2.1 FILARIASIS

Lymphatic filariasis (LF) is a debilitating disease affecting approximately 120 million people in tropical and subtropical areas. Of these infections, 90% are caused by *Wuchereria bancrofti* and 10% by *Brugia malayi*. Lymphatic filariasis is endemic in 80 countries with 70% of infected cases concentrated in India, Nigeria, Bangladesh and Indonesia. However WHO has rated filariasis as one of only six “potentially eradicable” infectious diseases with two decades of elimination strategies being practiced (WHO 2001). The main parasitic agent of disease in Africa and India is the filarial nematode, *W.bancrofti*, while in south-east Asia the main agent is *B.malayi*. Progress has been made in mapping the distribution of lymphatic filariasis during the past decade. Globally, 1334 million people live in the 81 countries where the disease is known to be endemic and MDA has been implemented (Figure 2.1).

LF is the second leading cause of chronic disability worldwide due to its stigmatizing and disabling clinical manifestations, including 15 million people with lymphoedema (elephantiasis) and 25 million men with urogenital swelling, principally scrotal hydrocele. The Global Programme to Eliminate Lymphatic Filariasis (GPELF) has been one of the most rapidly expanding global health programmes in the history of public health. GPELF was launched in 2000 with the goal to eliminate LF (Fig 2.2) as a public health
problem by 2020. GPELF aims to (i) interrupt transmission using combinations of 2 medicines delivered to entire populations at risk - MDA, and (ii) manage morbidity and prevent disability. MDA aimed at eliminating LF, spearheaded by WHO’s Global Programme to Eliminate Lymphatic Filariasis continues to achieve unparalleled success since its official inauguration in 2000. MDA is recognized as the main strategy that will enable GPELF to achieve elimination by 2020.

Figure 2.1 Distribution and status of lymphatic filariasis worldwide, 2010

Figure 2.2 Steps taken by GPELF to interrupt transmission
The highest increase in the number of people treated was in the South-East Asia Region, where slightly more than 414 million were treated, compared with just over 380 million in 2010. Almost 4 billion doses of medicines for the treatment of LF have been delivered for the period 2000 – 2011 to a cumulative targeted population of 952 million people. A total of 73 countries are endemic for LF. Official estimates published by WHO in 2004 indicated that 1.34 billion people were at risk of infection in 83 countries. Of these 83 countries, two- China and Republic of Korea have been officially recognized as having eliminated LF as a public health problem, making the current total 81 endemic countries.

![Figure 2.3 Global distribution of lymphatic filariasis and status of MDA, 2009](image)

The results for 10 countries indicated that the transmission was at extremely low levels, indicating MDA not necessary. Figure 2.3 shows the geographical distribution of the disease as well as the status of programme implementation.
2.1.1 Filarial Parasite Biology

Lymphatic filariasis is mosquito-borne. The infective stage larvae (L3) enter the host following a mosquito bite and migrate through the subcutaneous tissue to the nearest lymphatic vessel (Fig 2.4). They lie in the afferent vessel of the lymph node and undergo maturation by moulting first to fourth stage larvae at around 7 days post-infection (p.i.) and then moulting to adulthood at around 30 days p.i. within the lymphatics, the adult male and female worms mate and begin to produce millions of first stage-larvae or microfilariae (Mf) by 60 days p.i (Scott & Nutman 2000). The Mf passes out of the lymphatic system and into the circulatory blood system, from which a tiny minority are taken up in the blood meal of feeding mosquitoes. Further maturation in the mosquito occurs when Mf leave the mid-gut and migrate into the flight muscles where they molt to form L3. The mature L3 then move from the flight muscles to the proboscis of the mosquito to be ready to invade a host when the mosquito next bites. Although this life-cycle superficially appears precarious (as male and female nematodes must find one another in the same lymphatic vessel and super-infection of the mosquito would clearly lead to lack of transmission), the extraordinary life-span of adult filarial nematodes within the mammalian host (estimated to be 8–16 years), combined with an estimated microfilarial life-span of up to 300 days, ensure the spread of disease (Fig 2.5).
Figure 2.4 Transmission of Filarial Parasite from Mosquito to Humans

a) *W. bancrofti* adult worms
b) *B. malayi* adult worms

Figure 2.5 Adult and microfilariae of *W. bancrofti* and *B. malayi*
During subsequent blood meal, mosquito ingests microfilariae from the blood. The mf undergoes differentiation in oesophagus, intestine and rectum in the thoracic flight muscle of the mosquito. Further, it undergoes molting to form L₁ and L₂ stages between 6-10 days, the infective form (L₃) of the parasite is developed from L₂ between 11 and 13 days. The L₃ larvae migrate from flight muscles to the proboscis of the mosquito that will aid in the transmission of the L₃ to the human host during feeding of the mosquito.

2.1.2 Morphology

A) Adult worms

The adult worms of *W. bancrofti* are long thread like, transparent and creamy white in color with a smooth cuticle (Fig 2.5a). They have a filiform shape with tapering ends. The male worm measures about 25-40 mm in length and 0.1mm in diameter. Its caudal extremity is curved ventrally and their copulatory spicules are unequal and dissimilar. The female adult worm varies in length from 80-100 mm in length and 0.24-1/3mm in thickness. The female vulva opens at the cervical positions (Craig et al 1970).

The *B. malayi* adult males vary in length between 13.5-23.3mm and in breadth from 0.07-0.08 mm in thickness (Fig 2.5b). The caudal part of the male is coiled and at the tip of the tail, there are 4 to 6 small papillae. The adult female worms vary in length from 43.5-55mm and in breadth from 0.13-0.17mm. The female vulva is survical in position and consists of transverse slit (Craig et al 1970).

B) Microfilariae

The *W. bancrofti* microfilariae appear as colorless and transparent bodies with blunt heads and rather tails. When stained, the embryo shows a hyaline sheath much larger than the larval body, representing the chorionic
envelope of the egg, somatic cells or nuclei appearing as granules in the central axis of the body, nerve ring, anterior V spot and posterior V spot.

(i) Hyaline sheath: This is a structureless sac, which is best seen where it projects slightly beyond the extremities of the embryo. The sheath is much longer (359µ) than the larval body so that the larva can move forward and backward with it. The shell represents the chorionic envelope of the eggs and it remains as a membrane surrounding the larvae.

(ii) Somatic cells or nuclei: These appear as granules in the central axes of the body and extend from the head to the tail end. The granules do not extend up to the tip of the tail and serve as a distinguishing feature of Mf of *W.bancrofti*. At the anterior end, there is space also devoid of granules, called the cephalic space which is as long as it is broad. The granules are broken at definite places serving as the landmark for the identification of the species which include the following.

- Nerve ring, and oblique space.
- Anterior ‘V’ spot that represents the rudimentary excretory system.
- Posterior ‘V’ spot or tail spot that represents the terminal part of the alimentary canal.
- G-cells: A few G-cells (The so called “genital cells”) are present posteriorly. While G-cells 2, 3 and 4 are just infront of the anal pore, G-cell 1 is situated further in the front.
- Innen Korper of Fulleborn: Also called as central (Internal) body of Manson that extends from the anterior V spot to the G-cell. It represents the rudimentary alimentary canal. The cuticle, which is seen only with the vital stains, has numerous fine transverse striae.
In *B. malayi* Mf, the nuclei are oval, irregularly spaced, overlapping and difficult to distinguish. It has very slender tail containing two small caudal nuclei and a single row of nuclei up to the tail’s end.

The larval forms do not undergo any further development in the human body unless they are taken up by their appropriate intermediate host (mosquito). If the mosquito does not suck up these microfilariae, they die in course of time. The life span of Mf in the human body has been found to be as long as 70 days.

**C) Infective Larvae (L₃)**

The mature larva has a well developed three caudal papillae that are sub equal in size and bubble life. The ‘Anal ratio’, as defined by Wharton (1957) as the distance from the anus to the caudal extremity, divided by the breadth of the larvae half way from the anus to the caudal extremity, is about 4.5 in *W. bancrofti* (Fig 2.6a). *B. malayi* L₃ however have three ill defined caudal papillae of which the central papilla is most prominent while the two ventro-lateral ones are less conspicuous. The anal ratio is about 4 in *B. malayi* (Fig 2.6b).

![Figure 2.6 Infective larvae of *W. bancrofti* and *B. malayi*](image)

a) *W. bancrofti* infective larvae          b) *B. malayi* infective larvae

**Figure 2.6** Infective larvae of *W. bancrofti* and *B. malayi*
2.1.3 Periodicity

In most parts of the world, including India, lymphatic filarial parasite is of nocturnal periodic form. During daytime, microfilariae are present in very low numbers in the peripheral blood and are virtually undetectable. They live mainly in the pulmonary capillaries, from where a proportion of them escape into the peripheral blood where they may be detected during the night hours of periodicity.

The nocturnally sub periodic and diurnally sub-periodic strains found mainly in the pacific islands show mf all hours, but their density increase slightly during the night or day respectively. The basis of filarial periodicity remains largely unknown; it may be altered by change in habits of the host, if the waking and sleeping hours are transposed. It is likely that microfilarial periodicity and the biting habits of the local vectors have influenced the development of the geographically distinct forms of the disease.

