CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF THESIS

"Filariasis" a chronic helminth infection is caused by tissue dwelling nematodes of the super family Filarioidea. The common filarial parasites affecting humans are Mansonella ozzardi, Loa loa, Dipetalonema perstans, Onchocerca volvulus, Brugia malayi and Wuchereria bancrofti. Over 900 million people in tropical and subtropical regions of the world are exposed to lymphatic filarial infection caused by the two major filarial parasites Wuchereria bancrofti and Brugia malayi (WHO, 1984).

Wuchereria bancrofti is the major parasite causing lymphatic filariasis in the South Asian region. About 304 million Indians are exposed to the risk of filarial infection (Sharma et al 1983). The principal vector transmitting the disease in this region is Culex quinquefasciatus. The disease though not fatal is debilitating one and drains out the normal health and energy from the affected individuals. The period of development of the clinical symptoms by these individuals depends on how successful the host parasite relationship remain. Some proportion end up in elephantiasis and others in filarial related symptoms. A section of the population living in the endemic regions develop an interstitial lung disease, known as Tropical Pulmonary Eosinophilia (TPE) resulting from immunologic hyper responsiveness to the W. bancrofti parasites.

An unequivocal diagnosis of filariasis is still based on the demonstration of microfilariae in peripheral blood by various parasitological examination techniques such as direct microscopic examination (wet blood smear, staining dried smear,
counting chamber method etc), concentration methods (dextran sedimentation, membrane filtration etc.), DEC provocation test etc.,. However parasitological methods are not reliable because the negative results from any of the above methods does not necessarily indicate a person to be free from filarial infection. Presence of Mf at densities below the threshold of detectability or single sex infections or rapid clearance of microfilaria due to immunity (?) or lack of circulating microfilaria as in tropical pulmonary eosinophilia syndrome or in "cryptic infection", a state of filariasis without microfilaria (Beaver, 1970) made the parasitological test (negative) nebulous and invalid. Hence there is a need to develop suitable immunological methods for diagnosis. One of the major problems of filarial immunodiagnosis is that the normal individuals from endemic regions showing a marked immune responsiveness to filarial antigens, eventhough they do not have any parasitological evidence of infection (Ottesen, and Hussain 1982). Individuals from this group are also exposed to other helminthic infections such as trichuris, hookworm etc (Lobos and Weiss 1986). And such intestinal helminthic infections are known to cross react with W.bancrofti antigens (Kaliraj and Harinath 1981). Therefore the extensive cross reactivity of antibodies induced by diverse species of filariae and intestinal nematodes has impeded the diagnosis of filariasis (Oliver et al, 1945, Ambroise-Thomas, 1974 and Maizels et al, 1985). The detection of filarial specific IgG4 subclass antibodies have been reported to enhance specificity in diagnosis (Lal and Ottesen 1988). Similarly detection of circulating filarial antigen has been proved to reveal active filarial infection and probabaly the occult state of infection.

For developing any of the sensitive immunological tests, there is a need to isolate and employ specific parasite antigens for diagnosis. The non-availability of W.bancrofti in quantities required for antigen extraction has become an obstacle for progress in filarial diagnosis. Purification of the heterologous filarial antigens by fractionations showed improved specificity for use in immunodiagnosis (Ottesen 1984). Although the fractionation has reduced the cross reaction, so far no antigen has been identified or isolated with clear cut species specificity for human lymphatic filariasis (Ottesen, 1984).
The objectives of the ongoing studies in our laboratory are to identify, isolate, purify and characterize the filarial specific recombinant antigens expressed in the *W. bancrofti* genomic DNA library, for the detection of class or sub class specific filarial antibody or the circulating antigen detection assays by employing the antibodies developed against these antigens. The specific objective of the present study is to employ the antigens isolated from easily available heterologous filarial parasite *S. digitata* to set up an "epitope search" of the similar antigens expressed in the *W. bancrofti* genomic library.

The first part of the study deals with differential extraction, affinity purification and characterization of the *Setaria digitata* cuticular antigens in order to isolate and identify specific antigens. *S. digitata* adult filarial parasites were easily obtainable from the peritoneal cavity of cattle. The cuticular mass separated from the adult worms of *S. digitata* was extracted using varying concentrations of EDTA. The total crude cuticular extract was purified through an immuno adsorbant prepared by coupling human bancroftian filarial antibodies, to improve specificity.

By a stepwise increase of the EDTA concentration during the antigen extraction from the cuticles of *S. digitata* resulted in a greater release of a 15 KDa peptide. The specificity of this 15 KDa peptide with the filarial sera and not with the non filarial helminthic and non helminthic parasitic sera became an exciting feature. However, fractionation of this 15 KDa peptide for diagnostic purposes in large quantities, without the co-migrating peptides, was not a feasible task. Therefore a need for a continuous supply of similar antigens was realized and *W. bancrofti* genomic library constructed earlier in our laboratory (Raghavan, 1991) became the source for isolating antigens with similar specificity. For this an initial exercise of isolating monospecific serum antibodies from filarial patients, reacting with the 15 KDa *S. digitata* peptide became essential for screening the library. This was accomplished by preparative Western blots.
The second part of the thesis deals with the screening of *W.bancrofti* genomic library for isolation of antigen(s) having the immunoreactivity homologous with 15 KDa peptide of *S.digitata*. Amongst several potential clones A single clone λWbG7 showed specific reaction with filarial sera and was negative with non-filarial and non-helminthic parasitic sera. The λWbG7 clone had a 2 Kb *W.bancrofti* DNA insert. The insert was recloned in the EcoRI site of the pMAL (683) vector (pGT7) for higher expression. The fusion protein obtained with the maltose binding protein (MBP) was approximately 105 KDa in molecular weight and was reactive with filarial sera only but not with other parasitic infection sera. The fusion protein was purified on a cross linked amylose column, for further studies.

The third part of the study deals with antibody detection assays, using the expressed fusion protein for diagnosis. The filarial specific IgG, IgG4 and IgE antibodies were detected using this (pGT7) antigen and was compared with the *S.digitata* affinity purified cuticular antigen at different stages of bancroftian filarial infection such as microfilaremia, pulmonary eosinophilia and chronic pathology.

The next part of the thesis was focussed on developing monoclonal and polyclonal antibody reagents for the purified recombinant pGT7 antigen. The murine myeloma cell line SP2/0 was used to immortalize the hyperimmunized spleen cells with the recombinant antigen pGT7 and was propagated in vitro. Four positive clones were isolated at the end of rigorous screening. However one monoclonal (McAbGT7-201) antibody was most effective in capturing circulating antigen in patients serum and was further studied. Simultaneously rabbit and murine polyclonal antibodies were also employed here.

The final part of the study deals with the free antigen(s) present in the bancroftian filarial sera. Anti *S.digitata* rabbit polyclonal antibodies were employed initialy to capture the circulating filarial antigens from the bancroftian
patients serum. The mouse polyclonal or monoclonal pGT7 antibodies were employed as the revealing antibodies.

The outcome of the present study is summarized below:

1. A 15 KDa cuticular antigen of *S. digitata* was identified to show filarial specificity.
2. The human filarial antibodies binding to this 15 KDa peptide were eluted and employed in the screening of the *W. bancrofti* genomic DNA library in order to isolate similar antigens.
3. The identified recombinant *W. bancrofti* antigen was recognized specifically by filarial IgG₄ antibodies in patients sera. This subclass response proved to be in concomitant with active filarial infection and is thus may be a marker, a marker not currently available for distinguishing between persistant and past infections.
4. Some "endemic normals" positive in the above testing showed microfilaria in the membrane concentration method, (a method not commonly used for routine epidemiological surveys) which further confirmed the validity of the assay as superior to the night blood wet smear observations.
5. Monoclonal and polyclonal antibodies developed against the recombinant antigen, pGT7 detected the circulating antigen not only in proven Mf + ve cases but also in the so called "normals" from endemic regions. Above 40% of the normals positive in the above assay were identified to be Mf + ve by membrane filtration method.
6. The quantification of the antigen concentrations correlated well with number of microfilaria in the peripheral blood, an ideal scale in the monitoring of the degree of active infection. Such reagents are needed for the evaluation of drug therapy and epidemiological surveillance. These reagents are the major outcome of the present study.
These have already been evaluated with a number of human filarial sera from India and from WHO. As the diagnosis procedure involves a pin prick of blood and which can be done during routine working hrs it presents an improved means of aiding chemotherapy in the control of infection.

