CHAPTER 1.
(A) INTRODUCTION
1. FILARIA AND HUMAN HEALTH

Lymphatic filariasis (LF) is an infectious disease caused by lymph-dwelling nematode parasites and transmitted by mosquitoes. It is one of the oldest and most debilitating of all the neglected tropical diseases. An estimated 120 million people in 73 countries are infected (WHO, 2012), and an estimated 1.39 billion live in areas where filariasis is endemic. Approximately 40 million people suffer from the stigmatizing and disabling clinical manifestations of the disease, including 15 million who have lymphoedema (elephantiasis), and 25 million men who have urogenital swelling, principally scrotal hydrocele (WHO, 2012). The Global Programme to Eliminate Lymphatic Filariasis (GPELF) is a rapidly growing worldwide public health programme which was launched in 1999, for its elimination as a public health problem by the year 2020 (Freedman, 1998; Michael, 1999; Addiss, 2010). In response, governments of the countries, where the disease is endemic have launched different programmes to eliminate LF as a global public health problem and have made significant progress (WHO, 2012). Of the 73 countries where lymphatic filariasis is considered endemic, 53 have implemented mass drug administration (MDA) to stop transmission. During 2000–2011, >3.9 billion doses of medicine were delivered to a targeted population of 952 million people (WHO, 2012). According to the World Health Organization, India, Indonesia, Nigeria and Bangladesh alone contribute about 70% of the infection worldwide.

In India, which carries 40% of the global LF burden, initiated an ambitious programme to eliminate LF. The programme envisages the yearly mass anti-filarial treatment of 590 million people living in ~300 000 villages and 1450 urban areas in 250 districts (the National Vector Borne Disease Control Programme website, www.nvbdcp.gov.in). LF is found to be endemic in 17 states and 6 Union Territories including the populous states of Uttar Pradesh and Bihar. Eastern coastal line is particularly more prone to lymphatic filariasis. In India alone, the disease causes an annual economic loss of nearly US $ 1 billion (Ramaiah et al., 2000a,b).
2. PREVALENCE AND DISTRIBUTION OF HUMAN FILARIASIS

Lymphatic filariasis (LF) is a major public health problem and inflicts a considerable social and economic burden on many developing countries. *Wuchereria bancrofti*, *Brugia malayi*, *Brugia timori*, *Onchocerca volvulus*, *Dipetalonema perstans*, *D. streptocerca*, *Loa loa* and *Mansonella ozzardi* are the species responsible for producing infestations in man (Manson- Bahr and Alcock, 1927). *Wuchereria bancrofti* is responsible for 91% of cases and is found throughout the tropics and in some subtropical areas world-wide. *Brugia malayi* is confined to Southeast and Eastern Asia (WHO, 2012; Bockarie and Deb, 2010). *Brugia timori* is found only in Timor and its adjacent islands. *B. malayi* is also found in monkeys, cats and other small animals but it is not known how important this is in the epidemiology of human disease. Unlike malaria where the only vectors are Anopheline mosquitoes, lymphatic filariasis can be transmitted by various species of the genera *Anopheles, Culex, Aedes, Ochlerotatus*, and *Mansonina*. The biting-time of the mosquito correlates with the periodicity of the microfilariae. In most parts of the world, the vectors are nocturnal feeders and the microfilariae exhibit nocturnal periodicity. In areas such as the Central Pacific where the mosquito feeds during the day, there is a diurnal periodicity where the highest number of microfilariae occurs at midday. In some areas of Southeast Asia, a subperiodic pattern is found, where some microfilariae are found in the blood at all times but there is a nocturnal peak. The filaroid parasites that commonly cause human disease and their characteristics are summarised in Table 1.1.
### Table 1.1: The filaroid parasites of humans.

