % of the MF, 16 % of acute and 8 % of chronic filarial disease contained IgG antibodies to rSXP (Dissanayake et al 1994). Further, the SXP antibody ELISA was evaluated and it was observed that 80 % of MF and 33 % of CP were positive. The W. bancrofti (WbSXP-1) orthologue of BmSXP-1 was identified and cloned by Rao and Eswaran (2000) from W. bancrofti L3 cDNA library. WbSXP-1 was shown to specifically recognize IgG4 antibodies in MF and it shared 84 % homology at amino acid level with BmSXP-1. The WbSXP-1 encoded a 26 kDa His tagged fusion protein was shown to be 91 % sensitive and 100 % specific for patients with both Brugian and Bancroftian filariasis. The WbSXP-1 was further evaluated in Nigeria and it was observed that > 91 % of sera from Wb MF were positive (Engelbrecht et al 2003). A recombinant antigen, L1 SXP-1 identified from Loa loa L3 cDNA library, preferentially recognized sera from experimentally infected rhesus monkey and was observed that > 56 % of sera from MF were positive for loiasis (Klion et al 2003). rWbSXP-1 based rapid
kit was extensively tested and evaluated in national and WHO based trials (Basker et al 2004, Lammie et al 2004).

In a significant work, Chandrasekhar et al (1994) identified and cloned an antigen Bm14 by screening a B. malayi cDNA library. The recombinant Bm14 specifically reacted with sera from filarial patients. Bm14 is a 130 amino acid protein, having an endoplasmic reticulum targeting sequence. The Bm14 antibody test was positive for > 90 % of sera from MF cases and 60 % of CP cases (Ramzy et al 1995). Further Evaluation of Bm14 antibody test at Nile delta of Egypt showed positive for Mf sera 88.9 % and with the endemic normal 77.8 % who are also positive by antigen tests (Weil et al 1999). The W. bancrofti (Wb14) orthologue of the Bm14 was identified, cloned and was shown to specifically recognize IgG4 antibodies in MF and it shared 91 % and 81 % homology at nucleotide and amino acid level with Bm14 antigen. A recombinant antigen Ov.16 identified from cDNA library encoded a 52 kDa MBP fusion protein was shown to specifically recognize IgG4 antibodies from Onchocerciasis MF patients. The further development of a rapid card test to detect IgG4 antibodies using the Ov.16 antigen could detect around 90 % of sera from MF (Weil et al 1999).

A recombinant clone pGT7 identified from genomic expression library of W. bancrofti mf in lgt11 (Raghavan et al 1991) could specifically recognize IgG4 isotype antibodies by MF (Theodore et al 1993). Raghavan et al (1992) had isolated a recombinant antigen, WbN1 from the genomic expression library of W. bancrofti and found the sequence similarities with myosin. Further, they have demonstrated the presence of this antigen in the muscle of the adult filarial parasite and microfilariae. A recombinant clone BmR1 was shown to specifically recognize IgG4 isotype antibodies in brugian MF sera by ELISA and shown to be about 96 % sensitive and 94 % specific for brugian filariasis (Rahmah et al 2001). The further development of a rapid
dipstick test to detect IgG4 antibodies using the BmR1 antigen was shown to detect around 95% of sera from brugian MF and only 54% of sera from bancroftian MF (Rahmah et al 2001). The multi center evaluation of the BmR1 dipstick test has shown to be 97% sensitive with brugian MF sera, 15% positive with L. loa MF and O. volvulus sera and only 53.6% with bancroftian Mf sera (Rahmah et al 2003).