1.2 LITERATURE REVIEW

1.2.1 Filariasis

The filarial group of Helminths transmitted by insects, belonged to the super family Filarioidea, order Spirurida, class Nematoda (CIH Key to the Nematode parasites of vertebrates) (Anderson and Bain, 1976). The WHO estimated (1984) that at least 905 million persons are at risk of infection with filariasis in the endemic areas although the number with overt disease is much less approximately 81.6 million. The Filarioidea contains two families, the Onchocercidae and the Filariidae. All the medically important species are in the Onchocercidae, including *Wuchereria bancrofti* and *Brugia malayi*, the causative organisms of bancroftian and brugian filariasis; *Onchocerca volvulus* the cause of onchocerciasis or river blindness; *Loa loa*, *Dipetalonema perstans*, *D.streptocerca* and *Mansonella ozzardi* which cause relatively benign filariasis in man.

1.2.1.1 *Wuchereria bancrofti*

*W.bancrofti* which lives in the lymphatics, produces "sheathed" microfilariae that exhibit nocturnal periodicity (except in the South Pacific), and is widely distributed throughout the tropics and subtropics (Beaver *et al.*, 1984) (Figure 1.1). In India it is transmitted by *Culex quinquefasciatus*. The major symptoms are lymphadenitis, lymphangitis, eosinophilia and lymph stasis (elephantiasis).
Figure 1.1 World distribution of *Wuchereria bancrofti* and *Brugia malayi*. 
Reproduced from clinical parasitology (Beaver et al., 1984).
1.2.1.2 *Brugia malayi*

*B. malayi*, which also lives in the lymphatics, produces 'sheathed' microfilariae that in some areas exhibit nocturnal periodicity. It is an important parasite in Eastern Asia, South Western Pacific Islands, parts of India and elsewhere. A closely similar species, *Brugia timori*, is found in people on the island of Timor and neighbouring islands (3). The typical vector transmitting *B. malayi* is *Mansonia sp.* in Asia and Indonesia. The symptoms of the disease is similar to *W. bancrofti* infection outlined above.

1.2.1.3 *Onchocerca volvulus*

*O. volvulus*, which lives in subcutaneous nodules, produces "unsheathed" microfilariae that distribute themselves in the cutaneous lymphatics (rarely they are found in the blood). It is widely distributed in tropical Africa, North Yemen parts of Mexico, Guatemala, Venezuela, Brazil, Colombia and Equador (Beaver et al., 1984). The black fly *Simulium damnosum* and *S. neavei* serve as the intermediate host and transmit the disease. The major clinical manifestation include Onchocercal nodules (Onchocercoma), Onchocercal Dermatitis and ocular manifestations.

1.2.1.4 *Loa loa*

*Loa loa*, which lives in the cutaneous and subcutaneous tissues, produces ‘sheathed’ microfilariae that exhibit diurnal periodicity, and is limited in distribution to tropical Africa (Beaver et al., 1984). Humans in endemic foci acquire infection when bitten by infected *Chrysops silacea* and *C. dimidiata*. The clinical symptoms include Calabar swelling, urticarial swelling and ocular manifestations (such as conjunctival granulomata etc),
1.2.1.5 *Mansonella perstans*

*Mansonella* (syn. *Dipetalonema*) *perstans*, which lives in the body cavities, produces microfilariae that are ‘unsheathed’ and are non periodic microfilariae, and is found only in tropical Africa (Beaver *et al*, 1984). Human infection is produced by bites of infected *Culicoides* in endemic foci. Abdominal pain, subcutaneous swelling, rashes and pain in serous cavities are the general clinical symptoms.

1.2.1.6 *Mansonella ozzardi*

*Mansonella ozzardi*, which lives in subcutaneous tissues and possibly in the body cavities, produces ‘unsheathed’ non periodic microfilariae and is found only in tropical America (Beaver *et al*, 1984). The species of *Culicoides* are apparently vectors in Surinam, Argentina and Haiti. In Amazon in Brazil the vector is *Simulium amazonium*. There are no manifest symptoms in infected individuals. The adult worms apparently produce little tissue reaction.

In addition numerous filariae of animals are occasionally found in man in various parts of the world.

1.2.1.7 *Setaria digitata*

The parasite is largely confined to the tropics and subtropics. The adult parasites were found in the lymphatic vessels and in the peritoneal cavity of the cattle. They are mostly commensals and no zoonotic infection of man is reported. The adult parasite is often creamy white in color, are thread like structure. They are filiform in shape and both the ends are tapering, the head end terminating in a slightly rounded swelling. The male measures 2.5 to 4 cm in length by 0.1 mm in thickness. The female measures 8 to 10 cm in length by 0.2 and 0.3 mm in thickness.
1.2.2 Bancroftian Filariasis

As mentioned earlier the bancroftian filariasis is caused by the parasite *W.bancrofti*. Microfilariae of the parasite were first found by Wucherer in the urine of a patient suffering from chyluria in Brazil in 1866. Microfilariae were seen in the blood of patients in India by Lewis in 1872. In 1876, Bancroft found the adult worms in humans. India has over 22 million microfilaria carriers and 18 million diseased persons as of 1983 survey (Katiyar and Murthy, 1990) (Figure 1.2).

1.2.3 Clinical manifestations and treatment

Clinical manifestations of infection are variable and probably depend upon constitutional factors in the host, numbers of infecting organisms and possibly strain differences in the parasite itself. The range of clinical manifestations of lymphatic filariasis is broad, and in brugian or bancroftian filariasis these include, 'filarial fevers', the syndrome of tropical pulmonary eosinophilia, a condition in which individuals are entirely asymptomatic yet have persistent microfilaremia, and lymphatic pathology.

1.2.3.1 Filarial fever

Frequently early manifestations of filariasis are fever, lymphangitis, and lymphadenitis. Febrile attacks may be seen with or without associated lymphangitis, although the latter are less common. These attacks are sometimes referred to as 'filarial' or 'elephantoid' fever. The attack usually subsides within 5 days.

1.2.3.2 Tropical pulmonary eosinophilia

Tropical pulmonary eosinophilia individuals comprise fewer than 1% of all patients with lymphatic filariasis and who clinically appear entirely distinct from the other groups of patients. Immunologically they are extremely hyperresponsive
Figure 1.2 Spectrum of clinical outcomes due to infection with *Brugia malayi* or *Wuchereria bancrofti.*
to all filarial antigens but especially to those derived from microfilariae (Ottesen et al., 1979). Antifilarial antibodies of all classes are markedly elevated and the IgE and eosinophil levels are as high in this condition as in any other non-neoplastic disorder (Neva and Ottesen, 1978). This clinical syndrome is now generally regarded as a form of ‘Occult filariasis’ in which the absence of circulating microfilariae reflects an immunological hyper responsiveness on the part of the host that leads to very effective clearance of these parasites from the blood. Most of this clearance is probably mediated by IgG antibodies and effected preferentially by the lungs, with the asthmatic symptoms probably resulting from allergic responses mediated through the specific IgE antibodies bound to lung mast cells.

1.2.3.3 Asymptomatic microfilaremia

Individuals with microfilaremia (harbouring Mf) but who are asymptomatic and without acute or chronic lymphatic involvement, form the group that is immunologically least reactive. Their lymphocytes generally fail to respond significantly to filarial antigens in vitro (Ottesen et al 1977 and Piessens et al 1980a,b) and their levels of serum antibodies to both adult and microfilarial antigens are minimal or absent (Ottesen et al 1982; McGreevy et al., 1980). This hypo responsiveness is probably a manifestation of hyper reactive suppressor mechanisms limiting responsiveness to the parasite; it is probable that the clinical hypo-reactivity of the host is a direct consequence of this concomitant immunological hypo responsiveness.

1.2.3.4 Lymphatic pathology

Some microfilaremic patients remain unaffected for decades, others develop intermittent, filarial fevers which ultimately lead to hydrocele, chyluria or/and elephantiasis which are the main characteristics of chronic pathology of bancroftian filariasis.
1.23.4.1 Hydrocele

In endemic areas such as East West tropical Africa, India and Indonesia, hydrocele is the most common sign of filariasis. Formation of the hydrocele is generally preceded by frequent episodes of fumiculitis or orchitis. A lymph varix of the spermatic cord may appear and rupture into the scrotal sac, leading to a condition known as lymphocele. Lymphocele, is one type of hydrocele, condition that may also develop gradually as a result of recurrent attacks of Orchitis.

1.23.4.2 Chyluria

Chyluria is highly prevalent in East Africa and China but less common in India and Pacific. In chyluria the chyle escapes through the urine due to rupture of varicose chyle vessels through the mucous membrane of the urinary tract. In some cases the microfilariae may be found in the urine.