2.2 FILARIASIS – SPECTRAL DISEASE

Lymphatic filariasis is characterized by a wide spectrum of clinical manifestations in individuals from endemic region with signs and symptoms often varying from one endemic area to another (Ottesen & Froysaker 1982, Ottesen et al 1992). They result from the outcome of complex interactions of several underlying factors and processes related to the human host, parasites and environment. Based on the immune response spectrum filariasis can be divided into five infection group categories.

2.2.1 Endemic Normal Individuals

These individuals reside in endemic area, being exposed to infection still not harboring Mf, remain free of infection suggesting evidence that they
may be immune to invading L3 (Maizels & Lawrence 1991). The protective immunity exhibited by EN reflects the existence of anti larval immunity—presumably mediated by a phenomenon of antibody dependent cell mediated cytotoxicity (ADCC) involving macrophages and eosinophils (Rajan et al 2002).

### 2.2.2 Microfilaraemic Individuals

Individuals belonging to this group harbor microfilariae but remain asymptomatic. However this state tends to serve as reservoir of infection carrying the microfilariae in continuing the transmission. Although they may be clinically asymptomatic, virtually all individuals with *W. bancrofti* or *B. malayi* microfilaraemia have some degree of subclinical disease that includes microscopic haematuria and/or proteinuria dilated (and tortuous) lymphatics when imaged and, in men with *W. bancrofti* infection, scrotal lymphangietasia (by ultrasound). The characteristic feature of this clinical population is filarial antigen specific hyporesponsiveness (Mahanty et al 1997, Ravichandran et al 1997). 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 & Nutman 1992).

### 2.2.3 Acute Manifestation

During acute infection, antigen specific T cell response is stimulated and cells proliferate in response to parasite antigen. With increasing exposure of the immune system to parasite antigens that are released from metabolically active worms, the immune system becomes increasingly hyporesponsive, first to specific parasite antigens and subsequently, when high worm burden occurs, to bystander antigen (Maizels & Yazdanbakhsh 2003). The most common manifestation of acute filariasis is Adeno-lymphadenitis (ADL), 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 males there may be orchitis, epididymitis and acute transient hydroceles (Wartman 1947, Nanduri & Kazura 1989, Roberts et al 1996).

2.2.4 Chronic Disease

Individuals with chronic disease are amicrofilaraemic, with the onset of persistent limb lymphodema or hydroceles after an acute attack (Evans et al 1993). The lymphatic dilation, co-channeling and rapid lymph flow seen in asymptomatic microfilaraemic is replaced by tortuosity, dermal back-flow, obstruction, stasis and poor regional node visualisation (Witte et al 1993) The first evidence of impending elephantiasis is persisting lymphodema. Fissure and ulcer formation allows access of bacteria and fungi, which have been shown to play an important role in the pathogenesis of elephantiasis. The switch from asymptomatic filariasis to chronic pathology is also accompanied by profound changes in cellular and humoral immunity.

2.2.5 Tropical Pulmonary Eosinophilia (TPE)

It is one of the least common manifestations of filariasis with immunological spectrum different from asymptomatic microfilariae. Circulating microfilariae are not usually found (Van der Sar & Hartz 1945, Ottesen et al 1982) but adult worms may be detected by ultrasound (Dreyer et al 1996). Clinically TPE is characterized by nocturnal coughing and asthma. In TPE there is a severe hypersensitivity response with marked eosinophilia, extreme levels of serum IgE and high titres of antifilarial IgG and IgE.
2.3 HOST SYSTEM

*W. bancrofti* has a very specific host system. Human beings serve as the definitive host and mosquitoes as their intermediate hosts. There are no known reservoirs for *W. bancrofti* whereas *B. malayi* has been found in macaques, leaf monkeys, cats and civet cats.

2.3.1 Animal Models for Filariasis

Immunisation with various parasites and parasite material has been observed to induce a good level of protection in animal models. However, no commercial trials have been successful due to difficulties with reproducibility, pathological manifestations, insufficient protection, and the lack of correlation between human immunity and that in animal models. The non-availability of animal models for *W. bancrofti* and *O. volvulus* as well as the limited development of the Brugian parasite in mice has severely hampered the immunological investigations using animal models.

Murine models allow limited development of parasite and are being used to study acquired resistance to L3. The only reported model for *W. bancrofti* is the infection in silver leaf monkeys which serves as a tool for immunological studies. *B. malayi* can be maintained in small animals like rodents and the susceptibility of infection *B. malayi* has been reported to vary in different laboratory hosts ranging from fully susceptible gerbils (*Meriones unguiculatus*) to semi-permissible mice. However, among the currently available animal models, gerbil or jird is a permissive host for *B. malayi* and is a well established animal model for *B. malayi* infection.
2.3.2 Mouse as a Model

Semi permissive host such as Balb/c mice have been proposed as suitable models to assess immunity against developing larvae of *B. malayi*. The problem inherent in these systems is the progressive attrition of parasites in naïve mice so that in general 100% of the inoculated parasites are killed by day 30- post- infection. This leaves one with a very short period to assess vaccine efficacies, often with a high standard deviation. However, immunological parameters like antibody isotyping, splenocyte proliferation studies, cytokine analysis at the message level by RT-PCR and at the protein level by ELISA is quite possible in mice models, by the use of commercially designed reagents and kits.

So a certain level of immunological characterisation of the antigens can be achieved using this model.

2.4 IMMUNE RESPONSE TO FILARIASIS

Human lymphatic filariasis is a complex disease where many of the infected subjects do not display overt clinical manifestation while a majority of patients with acute and/or chronic disease are largely free of demonstrable infection (Kumaraswami 2000). Filariasis being a spectral disease affects different patient populations, with different set of immune response elicited against each stage (Fig 2.7). Broadly, the immune response can be classified into: antibody response, cellular response.
2.4.1 Antibody Response

The endemic normal individuals produce antibodies against L3 antigens. Some authors have claimed endemic normal as “putatively immune” to filarial worms and many studies are being carried out to analyse the immune response in this group in filariasis and onchocerciasis (Nutman et al 1991, Steel et al 1996). In humans IgG4 isotype constitute only 5% of total antibody, however, in filarial patients IgG4 can be as much as 95% of the blood immunoglobulin. Mf+ individuals have active microfilaraemic infection and extremely elevated levels of filarial-specific immunoglobulin of the IgG4 isotype (Kurniawan et al 1993). Type 2 cytokine IL-4 is responsible for production of IgG4 and IgE. The raised levels of IgG4 in MF carriers have been exploited as a diagnostic marker in detecting active infection (Atmadja et al 1995). Active microfilaraemic infection is also associated with lower levels of IgG1, IgG2 and IgG3 than in other patient categories (Kurniawan-
Atmadja et al 1998). IgE levels, which are also up-regulated by IL-4, are particularly raised in patients with chronic pathology. However, while IgE levels are generally raised in Mf+ individuals, these people have a higher IgG4: IgE ratio than those with chronic pathology (Kurniawan et al 1993). IgG4 and IgE recognize similar epitopes of filarial antigens which suggest that IgG4 may block sites that if recognized by host IgE lead to pathology, nematode killing or both. Elevated levels of IgE was observed in Endemic normal control group, asymptomatic microfilaraemic or chronic patient which was high or higher than those reported for ragweed or grass allergic individuals, yet none of these filariasis patients exhibited any symptoms of allergy (Gleich et al 1977), yet the only group of patients with allergic manifestations like nocturnal asthma was that group with the tropical eosinophilia syndrome. Chronic patients have enhanced IgE antibody responses to certain L3 antigens compared with MF carriers. Patients with lymphoedema tend to have Th1like cytokine responses to filarial antigens in vitro and high levels of antifilarial IgE antibodies. Microfilaria carriers tend to have Th2 like responses to filarial antigens and their antibody responses are dominated by the IgG4 isotype. These results were consistent with the notions that IgE antibodies are involved in pathogenesis and protective immunity in filariasis and that IgG4 antibodies interfere in some way with immunity and immunopathogenesis (Kurniawan et al 1993, Mahanty et al 1994, Atmadja et al 1995).