<table>
<thead>
<tr>
<th>Species</th>
<th>Location of Adult</th>
<th>Major Pathology</th>
<th>Diseases</th>
<th>Location of Microfilariae</th>
<th>Major Vector</th>
<th>Geographical Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major Filariae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Wucherea bancrofti</em></td>
<td>Lymphatics</td>
<td>Lymphangitis</td>
<td>Lymphatic filariasis</td>
<td>Blood: may exhibit nocturnal periodicity</td>
<td>Species of <em>Culex, Aedes</em> and <em>Anopheles</em> Mosquitoes</td>
<td>Widespread in tropical and subtropical countries</td>
</tr>
<tr>
<td><em>Brugia malayi</em></td>
<td>Lymphatics</td>
<td>Lymphangitis</td>
<td>Lymphatic filariasis</td>
<td>Blood</td>
<td>Species of <em>Mansonia</em> Mosquitoes</td>
<td>Southeast Asia</td>
</tr>
<tr>
<td><em>Brugia timori</em></td>
<td>Lymphatics</td>
<td>Adino lymphangitis</td>
<td>Lymphatic filariasis</td>
<td>Blood: may exhibit nocturnal periodicity</td>
<td>Species of <em>Anopheles</em> Mosquitoes</td>
<td>Indonesia</td>
</tr>
<tr>
<td><em>Onchocerca volvulus</em></td>
<td>Subcutaneous nodules</td>
<td>Loss of vision, dermatitis</td>
<td>Onchocerciasis</td>
<td>Tissue fluid in the skin</td>
<td><em>Simulium</em> spp. (Black flies)</td>
<td>Africa, Mexico, Guatemala, loci in central and south America</td>
</tr>
<tr>
<td><em>Dracunculus medinensis</em></td>
<td>(Guinea)</td>
<td>Subcutaneous</td>
<td>Subcutaneous</td>
<td>Dracunculiasis</td>
<td>None. 1st stage larvae are</td>
<td>Copepod</td>
</tr>
<tr>
<td>Minor Filariae</td>
<td>Tissue Location</td>
<td>Clinical Symptoms</td>
<td>Site of Disease</td>
<td>Vector</td>
<td>Geographical Distribution</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
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<td></td>
</tr>
<tr>
<td><strong>Loa Loa</strong></td>
<td>Subcutaneous nodules</td>
<td>Transient swelling, temporary loss of vision</td>
<td>Blood: exhibit diurnal periodicity</td>
<td><em>Chrysops</em> spp. (Deer flies)</td>
<td>Tropical Africa</td>
<td></td>
</tr>
<tr>
<td><strong>Mansonella streptocerca</strong></td>
<td>Skin</td>
<td>Dermatitis</td>
<td>Mansonellosis</td>
<td>Skin</td>
<td>Small biting flies</td>
<td>West Africa</td>
</tr>
<tr>
<td><strong>Mansonella pertans</strong></td>
<td>Body cavities</td>
<td>Not well defined</td>
<td>Mansonellosis</td>
<td>Blood</td>
<td>Small biting flies</td>
<td>Africa and South America</td>
</tr>
<tr>
<td><strong>Mansonella ozzardi</strong></td>
<td>Subcutaneous and connective tissue based on experimental animal studies</td>
<td>Not well defined</td>
<td>Mansonellosis</td>
<td>Blood</td>
<td>Small biting flies in the genera <em>Simulium</em> and <em>Culicoides</em></td>
<td>West Indies, Central and South America</td>
</tr>
<tr>
<td><strong>Dirofilaria spp</strong></td>
<td>None in human</td>
<td>Subcutaneous nodules, lung lesions</td>
<td>Dirofilariasis</td>
<td>None in human</td>
<td>Many species of Mosquitoes</td>
<td>Cosmopolitan</td>
</tr>
</tbody>
</table>
A district-level endemcity map created for India in 2000 shows that of the 289 districts surveyed up to 1995 (62% of all districts), as many as 257 were found to be endemic (Sabesan et al., 2000). Seventeen states and six Union Territories were identified to be endemic with about 553 million people exposed to the risk of infection; and of them, about 146 million live in urban and the remaining in rural areas. About 31 million people are estimated to be the carriers of mf and over 23 million suffer from filarial disease manifestations in India (WHO, 2005). Bihar has highest endemcity (over 17%) followed by Kerala (15.7%) and Uttar Pradesh (14.6%). Andhra Pradesh and Tamil Nadu have about 10% endemcity. Goa showed the lowest endemcity (less than 1%) followed by Lakshadweep (1.8%), Madhya Pradesh (above 3%) and Assam (about 5%). B. malayi is prevalent in the states of Kerala, Tamil Nadu, Andhra Pradesh, Orissa, Madhya Pradesh, Assam and West Bengal. The single largest tract of this infection lies along the west coast of Kerala, comprising the districts of Trichur, Ernakulum, Alleppey, Quilon and Trivandrum, stretching over an area of 1800 sq km. The infection in the other six states is confined to a few villages. Surveys undertaken recently in Kerala and a few villages in other states revealed either a reduction of foci or complete elimination of the parasite as well as the vector(s) in many villages which were known to be endemic for B. malayi infection four decades back (Regu et al., 2005; Sabesan et al., 2005).