1.23.4.3 Elephantiasis

Elephantiasis, the enlargement of one or more limbs, scrotum, breasts or vulva with dermal hypertrophy and verrucous changes, is a relatively uncommon and late complication of filariasis. The classical view is the obstruction of the lymphatics, particularly as they pass through lymphatic nodes, impede the flow of lymph and cause it to be retained in the affected limb. In the initial stage the swelling is best observed around the ankle which gradually spreads up the thigh. The affected limb increases 3-4 times the original size. But it is reasonably clear that both the humoral and cellular responses to filarial antigens is greater than in patients with asymptomatic microfilaremia (Ottesen 1984). The lymphatic damage and the obstruction in the lymph nodes is due to the immunological recognition of parasite which brings about the increased local inflammation and subsequently leads to the obstructive pathology.
1.23.5 Endemic normals

Individuals living in endemic regions but with absolutely no clinical or parasitological evidence of infection, form a particularly important group for study. As a group, their immune responsiveness to parasite antigens is significantly greater than that found in patients with microfilaria (Ottesen et al., 1977). The endemic normals is a very heterogeneous group with some individuals being immune to the parasite and others harbouring the post-patent or other occult infections described in numerous animal models (but not yet in man).

1.23.6 Treatment

The drug that is used for the treatment of bancroftian filariasis is Diethyl carbamazine (DEC) citrate. The mode of action of DEC is not clearly known but, it is predicted into

a) bring about a modification of the surface layer of the microfilarae which exposes them to immunological cell mediated lysis (Cesborn et al., 1987);

b) DEC acts in combination with the blood platelets, and additional triggering occurs by the excretory secretory (ES) antigens of the filarial nematodes and the involvement of certain free radicals (Cesborn et al., 1987).

As the DEC drug regimen requires daily administration for twelve days to achieve maximum effect and as it causes heavy side effects in the individuals, a more potent drug namely, Ivermectin, a semi-synthetic macrolide antibiotic produced by *Streptomyces ivermectilis* is presently found to be promising as a alternate drug (Kumaraswami et al., 1988). It has a wide helminthicidal spectrum for parasites of animals. It was also shown to be an extremely effective microfilaricidal agent in humans with onchocerciasis, even after administration of a single oral dose. In double-blind clinical trials, Ivermectin treatment has proved
to be superior to DEC treatment with respect to both efficacy and side effects (Diallo et al, 1987).

1.2.4 Parasite morphology and life cycle

The adult worms are minute and thread-like in form, with a smooth cuticle. Although tapering towards both ends, their terminations are bluntly rounded. The head is slightly swollen and is surmounted by two rings of small sessile papillae. The mouth is unarmed, a buccal vestibule is lacking. The male measures about 4 cm in length by 0.1 mm in diameter and its tail is curved vertically. The female measures 8 to 10 cm and the tail is narrow and abruptly pointed. The young embryos in the inner portion of the uterus are confined within ovoid shells and as they progress towards the outer portion of the uterus the shells become, elongated to accommodate the uncoiling embryos and becomes known as the sheaths of microfilariae. In this stage they are discharged from the worm. The microfilariae that migrate into lymph and blood channels measures an average of 244 to 296 µm in length by 7.5 to 10 µm in diameter. They are bluntly rounded anteriorly and pointed caudally. Their internal structure can be studied only by the use of fixed stained preparations or by vital dyes, which show a central column of nuclei, among which are important anatomical ‘landmarks’ used in differentiating this species from other macrofilariae in blood and tissue films. These ‘landmarks’ consist of the nerve ring, excretory pore, excretory cell, ‘G’ cells and anal pore. The percentage distances, of these ‘landmarks’ from the anterior end of the microfilaria provide a formula that is quite constant for a species (Beaver et al., 1984).

The life cycle of *W. bancrofti* passes through a definitive host, humans and the intermediate host, the mosquitoes (Figure 1.3). The sheathed microfilariae ingested by the mosquito during the blood meal cast off their sheaths quickly, penetrate the gut-wall within an hour or two and migrate to the thoracic muscles. Here they begin to rest and grow. In the next few days they develop rudimentary digestive tracts and within 3-7 days the larvae grow rapidly and shed off the cuticle.
Develops to maturity in lymphatics infective larvae enter through skin. Enter lymph vessels and lodge in LYMPH NODES.

Female gives birth to living embryo (microfilaria).

INFECTIVE LARVA penetrates skin of man after being deposited there when mosquito bites.

MICROFILARIA ingested by mosquito during blood meal loses sheath.

Figure 1.3 Life cycle of *Wuchereria bancrofti*. Reproduced from clinical parasitology (Beaver *et al*, 1984).
This stage is referred as the second stage larvae. By the 10th or 11th day the metamorphosis is complete. The digestive system, the body cavity and genital organs are now formed. They are referred to as third stage larvae and at this stage they are infective to humans. When the infected mosquito bites a human being, the 3rd stage larvae are not directly injected into the blood stream but are deposited on the skin near the site of puncture. The larvae either enter through the puncture wound, or penetrate through the skin, on their own. The 3rd stage larvae having penetrated the skin, reach the lymph node and begin to grow into adult worms. In about 5 to 18 months they become sexually mature and thus a new generation of microfilariae are produced which passes either through the thoracic duct or the lymphatic duct to the venous system and to the peripheral circulations thus completing the cycle (Manosn-Bahr and Apted, 1982).

1.2.5 Microfilarial periodicity

In most regions of the world there is a marked periodicity in the circulation of *W.bancrofti* microfilariae in the blood. The numbers are high during a 4 hour period at midnight and scanty or absent during day light hours. This type of circadian fluctuation is referred to as nocturnal periodic. In parts of the pacific region *W.bancrofti* microfilarial fluctuation is relatively slight, with an increase in numbers during the afternoon and evening, but with microfilariae present in the blood continuously throughout the day and night. This type, therefore is referred to as diurnal subperiodic. In other filaria and presumably in *W.bancrofti* only a small part of the total microfilaria population is in the circulating blood, even at the peak levels of microfilaremia (Pacheco Orihel, 1968). A large part of the non-circulating population is sequestered in the lungs. Whether they remain in the blood and lymph capillaries and larger vessels or lie free in the tissues is unknown. They readily migrate from midgut to hemocoel through tissues of the vector. It is possible that they move freely in and out of the circulation in the vertebrate host (Bearer, 1970). The stimuli that send the microfilariae from the lungs and other tissues into the circulating blood and later back into the tissues are essentially unknown (Hawking *et al.*, 1981). Body temperature and oxygen tension have been
proposed as factors that determine the circadian rhythm (Hawking, 1967). The periodicity of *W. bancrofti* microfilariae can be reversed by reversing the periods of sleep and activity of the host (Hunter and Warren, 1950).

1.2.6 *In vitro* cultivation of filarial parasites

*In vitro* culture system for filarial parasites permit development from the third to the fourth larval stage (Franke *et al.*, 1984, 1987). The most advanced development previously achieved has been to the fifth stage of larval development (sexually immature adult worms) of *Brugia malayi* in a system employing co-culture of mammalian cells (Mak *et al* 1983). Copious production of microfilariae *in vitro* by the adult *B. malayi* was reported entirely from commercially available culture system (Ribern *et al* 1990). Third stage (L₃) larvae of *W. bancrofti* obtained from laboratory infected mosquitoes, grew and moulted to the fourth stage (L₄) *in vitro*. The L₄ larvae were motile having excellent morphological condition with the development of the reproductive system in males and females (Franke, 1987).

1.2.7 Diagnosis

1.2.7.1 Parasitological diagnosis

1.2.7.1.1 Simple direct blood observation

The diagnosis was based on demonstration of microfilariae in the blood or on clinical evidence. Most commonly used test for the detection of microfilaremia is the thick film containing 20 to 60 mm³ blood, air dried, laked, fixed and stained with hematoxylin or Giemsa (Ramachandran, 1970) and observed under microscope.
1.2.7.1.2 Knott concentration method

A method was developed by Knott (1939) to concentrate the microfilariae from 2 ml of blood samples in 2% formalin followed by observing the parasite under microscope.

1.2.7.1.3 Concentration by Microfilters

Filtration of venous blood through microfilters has come into common use (Desowitz and Hitchcock, 1974). However, the necessity for venipuncture reduces the number of people who are willing to be examined.

1.2.7.1.4 DEC provocation test

In areas where microfilaria is nocturnal or is present throughout the day but at low levels, microfilaria may be induced to appear in the blood during the day by giving Diethylcarbamazine in a single dose of 2 mg per kg body weight. Usually peak numbers is reported to occur 45 min after administration (McMohan et al., 1979).

1.2.7.2 Immunodiagnosis

Definitive diagnosis of filarial infections requires direct demonstration of the parasite in the host. However because filariae can be present in small numbers or sequestered in inaccessible sites and because the means to detect them parasitologically are relatively insensitive, immunodiagnostic techniques have been greatly realized as the essential tool for diagnosis (Ottesen, 1984).