2.4.2 Cellular Response

Filarial parasites induce significant immune responses to a myriad of parasite antigens, but successfully survive and procreate in mammalian hosts for several years without being recognized by the immune system. Many animals, from rodents to primates, that harbor adult parasites, display immunological hyporesposiveness characterized by: (1) intense down
regulation of filarial-specific T-cell proliferation; (2) decreased production of T helper cell type (Th) 1 cytokines such as IFN-γ; and (3) enhanced release of anti-inflammatory cytokines such as IL-10. In filariasis, T-cell responses of microfilaremic individuals are reported to be Th2-like (Ottesen 1992, King et al 1993), whereas those of patients with chronic lymphatic dysfunction are described as being Th1-like (Dimock et al 1994, Maizels et al 1995). Filarial-specific T-cell responses are characterized by rapid induction of anti-inflammatory cytokines such as IL-4 and IL-10 within a few days of larval inoculation (Osborne & Devaney 1999). Antigen-presenting cells and IL-4/IL-10 actively suppress induction of Th1 cytokines and filarial specific T-cell proliferation in infected mice. Lymphocytes from patients with asymptomatic microfilaremia (associated with either Brugia malayi or Wuchereria bancrofti infections) fail to respond to filarial antigens in cellular proliferation assays though they retain their reactivity to nonparasite antigens. Serum suppressor factors (Piessens et al 1980), suppressor adherent cells (assumed to be monocytes), and T-lymphocyte suppressor cells (Piessens et al 1982) each have been implicated as mediators of this parasitic-specific immunosuppression. The presence of microfilariae can lead to inhibition of splenic antigen-specific lymphocyte responses ex vivo (O'Connor et al 2000) and in addition, microfilaria-derived molecules can able to inhibit lymphocyte proliferative responses to concanavalin A (Wadee et al 1987). Reduced parasite specific lymphocyte precursor frequency was reported in microfilaraemics, which did not extend to nonparasite antigens or mitogens (King et al 1992). Individuals with lymphaedema and elephantiasis show increased proliferation, correlated with absence of antigen rather than presence of the disease (Addiss et al 1995). Individuals free of current active infection display a Th1 biased response, moved from a state of infection to become free of infection, as a consequence of (1) induction of Th1 cytokines or (2) elimination of parasites, which results in the stimulation of Th1-dominant responses. Patients with clinical filariasis and 'endemic normals’
display a Th1 skewed filarial specific immune response – a susceptible state of Th cell activity for growth and development of filarial larvae.

2.5 DIAGNOSIS

Traditionally diagnosis of lymphatic filariasis was performed by detection of microfilaria in blood, which would be collected around midnights in areas were microfilariae exhibit nocturnal periodicity and during midday were periodicity is diurnal. Giemsa staining of thick blood smear a simplest method to detect microfilariae has been an age old practice (Khamboonruang et al 1987, Schultz 1988, Schuurkamp et al 1990, Sabry 1992). The disadvantage of thick films was that they underestimate the prevalence of microfilaraemia if microfilariae densities were low because the theoretical detection limit for such procedures are between 15 and 60 microfilariae per ml (Panicker et al 1991, Turner et al 1993, Faris et al 1998). Another problem is loss of microfilariae from the film during processing especially if anti coagulated blood is used. Knott’s method of a concentrating technique increases sensitivity, which works by lysing red blood cells using 1% formalin and the deposit, was examined for microfilariae (Knott 1935). Formalin precipitates the protein making the observation of microfilariae difficult, which was overcome by the use of mild detergent Triton X-100 which dissolves most of the protein deposit and enhances the visibility of the microfilariae (Melrose et al 2000). Membrane filter technique a widely used concentration method uses polycarbonate filters to trap the microfilariae later counted (Nathan et al 1982, Moulia-Pelat et al 1992).

The modern techniques enable the detection of filarial infection by the presence of circulating filarial antigen, which has replaced the cumbersome process of night blood collection for the identification of microfilariae. Currently there are two commercially available tests, one in an enzyme linked immunosorbent assay format and other a rapid format
immune-chromatographic card test. Trop Bio Og4C3 Antigen Test-monoconal antibody is raised against *Onchocerca gibsoni* antigen and shows very strong specificity for *W. bancrofti* antigen. Since it detects antigen from both adult worms and microfilariae the Og4C3 assay will detect amicrofilaraemic and microfilaraemic infections (More & Copeman 1990, Turner et al 1993) and is a very good marker of active filarial infection with adult worms (Chanteau et al 1994).

Filarial-specific enzymes have been characterised and show diagnostic potential either as antigens for antibody assays or by the detection of the enzyme itself. Filarial acetylcholinesterase has been identified in the serum of infected people (Misra et al 1993) and in in-vitro culture fluid (Rathaur et al 1987) and has inter-species cross-reactivity (Sharma et al 1998).

Anti-filarial IgG4 antibody is produced in abundance during filarial infections and unlike broad-spectrum IgG antibody shows little cross-reaction with non-filaroid helminthes. Antifilarial IgG antibody reacts with a wide range of other helminths and protozoa due to shared phosphocholine antigens. Because humans are not able to synthesise anti-phosphocholine or anti-carbohydrate IgG4 (Maizels et al 1987, Scott et al 1987, Lal et al 1991) the assay of filarial IgG4 antibodies greatly increases specificity and enhances the diagnostic ability of the test (Lal & Ottesen 1988, Rahmah et al 1994, Chanteau et al 1995, Haarbrink et al 1995, Terhell et al 1996) and Mahanty *et al.,* (1994) have shown that antifilarial IgG4 is a good index of the intensity and duration of filarial exposure in endemic populations, and (Maizels et al 1995) found that the level of IgG4 antibody correlates with microfilariae counts.

In this regard, the rWbSXP-1 antigen based immunoassay was converted to a more specific, simple and rapid field applicable format,
qualitative flow through immuno filtration test (3 min) for diagnosis and surveillance of filarial elimination programme by supervisor (Dr. Kaliraj) group in our laboratory. The evaluation studies of the test kit at different centers in India had demonstrated the high sensitivity for Bancroftian (91.4%) and for Brugian (90.8%) microfilaremic cases. The development of immunodiagnostic method using rWbSXP-1 was carried out with a MoU with M/s SPAN diagnostics Ltd., Surat and the technological knowhow was transferred in 2007. Further, the Wb-SXP peptide based ELISA was also shown to detect mf specific antibody reactivity (Pandiaraja et al 2010).

PCR methods have been successfully used for the detection of W. bancrofti DNA in blood, plasma, paraffin embedded tissue sections (McCarthy et al 1996) and sputum (Abbasi et al 1996, Abbasi et al 1999), and B. malayi DNA in blood (Lizotte et al 1994, Rahmah et al 1998) and urine (Lucena et al 1998). It was demonstrated that PCR technique of detection of Wuchereria bancrofti was as sensitive as the filtration technique with the ability to detect even 1 microfilaria in the blood collected for diagnosis (Dissanayake et al 2000).

2.6 CHEMOTHERAPY OF LYMPHATIC FILARIASIS

Treatment of lymphatic filarial infection can be approached in two ways: (1) treatment of an individual patient to relieve or prevent symptoms, and (2) treatment of an entire population in an endemic area to decrease transmission. In selective chemotherapy, the lost infections are balanced by new infections to produce a dynamic equilibrium and there needs to be continual reassessment of the filarial status of the community to identify the newly infected. Mass chemotherapy regardless of parasite status excludes the problems of selective therapy. Mass treatment aims to treat all members of the endemic community at the same time and will therefore treat pre-patent as well as patent infections.
Antimony and arsenic based drugs and naphthalene sulfoic acid (Suramin) were used to treat filariasis but with limited success (James & Gilles 1985, Campbell & Rew 1986). Suramin has been shown to be moderately active against adult *W. bancrofti* and *Brugia phahangi* (Howells et al 1983) but it has no effect on microfilariae and will therefore not interrupt the transmission cycle in the short term. For the last 50 years the piperazine derivative diethylcarbamizine (DEC, proprietary names: Hetrazan, Banocide and Notezine) has been, and still is, the most widely used drug for the treatment of lymphatic filariasis (James & Gilles 1985, MacKenzie & Kron 1985). Diethylcarbamazine is given orally and is rapidly absorbed reaching peak blood levels one to hours after ingestion. DEC is a highly effective microfilaricidal drug for *W. bancrofti*. DEC treatment and residual spraying in China, eliminated Bancroftian filariasis and subsequent surveillance showed no signs of resurgence (Liu et al 1992).

Ivermectin has been used very successfully for the treatment of oncocerciasis for a number of years (Goa et al 1991, Townson et al 1994) and a single annual dose of 400 µg/kg, either alone or in combination with Diethylcarbamazine, has proved to be very effective producing long-term suppression of microfilaraemia in Bancroftian lymphatic filariasis in a number of countries and is equally effective against brugian filariasis (Shenoy et al 1993). Albendazole is also effective in conjunction with either ivermectin or DEC in LF control.

2.7 HISTORY OF VACCINE AND VACCINATION

The word “vaccine” comes from the Latin word *vaccinus*, which means “pertaining to cows.” The history of the vaccine against smallpox, a human disease with no known animal reservoir, can be summed up as the replacement of inoculation with human smallpox (variolation) with inoculation with cowpox, a procedure invented by an English doctor, Edward
Jenner (1749-1823). The first human vaccines against viruses were based using weaker or attenuated viruses to generate immunity. The smallpox vaccine (Table 2.1) used cowpox, a poxvirus that was similar enough to smallpox to protect against it but usually didn’t serious the illness. Rabies was the first virus attenuated in a lab to create a vaccine for humans.