LF mapping by mf prevalence has been generated to depict the present scenario of human infection prevalence in India. The results of survey carried out in 443 districts out of a total 593 districts in India at different time points up to 2006 were considered for mf distribution mapping (WHO, 2005). In 239 (54%) districts the survey was carried out between 1960 and 1990. In the remaining 204 (46%) districts the survey results were updated after 1990. Accordingly, the mf prevalence map at the district level is made up to the year 2006 and is shown in (Figure 1.1). It is observed that the level of mf prevalence is not known in 150 (25.3%) districts. Among the surveyed districts, 172 were found to be with over 1% mf prevalence. Many of these districts (58%) were detected of this status after 1990. Maximum mf prevalence (12%) was recorded from Nicobar Islands during 1996. The National level average mf rate showed a declining trend from 1.24% in 2004 to 0.63% in 2008 (see the National Vector Borne Disease Control Programme website at http://nvbdcp.gov.in/filariasis-new.html) (Sabesan et al., 2010).
Besides India, LF has been found endemic in many areas of tropical and subtropical countries worldwide. Sri Lanka, the neighbouring country to India had a long history of prevalence as reported by Schweinfurth (1983) and the present reports have shown that nearly 10.5 million people are at risk of LF infection. The disease has also been detected in significant proportions of Nepal in the recent time (Watanbe et al., 2003). Randomly collected serum and urine samples from residents in two rural areas at different altitudes in Nepal showed the presence of *W. bancrofti* antigens and antibodies (Watanbe et al., 2003). LF infection is concentrated to 39 of 77 provinces of Phillipines, accounting for more than 90% of the entire population at risk in the Mekong-Plus subregion (WHO, 2007).

In the South East Asian country, Vietnam, the prevalence and distribution of lymphatic filariasis existed from the early 1900s. A survey carried out on some 135000 individuals in 24 provinces of Vietnam showed highest prevalence of *B. malayi* microfilaraemia in lowland areas of the Red River Delta and Quang-binh province (Meyrowitch et al., 1998). The 2007 survey has shown that nearly 0.68 million people in Vietnam are at risk of LF infection (WHO, 2007).
There have been number of reports on the prevalence and distribution of lymphatic filariasis in Ghana and the African sub-region (Grenfell et al., 1990; Gyapong et al., 1996) (Figure 1.2). Serum samples from 341 individuals in Nigeria showed the prevalence of *W. bancrofti* infection by 10% (Engelbrecht et al., 2003). In south eastern Kenya serological studies revealed circulating microfilariae in 13.7% of the study group (Estambale et al., 1994). A high frequency of people with swollen legs/feet was observed in Pawe settlement area of Region 6 in northwest Ethiopia (Birrie et al., 1997). The estimated mass drug administration against LF by WHO programme is shown in Table 1.2.
Table 1.2: Mass drug administration (MDA) implemented for lymphatic filariasis, by WHO region or regional programme review group, 2011