An immunodiagnostic test to be acceptable as a tool for diagnostic and epidemiological purposes, it should satisfy certain requirements in sensitivity, specificity, predictive value and reliability. Further it should be simple to perform,
economic costwise, acceptable to population and should be adaptable for field study (Harinath, 1984).

1.2.7.2.1 Antibody assay

Tests which assay antibody must have low, moderate and high sensitivity (Kagan, 1983). Complement fixation and gel diffusion tests fall under low reactivity. These tests require relatively high concentrations of antibodies and the titres obtained are usually low. In the beginning of infection when the antibodies are absent or their level is appreciably low, these tests are usually non reactors. Tests of moderate reactivity are indirect haemagglutination (IHA) (Kharat et al 1981 and Naidu et al 1984) and indirect immunofluorescent (IIF) techniques (Piezens et al., 1980). These tests are reasonably acceptable but have now been replaced by more sensitive ones. Test of high reactivity are immediate hypersensitivity reaction (Smith et al.,1971 and Grove et al., 1977), radio immunoassay (RIA) (Hamilton et al., 1981; Hamilton and Ward 1982 and Maizels et al., 1983) and enzyme linked immunosorbant assay (ELISA) (Weiss et al., 1987; Kaliraj et al 1981a and Kharant et al., 1982).

1.2.7.2.1.1 Complement fixation test (CFT)

Complement is a group of proteins present in the serum which acts sequentially to lyse the antibody reacted bound organisms. Complement fixation is used to demonstrate the presence of complement fixing antibody in the serum. The fact that antibody, once it combines with antigen, is liable to activate the complement system and is used as a method for showing the presence of a particular antibody in the serum. Generally the CFT is performed by using the 50\% end point method, titrating the serum against a constant amount of complement. The earliest report available in this regard was of Rohain and van den Branden and Fairley (1962), used an alcoholic extract of Onchocerca cyst in complement fixation test (CFT) for diagnosis of onchocerciasis and reported that this test was not suitable for diagnostic purposes. Since then a number of workers
have tried CFT for the diagnosis of filariasis using different filarial antigens. However the sensitivity of these antigens in CFT has not been uniform.

It was reported by Connor (1932) that an alcoholic extract of *D.immitis* antigen in CFT gave positive reaction against sera of patients with different filarial infections and concluded that this test was specific for *Loa loa*, less sensitive for *W.bancrofti* infections. In recent years workers seem to have attached less importance to this test because of the discrepancy of results in many instances which might be associated with either the poor preservation or the poor complementary potency of some sera. However the test was reported to be more useful for the diagnosis in some other parasitic infections. Sleeman (1960) has prepared a specific antigen for CFT and employed for the diagnosis of Schistosomiasis. Pellegrino and Pedreira de Freitas (1961) developed and employed a quantitative complement fixation test for *Schistosomiasis mansoni* infection. The CFT gave promising results in the diagnosis of "chagas" disease (Kagan, 1974 and Maekelt 1960).

1.2.7.2.1.2 Precipitation test (ID,IE,CIEP)

At its early stages precipitin test was performed in liquid medium which was of limited use in the diagnosis of bancroftian and other filarial infections. In a comparative study it was observed (Culbertson et al 1944, 1944a), that the precipitin test using 0.5% carbol saline extract of *L.carinii* antigen was comparable in sensitivity to that of complement fixation test and lower than that of intradermal test for the diagnosis of bancroftian filariasis.

In recent years after the development of double diffusion (D.D or I.D) and immunoelectrophoresis (IE) etc., the precipitation test on liquid medium is found to be of limited value for serodiagnosis. Capron et al (1968) applied D.D and I.E using *D.vitae, O.volvulus and D.immitis* antigens for diagnosis of filariasis and observed the characteristic precipitin arcs in immunoelectrophoresis for loiasis, Wuchereriasis and onchocerciasis.
Desowitz and Una (1976) observed the presence of circulating antigen and stage specific antibodies in the sera of *D. immitis* infected cats and humans infected with *W. bancrofti* by counter immunoelectrophoresis (CIEP) using *D. immitis* antigen and rabbit anti *D. immitis* sera. The same technique was satisfactorily employed for the detection of antibody in *B. pahangi* infected cat using homologous antigen (1976).

Though the precipitation techniques such as agar gel diffusion, immunoelectrophoresis and CIEP have a wide application, they require concentrated form of antigens which cause practical difficulties in using these techniques on a large scale for serodiagnosis.

**1.2.7.2.13 Indirect haemagglutination test (IHAT)**

Some soluble antigens can be coated on to red blood cells of either sheep, horse or man which are then found to react as if they were antigens. Red blood cells have been found to be extremely convenient passive carriers of antigen (Talwar, 1983). Agglutination of the particulate antigen in the presence of the specific antibody constitutes the basic principle of indirect haemagglutination test (IHAT). Nearly 20 years have passed since IHAT was first applied for the diagnosis of filariasis. The earliest study was reported in 1960 by Jung and Harris who used this technique for the diagnosis of *D. immitis* infections in humans. Healy and Kagan (1961) observed positive haemagglutination and bentonite flocculation reactions with the sera of 24 dogs collected in Atlanta, Georgia of which 44% of the animals showed Mf of *Dipetalonema* sp. in their blood. Though the test on the whole showed fairly reliable results for the different types of filariasis, unfortunately its specificity was found to be low in many instances due to the cross reactions between filarial antigen and other parasites. Pacheco (1961) carried out a study on the specificity and sensitivity of *D. immitis* antigen by IHAT and found that crude extracts of adult worms were not specific while ethanol extract of antigen was more specific but showed on the whole less reaction and a
few cross reactions against Ascaris infection, tropical eosinophilia and Schistosomiasis.

It appears that this test would be more useful with the development of more specific antigen. Mantovani and Kagan (1967) used a specific fraction of *D. immitis* antigen in IHAT and skin test and observed the positive reaction only against the sera of dogs infected with homologous parasites. In a similar test Fujita *et al* (1970) observed a promising result using *L. cairinii* and *Setaria cervi* antigens for the diagnosis of experimental and human filariasis.

Though the agglutination tests are reported to be sensitive for the diagnosis of many parasitic infections, they seem to be limited in detection of preferential antibodies of the IgM class.

1.2.7.2.1.4 Fluorescent antibody techniques

There are two types of fluorescent antibody techniques. Direct fluorescent antibody technique (DFAT) consists of bringing fluorescein-tagged antibodies into contact with antigens fixed on a slide, allowing them to react, washing off excess antibody and examining the fluorescence at the site of antigen antibody interaction under the fluorescence microscope. In indirect fluorescence antibody test (IFAT) the serum is layered on antigen smear and after removal of non-specific immunoglobulin by washing, the smear is treated with fluorescein tagged antiglobulin serum, specific for the globulin of the serum applied earlier. Finally the presence of antibody is confirmed by observing the smear under fluorescence microscope. The IFAT can be used both for detecting specific antibodies in sera or other body fluids and also for detection of antigens.

Fluorescent antibody studies were made on protozoa by Goldman (1953) who demonstrated the value of this technique in parasitology. Jackson and Lewert (1957) employed this technique in helminthes infections for the diagnosis of *Trichinella spiralis* in man. Both direct and indirect fluorescent antibody tests
(DFAT and IFAT) have been employed in many parasitic infections. Most of the workers have been attracted by IFAT because of its simplicity and this test had been widely employed in many protozoan infections (Fige and Muschel, 1959 and Boonpucknavig and Nairn, 1967) and helminthic infections such as *Hymenolepis nana* (Coleman, 1961), *Ascaris lumbricoides* (Taffs and Voller, 1963) *Toxocara canis* (Mitchell, 1964) *Trichinella spiralis* (Sulzer and Kagan, 1967) *Schistosoma sp.* and (Sadum et al., 1960) filariasis (Van Weeman and Schuurs, 1971).