1.4.9 Monoclonal Antibodies for Diagnosis

Monoclonal antibodies produced by hybridoma technology have been extensively used for diagnosis of active filarial infection. MAb (Gib13) raised against O. gibsoni could detect circulating antigen in the sera or urine samples of W. bancrofti infected persons in an immuno-radiometric assay (Forsyth et al 1985). Another MAb, E34, raised against W. bancrofti mf ESantigens were able to detect filarial antigen associated with active infection (Reddy et al 1989). mAb raised against a major 200 kDa circulating antigen was directed against phosphocholine (PC) epitopes of W. bancrofti. Though this PC determinant is not filarial specific, its abundance in PC-bearing filarial antigen in circulation makes it a useful target for immuno diagnosis (Lal et al 1987).

The use of MAb-Og4C3 of O. gibsoni for the detection of circulating antigen in human sera was found to be highly specific to W. bancrofti infections and could detect circulating ES antigens from W. bancrofti adult worms. Further it does not cross react with the sera of patients infected with B. malayi, B. timori, O. volvulus or Loa loa (More and Copemann 1990). The MAb AD12 raised against a 200 kDa protein was used to develop an antigen assay which has been shown to detect specific antigen in MF positive patients (Weil et al 1996).
Janardhan et al (2010), report shows that the monoclonal antibodies could be raised against rWbSXP-1 and was tested to show the possible detection by ELISA. However, the monoclonal had low affinity and breaking the circulating immune complex holds the key in giving stable and effective results.

1.4.9.1 Antigen Detection Assays

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, Reddy et al 1984, Hamilton et al 1984). Several antigen preparations have been used for MAb production and are apparently useful for detecting active filarial infection. Several investigators demonstrated circulating antigens in filariosis using monoclonal antibodies. Au et al (1981) demonstrated the presence of circulating worm antigens in human sera with Brugian or bancroftian infections, using a double antibody sandwich ELISA developed with rabbit antisera to B. pahangi adults. Presence of circulating antigen in patients with bancroftian filariosis by immunoradiometric assay (IRMA) using rabbit polyclonal antisera labelled with Iodine125 was demonstrated by Paranjape et al (1986). Forsyth et al (1985) have used MAb GIB-13 raised against Onchocerca gibsoni egg and mf antigens to detect bancroftian-circulating antigen in human sera using IRMA assay. Another MAb, E34 raised against W. bancrofti mf ES antigens was able to detect filarial antigen associated with active infection (Reddy et al 1986). A MAb raised against a major 200 kDa circulating antigen was directed against phosphocholine (PC) epitopes of W. bancrofti (Lal et al 1987). Wuchereria bancrofti microfilarial SDS soluble antigen based filarial antigen dipstick ELISA developed by Cheirmaraj et al (1992) was found be positive in MF cases. MAb raised to ES antigen were used to screen the
cDNA library of *B. malayi* and two important diagnostic recombinant protein, Bm12 and Bm14 were identified (Kumari et al 1994). Monoclonal antibodies K3AE7 and K3BDS raised against excretory-secretory (ES) antigens of *S. digitata* were shown to be promising in the diagnosis of *W. bancrofti* infection (Dhas and Raj 1995). Another filarial antigen detection assay was developed based on *B. malayi* mf ES antigen can detect occult filarial infections (Harinath et al 1996).

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). Several attempts have been made to evaluate the validity of this MAb Og4C3 assay by using whole blood, collected onto filter paper strips to suit field conditions (Lalitha et al 1998, Itoh et al 1998, Gyapong et al 1998). Lalitha et al (1998) has observed that the filter paper method and the serum method gave comparable results. Poly and monoclonal antibodies derived from pGT7 were used in a sandwich ELISA for the detection of circulating parasite antigens (Theodore et al 1996). Ramzy et al (1991) have evaluated the performance of antigen detection in the sera from an endemic area of *W. bancrofti* using MAb raised to *Dirofilaria immitis*. Yet another MAb raised against *W. bancrofti* L3 larvae recognized 93 kDa antigen and did not react with other nematode species (Burkot et al 1996).