<table>
<thead>
<tr>
<th>WHO region or regional programme review group</th>
<th>No. of endemic countries –</th>
<th>Estimated population requiring MDA</th>
<th>No. of implementation units delivering MDA</th>
<th>No. of countries delivering MDA</th>
<th>Total population estimated to have been covered by MDA</th>
<th>Total population reported to have ingested medicines as part of MDA</th>
<th>Reported coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>African</td>
<td>34</td>
<td>442 546 242</td>
<td>603</td>
<td>19</td>
<td>124 851 911</td>
<td>94 235 846</td>
<td>75.48</td>
</tr>
<tr>
<td>Americas</td>
<td>4</td>
<td>12 377 684</td>
<td>126</td>
<td>3</td>
<td>10 168 045</td>
<td>8 973 982</td>
<td>88.26</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>4</td>
<td>22 133 780</td>
<td>30</td>
<td>2</td>
<td>580 443</td>
<td>531 356</td>
<td>91.54</td>
</tr>
<tr>
<td>Mekong-Plus</td>
<td>6</td>
<td>30 180 699</td>
<td>77</td>
<td>5</td>
<td>29 985 592</td>
<td>19 963 359</td>
<td>66.58</td>
</tr>
<tr>
<td>Pacific Programme to Eliminate Lymphatic Filariasis</td>
<td>16</td>
<td>6 747 361</td>
<td>14</td>
<td>14</td>
<td>1 144 048</td>
<td>796 789</td>
<td>69.65</td>
</tr>
<tr>
<td>South-East Asia</td>
<td>9</td>
<td>878 763 716</td>
<td>434</td>
<td>9</td>
<td>570 137 194</td>
<td>414 076 329</td>
<td>72.63</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>73</strong></td>
<td><strong>1 392 749 482</strong></td>
<td><strong>1 284</strong></td>
<td><strong>53</strong></td>
<td><strong>736 867 233</strong></td>
<td><strong>538 577 661</strong></td>
<td><strong>73.09</strong></td>
</tr>
</tbody>
</table>
Figure 1.2: Status of mass drug administration (MDA) in lymphatic filariasis endemic countries, 2011.
3. HUMAN DISEASES CAUSED BY FILARIAL PARASITES

3.1. Lymphatic Filariasis

*W. bancrofti, B. malayi and B. timori* are mosquito-borne parasites and cause LF. In most endemic areas elephantiasis only occurs in a small proportion of the people suffering from LF. *W. bancrofti* occurs in sub-Saharan Africa, Southeast Asia, the Indian subcontinent, many of the Pacific islands, and focal areas of Latin America. *B. malayi* occurs mainly in China, India, Malaysia, the Philippines, Indonesia, and various Pacific islands. *B. timori* occurs on the Timor Island of Indonesia. Overall, approximately two-thirds of individuals infected with lymphatic filariasis are in Asia. (McMahon and Simonsen, 1996; Bockaire and Deb, 2010; WHO, 2012).

3.2. Onchocerciasis

Onchocerciasis is the second-leading infectious cause of blindness worldwide: approximately 500,000 people are blind due to onchocerciasis. Onchocerciasis (also called river blindness) is caused by *O. volvulus* and the vector is the *Simulid* black fly. More than 99% of cases occur in 27 countries in sub-Saharan Africa. Overall, 120 million people live at risk of infection in endemic countries in Africa. Smaller foci of infection have been found in Yemen and Central and Southern America (Mexico, Guatemala, Ecuador, Colombia, Venezuela, and Brazil). Transmission has now been eliminated or interrupted in nine of the foci in the Americas, is suppressed in one Guatemalan focus, and is ongoing in two foci in Venezuela and one in Brazil (WHO, 2011). Microfilariae invade the skin and give rise to dermatitis premature aging of the skin and skin atrophy. Development of the adult worm leads to nodule formation. Microfilariae invade the eye and cause an inflammatory reaction that can lead to blindness (Murdoch *et al.*, 2002; Basanez *et al.*, 2006).