### 1.2.7.2.1.5 Skin test

Skin tests appear to be very suitable for field use. Since the test is based on immediate hyper sensitivity of the patient, the positivity appears within few minutes of antigen injection and the result can be obtained within 15 min. The test neither demands much of technical skill nor sophisticated laboratory facilities. The main difficulty has been the use of proper antigen. On earlier occasions heterologous filarids such as *Dracunculus medenensis* (Fairley and Liston, 1924), *Setaria cervi* (Goodman et al., 1945 and Ridley and Scott, 1961) and *Dirofilaria immitis* (Sawada et al., 1969) were used as the source of test antigen. But these ended in non specific and false positive reactions. Sawada et al (1969) have claimed that the highly purified *D.immitis* antigen is useful in the detection of lymphatic filariasis. This antigen contains approximately twelve proteins detectable by electrophoresis. The *D.immitis* antigen was evaluated in many countries by WHO and the results were equivocal (Smith et al., 1971). The sensitivity of this antigen was questioned by Grove et al (1977) who stated that *B.malayi* skin test antigen was much more sensitive. They further claimed that *D.immitis* antigen was more reactive in patients with Malayan filariasis. Because of these limitations further work with *D.immitis* antigen was discontinued. The overall consensus is that for *W.bancrofti* infection, *D.immitis* skin test antigen is not as sensitive as that made from *B.malayi*. Grove et al (1977) have employed saline extract of Mf, adult worms and L3 of subperiodic strain of *B.malayi* and evaluated these antigens in skin test in Philippines. They found that over 90% of the filaria
infected patients reacted to all three antigens in filaria skin test, while *D. immitis* antigen was much less sensitive. The skin test antigen has significant value in measuring the exposure rate, and needed characterization and identification of the specific reactive antigenic components, for improvement. However as presently used in lymphatic filariasis with antigens of inadequate specificity they are of little help in immunodiagnosis of patients in endemic regions (Weller *et al.*, 1980). The use of more purified antigens has led to greater success in the diagnosis of human onchocerciasis (Schiller, 1980). A skin test was developed using the *B. malayi* L₄ parasite antigen for the diagnosis of bancroftian and brugian filariasis, at the Central Drug Research Institute (CDRI), UP, India. However the test was unable to distinguish the stage specific infection.

1.2.7.2.1.6 Enzyme linked immunosorbent assay (ELISA)

In enzyme immunoassay developed by Van Weeman and Schurs (1971) and Engvall and Perlmann (1972), the detection and assay of one immune component either antigen or antibody is made possible by the interaction of a substrate with the enzyme linked to the other immune component. Based on this principle various enzyme immunoassay techniques such as defined antigen substrate sphere (DASS) enzyme immunoassay (Deelder and Streejkkerk, 1975), enzyme linked immunosorbent assay or ELISA (Ruitenberg *et al.*, 1975) and stick ELISA (Felgner, 1978) had been developed and employed for the detection of antigen or antibody in many diseases (Voller *et al.*, 1976).

Since ELISA using tubes or microtitre plates is more simple for large scale application, it has been recently employed in many parasitic diseases such as malaria (Voller *et al* 1975), African trypanosomiasis (Voller *et al.*,1975b), "Chagas" disease (Voller *et al*, 1975a), Leishmaniasis (Hommel, 1976), Trichinosis (Ljungstrom *et al*, 1974) and Schistosomiasis (Kelsoe and Weller, 1978).

Bartlett *et al* (1975) applied micro-ELISA using the enzyme alkaline phosphatase in the detection of *O. volvulus* infections. It was not possible to use
the homologous antigen extracted from *O. volvulus* adult worm, because contaminants of host origin reacted with the conjugate. But the cross reaction with parasitic nematodes suggested that *O. gutturosa*, a cattle parasite could be used as antigen. Sera from people with onchocerciasis gave higher values than control African sera from non-endemic area. The authors suggested that purified antigen will be more useful for developing a specific ELISA test. Marcoullis *et al* (1978) purified the *O. volvulus* adult worm antigen by sequential affinity chromatography and reported that this purified antigen should be used in ELISA technique for the specific diagnosis of onchocerciasis. ELISA seems to be quite sensitive and can make use of purified antigens thus increasing the specificity of the immune reaction.

The dramatically increased sensitivity of this assay (detection of femtogram or attogram levels of antibody) has brought with it the need to develop assay reagents of equally enhanced specificity than the older techniques (Ottesen, 1984). Detection of antibodies to the parasite, in ELISA as an evidence of previous exposure to the parasite, allows calculations to be made for exposure rates within a given population. The fact that the quantity and quality (Ig class) of antibodies synthesized to parasite antigens may vary, sometimes due to genetic differences between outbred hosts and often with time-dependent characteristics, has two importance consequences for the construction of diagnostic tests based on the detection of anti-parasite antibodies.

First, the probability of a given antigenic determinant being universally recognized by all sera from infected individuals at all times, during and after infection must be low and thus the antigen mixtures will probably be required for diagnostic tests with 100% sensitivity.

Secondly, a thorough knowledge of the kinetics and classes of antibodies synthesized within a population will allow the construction of prognostic tests, or antibody windows indicative, for example of early versus late infections, susceptible versus resistant hosts or probability of pathological consequences in particular host.
Thus an immunochemical dissection of humoral anti-parasite responses may help in the understanding of protective versus pathological responses. The first objective is to identify appropriately specific parasite antigen-host antibody systems (Parkhouse and Harrison, 1989 and Cheirmaraj et al., 1991).

1.2.7.2.1.6.1 Heterologous somatic antigens

It is likely that homologous (Wuchereria, Brugia or Onchocerca spp.) antigens are preferable to heterologous ones for developing specific immunodiagnostic techniques. Successful maintenance of Brugia infections in jirds has permitted use of *B. malayi* and *B. timori* parasites to develop antigens for diagnosing filarial infections, but the general non-availability of *W. bancrofti* and *O. vulvulus* parasites has led to many attempts to purify heterologous filarial antigen to obtain fractions that show specificity for these human parasites. Such studies have included use of antigens of *S. digitata* (Dissanayake and Ismail, 1980), *D. viteae* (Baschong et al., 1982), *O. gibsoni* (Forsyth et al., 1981), *Litmosoides carinii* (Dasgupta and Bala, 1978) and *Dirofilaria immitis* (Sawada et al., 1969). Although fractionation of the crude antigens does diminish cross reactivity, it is fair to say that no antigens from these heterologous parasites have yet been isolated with clear state or species specificity for the human lymphatic filariae (Ottesen, 1984).

1.2.7.2.1.6.2 Homologous antigens (Somatic or excretory-secretory antigens)

Homologous (Wuchereria, Brugia or Onchocerca spp) antigens are preferable to heterologous ones for developing specific immunodiagnostic techniques. Using soluble *W. bancrofti* Mf antigen, the efficiency of IHAT, IFAT and ELISA tests has been compared for the detection of antibody in filarial sera. Filarial antibody could be detected in 93%, 100% and 81% of the microfilaremics, 75%, 90% and 100% of chronic pathology and none of the non-endemic sera was detected by IHAT, IFAT and ELISA respectively. However 45-65% endemic normal sera showed the presence of filarial antibody by these tests (Kaliraj et al., 2007).
The soluble antigens (MfS) isolated from *W. bancrofti* microfilariae were fractionated by Sephadex G-150 gel filtration into 3 antigenic fractions (MfS 1, 2 and 3). The MfS3 fraction was weakly reactive in IHA but the same was found to be highly reactive in ELISA. The MfS2 antigen fraction showed cross reaction with non-filarial helminth infected sera similar to the crude soluble antigen (MfS). The antigenic fractions (MfS1 and MfS3) were further fractionated by DEAE cellulose chromatography. Analysis by ELISA showed that MfS1b and MfS3e antigen fractions were highly active in the detection of filarial antibody in chronic filariasis (85%) and microfilaraemia (88%) sera respectively (Kaliraj et al., 1982). However, processing of microfilariae from 100 ml of blood sample containing 50 Mf/20 mm³ gives antigen fraction (MfS3e) just sufficient for about 2000 tests, which is not a practical proposition for large scale isolation of antigen for field surveys (Harinath, 1984). The excretory and secretory (ES) antigens from *in vitro* maintained *W. bancrofti* microfilariae, showed highly sensitive and fairly specific reactions in ELISA. As little as 0.35 ng ES antigen protein per well was found to be sufficient in detecting filarial antibody (Kharat et al., 1982) when compared to the soluble microfilarial antigen (1.5 μg/well) or fractionated antigen (0.1 μg/well). These studies showed that detection of ES antigen specific IgG or IgM or IgA antibodies will be more useful than specific IgE for the immunodiagnosis of filariasis (Harinath, 1984).

The execution and evaluation of onchocerciasis control programme is hampered by the lack of adequately specific diagnostic tools to measure exposure to the parasite. Five ELISA based systems for the detection of antibodies to *O. volvulus* were compared. Four were conventional antibody detection ELISA assays and the fifth was an inhibition type ELISA assay using a monoclonal antibody (NIM-M8) directed at an *O. volvulus* specific somatic antigen of the adult worm. The antigens for the conventional ELISA assays consisted of a conventional aqueous extract of adults of *O. volvulus* and a low molecular weight surface fraction of the adult (Cabrera and Parkhouse, 1987). In addition, following reports of an antibody response to phosphorylcholine (PC) in nematode infections (Pery et al., 1974; Forsyth et al., 1985) a PC antigen as a control was also
employed. Finally a human IgG4 subclass specific ELISA assay was used in conjunction with the total aqueous extract of adult worms. The assay systems were evaluated for specificity and sensitivity using panels of sera from clinically diagnosed infections with *O. volvulus* in Venezuela. The results obtained indicate that the low molecular weight, surface derived fraction is a considerably more specific trapping layer for antibody assays than the conventionally used PBS extract or phosphorylcholine. Equally specific was the monoclonal antibody-based inhibition type ELISA assay. The main IgG subclass response was IgG4, and restricting the assay to this antibody subclass resulted in an improved specificity when crude PBS extracts of the parasite were used as antigen. The study therefore stresses the importance of using a previously defined and specific system for immunodiagnosis, and also provides an example of how restricting assays to one Ig subclass here IgG4 may improve specificity (Parkhouse and Harisson 1989).