Table 2.1 Viral vaccines

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<th>S.no</th>
<th>Type</th>
<th>Established</th>
<th>Experimental</th>
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<tr>
<td>1</td>
<td>Live attenuated</td>
<td>Vaccinia</td>
<td>Cytomegalovirus</td>
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<td></td>
<td></td>
<td>Measles</td>
<td>Hepatitis A</td>
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<td>Rubella</td>
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<td>Polio (Sabin)</td>
<td>Dengue</td>
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<td>Varicella Zoster</td>
<td>Rota</td>
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<td>Adeno</td>
<td>Para influenza</td>
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<td></td>
<td></td>
<td>Yellow fever</td>
<td>Japanese encephalitis</td>
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<tr>
<td>2</td>
<td>Inactivated</td>
<td>Polio (Salk)</td>
<td>HIV</td>
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<td>Influenza</td>
<td>Influenza</td>
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<tr>
<td>3</td>
<td>Subunit</td>
<td>Hepatitis B, Influenza</td>
<td>HIV</td>
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<td>Cytomegalovirus</td>
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The next seminal development in vaccines came through the research of Louis Pasteur who had developed the germ theory of disease. A culture of chicken cholera bacillus, which had accidentally been left on a bench during the warm summer months, lost much of its ability to cause disease; nonetheless, birds which had been inoculated with this old culture were resistant to fresh virulent cultures of the bacillus. This attenuation of virulent organisms was reproduced by Pasteur for anthrax and rabies using abnormal culture and passage conditions. Recognizing the relevance of Jenner's research for his own experiments, Pasteur called his treatment vaccination, a term which has stood the test of time.
In 1882, Robert Koch (1843-1910) described the tubercle bacillus responsible for tuberculosis in humans. In 1897, Albert Calmette and Camille Guérin, a veterinary pupil of Nocard, began working together. A bovine bacillus, isolated by Nocard in a sample taken from the udder of a tuberculous cow, was cultured by passages through glycerinated bile potato medium, eventually resulting in an attenuated form. The tubercular bacillus has a fatty capsule which makes it difficult to blend. The idea of using bovine bile in the culture medium most likely came from the veterinarian Vallée, who had used delipidated bacilli in his vaccination trials: at that time, ideas were readily passed from team to team. The bacillus, from 1908 to 1921, was subsequently transformed by serial passages (230 passages) without regaining virulence in susceptible animals. The vaccine was called ‘BCG’ (which stands for ‘vaccin bilié de Calmette et Guérin’).

Vaccines are crucial to maintaining public health: They are a safe, cost-effective, and efficient way to prevent sickness and death from infectious diseases. Vaccines have led to some of the greatest public health triumphs ever, including the eradication of naturally occurring smallpox from the globe. Vaccines have saved countless lives, lowered the global incidence of polio by 99 percent and reduced illness, disability and death from diphtheria, tetanus, whooping cough, measles, *Haemophilus influenzae* type b disease, and epidemic meningococcal A meningitis (Table 2.2).
Table 2.2    Bacterial vaccines

<table>
<thead>
<tr>
<th>S.No</th>
<th>Type</th>
<th>Established</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Live attenuated</td>
<td>Mycobacterium tuberculosis (BCG)</td>
<td>Vibrio cholera, Salmonella typhi (Ty21a: Vi⁻ mutant) S.typhi (aro A: aromatic pathway mutant)</td>
</tr>
<tr>
<td>2</td>
<td>Inactivated</td>
<td>V.cholerae, B.pertusis, S.typhi</td>
<td>V.cholera + toxin B subunit M.lepraex</td>
</tr>
<tr>
<td>3</td>
<td>Subunit</td>
<td>Haemophilus influenzae, Neisseria meningitidis, Streptococcus pneumoniae</td>
<td>S.typhi (capsular Vi⁻ carrier) H.influenzae (dip/tet toxoids) M.tuberculosis (naked DNA)</td>
</tr>
<tr>
<td>4</td>
<td>Toxoid</td>
<td>Tetanus, dipheria</td>
<td></td>
</tr>
</tbody>
</table>

In recent years, a number of research groups around the world have begun to focus on creating vaccines against some of the most serious and deadly fungal infections (Table 2.3). There are a number of technical and economic barriers remain to be overcome before the first such fungal vaccine is available for use in humans. Candidiasis, an infection caused by one of several species of the yeast Candida, is now the fourth most common bloodstream infection in hospitalized patients both in the United States and in many European countries. And the death rate from such Candida infections remains about 30 to 40 percent, even after treatment with antifungal therapy. The recent vaccine approaches have shown success in animal models, which include conjugating a fungal surface protein or a polysaccharide to a non-fungal protein. Antonio Cassone, at the Istituto Superiore di Sanità in Rome, developed laminarin vaccine which was not only protective against candida infection in mice but also against Aspergillus and Cryptococcus (Cassone & Cauda 2012).
Table 2.3  Fungal vaccines

<table>
<thead>
<tr>
<th>S.No</th>
<th>Disease</th>
<th>Established</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Candidiasis</td>
<td>β-mannan-peptide or protein conjugates</td>
<td>Recombinant Als 3* proteins.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HyR1</td>
<td>Recombinant Sap2*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laminarin – CRM197 conjugate</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Cryptococcosis</td>
<td>Laminarin-CRM197 conjugate GXM conjugate peptide mimotopes</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Aspergillosis</td>
<td>Laminarin – CRM197 conjugate AspF antigens Cell wall glucanase Crf1</td>
<td></td>
</tr>
</tbody>
</table>

Parasite vaccine development (Table 2.4) is highly dependent on the use of animal models to define the immune response which may mediate protection and to evaluate the protective efficacy of candidate antigens prior to clinical trials. A single genus of parasite, Plasmodium, is responsible for the third most common cause of human mortality globally; malaria (Gardiner et al 2005). Other similarly devastating parasitic diseases of humans include cysticercosis (including neurocysticercosis), toxoplasmosis, schistosomiasis and leishmaniasis, which cause significant morbidity and mortality. Malaria is the tropical parasite disease that claims a death toll of over 1.1 million deaths. The PfSPZ Vaccine, developed by scientists at Sanaria Inc., is composed of a live but weakened version of *Plasmodium falciparum*. This candidate malaria vaccine is safe and protects against infection in adults, according to the results of an early-stage clinical trial (Epstein et al 2011). Other similarly devastating parasitic diseases of humans include cysticercosis (including neurocysticercosis), toxoplasmosis, schistosomiasis and leishmaniasis, filariasis, which cause significant morbidity and mortality.
2.7.1 Filarial Vaccine Development Studies

Though antifilarial drugs are available several rounds of mass treatment are necessary to reduce the levels of infection below those necessary to sustain transmission (Fan 1992). Additional preventive measures such as vector control and vaccine development are crucial to control the infection in endemic regions. Certain population of individuals residing in the endemic area tend to be refractory to the infection and these individuals carry high levels of antibodies against the parasite antigens which are believed to be protective (Freedman et al 1989). Antigens of the infective third stage larvae of filarial parasites are of special interest since they represent the first larval stage that enters into the human host which can be ideal targets as vaccine candidate.

There is no established and convenient animal model for the major lymphatic filarial parasite W.bancrofti. Attempts to obtain full development of W. bancrofti even in congenitally athymic (nude) mice and immune suppressed gerbils were not successful (Philipp et al 1984). Palmieri et al, (1982) reported that W. bancrofti reaches reproductive immunity in primates

<table>
<thead>
<tr>
<th>S.No</th>
<th>Vaccine type</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Non attenuated</td>
<td>Eimeria spp Theileria parva</td>
</tr>
<tr>
<td>2</td>
<td>Attenuated</td>
<td>Toxoplasma gondii Babesia bovis and B. bigemina Theileria annulata</td>
</tr>
<tr>
<td>3</td>
<td>Sub-unit vaccine</td>
<td>Leishmania infantum</td>
</tr>
<tr>
<td>4</td>
<td>Recombinant antigen</td>
<td>Taenia ovis Boophilus microplus Schistosomiasis japonica</td>
</tr>
</tbody>
</table>
i.e. leaf monkeys. As such modeling of lymphatic filariasis is complicated by the diversity of infection in natural host, human. To identify the protective immunogens and study the mechanism involved surrogate host with varying permissiveness like those of fully permissive (e.g. Jirds, cats, nude mice for Brugia), semi permissive (BALB/c mice) and non-permissive have been explored.

In early experiments, irradiated L3 in L. sigmodontis BALB/c mice model was immunized and contributed 70% reduction in worm burden 2 weeks post immunization (Le Goff et al 1997, Martin et al 2001). Similar kind of experiment carried out with irradiated L3 worms, the duration of immunization was prolonged for 5 months and parasite challenge study showed 54-58% protection 10 days p.i. Thus, long-term vaccination protocols generated a strong memory response that led to significant but incomplete protection that was limited to the infective larval stage suggesting alternative vaccination strategies are needed (Babayan et al 2006). BmA2 a 120Kda protein from adult worm induced protection against Mf and L3 larvae in in vitro ADCC and offered 88% reduction in development of parasites to adult worms (Chenthamarakshan et al 1995).