3.3. Loasis

Loasis is caused by *Loa loa* (sometimes called the African eye worm) and is spread by *Chrysops* flies which breed in the high-canopied rain forest of west and central Africa, including the coastal plains of northern Angola, southeastern Benin, Cameroon, Central African Republic, Chad, Republic of the Congo, Equatorial Guinea, Gabon, Nigeria, Sudan, and the Democratic Republic of Congo. Rare cases have been reported in the region from Ghana to Guinea and in Uganda, Mali, Zambia, and Ethiopia (Klion and Nutman, 2011). Signs and symptoms include fugitive or "Calabar"
swellings, itching and joint pains. Sometimes an asymptomatic invasion of the eye surface occurs hence the name "eye worm" (Negesse et al., 1985). It is the most common filaroid infecting travellers from non-endemic areas (Zoure et al., 2011; Wanji et al., 2012).

3.4. Miscellaneous Filaria Infections

Mansonelliasis (or mansonellosis) is the condition of infection by the nematode Mansonella. The disease exists in Africa and tropical Americas, spread by biting midges (Culicoids) or blackflies (Simulium). M. ozzardi is found only in the New World, M. steptocerca is found only in the Congo basin, and M. perstans is found in both the previously described areas of Africa and Latin America. Prevalence rates vary from a few percent to as much as 90% in areas like Trinidad, Guyana and Colombia (John and William, 2006). Most infections are asymptomatic but loiasis-like signs and symptoms can occur (Simonsen et al., 2011).

3.5. Zoonotic Filarial Infection

Filarial infections with various known and unknown animal filarial parasites have regularly been reported throughout the world (Tobie and Beye, 1962; Beaver and Orihel, 1965; Nelson, 1965; Orihel and Eberhard, 1998). D. immitis is the cause of heartworm in dogs and occasionally effects humans (Rodrigues-Silva et al., 1995). Most cases are asymptomatic and the worm becomes calcified in the lung resulting in a "coin lesion", which may be mistaken for carcinoma or tuberculosis. Occasionally asthmatic-like symptoms occur and there may be a marked eosinophilia. Another dog filaroid D. repens can cause subcutaneous nodules, peri-orbital lesions, coin lesions in the lungs, and breast lump in humans and rare cases have been reported throughout the world (Bennett et al., 1989; Pampiglione et al., 1995). High levels of antifilarial IgG, IgE and IgM antibodies are present in patients with dirofilariasis (Simon et al., 1997). The prevalence of D. immitis antibodies in humans in closely related to the number of infected dogs in the community (Welch and Dobson, 1974; Theis, 2005).

3.6. Dracunculiasis

D. medinensis is a large filarial worm that causes Dracunculiasis or Guinea worm disease and reported in humans, dogs, cats, horses, cattle, and other animals in Africa and Asia. A similar species of the Dracunculus genus, D. insignis, is a parasite which causes Dracunculiasis in dogs, raccoons, mink, fox, otter, and skunks of North
America. Its vector is a fresh water copepod and its life cycle differs from that of a typical Filaroids. Instead of invading the tissues the microfilariae are liberated directly from the uterus of the female worm into water. The female worm migrates to the lower leg or foot and an ulcer is formed from which the microfilariae are discharged. These ulcers often become infected with bacteria resulting in cellulitis or if in the vicinity of a joint, arthralgia. The worm causes ulcers on the lower leg or foot, which often become infected with bacteria. Chronic inflammation of the joints can lead to stiffness and permanent disability (McMahon and Simonsen, 1996; WHO, 2010).