1.4.9.2 Rapid Tests

A rapid test is an inexpensive, disposable, membrane-based assay that provides visual evidence of the presence of an analyte in a liquid sample. These tests must be simple, convenient, accurate, reliable, inexpensive and disposable. They must also be easily and unambiguously interpreted, even by
users without experience. The tests may be formatted either as dipsticks or as devices enclosed within plastic housings (Chandler et al 2000). Liquid sample required to perform the test is as little as 200 μL which is usually completed within few minutes. In clinical assays, the sample may be urine, blood, serum, saliva, or other body fluids. No instrumentation is required to perform such tests, which can be used in clinics, laboratories, field locations, and at home often by inexperienced personnel. The rapid membrane-based format application are low-cost alternatives to expensive instrumented methods of testing and come as Lateral flow are immunochromatographic Strips and flow-through devices which are membrane immunofiltration kits. Rapid tests are currently applied for a variety of clinical applications and diseases, including multi drug screen, pregnancy test, forensic, isotyping of monoclonals, stress indicators, cancer and tumor markers.

A rapid form of filarial antigen card test was developed by ICT diagnostics, Balgowlah, New south Wales, Australia based on AD12 MAb (Weil et al 1987a, Weil et al 1997) specific for bancroftian filariasis. Several studies have been made to evaluate the ICT card test (Freedman et al 1997, Bhumiratana et al 1999, Nguyen et al 1999, Phantana et al 1999, Simonsen et al 1999, Omar et al 2000). The ICT card test has been modified to suit the field conditions performed with whole blood samples and it has been evaluated by several research groups (Ramzy et al 1999, Pani et al 2000, Chandrasena et al 2002). The circulating antigen detection assays are particularly useful in the assessment of treatment and drug withdrawal in the infected patients (Eberhand et al 1997, Weil et al 1998).

1.4.9.3 Lateral Flow (Immuno-Chromatographic) Strip

The lateral-flow format is the most common since it is easy to manufacture, store, use and provides results in a short time, preferably
minutes. The formats are typically a nitrocellulose strip on which a capture antibody is immobilized. Usually a pad made of glass fiber, containing dried conjugate is attached to the membrane strip. The conjugate pad often contains gold particles adsorbed with antibodies specific to the analyte being detected (Vesey et al. 1998). A sample pad, usually absorbing paper, is attached to the conjugate pad. On sample application, the liquid sample migrates by capillary diffusion through the conjugate pad, rehydrating the gold conjugate and allowing the interaction of the sample analyte with the conjugate (Dar et al. 1994). The complex of gold conjugate and analyte then migrates towards the capture antibody, where they become immobilized and produce a distinct signal in the form of a sharp red line. The second line is a control, will be formed on the membrane by excess gold conjugate, indicating the test is complete. Many can be used with whole blood, serum, or plasma and some can be used with finger-stick specimens, saliva or oral fluids. In some lateral-flow devices, the test strip is encased in a plastic cartridge.

Figure 1.4 Immunochromatographic strip test
(Source: www.rapid-diagnostic.org)
1.4.9.4 Flow Through Devices (Membrane Immunofiltration)

Membrane immunofiltration devices employ solid-phase capture technology, which involves the immobilization of antigens on a porous membrane (Xiao et al 1995). The specimen flows through the membrane and the antibody gets absorbed into an absorbent pad. A dot or a line visibly forms on the membrane when developed with a signal reagent (usually a colloidal gold- Protein A conjugate). The flow-through tests require some steps for the addition of specimen, wash buffers, and signal reagent, and they can usually be performed within 5 min. Many tests use serum or plasma, though some are equipped with a filter to allow the use of whole-blood specimens. The devices or reagents typically require refrigeration. A simple and sensitive rapid format immuno-diagnostic test for the detection of circulating antibody for the diagnosis of filarial infections was developed to assess exposure in individuals with a phenomenal performance of 91% sensitivity and 100% specificity (Basker 2004).