Recombinant proteins such as ALT-2 showed over 73% protection against a challenge infection in the jird model and over 64% protection in the mouse model (Gnanasekar et al 2004). Many other recombinant proteins studied, like Chitinase and SXP, have shown partial protection (Wang S et al 1999b). Veerapathran et al, (2009) have shown rWbGST as a potential vaccine candidate against lymphatic filariasis which offered 61% protection against a B.malayi challenge infection in jird model. Multivalent fusion protein vaccine using three well characterized antigens of Brugia malayi, heat shock protein 12.6 (HSP12.6), Abundant Larval transcript-2 (ALT-2) and tetraspanin large extra cellular loop (TSP-LEL) elicited both Th1 and Th2
mixed type immune response. Vaccination trials in mice showed >95% protection with rBmHAT (Dakshinamoorthy et al 2013).

2.7.2 Peptide Vaccine

Peptide vaccines have been extensively studied for various parasitic diseases. The only antimalarial vaccine subjected to large scale clinical trials is a synthetic peptide based vaccine, spf66 (Amador et al 1992). The S3Pvac synthetic vaccine composed of three peptides (GK1, KETc1 and KETc12) was reported to effectively protect against pig cysticercosis (de Aluja et al 2005). Peptides containing protective epitopes of malarial antigens have shown more promise than the whole recombinant antigenic proteins (Chatterjee et al 1995). Glutamate-Rich Protein (GLURP) is a blood stage antigen and is present on the parasitopherous membrane of mature schizonts. The Statens Serum Institute and EMVI have developed a 128- amino acid synthetic peptide vaccine using the partial sequence from GLURP. The vaccine contains T cell epitopes as well as conserved antibody-dependent cytotoxic inhibitory epitopes. A Phase 1 trial in normal healthy malaria-naïve volunteers has been recently concluded using vaccine formulated with alum and Montanide ISA720 (Ballou et al 2004).

Epitope-based vaccines containing well-characterized immunogenic regions have been extensively shown to be promising in various diseases capable of inducing protective immunity (Srinivasan et al 2004). A number of such vaccines are currently under clinical trials against viruses (El Kasmi & Muller 2001), bacteria (Sabhnani et al 2003) and cancer (Kieber-Emmons et al 1999). These epitopes are also important for allergy research and in determining cross-reactivity of IgE-type epitopes of allergens (Selo et al 1999).
Madhumathi et al, (2010b) has shown that the TRX peptides give 75.14% protection when compared to the 63.05% of whole protein TRX, establishing the success of peptide vaccines in lymphatic filariasis. Combination of carefully selected immunodominant epitope peptides from vaccine candidates could be used to develop multi-peptide vaccine constructs which would further enhance the vaccine efficacy. The T epitopes identified by Madhumathi et al, (2010c) in ALT-2 can be incorporated in epitope based vaccine development for filariasis carrying immunodominant epitopes that elicits Th2 responses.

2.8  PUTATIVE VACCINE CANDIDATE- ABUNDANT LARVAL TRANSCRIPT-2 (ALT)

A gene first approach to identify vaccine candidates was adopted by Gregory et al, (1997) and through Expree Sequence Tag (EST) analysis of L3 stage of parasite two highly expressed genes designated as ALT-1 and -2 were identified. ALT-1 and ALT-2 represent closely related proteins (79% identity) and are homologous to an abundant immunogen from larvae of the dog heartworm Dirofilaria immitis (Di-20/22L) (Frank et al 1996) and to proteins from the additional filarial parasites Onchocerca volvulus (Ov-ALT-1) (Joseph et al 1998) and Acanthocheilonema viteae (Pogonka et al 1999). The alt genes represent attractive vaccine antigens for three reasons:

(i) Larva specific in immunological terms;
(ii) Highly expressed, offering an abundant target;
(iii) No known homolog in the mammalian host.

ALT proteins are stockpiled in the oesophageal glands of infective larvae and are secreted by the parasites when they encounter mammalian culture conditions. The conservation of alt genes in all the filarial nematodes
so far studied and the very weak similarity to a single *C. elegans* locus imply that ALT products are critical in a filaria-specific role. The biological function of the ALT proteins is not known, but their highly regulated expression, abundance, and presence in excreted-secreted products of mammalian stage nematodes (Gomez-Escobar et al 2005) suggest that they may play an important role in establishing and maintaining infection.

The establishment of infection evidently requires a remarkably high degree of expression at the point of initial entry by parasites into the mammalian host. If this role is essential to parasite survival, neutralization by the immune response may be sufficient to ensure protection. It has been established that larval stages rapidly elicit a strong Th2 response in mice and induce host macrophages to adopt a counter-inflammatory phenotype. It was reported that neutralization of ALT function may be sufficient to protect the host from infection (Gomez-Escobar et al 2005).

### 2.8.1 ALT Attributes

The *alt-1* gene was originally identified as a prominent trans-spliced mRNA from L3 larvae of *B. malayi* (Gregory et al 1997), and *alt-2* was identified as a closely related expressed sequence tag (EST) from the same stage. *Alt* like genes has been found in other filarial nematodes and is characterized by a signal peptide, variable acidic domain and a conserved cysteine rich domain (Fig 2.8). The alignment of ALT sequences shows that all transcripts show variations in the acidic domain but the remaining protein is conserved. The alt-2 genes of *B. malayi* and *W. bancrofti* share 79% and 73% identity at the amino acid level with the alt-1 gene (Ramachandran et al 2004). *alt-1* and *alt-2* genes differ in the highly acidic domain of the protein. *W. bancrofti ALT*-2 differed from Brugia malayi *ALT*-2 in the variable acidic domain. A distantly related *C. elegans* and *C. briggsae* both lack the acidic domain.
2.8.2 ALT Sequence

AAC35355. Reports abundant larval t... [gi: 3599467]
>gi|3599467|gb|AAC35355.1| abundant larval transcript-2 protein

MNKLIATGFGLVILLVTLPASESDEEFDDGSNDETDDEDEGNSEQGDD
EYVTKGVEVETDGKKKECSSHACEDQREPQAWCRPNENQSWTDDK
GCFEDKLVSCVIERKNNGKLEYSYCAEGWQCA

It has an N-terminal signal peptide, a variable acidic domain, and a conserved, cysteine-rich domain. It has 9.4% Aspartic acid and 14.1% Glutamic acid residues which is the highest of all amino acid residues. The acidic domain is unique to filarial species and is reported to be essential for biological function. Alignment of the protein sequence shows that among the filarial parasites the acidic tract is highly variable but the remainder of the protein is conserved (Fig 2.9).

Figure 2.9 Comparison of ALT sequences with C.elegans, showing the signal sequence and variable domain.
Conserved residues are shaded in the picture.
2.8.3 Immune Response to ALT

Individuals from *B. malayi* endemic area were demonstrated to have an antibody response to recombinant ALT-1 in a hierarchal pattern with recognition greatest in endemic normals followed by patients with chronic pathology and least being microfilaraemics. The recombinant protein was also observed to induce 76% protection in a jird model of protective immunity (Gregory et al 2000).

In brugian filariasis endemic area the recognition of recombinant ALT protein was predominately by IgG3 and IgG1 followed by IgG4. The greater response among individuals with chronic pathology compared to microfilaraemics may be due to the presence of concomitant immunity, a characteristic phenomenon of helminth infections, where the presence of an active infection induces an immune response against the incoming infective larvae leading to resistance (Kurniawan-Atmadja et al 1998).

2.8.4 Immunomodulatory Role of ALT-2

ALTs may interfere with the critical first interaction between the innate immune system and the nematode invaders. Although ALT antigens are not expressed on the parasite surface, they can induce protective immunity in animal models (Abraham et al 2001, Gnanasekar et al 2004), indicating that neutralization of ALT function may be sufficient to protect the host from infection. Gomez-Escobar et al, (2005) transfected *alt* gene into *L. Mexicana* and showed that infection of macrophages *in vitro* is exacerbated by expression of either ALT protein. Mice infected with *alt*-transgenic parasites display more rapid lesion development and higher parasite burdens than controls. The *alt* transfected parasites were more resistant to INF-γ-induced killing by macrophages, supporting the hypothesis that ALT proteins act to modify host immune responses in filarial infection.
The conservation of *alt* genes in all the filarial nematodes so far studied and the very weak similarity to a single *C.elegans* locus imply that ALT products are critical in a filaria-specific role. The characteristic feature of chromadorea ALT family is the variable acidic domain, which is not present in *C.elegans* imply that the acidic domain could play a critical role in eliciting immune response. Gomez-Escobar et al, (2005) constructed a truncated mutant of ALT-2, named *acidic domain deleted (add)*, which was cloned into the pSSU vector and electroporated into *L. mexicana*. The *add* transfected parasites did not display the greater infectivity seen with *alt*-transfectants, but showed a phenotype indistinguishable from wild type parasites. *ADD* region of ALT showed a clearcut abolition of the *alt* phenotype, indicating the essential functional nature of this sequence and paving the way for a finer analysis of structure-function relationships in a tractable experimental system.