4. LIFE CYCLE OF FILARIAL PARASITES IN HUMANS

Filarial parasites (filaroids) are long hair-like tissue dwelling nematodes. All except the Guinea worm *Dracunculus medinensis* (which uses a copepod) employ arthropods as intermediate hosts. The life cycle of filarial parasite includes an obligatory maturation stage in a blood-sucking insect or copepod, and a reproductive stage in the tissues or blood of a definitive host (Figure 1.5). The adult parasite worms, male and female, live in the lymph vessels and lymph nodes by making nest in the dilated lymphatics. The adult worms survive for about 5-8 years and sometimes for as long as 15 years (Figure 1.3). After mating, the female worm parturates millions of microfilariae which finally migrate to blood circulation. The sheathed microfilariae begin to appear in the blood circulation in six months to one year after infection (prepatent period). The microfilariae remain in the arterioles of the lungs during the day and emerge into the peripheral circulation at night (nocturnally periodic). The periodicity of mf coincides with the biting activity of the vector. The sexual cycle of the parasite takes place in the human host, where the adult worms ultimately die. The life cycle of the parasite is cyclodevelopmental in the vector where the parasites do not multiply.

Figure 1.3 : Morphological differences between *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. 
Although very similar in morphology, *W. bancrofti*, at left, has no nuclei in the tail, whereas *B. malayi*, center, and *B. timori*, not pictured, have nuclei that extend to the tip. Nuclei in the tip of the tail can be seen as swelled areas, highlighted by arrows in the right picture.

Microfilariae, (when picked up by the mosquito during blood meal) undergo development in mosquitoes (intermediate hosts) to form infective larvae which usually takes about 10 to 14 days. The ingested microfilariae first shed their sheaths, penetrate the stomach wall, migrate to the muscles of the thorax and develop there without multiplication. The slender and tiny microfilariae (mean length of mf in Wb 290 µ, Bm 222 µ and Bt 310 µ) transform into immobile and inactive sausage stage (L1) larva, which has a cuticle that forms a conspicuous slender tail with specific identification characters. The larvae grow rapidly in length and breadth after their first moult to become L2 or pre-infective larva, which is recognised by the presence of one or two papillae at its caudal end and by its short tail. This L2 stage mouls to become L3 which is infective. It is slender and thread like, measuring about 1500-2000 microns in length. It is highly motile which is a unique phenomenon used for identification (Figure 1.4).

**Figure 1.4 : Different stages of filarial parasite larva in mosquito**

![mf in mosquito vector](image1)

![L1 in mosquito vector](image2)

![L2 in mosquito vector](image3)

![L3 in mosquito vector](image4)
When the infective mosquitoes (harbouring L3 larvae) bite, some or all of the infective larvae escape from the proboscis and actively enter the human host through the wound made by the mosquito bite or penetrate the skin on their own and migrate into lymphatic system (McMahon and Simonsen, 1996). Unlike malaria, the infective stage is not directly injected into the skin of the new host. It is deposited onto the skin whilst the mosquito is feeding and finds its own way through the skin, usually via the puncture made by the mosquito. In the lymphatic system of the infected persons, the infective larvae develop into adult male and female worms (Figure 1.5).

Figure 1.5 : Life cycle of filarial parasite in humans (adapted from http://en.wikipedia.org/wiki/filariasis).

WOLBACHIA ENDOSYMBIONT

Several studies have demonstrated presence of Wolbachia, bacterial endosymbionts in the adult filarial worms and microfilariae of both W. bancrofti and B. malayi. This bacterium is necessary for the development, viability and fertility of the adult parasites. Drug interventions directed against Wolbachia cause deleterious effect on the survival of the adult worms.
5. CLASSIFICATION OF LYMPHATIC FILARIASIS:

It has been reported that the three major groups of people are found in a filarial-endemic area which are associated with specific symptoms and immune responses (Evan *et al.*, 1993; Ottesen, 1993.)