Figure 1.5 Flow through immuno filtration test developed under MoU at SPAN Diagnostics Ltd.
1.4.9.5 Application of Gold in Rapid Tests and Diagnostics

For rapid tests, the stability of the conjugate is critical for avoiding false positives. The predisposition to agglutinate can become a major problem, as in the case of latex based applications. Gold labels were introduced into membrane-based rapid tests because of their greater potential in being stable. Gold labels have superior stability, sensitivity, precision and reproducibility making it suitable for use in rapid tests. Gold is essentially inert and forms almost perfectly spherical particles (Chandler et al 2000). Proteins bind to the surfaces of these gold particles with enormous strength, thus providing a high degree of long-term stability in both liquid and dried forms.

The benefits of different ‘labels’ used to mark antibodies and antigens have been studied. It has been researched in detail and found that colloidal gold label is outstanding in most tests application compared to other colloids based on silver, carbon, latex, dye or enzymatic in features like visibility, sensitivity, stability, coloration, reproducibility of results and scale up for industrial use. The ease of preparation, use, and adaptability, low cost and clear result makes it the most preferable in rapid tests (Handley 1989).

In general, all the colloidal production method use a reducer to donate electrons to the positively charged gold ions in solution and produce atomic gold (Hayat et al 1989). Commonly used reducers include sodium citrate, yellow phosphorus, sodium borohydride, and sodium thiocyanate.

This reaction is shown as follows:

\[ \text{H AuCl}_4 + \text{e}^- \text{ (reducer)} = \text{Au0 (colloid)} \]

The process of reduction from gold ions to gold atoms in the presence of reducer and the super saturation have been described in detail by...
Handley (1989). A gold colloid comprises a suspension of gold particles individually surrounded by a negative charge layer arising from the residual negative ions in the solution. This charge layer, called the zeta potential, provides the means for the gold particles to repel one another and to stay in suspension indefinitely. The zeta potential change depending on the ionic strength of the solution (Frens 1973). The signal is generated on the test strip or flow through by the accumulation of gold particles at the test or control line/spot. The balance between required visibility and steric hindrance dictates that, for most immunoassay applications, the optimum particle size is 40 nm. In some cases where steric hindrance is a greater problem (e.g., for smaller antigens), particles of 20 nm are preferred (Hayat 1989).

1.4.10 Protein Expression Systems

Protein expression has become a major tool to analyse intracellular processes, both in vitro and in vivo. The choice of expression system depends entirely on the purpose of the study. For some cases, transient transfection is the most obvious choice because of its relatively short time investment. In other cases, homogeneous populations and large quantities of cells may be required, which involves making cell lines stably expressing the desired protein. It may also be advantageous to express proteins under inducible promoters, this is particularly true if the protein exerts pathological effects on the cell. Expression through virus infection is also possible. Here, large quantities of cells can be infected at the same time and the protein assayed for shortly after infection. Protein expression can also be achieved directly via microinjection of plasmid DNA directly into the nucleus of the host cell.

1.4.10.1 Expression of Recombinant Proteins in Baculovirus Expression system

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. The recombinant proteins are processed, modified, and targeted to their appropriate cellular locations, where they are functionally similar to their authentic counterparts. The Bac-to-Bac Baculovirus Expression System provides a rapid and efficient method to generate recombinant baculoviruses (Ciccarone et al 1997). This method was developed by researchers at Monsanto, and is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in E. coli (Luckow et al 1993).