Madhumati et al, (2010a) spanned eight epitopic regions of *W.bancrofti* ALT and checked reactivity of the synthetic peptides in clinical samples, which showed that the crucial epitope region 55-68 and 73-91 were recognized by MF sera with significantly high IgG4 isotype. MF-positive individuals have extremely elevated levels of filarial-specific immunoglobulin of the IgG4 isotype (Kurniawan et al 1993, Lawrence 2001). High IgG4 levels elicited by these peptides explain their possible role in modulating early immune response in host, since IgG4 secretion induced by Tr-1 cells in an IL-10-dependent mechanism is known to down-regulate the immune response observed in MF patients. Madhumati et al, (2010b) also showed that peptides 2, 3, 7 elicited predominant IgG1 response in EN, CP followed by IgG2 and IgG3, while IgG4 isotypes were not detectable. Peptides 1, 2, and 7 showed IgG1 and IgG2 antibodies predominantly. Both N-terminal and C-terminal region along with region of variable acidic domain carried both B and T
epitope region, which can be exploited as a multiple antigen construct vaccine design.

2.9 MULTIPLE ANTIGENIC PEPTIDES (MAP)

MAP introduced by Tam, (1989) was designed to eliminate the need for conjugation of synthetic peptides to carrier proteins. It involves attaching peptides to four or eight branched lysine core and elicited strong immune responses in the absence of carriers (Posnett et al 1988). A special type of tetravalent MAP was found to be immunogenic in the experimental Aotus monkey model and have been able to induce protective immunity when challenged experimentally with a highly infective \textit{P.falciparum} strain (Niederhafner et al 2005). T and B cell epitopes of the repeat region of the \textit{P. falciparum} CS protein had been constructed as MAP and immunogenicity was studied in different mice models.

The modern strategy of using MAP’s for many parasitic disease like schistosomiasis, malaria and other disease have made an impact in the vaccination. The recent development of MAP allows the incorporation of multiple T and B cell epitopes in a single immunogenic macromolecule (Tam 1988). MAPS containing multiple of the immunodominant B cell epitope plus a T-helper (Th) cell epitope or Th epitope alone, all from the \textit{P. berghei} circumsporozoite (CS) protein have shown the capacity to significantly protect immunized mice (Tam et al 1990).

MAPs containing peptides derived from 28 kDa glutathione S-transferase of \textit{S.mansoni} have been synthesized and the antigenicities and protective effects were examined (Huang et al 2005). The epitope mapping studies carried out by Madhumathi et al, (2010a,c) in ALT was instrumental in designing the ALT MAP construction, first of its kind for filariasis. The promising results in the preliminary studies in experimental filariasis and the
tremendous success of peptide vaccines in other diseases along with the need for prevention of this disease was the rationale behind this construction and evaluation of Multiple Antigenic Peptide Vaccines for Lymphatic Filariasis.

2.10 INTRODUCTION TO VACCINE ADJUVANT

Vaccine development has been advancing over the years. New types of vaccines, such as DNA-based, recombinant subunits, recombinant viruses, and conjugates have been developed and introduced commercially. These vaccines tend to be safer and less reactogenic than older-style vaccines made from live or killed whole organisms. Use of new adjuvants that work with these vaccine types and that are less reactogenic has not kept pace with vaccine technology. Part of the barrier is the stringent regulatory environment. The Food and Drug Administration (FDA) approves adjuvants only in combination with vaccine and does not approve adjuvants alone. Aluminium salts were the first adjuvants approved by the FDA for use in humans. Use of alum salts began in the 1930s, before regulatory guidelines became more stringent. More recently, approvals have been obtained in Europe for MF59 as an adjuvant component of flu vaccine for elderly patients (Fluad®; Novartis Vaccines) and AS04 (combination of alum and MPL, GlaxoSmithKline) as the adjuvant for a viral vaccines (hepatitis B, HPV).

Vaccine adjuvants are powerful stimulators of immune system and therefore raise safety concerns. The emphasis on boosting humoral immune responses understandably has led to the development of adjuvants with the ability to enhance antibody responses. As a consequence, many commonly used adjuvants are effective at elevating serum antibody titers, but do not elicit significant Th1 responses or cytotoxic T lymphocytes (CTLs). Table 2.5 provides a description of vaccine adjuvants (antigen delivery systems, immunopotentiators and combinations) that have undergone clinical testing (Pashine et al 2005).
<table>
<thead>
<tr>
<th>Adjuvant/ Formulations</th>
<th>Benefits</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mineral salts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminum salts</td>
<td>The adjuvant in &gt;80% of vaccines licensed for human use. Induction of strong antibody responses, independent of TLR signaling. Directly activate DCs to secrete IL-1β and IL-18</td>
<td>Alum has been used for years in vaccines for billions of people of all ages. Poor CD8 T-cell induction</td>
</tr>
<tr>
<td>(hydroxide, phosphate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Alum)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Li et al 2008)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>Has been used as an adjuvant in vaccine formulations against diphtheria, tetanus, pertussis and poliomyelitis; More efficient than aluminum hydroxide when tested as a booster with DT as an antigen; Has also been used for absorption of extracts for hyposensitization of allergic patients.</td>
<td>Potential alternative to aluminum salts.</td>
</tr>
<tr>
<td>(Gupta &amp; Siber 1995)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Emulsions</strong></td>
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<td></td>
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<tr>
<td>MF59</td>
<td>Increased flu vaccine immunogenicity in young adults and in elderly people broadens response against heterovariant strains. Improved immunogenicity over Alum when tested with HBV vaccine, HSV, HIV1 gp120, and CMV gB. IM injection in combination with a variety of subunit antigens results in elevated antibody response, increased T-cell proliferation and induction of cytotoxic lymphocytes.</td>
<td>Induces chemokines to increase recruitment of immune cells, enhances Ag uptake by monocytes and differentiation to DCs. MF59 is a component of Fludad®, a licensed subunit influenza vaccine in Europe with &gt;30 million doses distributed. Combination of MF59 with MTP-PE enhanced systemic reactogenicity, without improving immunogenicity in Ph1 flu vaccine study</td>
</tr>
<tr>
<td>(Microfluidized detergent stabilized squalene oil-in-water emulsion)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(O'Hagan et al 2013)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjuvant/ formulations</td>
<td>Benefits</td>
<td>Comments</td>
</tr>
<tr>
<td>------------------------</td>
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<td>----------</td>
</tr>
<tr>
<td>OM-174 (Lipid A derivative, E. coli), OM-triacyl (Meraldi et al 2003)</td>
<td>OM-triacyl adjuvants are synthetic analogs based on a common triacyl motif, which induce maturation of human dendritic cells in vitro.</td>
<td>Act as potent Th1 adjuvants in mice, chimpanzees and orangutans. Act as TLR-9 agonists, bias response to Th1 immunity with CD8 T cell induction.</td>
</tr>
<tr>
<td>CpG ODN (synthetic oligonucleotides containing immunostimulatory CpG motifs) (Bode et al 2011)</td>
<td>Phase 1 trials conducted in humans (in association with Alum) have shown enhanced antibody responses against HBsAg. At least 3 classes of oligonucleotides are now defined, with respect to their distinct capacity to activate either: human B, NK or dendritic cells in vitro.</td>
<td></td>
</tr>
<tr>
<td><strong>Combination Adjuvants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS04 (Alum + MPL) (Didierlaurent et al 2009)</td>
<td>When compared with Alum, increased antibody titers, seroconversion rates and lymphoproliferative responses.</td>
<td>Administered to &gt;30,000 subjects. Tested in combination with proteins (HBsAg, HSV gD, EBV gp350) and HPV16/18 L1 VLP. Component of Fendrix™ and Cervarix™, approved HBV and HPV vaccines in Europe.</td>
</tr>
<tr>
<td>AS01 (Liposomes + MPL + QS21) (Garcon &amp; Van Mechelen 2011)</td>
<td>Designed to improve CD8 T-cell responses. Data with malaria antigens indicate higher antibody and T-cell response with AS01 than with AS02</td>
<td>AS01 favors Th-1 responses, AS02 elicits more balanced Th-1/Th-2 responses. Used with malaria and TB antigens.</td>
</tr>
<tr>
<td>Adjuvant/ Formulations</td>
<td>Benefits</td>
<td>Comments</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Immuno-adjuvants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokines: (IL-2, IL-12, GM-CSF, Flt3) (Rizza et al 2002)</td>
<td>Enhancement of antibody responses with GM-CSF.</td>
<td>Administration of Flt3 with HBV antigen resulted in the accumulation of immature DCs in peripheral blood, without enhancement of antibody response.</td>
</tr>
<tr>
<td><strong>Accessory molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B7.1)</td>
<td>The accessory molecule B7.1 provides costimulatory signals to T lymphocytes, has been included in association with the CEA antigen within the canarypox vector ALVAC, thereby potentially enhancing cellular responses.</td>
<td></td>
</tr>
<tr>
<td><strong>Particulate formulations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomes (DNPC/Chol) (Zhao et al 2011)</td>
<td>Lipid-bilayer membranes enclosing aqueous compartments. Slight increase in CD8 + CTL response when combined with a flu vaccine. Can be freeze-dried</td>
<td>Fuse with cell membrane to deliver proteins to MHC class I pathway No increase in antibody titers (equivalent to vaccine alone). Limited by manufacturing and cost issues.</td>
</tr>
<tr>
<td>PLA (polylactic acid) PLG (poly[lactide-co-glycolide]) microparticles (Jain et al 2011)</td>
<td>PLGA particles were shown to elicit Th1 (presentation of CTL epitopes) and Th2 responses in mice.</td>
<td>Microparticles function mainly as delivery system. Clade B, Gag, DNA/PLG and Env DNA/PLG Microparticles Vaccine is in an ongoing Ph1 trial in HIV-negative adults. Ongoing trial with the tetanus toxoid</td>
</tr>
</tbody>
</table>
Table 2.5 Continued