5.1. Asymptomatic microfilaraemic (Mf positive): Those people who have microfilariae (Mf) in their bloodstream and generally showed no outward signs of filarial disease. Maizels and Lawrence, (1991) have suggested that the Mf positive people are likely to harbour fecund adults in the lymphatics and appear to be immunologically tolerant to the parasite. They are susceptible to infection and have immunological responses described as modified T helper 2 (TH2)-cell responses. They have TH2-cell responses, with low levels of TH1 cells responses, with low levels of TH1 cells responses, and express high levels of interleukin-10 (IL-10) (Mahanty and Nutman, 1995), which might indicate a strong regulatory T (TReg)-cell activity. The TH2-type antibody profiles are dominated by the IgG4 isotype with relatively little TgE (Kurniwan *et al.*, 1993). These individuals often have clinically silent infections and are the main reservoir for onward transmission.

5.2. Chronic patients (CH): the second group of individuals displayed chronic pathology, such as lymphoedema, hydrocele and elephantiasis. Individuals of this group are generally amicrofilaraemic. In chronic patients, strong type-1 immune responses are associated with lymphatic inflammation. This leads to pathological outcomes, such as elephantiasis, which are caused by the failure of lymphatic drainage and secondary infection. In such cases, it would be expected that a low activity of regulatory T cells occurred (Maizel and Yazdanbakhsh, 2003).

5.3. Endemic normal (EN) or putatively immune (PI): This group represents the population who, are continually exposed to infective mosquito bites but remain both symptom and Mf-free, suggesting that they may be immune to invading L3 infection (Maizels and Lawrence, 1991). This group has well-balanced immune responses which are characterised by the presence of TH1 and TH2 cell responses. These responses are controlled due to the presence of T Reg-cell activity. It is imagined that the balanced TH1 and TH2 cell responses are of sufficient magnitude to kill the invading helminthes. This is also reflected in a less skewed distribution of IgG4 and IgE isotypes in the TH2-type antibody profile.
6. LYMPHATIC FILARIASIS: CLINICAL FEATURE

The majority of infected people exhibit few if any obvious clinical signs, even though they can have microfilariae in their peripheral blood. Man is the natural host. All ages and genders are susceptible to infection. In endemic areas, the youngest age recorded with filarial infection was infant aged 6 months. The infection increases with age reaching a peak between 20 and 25 years. Disease manifestation appears in a small proportion of infected individuals, commonly over 10 years of age. The disease spectrum of LF ranges from the initial phase of asymptomatic microfilaraemia to the later stages of acute, chronic and occult clinical manifestations.

6.1. Asymptomatic Parasite Carrier State

Some of the infected individuals continue to harbour the parasite for many years without any sign and symptoms of disease. Even at this stage subclinical changes like lymph vessel dilation and tortuosity are shown by ultrasonography and lymphoscintigraphy. Only some among these infected asymptomatic individuals progress to clinical disease in course of time.

6.2. Acute Disease

1. Adenolymphangitis:

   (i) Acute dermato-adeno-lymphangitis (ADLA)

   (ii) Acute filarial lymphangitis (AFL)

2. Acute epididymo-orchitis and funiculitis:

   6.2.1. Adenolymphangitis

6.2.1.1. Acute dermato-adeno-lymphangitis (ADLA): Attacks of ADLA associated with fever and chills are the common acute manifestations for which the patients seek medical intervention. It occurs both in early and late stages of the disease progression, it is more frequent in higher grades of lymphoedema. The affected area, usually in the extremities is extremely painful, warm, red, swollen and tender, the draining lymph nodes in the groin or axilla become swollen and tender. There may be lymphangitis, lymphadenitis, cellulitis or abscess. Depending upon the precipitating factors, the frequency and duration of each episode vary. Entry of bacteria and pathogens through the lesions of the affected parts is responsible for the acute episodes. Retrograde lymphangitis often occurs after 4 to 8 hours. There is centrifugal redness, pain and heat
over the course of the lymph vessels. Pyogenic lymphangitis proceeds centripetal, not centrifugal. In most cases, the symptoms last for 3-4 days.