The isotype or subclass of host antibody may be important in conferring greater or lesser specificity on immunodiagnostic techniques. For example IgE antibody responses have greater specificity than IgG responses in human *Onchocerca* and lymphatic filarial infection according to Weiss *et al* (1982). This is in contrary to the reports of Harinath (1984). Qualitative analysis of these IgE antibodies has only recently begun and the implications of the findings for immunodiagnosis are still uncertain (Hussain and Ottesen, 1983).

*Brugia timori* infected individuals were surveyed with radiolabelled surface antigen from the same species, prepared from infective larvae. Antibody reactivities show a general rise as severity of disease increases with microfilaremics being generally low, acute symptomatic individuals intermediate and elephantiasis sufferers the highest. The asymptomatic, apparently uninfected group, however showed a bimodality with some individuals bearing antibody levels characteristic of chronic infection (Maizels *et al*., 1983). These results illustrate the ambiguity of tests aimed at detecting host antibody to a chronic parasitic infection (Cruickshank and Mackenzie, 1981). The asymptomatic individuals with high antibody may fall into any of three groups, with fundamentally different
implications: Firstly, this antibody may be fully protective against infection and these individuals may never have contracted filariasis. Secondly the antibody may be the result of a past infection, which has now been eliminated or thirdly these levels of antibody may be truly diagnostic, of a prepatent infection which will subsequently become microfilaremic or symptomatic (Maizels et al., 1983).

An ELISA for bancroftian filariasis marketed by Cadila Inc, India, was unable to distinguish the active filarial infections from the normals from endemic regions.

1.2.7.2.1.6.3 Recombinant antigens

"Current state of helminth diagnosis has recently been reviewed and makes depressing reading, with most tests focusing on crude parasite extracts and little emphasis on working with defined systems", according to Walls and Schantz (1986). The 1988 international congress for tropical medicine and malaria held in Amsterdam reiterated the need for cloned and expressed antigens in parasite diagnosis (Parkhouse and Harisson, 1989). The new generation of immunodiagnostic tests will permit individual epitopes to be used as target, although indications are that a 'cocktail' of such epitopes will be necessary to cover wide individual variations in the antibody response in human population.

It is desirable to identify immunogens that will not exacerbate immunopathology but which could lead to the development of vaccine conferring long-lasting protection (Denham et al., 1983; Selkirk et al., 1986). DNA technology has provided an avenue for purifying substantial quantities of defined parasite antigens which is a precondition for any strategy aimed at identifying diagnostic antigens. The availability of well characterized proteins had facilitated the search for species specific diagnostic reagents.

A Brugia malayi geneomic DNA library was constructed by Arasu et al. (1987) in bacteriophage vector λgt11 and was screened for species specific clones. An advantage of a genomic parasite library over a cDNA library was that the recombinant proteins from all stages of a parasite's life cycle could be obtained.
from a genomic library. On the other hand the disadvantages of genomic cloning via restriction endonucleases include the non random distribution of recognition sites and hence a biased selection for genes with sites close to or within coding regions and with correct in-frame reading frames. The screening of different constructs of genomic libraries would help to alleviate this problem.

The cloning of species specific L3 antigens that are also expressed by adult worms (or microfilaremia) of *B. malayi* was reported to be a potential immunodiagnostic reagent (Arasu *et al.*, 1987). Using such recombinant antigens as immunogens species specific polyclonal antisera could be produced and used to detect parasite components in bodily fluids of infected individuals. The converse will also be possible; that is, recombinant antigens could be used to screen human sera for *B. malayi*-specific antibodies.

Two clones isolated from an expression library constructed (Werner *et al.*, 1989) from *B. malayi* genomic DNA were recognized by two distinct immunoglobulin classes. A λgt11 fusion protein containing part of the *B. malayi* myosin tail region is recognized by antibodies of the IgG class from a high percentage of bancroftian filariasis patients. A fusion protein containing a collagen-like sequence is less frequently or weakly recognized by IgG class but was found specific for IgE (Werner *et al.*, 1989).

A λgt11 cDNA library constructed from *B. malayi* adult mRNA, yielded a clone expressing 63 kDa protective antigen. Infected animals injected with the 63 kDa molecule demonstrated 50-70% reduction in parasitemia (Nilsen *et al.*, 1988).

A genomic DNA library constructed from *W. bancrofti* in λgt11 (Raghavan *et al.*, 1991) showed restricted specificity to the major lymphatic filarial parasites *W. bancrofti* and *B. malayi* and not to other filarial and non-filarial species tested. However it is reported that the cloned DNA was not a repetitive one and the protein expressed had homology to the intracellular molecules such as myosin, a
highly immunogenic and prevalent molecule in many helminthic and non-helminth parasitic organisms and hence its diagnostic validity is yet to be determined.

"The potential of such recombinant DNA approach is great, the strategy good and the optimism high, an usable product still lies only in future", says Ottesen (1989).

1.2.7.2.2 Antigen assays

The major short coming of immunodiagnostic techniques based on detection of antibody in the infected host is their inability to distinguish past exposure from current infection. Detection of parasite material (antigen or other products) in blood or urine should be much more effective for assessing the status of individual infections. Confirmation of current infection would allow:

a) recognition of asymptomatic cases for drug or other treatment,
b) recognition of re-establishment of the parasite following drug treatment or evaluation of ecological control measures and
c) classification of areas with low, intermediate or high prevalence.

Paradoxically, parasite antigens as such are not ideal probes, since by definition they provoke antibody responses and thus are rapidly cleared from the circulation of the host. Non-antigenic or poorly antigenic, stage-specific secreted components on the other hand, would form an ideal focus for the construction of serum or urine based immunochemical tests for parasites, since they are continuously released by live parasites and may not be rapidly cleared by the humoral immune system of the host (Parkhouse and Clark, 1983). The clearance from the peripheral circulation of antibody, complexed in vivo with antigen, is unpredictable. Techniques for correlation of immunoassays results for interference by host antibody have unfortunately not been successful because of lack of correlation between the amount of specific human antibody in serum as measured by solid phase immunoassays and with degree of interference as shown by
inhibition antibody add-back experiment (Hamilton and Scott, 1984). Thus filarial antigen assays should be considered semi-quantitative tool for defining the absence or the presence of antigen as diagnostic indicator. Given an appropriately defined parasite product secreted by a viable parasite at a constant rate and a complementary monoclonal antibody, it would be possible to construct a dose-dependent (and thus quantitative) ELISA for the detection of parasites (Parkhouse and Harisson, 1989).

Though several antigen detection assays have been described for bancroftian filariasis their prediction value has not been assayed in endemic population. In determining circulating antigens, it is important to consider the possible source of antigens (for antibody production). In endemic areas the entire population is exposed to the vector and is likely to produce antibody to larval antigen. But members of the exposed population are unlikely to have detectable levels of circulating larval antigens because of the physical size and number of infective larvae to produce persistence and detectable antigenemia. The excretory and secretory (ES) products of adult filariae can result in antigenemia if the worm load is sufficiently high but this is unlikely in acute and early infections. In contrast to animal filarial infections where high load of adult worms are not uncommon, there may be very few adults in *W. bancrofti* infections in humans. The other possibility which may produce antigenemia is the shedding of cuticle or cuticle associated components during larval development and the adult stages of parasites and the shedding of egg debris and other components associated with reproduction. But there appears to be only hypothetical considerations as the antigens produced from those stages would not be sufficient to be used for antibody production.

Antigen epitope sharing between filarial parasite and the host is probably the most serious limitation in antigen determination assays. Sharing of host antigen by filarial parasites has been reported in relation to evasion of the host immune responses by Court and Storey (1981) and McGreevy *et al.* (1975). Such antigenic similarities have been demonstrated in *O. volvulus* (Oliver-Gonzalez and Morales,
As such any assay method based on antigen detection has a caution for practical utility.