<table>
<thead>
<tr>
<th>Adjuvant/formulations</th>
<th>Benefits</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISCOMS® (structured complex of saponins and lipids) (Lovgren Bengtsson et al 2011)</td>
<td>Increase of influenza-specific CD8 + CTL response (when compared with flu vaccine alone). Allows reduction in QS-21 dose. Adjuvant activity from induction of cytokines (IFN-g and IL-12)</td>
<td>SCOMS has been used in vaccines for &gt;1,000 people; with flu, HPV, HCV and cancer antigens Second generation (ISCOMATRIX) adjuvants based on purified saponins are currently being tested with HPV16 (E6/E7) fusion protein.</td>
</tr>
</tbody>
</table>

2.10.1 Enhance the Immune Response—With the Use of Adjuvants

Adjuvants are defined as compounds that can increase and/or modulate the intrinsic immunogenicity of an antigen, and the term adjuvant is itself derived from the latin *adjuvare*, meaning to help. Purified protein antigens and peptides, the currently favored subunit vaccine candidates, are poor immunogens. Injection of these substances usually induces immunological tolerance unless administered with an adjuvant (Waksman 1979, Warren et al 1986, Gupta et al 1993), traditionally CFA. The addition of adjuvants to vaccines enhances, sustains and directs the immunogenicity of antigens, effectively modulating appropriate immune responses, reducing the amount of antigen or number of immunizations required and improving the efficacy of vaccines in newborns, elderly or immuno-compromised individuals (Kenney & Edelman 2003). However, the mode of action followed by many adjuvants largely remains mysterious and empirical.

Adjuvants have limited or no efficacy unless properly formulated, therefore both adjuvant components and formulation (e.g. oil in water,
particle size, charge, etc.) are crucial for enhancing vaccine potency. Traditional live vaccines based on attenuated pathogens typically do not require the addition of adjuvants. Vaccines based on inactivated viruses or bacteria are often sufficiently immunogenic without added adjuvants, although some of these (e.g. split flu virus, Hepatitis A virus or whole cell Pertussis) can be formulated with adjuvants to further enhance the immune responses. By contrast, protein-based vaccines although offering considerable advantages over traditional vaccines in terms of safety and cost of production, in most cases have limited immunogenicity and require the addition of adjuvants to induce a protective and long-lasting immune response.

Recombinant protein-based vaccines, including those for Hepatitis B and human papilloma virus, have been successfully developed to elicit protective antibody responses using only aluminum salts (Alum) as adjuvant. The next generation of recombinant vaccines, aimed at diseases such as malaria, tuberculosis and HIV and/or AIDS, will require not only very strong and long lasting antibody responses but also potent cell mediated immunity based on CD4 and CD8 T-cell responses. For an effective adjuvant formulation the dose, immune response, therapeutic vaccine requirement all has to be taken into consideration (Fig 2.10).

Vaccine adjuvants influence the immune response to our benefit in one or more ways. The ability of adjuvants to influence so many parameters of the immune response greatly complicates the process of finding an effective adjuvant. The knowledge that we have acquired on how an adjuvant works in a cellular level is insufficient to design a rational approach for matching the vaccine antigen with the proper adjuvant. Many investigators have advocated an empirical approach for antigen selection based on the balance between toxicity, adjuvanticity in animals, and whether one wishes to
stimulate a cellular (Th1) response, a humoral (Th2) response, or a balance of the two responses.

**Beneficial Effects of Vaccine Adjuvants**

1. Increase the potency of antigenically weak peptides.
2. Enhance the speed, vigor, and persistence of the immune response to stronger antigens.
3. Modulate antibody avidity, specificity, quantity, isotype, and subclass.
4. Select for or enhance the cytotoxic T-cell response.
5. Increase the immune response to vaccines in immunologically immature, suppressed or senescent individuals.
6. Decrease the amount of antigen required, thus reducing the cost and the likelihood of antigen competition in combination vaccines.

![Figure 2.10: Roles of adjuvants in future vaccines](image-url)
2.10.2 Where Do Adjuvants Act?

Novel adjuvants aim at tailoring the vaccine induced immune responses to achieve maximal efficacy, through optimization of B-cell responses (in terms of level, quality, duration and memory) and generating appropriate T-cell responses (in terms of effector functions and memory). Understanding which innate immune receptors are involved in the response to different classes of adjuvants, and which signals convert dendritic cells (DC) and monocytes into immunostimulatory antigen-presenting cells (APCs), will provide a scientific rationale for their use, and reduce the risk of unintended adverse reactions. Adjuvants have strengthened the link between the initial innate and subsequent adaptive response. The crucial steps and signals required to induce T- and B-cell responses have been defined, demonstrating that innate immune signals modulate not only the magnitude of the adaptive response but also the quality of this response. Secretion of pro-inflammatory cytokines such as interleukin (IL)-12 will drive TH1-cell polarization and subsequent interferon (IFN)-\(\gamma\) secretion by T cells. Fig 2.11 represents different signals required to initiate a potent immune response as signal 0 - antigen recognition and APC activation, signal 1 - antigen presentation and signal 2-co-stimulation (Schijns 2001). Adjuvants can act on each of these three signals. Although targeting co-stimulatory molecules (signal 2) directly through antibodies, cytokines or chemokines is an interesting possibility (Barr et al 2006), this might result in non-focused over-stimulation. There are different modes of action of based on the type of adjuvants (Table 2.6) which can be used as per the demand of the vaccine used for the disease.
Figure 2.11: Different signals required to initiate potent immune response

Table 2.6: Modes of adjuvant action

<table>
<thead>
<tr>
<th>Action</th>
<th>Adjuvant type</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Immunomodulation</td>
<td>Generally small molecules or proteins which modify the cytokine network</td>
<td>Upregulation of immune response. Selection of Th1 or Th2</td>
</tr>
<tr>
<td>2. Presentation</td>
<td>Generally amphiphatic molecules or complexes which interact with immunogen in its native conformation</td>
<td>Increased neutralizing antibody response. Greater duration of response</td>
</tr>
<tr>
<td>3. CTL induction</td>
<td>Particles which can bind or enclose immunogen and which can fuse with or disrupt cell membranes.</td>
<td>Cytosolic processing of protein yielding correct class I restricted peptides</td>
</tr>
<tr>
<td>4. Targeting</td>
<td>Particulate adjuvants which bind immunogen, Adjuvants which saturate Kupffer cells</td>
<td>Simple process if promiscuous peptide(s) known</td>
</tr>
<tr>
<td>5. Depot generation</td>
<td>w/o emulsion for short term. Microspheres or nanospheres for long term</td>
<td>As above. May also determine type of response if targeting selective</td>
</tr>
</tbody>
</table>

2.10.3 Adjuvants Classification

Adjuvants are classified according to their chemical nature, origin or physical chemical properties (Kwak & Longo 1996, Cox 1997) yet, related compounds frequently have divergent immunomodulating properties. Immunological adjuvants can be defined as compounds that bias the immune system towards Th1 or Th2 immunity and significantly enhance the immune response against an antigen. In this study to enhance the efficacy of BmALT
different adjuvants have been administered to animal models (Table 2.7). Since filariasis is associated with T cell hypo responsiveness attempt has been made to administer antigens with adjuvants that restore Th1 effector cells and or elicit protective immunity Th1/Th2 balance.

Table 2.7: Adjuvants used in the present study

<table>
<thead>
<tr>
<th>Adjuvant categories</th>
<th>Adjuvants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral based adjuvants</td>
<td>Alum</td>
</tr>
<tr>
<td>Bacterial derived adjuvants</td>
<td>MPLA, MDP</td>
</tr>
<tr>
<td>Carbohydrate adjuvants</td>
<td>Chitosan, Inulin</td>
</tr>
<tr>
<td>Saponin</td>
<td>QS21</td>
</tr>
</tbody>
</table>

2.10.4 Mineral Based Adjuvant

Alum:

Aluminium salts, principally aluminium hydroxide or phosphate, have been the most widely used adjuvants in humans. Alum adjuvants are components of several licensed human vaccines, including diphtheria-pertussis-tetanus, diphtheria-tetanus (DT), DT combined with Hepatitis B (HBV), Haemophilus influenza B or inactivated polio virus, Hepatitis A (HAV), Streptococcus pneumonia, meningococcal and human papilloma virus (HPV) (Clements & Griffiths 2002). Proteins can bind to aluminium adjuvants by two ways: (i) electrostatic interaction between proteins and positively charged aluminium hydroxide (Iyer et al 2003) (ii) ligand exchange between hydroxyl and phosphate group (Morefield et al 2005). The mechanisms of action of the aluminum salts frequently cited include: (i) depot formation facilitating continuous antigen release; (ii) particulate structure formation promoting antigen phagocytosis by antigen presenting cells (APC) such as DC, macrophages and B cells and, (iii) induction of inflammation
resulting in recruitment and activation of macrophages, and increased major histocompatibility complex (MHC) class II expression and antigen presentation (Ulanova et al 2001). Alum has been shown to boost humoral immunity by providing Th2 cell help to follicular B cells (Brewer et al 1999). Studies have demonstrated that while aluminum adjuvants can stimulate Th2 type responses and the production of cytokines, such as IL-4 and IL-5, as well as B cell production of IgG1 and IgE, it fails to stimulate Th1 responses, such as IFN-γ production and B cell IgG2a secretion (Brewer & Alexander 1997).