1.4.10.2 Overview of Baculovirology

Baculoviruses are the most prominent viruses known to affect the insect population. They are double-stranded, circular, supercoiled DNA molecules in a rod-shaped capsid (Summers et al; 1972) that can be divided to two genera: nucleopolyhedroviruses (NPV) and granuloviruses (GV). While GVs contain only one nucleocapsid per envelope, NPVs contain either single (SNPV) or multiple (MNPV) nucleocapsids per envelope. The enveloped virions are further occluded in granulin matrix in GVs and polyhedrin for NPVs. Moreover, GV have only single virion per granulin occlusion body while polyhedra contain multiple embedded virions. More than 500 baculovirus isolates (based on hosts of origin) have been identified, most of which originated in arthropods, particularly insects of the order Lepidoptera (Matthews; 1982, Longworth; 1983). Two of the most common isolates used in foreign gene expression are Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) and Bombyx mori (silkworm) nuclear polyhedrosis virus (BmNPV).
1.4.10.3 Baculoviruses as Expression Vectors

The major difference between the naturally occurring in vivo infection and the recombinant in vitro infection is that the naturally occurring polyhedrin gene within the wild-type baculovirus genome is replaced with a recombinant gene or cDNA. These genes are commonly under the control of polyhedrin and p10 promoters. In the late phase of infection, the virions are assembled and budded recombinant virions are released. However, during the very late phase of infection, the inserted heterologous genes are placed under the transcriptional control of the strong AcNPV polyhedrin promoter. Thus, recombinant product is expressed in place of the naturally occurring polyhedrin protein. Usually, the recombinant proteins are processed, modified, and targeted to the appropriate cellular locations.

1.4.10.4 Cytopathogenesis of Recombinant Baculovirus

As the recombinant infection advances, several morphological changes take place within the cells. The timing of the infection cycle and the changes in cell morphology vary with the insect cell line and strain of baculovirus used. The metabolic condition of the culture and growth medium used also can affect the timing of baculovirus infection.

**Early Phase:** Infection begins with the adsorptive endocytosis of one or more competent virions by a cell in a high metabolic state (peak replication rate). The nucleocapsids pass through the cytoplasm to the nucleus. When the virions enter the nucleus, they release the contents of the capsid. Within 30 min of infection, viral RNA is detectable. Within the first 6 h of infection, the cellular structure changes, normal cellular functions decline precipitously, and early-phase proteins become evident.
Late Phase: Within 6 to 24 h after infection, an infected cell ceases many normal functions, stops dividing, and is logarithmically increasing production of viral genome and budded virus. The virogenic stroma (an electrondense nuclear structure) becomes well developed. Infected cells increase in diameter and have enlarged nuclei. Infected cultures stop growing.

Very Late Phase: Within 20 to 36 h after infection, cells cease production of budded virus and begin the assembly, production, and expression of recombinant gene product. In monolayer cultures, areas of infection display decreased density as cells die and lyse. Likewise, in suspension cultures, cell densities begin to decrease. Infected cells continue to be increased in diameter and have enlarged nuclei. The cytoplasm may contain vacuoles, and the nuclei may demonstrate granularity. As the infected cells die, plaques develop in immobilized cultures. The plaques can be identified under a microscope as regions of decreased cell density, or by eye as regions of differential refractivity.

1.4.10.5 Generating a Recombinant Virus by Site-Specific Transposition

A faster approach for generating a recombinant baculovirus (Luckow et al 1993, Anderson 1996) uses site-specific transposition with Tn7 to insert foreign genes into bacmid DNA propagated in E. coli. The gene of interest is cloned into a pFastBac vector, and the recombinant plasmid is transformed into DH10BAC competent cells which contain the bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac plasmid can transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by antibiotic selection and blue/white screening, since the transposition results in disruption of the lacZα gene. High molecular weight mini-prep DNA is
prepared from selected E. coli clones containing the recombinant bacmid and this DNA are then used to transfect insect cells. The steps to generate a recombinant baculovirus by site-specific transposition using the BAC-to-BAC Baculovirus Expression System are outlined in Figure 1.6.