Circulating filarial antigen was first demonstrated in bancroftian filariasis in 1946 by passive cutaneous anaphylaxis (Franks 1946). Attempts to detect circulating antigens were met with partial success. Kaliraj et al. (1981) carried out counter current immunoelectrophoresis (CIEP), indirect haemagglutination test (IHAT) by using human filarial serum immunoglobulins in patients with *W. bancrofti* infections. CIEP was found to be better than IHAT and 23 of 30 sera from persons with microfilaraemia showed the presence of circulating antigens. In general these tests were not sensitive enough to be practically useful (Ottesen, 1984). Later in similar study employing ELISA as the test model, 27 out of the 33 microfilaremic sera and 19 out of the 30 from clinical cases were found positive for circulating antigens (Kaliraj et al 1981).

Several groups have reported that filarial antigens are detectable in urine as well as in serum (Reddy et al, 1984; Zheng et al, 1987 and Lutsch et al, 1988). The assumption that urine will be easier to obtain than blood for field studies is not necessarily valid (Weil,1990). Additional studies are needed to optimize urinary antigen assays and to systematically evaluate these assays in large field studies.

More recently technical refinements (monoclonal antibodies, pretreatment of sera to release antigen from immune complexes) have resulted in the development of more sensitive assays. For example monoclonal antibodies to phosphorylcholine (PC) have been developed to detect parasite antigens in filarial sera. Forsyth et al (1985) have developed monoclonal antibody to PC present on *O.gibsoni* egg antigen (McAb Gib 13) and Lal et al (1987) to a circulating glycoprotein antigen of 200 kDa, present in *W.bancrofti* infected sera (McAb CA10). (The PC determinant widespread in nature, is strongly represented in helminth antigens). The sensitivity of PC assays for *W.bancrofti* infection has ranged from excellent to fair (93-85% for sera from microfilaraemic patients)
depending on the population surveyed. This variability is believed to be related to
the variable prevalence of high levels of anti-PC antibodies in different
populations. Studies of PC antigenemia in filariasis have reported conflicting
specificity results. PC assays can not differentiate among filarial species, and some
authors have detected PC activity in sera from patients with non filarial helminth
infections (Lal and Ottesen, 1989).

A monoclonal antibody (AD12) was reported that it binds to a repeated
non-PC determinant of a 200 kDa circulating *W. bancrofti* antigen which is an
excretion products of adult worms (Weil and Liftis, 1987 and Weil et al., 1987).
Field studies performed in India and Egypt indicate that this assay is specific for
*W. bancrofti* infection and more sensitive for active infection than Mf detection.
The sensitivity of the AD12 assay for sera from microfilaremic individuals is
approximately 95% but most sera from amicrofilaremic patients with clinical
filariasis are negative in the assay (Weil and Liftis, 1987). The chemical stability
of the 200 kDa antigen and the fact that blood collected by finger prick at any
time of the day or night can be processed for the assay facilitate its use in large
scale field studies. The assay has also been used successfully to evaluate the
microfilaricidal effect of diethylcarbamazine therapy for bancroftian filariasis (Weil
et al., 1988).

Another promising filarial antigen assay is based on a monoclonal antibody
(ES34) to stage specific *W. bancrofti* Mf excretory products with molecular weights
of 55 to 63 kDa (Reddy et al., 1989). This antibody has been used in several
different assay formats to detect *W. bancrofti* antigen in sera from India and
China. Sensitivity results have ranged from 68 to 96% for sera from microfilaremic
subjects. To date, insufficient data have been published to establish the specificity
of ES34 antigen assays. Poor specificity results were obtained with another
monoclonal antibody based filarial antigen assay Lutsch et al., (1988) suggested
that the results obtained with ES34 should be viewed with caution because of
pending additional experience with these assays.
All filarial antigen assays detect parasite antigen in sera from asymptomatic, amicrofilaremic subjects who reside in endemic areas (endemic normals). Additional studies are needed to determine the significance of parasite antigenemia in endemic normal subjects.

However, it is important to realize the earlier mentioned limitation of antigen detection, especially in bancroftian filarial infections. First antigen detection is not sensitive for diagnosis of clinical filariasis in amicrofilaremic individuals. Species-specific antibody assays are needed for confirmatory testing of individual patients with clinical findings that are suggestive but not diagnostic for lymphatic filariasis. Secondly it should be emphasized that antigen detection is complementary to and not a substitute for Mf detection. Microfilaria prevalence and density data are necessary for understanding the dynamics of filariasis transmission in populations. However since antigen testing detects essentially all Mf carriers, one could consider using a strategy of screening blood collected during the day, for filarial antigen to determine infection prevalence and intensity. Night blood examinations could then be limited to those with positive antigen tests to determine Mf prevalence rates and densities (Weil, 1990).

1.2.8 Antigen characterization

The antigens of the filarial origin are of greatest value in terms of immunodiagnosis, immunopathology and protective immunity. As multicellular organisms, the filarial nematodes contain hundreds of potential antigenic molecules (Lobos and Weiss, 1986). Though many of the antigen extraction techniques are common and which are also applied to filarial parasites, the work has been severely impeded by the lack of animal models for the maintenance of O.volculeulus and W.bancrofti. Secondly all laboratory-derived parasites must be produced in arthropod vector colonies, which also severely limits the material availability (Maizels and Selkirk, 1988). Brugia malayi and B.timori could be reared in Mangolian jirds ( McCall et al, 1973) and hence many studies have been conducted in the rodent systems. Broadly the filarial antigens can be dealt with as
surface antigens, secreted antigens, phosphorylcholines and structural and developmental antigens (Table 1.1).

1.2.8.1 Surface antigens

The surface molecules of the parasite are in contact with the host and hence are in the range of cellular and immune responses and could easily be the targets of lethal immune effector mechanisms. These molecules have been analysed extensively by the surface directed radio-labelling techniques for a range of filarial species and instead of simplicity of the antigenic profiles which were expected, there was an appreciable complexities in the surface protein antigens among the filarial species.

There was a general cross-reactivity of the surface antigen from the different life cycle stages i.e. adult, third stage larvae and the microfilaria of *B. timori* (Maizels et al, 1983). The surface antigens of *B. malayi*, *B. timori* and *B. pahangi* were shown to be closely related in structure. Immunoprecipitation techniques revealed that the antibodies raised in mice against the antigens derived from one stage of species reacted with the surface antigens from other stages and species. The major microfilarial surface antigens of *B. malayi* of molecular weight range 65-70 kDa were recognized by serum antibodies from Mf, infective larvae or adult worm infected animals. The majority of the surface antigens are collagens, which are not exposed on the epicuticular surface (Maizels and Selkirk, 1988 and Selkirk et al, 1986). The prominent protein, which is externalized, is the 29 kDa major surface glycoprotein of *Brugia* and *Wuchereria* adults (Maizels et al, 1989 and Devancy, 1988). It is also quite interesting to note that the prominent surface glycoprotein of other filarial nematodes fall in the small molecular weight range as 20 kDa in onchocerciasis (Philipp et al, 1984 and Taylor et al, 1986) and 35 kDa of the infective larval stage (L3) of *D. immitis* (Philipp and Davis, 1986). The microfilariae of *B. malayi* also have a range of surface associated proteins from 30 kDa to 150 kDa (Maizels et al, 1983 and Egwang and Kazura, 1987). Most of
<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Molecular Mass</th>
<th>Species and Life Cycle Stages</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>15 KDa</td>
<td>Adult Brugia</td>
<td>Non-glycosylated cuticle antigen</td>
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<tr>
<td>2.</td>
<td>17-200</td>
<td>L3 and Adult Brugia</td>
<td>Ladder profile on gel might be epicuticular (?)</td>
</tr>
<tr>
<td>3.</td>
<td>25</td>
<td>B. malayi Mf</td>
<td>Stimulates antimicrofilarial immunity</td>
</tr>
<tr>
<td>4.</td>
<td>29-30</td>
<td>Mammalian stages of lymphatic filariae</td>
<td>Major surfaces labelled glycoprotein: exposed on surface and also secreted</td>
</tr>
<tr>
<td>5.</td>
<td>36</td>
<td>Adult Brugia</td>
<td>Collagen (probably Type IV)</td>
</tr>
<tr>
<td>6.</td>
<td>65-70</td>
<td>B. malayi Mf</td>
<td>Related to maturation and infectivity</td>
</tr>
<tr>
<td>7.</td>
<td>65-75</td>
<td>Mf of Brugia</td>
<td>Major surface antigen</td>
</tr>
<tr>
<td>8.</td>
<td>65-160</td>
<td>Adult Brugia</td>
<td>Cuticular collagens, not exposed</td>
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<td>71-107</td>
<td>L3 and adult Brugia</td>
<td>Four secreted collagenase bands</td>
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<tr>
<td>13.</td>
<td>100</td>
<td>Adult B. malayi</td>
<td>Secreted acetylcholine esterase</td>
</tr>
<tr>
<td>14.</td>
<td>110</td>
<td>B. malayi Mf</td>
<td>Target of antibody mediating clearance</td>
</tr>
<tr>
<td>15.</td>
<td>150</td>
<td>B. malayi Mf</td>
<td>Surface labelled glycoprotein</td>
</tr>
<tr>
<td>16.</td>
<td>200</td>
<td>Adult W. bancrofti</td>
<td>Circulating PC</td>
</tr>
<tr>
<td>17.</td>
<td>200-210</td>
<td>All filariae</td>
<td>Myosin heavy chains</td>
</tr>
</tbody>
</table>

Reproduced from Maizels and Selkirk (1988).
these antigens are water soluble and do not require detergents for their release from the epicuticle of the parasite.