### 2.10.5 Bacterial Derived Adjuvants

#### a) Monophosphoryl lipid A (MPLA)

The TLR4 agonist lipopolysaccharide (LPS), an outermembrane component of gram-negative bacteria, is a potent inducer of inflammation and innate immune responses and is an excellent immunological adjuvant, but is far too toxic for use in vaccines. The lipid A portion of LPS is responsible for the endotoxicity of LPS, and modification of the lipid A structure can thus result in an LPS-like molecule that is not very endotoxic. Monophosphoryl lipid A (MPLA) is a derivative of *Salmonella minnesota* R595 LPS. MPLA is made by removing a phosphate group and an acyl chain from the LPS molecule. Like LPS, MPLA activates TLR4, but it is over 100 times less toxic. It is less toxic than its parent molecule, demonstrating attenuated cytokine release and decreased, disseminated vascular coagulation and cell migration, which are usually associated with LPS (Yao et al 1994). MPLA activates TLR4 pathway, resulting in an enhanced production of cytokines leading to the maturation and leading to the maturation and migration of APCs to the lymph nodes. TLR4 stimulation can contribute to the activation of the innate immune response, by activating NF-κB transcriptional activity and the subsequent expression of pro-inflammatory cytokines, such as TNF-α and IL-6 (Iwasaki & Medzhitov 2004). These cytokines can in turn enhance
the adaptive immune response by stimulating the maturation of APCs while repressing the tolerance response through the inhibition of regulatory T cell (Treg) activity (Pasare & Medzhitov 2003). MPLA is generally reported to promote IFN-γ production by antigen-specific CD4+ T cells, therefore skewing the immune response towards a Th1 profile (Casella & Mitchell 2008). AS04 is a combination of MPLA and either aluminum hydroxide or aluminum phosphate (Garcon et al 2007). AS04 is used in the hepatitis B virus (HBV) vaccine FENDrix and in the human papilloma virus (HPV) vaccine Cervarix, both from GlaxoSmithKline Biologicals (Tagliabue & Rappuoli 2008).

b) N-acetylmuramyl-L-alanyl-D isoglutamine (muramyl dipeptide-MDP)

Muramyl dipeptide (MDP, N-acetylmuramyl-L-alanyl-D isoglutamine) has been shown to be the minimal structure of a bacterial cell wall peptidoglycan that can substitute for mycobacteria in Freund complete adjuvant. Myriad of studies in animals has established that MDP exhibits a broad array of immunological effects, including (i) enhancement or suppression of antibody levels dependent on time of administration relative to antigen; (ii) increased cell mediated immunity; (iii) increased nonspecific immunity to bacteria, viruses, fungi, and parasites; (iv) stimulation of natural resistance to tumors; (v) induction of autoimmune disease; (vi) increased cytokine release; (vii) increased IFN-γ; (viii) pyrogenicity; and (ix) somnogenic activity. Allison & Byars, (1990) developed an adjuvant formulation consisting of N-acetylmuranyl-L-threonyl-D-isoglutamine in a squalane pluronic polymer emulsion, which was effective in animals in increasing the response to influenza virus hemagglutinin, hepatitis B virus surface antigens, and Herpes Simplex Virus type 2. Different modifications of MDP have been reported to elicit different types of immune responses depending on antigen and administration route. This is attributable to the fact that MDP is known to be a pathogen-associated molecular pattern (PAMP).
Such structures are recognized by pattern recognition receptors (PRR), both extracellularly by toll-like receptors (TLR) and in the cytosol by nucleotide-binding oligomerization domain (NOD) proteins, leading to specific innate and adaptive immune responses. MDP is recognized in the cytosol by the NOD2 PRR, leading to caspase activation, apoptosis, pro-inflammatory cytokine production (TNF and IL-1$\beta$) and cytokine and costimulatory molecule production (e.g. IL-6, IL-12, B7.1 upregulation) involved in adaptive immunity (Fedorrocko et al 2003).

2.10.6 Carbohydrate Adjuvants

The need for safe and effective immune therapeutic agents and vaccine adjuvants has spurred interest in the use of plant or microbial-derived polysaccharides as human immune modulators. A major advantage of use of carbohydrate compounds as immunomodulators is that most carbohydrates are non-toxic and well tolerated in vivo.

a) Inulin

‘Inulin’ is a simple, inert polysaccharide consisting of a family of linear $\beta$ -D-(2→1) polyfructofuranosyl $\alpha$-D-glucoses. Inulin is the storage carbohydrate of Compositae and is obtained in high molecular weight from dahlia tubers. Inulin activates and exhausts complement when incubated with human serum, this property demonstrates alternative complement pathway (ACP) (Gotze & Muller-Eberhard 1971). Based on the solubility there are two different forms, precipitation from water referred to as alpha inulin ($\alpha$-IN), and the form obtained by precipitation from ethanol as beta inulin ($\beta$-IN). A third polymorphic form of particulate inulin was described and designated as gamma inulin ($\gamma$-IN) (Cooper & Carter 1986). $\gamma$-IN is virtually insoluble in water at 37°C, but is soluble in concentrated solution (>50 mg/mL) at temperatures in the range 70–80°C. Delta inulin (DI) obtained from gamma inulin at 55°C for 90 min has greater temperature stability and its enhanced
immune activity as measured by complement activation in vitro and by humoral and cellular immune responses in vivo. DI possessed potent vaccine adjuvant activity in vivo, enhancing antigen-specific humoral and cellular immunity when co-administered with vaccine antigens, including HBsAg and Japanese encephalitis virus (Lobigs et al 2010).

b) Chitosan

Chitosan derived by the deacetylation of chitin, is a polymer of D-glucosamine and N-acetyl D-glucosamine. The molecular unit of chitosan has one amino group and two hydroxyl groups that are potentially capable of reacting with an acidic medium. Chitosan is well known for its hydrophilic, biocompatible, biodegradable and non-toxic properties (Felt et al 1998, Illum 1998). Chitosan particle delivery system can reduce the clearance rate from the nasal cavity, thereby increasing the contact time of the delivery system with the nasal mucosa (Soane et al 1999). Chitosan suspensions or micro and nanoparticles have been reported to have immune stimulating activity such as increasing accumulation and activation of macrophage and polymorphonuclear cell, promoting resistance to infections of microorganisms, and inducing cytokines (van der Lubben et al 2001). The strong mucoadhesive property of chitosan is most important for drug delivery through the mucosal routes. In addition, the interaction of the positively charged chitosan with the negatively charged mucin layer and the tight junctions facilitates the paracellular transport of hydrophilic macromolecules by opening the tight junctions of the mucosal barriers (Dodane et al 1999). It was reported that chitosan solutions were able to enhance both the mucosal and systemic immune responses against influenza virus vaccines (Bacon et al 2000). The Bordetella pertussis filamentous haemagglutinin and recombinant pertussis toxins have been shown to induce strong antigen-specific systemic and mucosal immune responses after IN administration with chitosan (Jabba-

2.10.7 Saponin - QS21

Saponins are triterpene glycosides isolated from plants. The most widely used adjuvant Quil A and its derivatives were extracted from the bark of the *Quillaja saponaria* tree (Kensil et al 1995). Saponins have been widely used as an adjuvant in veterinary vaccines. QS-21 is a purified component of Quil A that demonstrates low toxicity and maximum adjuvant activity. In a variety of animal models, QS-21 has augmented the immunogenicity of protein, glycoprotein and polysaccharide antigens (White et al 1991). QS-21 has been shown to stimulate both humoral and cell-mediated Th1 and CTL responses to subunit antigens (Singh & O'Hagan 1999). Clinical trials are in progress with QS-21, alone or in combination with carriers and other immunostimulants for vaccines against infections including influenza, HSV, HIV, HBV and malaria, and cancers including melanoma, colon and B-cell lymphoma.

The review points out the need for effective prophylactics and enhancement of existing vaccine candidate in combating this neglected tropical disease. Hence, the present work is focused on:

1. Immunological characterization of ALT homologue Wb20/22 and their evaluation as a putative vaccine candidate.
2. Construction and evaluation of synthetic ALT MAP and encapsulation in microsphere.
3. Enhancing the efficacy of rALT using different adjuvants.