1.4.10.6 Insect Cell Lines Used in Baculovirus Expression Vector System

The most common cell lines used for BEVS applications are listed in table 1.2. Sf9 and Sf21 cell lines are the traditional cell lines used with baculovirus and originated at the USDA Insect Pathology Laboratory. These two cell lines originated from the IPLBSF-21 cell line, derived from the pupal ovarian tissue of the fall army worm, Spodoptera frugiperda (O’Reilly et al. 1992; Vaughn et al. 1977). Of these, Sf9, a clonal isolate of the Spodoptera frugiperda cell line IPLB-Sf21-AE, is probably the most widely used. The High Five cell line was developed by the Boyce Thompson Institute for Plant Research, Ithaca, NY and originated from the ovarian cells of the cabbage looper, Trichoplusia ni (Davis et al. 1992, Granados et al. 1994, Wickham et al. 1992, Wickham and Nemerow 1993). Ongoing research suggests that different insect cell lines may support varying levels of expression and differential glycosylation with the same recombinant protein (Hink et al. 1991).

Table 1.2 Insect cell lines commonly used in BEVS applications

<table>
<thead>
<tr>
<th>Insect Species</th>
<th>Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spodoptera frugiperda</td>
<td>Sf9</td>
</tr>
<tr>
<td>Spodoptera frugiperda</td>
<td>Sf-21</td>
</tr>
<tr>
<td>Trichoplusia ni</td>
<td>Tn-368</td>
</tr>
<tr>
<td>Trichoplusia ni</td>
<td>High-Five</td>
</tr>
</tbody>
</table>
1.4.10.7 Media and Growth Supplements for Maintenance of Insect Cells

Commonly used insect cell culture media are listed in table. Traditionally, Grace’s Supplemented (TNM-FH) medium has been the medium of choice for insect cell culture. However, other serum/hemolymph-dependent and serum-free formulations have evolved since Grace’s medium was introduced.

**Table 1.3 Insect cell culture media commonly used in BEVS applications**

<table>
<thead>
<tr>
<th>Serum/hemolymph-dependent media</th>
<th>Serum-free media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grace’s Supplemented (TNM-FH)</td>
<td>Sf-900 II SFM</td>
</tr>
<tr>
<td>IPL-41</td>
<td>EXPRESS-FIVE™ SFM</td>
</tr>
<tr>
<td>TC-100</td>
<td></td>
</tr>
<tr>
<td>Schneider’s Drosophila</td>
<td></td>
</tr>
</tbody>
</table>

Fetal bovine serum (FBS) has been the primary growth supplement used in insect cell culture medium. FBS has almost completely supplanted the first major supplement, insect hemolymph, which tended to melanize and deteriorates the quality of the culture medium (Mitsuhashi 1982). Of the more than 100 insect cell culture media described in the literature, a majority contain, or recommend, varying concentrations of serum as a growth supplement (Hink 1985). Serum and other undefined supplements, such as lactalbumin hydrolysate and yeastolate, provide cells with growth-promoting factors such as amino acids, peptides, and vitamins, which may not be available in defined, basal media formulations.

1.4.10.8 Environmental Factors for Maintenance of Insect Cells

**Temperature and pH:** The optimal range for growth and infection of most cultured insect cells is 25 °C to 30 °C. Healthy serum-supplemented monolayer cultures can be stored at 2 °C to 8 °C for periods up to 3 months.
The pH of a growth medium affects both cellular proliferation and viral or recombinant protein production. Although many values have been reported for invertebrate cells, in most applications a pH range of 6.0 to 6.4 works well for most lepidopteran cell lines. The insect media described in this guide will maintain a pH in this range under conditions of non-CO\textsubscript{2} equilibration and open-capped culture systems.

**Osmolality:** The optimal osmolality of medium for use with lepidopteran cell lines is 345 to 380 mOsm kg\textsuperscript{-1}. To maintain reliable and consistent cellular growth patterns and minimize technical problems, maintain pH and osmolality within the range.

**Aeration:** Invertebrate cells require sufficient transfer of dissolved oxygen by either passive or active methods for optimal cell proliferation and expression of recombinant proteins. Larger bioreactor systems using active or controlled oxygenation systems require dissolved oxygen at 10 % to 50 % of air saturation.