Among the surface proteins the most closely studied one in *B. malayi* is the 29 kDa glycoprotein and a complex of twelve components forming a regular series or 'ladder' between 17 and 200 kDa (Maizels *et al*, 1989). The 29 kDa peptide upon digestion with proteases and endoglycosidases revealed that it was closely conserved between *B. malayi* and *B. pahangi* and has two N-linked oligosaccharide chains each of 1.5 to 2 kDa. When the 17/200 kDa ladder was exposed to trypsin, all the peptides except the 17 kDa peptide had disappeared. (Both the 29 kDa and 17/200 kDa series were recovered as water soluble molecules by homogenization of the parasite (Maizels *et al*, 1989). Apart from these glycoproteins, the cuticle of the parasite has abundance of collagens linked by disulphide bonds and an unidentified additional component which resists the action of SDS and reducing agents (Betschart and Jenkins, 1987).

Studies on the three stages namely infective third stage larvae, adult worm and microfilaria of the filarial parasite of *B. malayi* antigens revealed that different stages showed different polypeptides. The three peptides namely 72 kDa, 30 kDa and 22 kDa were seen only in the infective stage larvae. The antigenic composition of these peptides when elucidated by immunoblot analysis revealed that 72 kDa and the 22 kDa were recognized by the rabbit hyperimmune sera to infective larvae but the antisera for the adult worms did not recognize these antigens. The sera from *W. bancrofti* patients recognized only the 72 kDa peptide and not the other two polypeptides revealing that the 72 kDa is a stage specific but not a genus specific antigen that is immunogenic in the infected host. The 22 kDa also is stage specific as it is not recognized by *W. bancrofti* sera, which might be either species specific antigen or a poorly immunogenic molecule of the parasite (Lai and Ottesen, 1988).
1.2.8.2 Secreted antigens

Apart from the surface antigens, a wide spectrum of molecules are released, secreted or excreted by the nematode parasites which represent major antigenic and functional challenges to the host. Studies were undertaken to characterize and define the potential use of excretory secretory antigens derived in vitro from *O. volvulus* (Schiller *et al*, 1980), *W. bancrofti* microfilariae (Kharat *et al*, 1982) and *B. malayi* adult worms and it was observed that these antigens were far simpler than the total worm extracts. Even in these preparations there seems to be a subset of antigens ranging from 10 kDa to 70 kDa which shared the identity to the surface antigens of the crude extracts (Kaushal *et al*, 1982).

Acetylcholine esterases were secreted by *B. malayi* during in vitro cultivation. A considerable amount of enzyme activity was detected both in the culture and worm extracts of adult and microfilarial stages of the parasite. As a proportion of protein the Mf produced more enzymes i.e.. three fold greater than the adult parasite. Immunoprecipitation techniques revealed the presence of 30, 40 and 200 kDa in Mf and 100 and 200 kDa in adult female worms were of ES origin and the physiological role of these enzymes in infection remains speculative (Rathaur *et al*, 1987).

Monoclonal antibodies raised against *W. bancrofti* ES antigens were studied for their diagnostic utility and one of the monoclonal antibodies against *W. bancrofti* was found to be relatively specific and sensitive in the detection of circulating filarial antigen (Reddy *et al*, 1984).

1.2.8.3 Phosphorylcholine

This represents a class of filarial secreted antigens which carry the phosphorylcholine (PC) determinant and which are heavily glycosylated. The PC epitopes are distributed widely in different nematode species and dominate the humoral antibody response. All nematodes tested till date have very high levels of
somatic PC antigens although some species also secrete PC positive molecules in vitro. The filaria secreted PC deserves further importance because of their potential significance as diagnostic target for general helminthic infections and their presence in the circulation of infected animals and human patients (Dissanayake et al, 1983; Selkirk et al, 1986). Only as a subset of the whole worm PC molecules are detected in the serum of the infected hosts and in each species examined multiple PC bearing antigens have been detected. Thus in Brugia species the dominant PC carrier in the infected serum is a 90 kDa molecular weight component (Maizels et al, 1987; Wenger et al, 1988). Upto 97% of total antifilarial antibody is mostly directed towards the PC specificity (Gualzata et al, 1986).

In B.malayi adults, three antigens namely a 90 kDa protein, 500 to 1000 kDa glycoconjugate which is protease resistant and a macromolecular population from 2 to 12 x 10^6 molecular weight that show partial protease sensitivity bears the PC (Maizels et al, 1987). The 90 kDa and the 500 to 1000 kDa components are also found in the infected jirds serum (Maizels et al, 1987 and Wenger et al, 1988) and in W.bancrofti patients serum a 200 kDa PC antigen is also reported in the sera (Lal et al, 1987). More recently a monoclonal antibody specific for a non specific PC epitope on W.bancrofti circulating antigen was reported by Weil (1987). But in most of these studies additional PC’s were detected and as yet there is no information relating these diverse antigens within filarial or nematode species.

1.2.8.4 Structural and developmental antigens

Most of the antigens produced by filarial nematodes are the structural proteins of the muscle, body wall and cell architecture. Even though the nematode myosin, tubulin and collagen are highly conserved through evolution they are easily detectable by the human sera. Selkirk et al (1989) was able to show the cross reactivity of a number of cuticular collagens with human type IV basement membrane collagen. Two dimensional electrophoresis revealed at least 16 acidic
components with molecular weights ranging from 35 kDa to 160 kDa. These proteins were localized in the basal and in the inner cortical layers of the cuticle which are cross linked by the disulphide bonds. Immunological cross reactivity was demonstrated between a 35 kDa component and human type IV (basement membrane) collagen. Autoreactive antibodies have been detected in individuals with filarial infection but with no correlation with the observed pathology of infection. Paramyosin, a 97 kDa invertebrate muscle protein stimulates cellular and humoral responses in filarial patients (Fuhrman and Piessens, 1985) and Chitin, a polymer of N-acetylglucosamine a non protein specificity absent in vertebrates forms the egg shell of filarial larvae and persists in the latter stages of the nematode life cycle (Maizels and Selkirk, 1988). The cuticular collagens are encoded by a multigene family and are expressed only during the period preceding each moult and different families of collagenous proteins can be seen in the cuticle at different developmental stages (Selkirk et al, 1989). One such developmental stage has been described in B.malayi Mf wherein a set of proteins from 65 to 75 kDa begins to be synthesized after the Mf emerges from the female. Until this antigen is expressed the microfilariae are unable to penetrate the mosquito midgut (Fuhrman et al, 1987).

1.2.8.5 Immune complexes

The role of the immune complexes in the regulation of host responsiveness in filariosis remains unclear. Circulating immune complexes have been shown to provoke immunological effects including the inhibition of T and B lymphocyte functions and interference with the antigen presentation by the macrophages. The immune complexes have been implicated in the initiation of inflammatory response to damaged microfilaria of O.volvulus (Steward,1982). Prasad et al (1983) has used ELISA to detect antigen in the immune complexes obtained from the filarial sera and was able to show that 90% of the clinical filarial sera showed the presence of the antigen in the immune complex. The antigen present in the immune complexes in sera obtained from W.bancrofti infected patients was
similar to that of \textit{S.digitata} antigens (Dissanayake, 1983). The circulating filarial antigens were also detected by using two site immunoassay (Hamilton \textit{et al}, 1984).

In general, with the advent of improved techniques the sensitivity of the assays in the detection of the disease had increased dramatically. However these tests lacked in specificity, the major requirement for immunodiagnosis of the disease. Isolation, characterization and evaluation of the heterologous, homologous or recombinant antigens for the diagnosis of filariasis have been reported constantly and were reviewed earlier. The complexity of the immune response for this disease, necessitates further understanding of the antigens eliciting the response and to design diagnostic tests with greater specificity. In that direction further work was performed to clone the \textit{W.bancrofti} genes and to isolate and evaluate the expressed antigens for the specific diagnosis of bancroftian filariasis.