**Shear Forces:** Suspension culture techniques generate mechanical shear forces. During suspension cell culture, most insect cell lines require shear force protection. Although serum concentrations between 5 % and 20 % in medium appear to provide some protection from shear forces, all suspension cultures, whether serum-free or serum-supplemented, be supplemented with a shear force protectant such as Pluronic F-68.

1.4.10.9 **Bac-to-Bac Baculovirus Expression System**

The Bac-to-Bac Baculovirus Expression System facilitates rapid and efficient generation of recombinant baculoviruses (Ciccarone et al 1997). Based on a method developed by Luckow et al (1993), the Bac-to-Bac
Baculovirus Expression System takes advantage of the site-specific transposition properties of the Tn7 transposon to simplify and enhance the process of generating recombinant bacmid DNA (Figure 1.6).

**Figure 1.6 Generation of recombinant baculoviruses and gene expression with the Bac-to-Bac Expression System**

The gene of interest is cloned into a pFastBac donor plasmid, and the recombinant plasmid is transformed into DH10Bac competent cells which contain the bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac donor plasmid can transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the lacZα gene. High molecular weight mini-prep DNA is prepared from selected E. coli clones containing the recombinant bacmid and this DNA are then used to transfect insect cells.
The first major component of the System is a pFastBac vector into which the gene of interest will be cloned. Depending on the pFastBac vector selected, expression of the gene of interest is controlled by the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) or p10 promoter for high-level expression in insect cells. This expression cassette is flanked by the left and right arms of Tn7, and also contains a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini Tn7.

The second major component of the System is the DH10Bac E. coli strain that is used as the host for your pFastBac vector. DH10Bac cells contain a baculovirus shuttle vector (bacmid) with a mini-attTn7 target site and a helper plasmid. Once the pFastBac expression plasmid is transformed into DH10Bac cells, transposition occurs between the mini-Tn7 element on the pFastBac vector and the mini-attTn7 target site on the bacmid to generate a recombinant bacmid. This transposition reaction occurs in the presence of transposition proteins supplied by the helper plasmid.

After transposition reaction was performed, isolate the high molecular weight recombinant bacmid DNA and transfec the bacmid DNA into insect cells to generate a recombinant baculovirus that can be used for preliminary expression experiments. After the baculoviral stock is amplified and titered, this high-titer stock can be used to infect insect cells for large-scale expression of the recombinant protein of interest.

**Baculovirus Shuttle Vector:** The baculovirus shuttle vector (bacmid), (136 kb), present in DH10Bac E. coli contains:

1. A low-copy number mini-F replicon
2. Kanamycin resistance marker
3. A segment of DNA encoding the LacZα peptide from a pUC-based cloning vector into which the attachment site for the bacterial transposon, Tn7 (miniattTn7) has been inserted. Insertion of the mini-attTn7 does not disrupt the reading frame of the LacZα peptide.

The bacmid propagates in E. coli DH10Bac as a large plasmid that confers resistance to kanamycin and can complement a lacZ deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer, IPTG. Recombinant bacmids are generated by transposing a mini-Tn7 element from a pFastBac donor plasmid to the mini-attTn7 attachment site on the bacmid. The Tn7 transposition functions are provided by a helper plasmid.

**Helper Plasmid:** DH10Bac E. coli also contain the helper plasmid, pMON7124 (13.2 kb), which encodes the transposase and confers resistance to tetracycline. The helper plasmid provides the Tn7 transposition function in trans. (Barry 1988).

**1.4.10.10 Post Translational Modification of Proteins in Insect Cells**

Because baculoviruses infect invertebrate cells, it is possible that the processing of proteins produced by them is different from the processing of proteins produced by vertebrate cells. Although this seems to be the case for some posttranslational modifications, it is not the case for others. For example, two of the three posttranslational modifications of the tyrosine protein kinase that occur in higher eukaryotic cells (myristylation and phosphorylation of serine 17) also take place in insect cells. However, another modification observed in vertebrate cells, phosphorylation of tyrosine 527, is almost undetectable in insect cells (Piwnica-Worms et al 1990).
In addition to myristylation, palmitylation has been shown to take place in insect cells. However, it has not been determined whether all or merely a subfraction of the total recombinant protein contains these modifications. Cleavage of signal sequences, removal of hormonal prosequences, and polyprotein cleavages have also been reported, although cleavage varies in its efficiency. Internal proteolytic cleavages at arginine- or lysine-rich sequences have been reported to be highly inefficient, and alpha-amidation, although it does not occur in cell culture, has been reported in larvae and pupae (Hellers et al 1991). In most of these cases a cell- or species-specific protease may be necessary for cleavage. Protein targeting seems conserved between insect and vertebrate cells. Thus, proteins can be secreted and localized faithfully to the nucleus, cytoplasm, or plasma membrane. Although much remains to be learned about the nature of protein glycosylation in insect cells, proteins that are glycosylated in vertebrate cells will also generally be glycosylated in insect cells. However, with few exceptions the N-linked oligosaccharides in insect cell–derived glycoproteins are only high-mannose type and are not processed to complex-type oligosaccharides containing fucose, galactose, and sialic acid. O-linked glycosylations have been even less well characterized in insect cells, but have been shown to occur. Davidson et al (1990), Jarvis and Summer (1992), Grabenhorst et al. (1993), James et al (1995), Jarvis and Finn (1995), Davis and Wood (1995), and Ogonah et al. (1996).

1.4.10.11 Influence of Signal Peptide on Expression of Extracellular Protein in Baculovirus Expression System

Recombinant baculoviruses are commonly used for the expression of foreign gene products in cultured lepidopteran insect cells (Luckow and Summers 1988a, Miller 1988, 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 over expression 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. The reason for this is unknown, but a widely discussed possibility is that heterologous signal peptides might be inefficiently recognized by the protein translocation machinery in lepidopteran insect cells. Theoretically, 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.

This idea was supported by the finding that secretion of plant propapain from baculovirus-infected insect cells could be enhanced by replacing its native signal peptide with the signal peptide from honeybee prepromellitin (Tessier et al 1991). Tessier and co-workers concluded that the amount of a recombinant protein secreted by the baculovirus system can be increased by simple manipulation of its signal peptide. However, they also found that propapain equipped with the drosophila α-amylase signal peptide and galactosidase equipped with the honeybee prepromellitin signal peptide was not secreted at all. Thus, it remained to be determined if insect-derived signal peptides could generally improve the expression and/or secretion of foreign secretory pathway proteins in the baculovirus system. Several different groups have expressed t-PA (tissue plasminogen activator) and other plasminogen activators in the baculovirus system, and it is well established that biologically active t-PA can be expressed and processed by baculovirus infected insect cells (Luckow and Summers 1988b, Devlin et al 1989, Jarvis and Summers 1989, King et al 1991). Human tissue plasminogen activator (t-
PA) is a secretory glycoprotein that is synthesized as a precursor with a 35-amino acid preprosequence (Pennica et al 1983). Co- and post-translational processing of this precursor results in the removal of the signal peptide and prosequence, N-glycosylation, the formation of 17 intrachain disulfide bonds, and ultimately, secretion of the processed end product (Wallen et al 1983, Pohl et al 1984). However, t-PA expression levels were always surprisingly low (less than 5 μg mL$^{-1}$ of infected cells), as compared with the levels usually obtained with this system (several hundred μg mL$^{-1}$ of infected cells; Luckow and Summers 1988a, Miller 1988, Luckow 1991, O’Reilly et al 1992). High level t-PA expression appeared to be blocked post-transcriptionally, as similar amounts of polyhedron and t-PA RNA, but not protein, could be produced by baculovirus-infected insect cells (Luckow and Summers 1988).