6.2.1.2. Acute filarial lymphangitis (AFL): At the location where adult worms die, small tender nodes are formed either in the scrotum or along the lymphatics of the limbs. Lymph nodes may become tender. Inflamed large lymphatics may stand out as long tender cords underneath the skin, usually along the sides of chest or medial aspect of arm, with restriction of movement of the affected limb. But these episodes are not associated with fever, toxaemia or evidence of secondary bacterial infection. Rarely abscess formation may be seen at the site of dead adult worms. This acute manifestation is directly caused by adult worms and is usually rare. This may occur due to death of adult worms either spontaneously or by antifilarial drugs.

6.2.2. Acute epididymo-orchitis and funiculitis: Inflammation of structures in the scrotal sac may result in acute epididymo-orchitis or funiculitis in bancroftian filariasis. This is characterised by severe pain, tenderness and swelling of scrotum usually with fever and rigor. The testes, epididymis or the spermatic cord may become swollen and extremely tender. This manifestation is also precipitated by secondary infections.

6.3 Chronic Disease

Lymphoedema, hydrocele, elephantiasis and chyluria are the main clinical pathological consequences of chronic bancroftian filariasis.

6.3.1. Involvement of Limbs

Lymphoedema of the extremities is a common chronic manifestation of LF, which on progression leads on to elephantiasis. The skin is then markedly thickened and can become wart-like. The oedema is "non-pitting" because there is also a proliferation of connective tissue. The tissue is fibrotic and hard. Gross increase in volume in a lymphoedema with dermatosclerosis and papillomatous lesions causing elephantiasis. Recurrent erysipelas (bacterial superinfection) can cause the elephantiasis to increase still further. Brugia infections mostly cause elephantiasis confined to the genitalia, lower legs and lower arms.

In the advanced stages of lymphoedema, the skin is thickened and thrown into folds, often with hypertrichosis, black pigmentation, nodules, warty growth, and Intertrigo in the webs of toes or chronic non-healing ulcers.
6.3.2. Genito-urinary Involvement

Hydrocele
Chylocele
Lymphoedema of the scrotum and penis
Lymph scrotum

Hydrocele is a common chronic manifestation of bancroftian filariasis in males. This is characterized by accumulation of fluid in the tunica vaginalis, the sac covering the testes (orchitis). This is very common in an endemic region. Microfilariae are often found in hydrocoele fluid. The swelling gradually increases over a period of time and in long standing cases, the size of the scrotum may be enormous. Lymphoedema of the scrotum and penis may occur in bancroftian filariasis. Lymph from the scrotum and the greater part of the penis drains towards the superficial inguinal lymph node that from the glans goes principally to the deep inguinal nodes while from the testis it flows to the pre-aortic and retroperitoneal lumbar lymph nodes. In some subjects, the skin of the scrotum may be covered with vesicles distended with lymph known as ‘lymph scrotum’. These patients are prone for ADLA attacks involving the skin of genitalia.

Chronic epididymitis, funiculitis (inflammatory) swelling of the spermatic cord, and lymphoedematous thickening of the scrotal skin are also genital manifestations of chronic filariasis. These manifestations are uncommon with brugian filariasis.

6.3.3. Other Manifestations

The other manifestations include chyluria, hematuria, Tropical Pulmonary Eosinophilia (TPE) and Filarial granulomata.

6.3.3.1. Chyluria: It is defined as the excretion of chyle in the urinary tract. The basic pathophysiology is related to blockage of the retroperitoneal lymph nodes below the cisterna chyli with consequent reflux and flow of the intestinal lymph directly into the renal lymphatics, which may rupture and permit flow of chyle into the urinary tract. The resultant “milky urine” contains considerable quantities of lymph originating from the gastro-intestinal tract. Long-term extensive chyluria results in hypoproteinaemia. The condition is usually painless but large amounts of dietary lipids, proteins, and possibly fat soluble vitamins are excreted leading to weight loss.
6.3.3.2. Occult filariasis and Tropical Pulmonary Eosinophilia: It is the condition in which the classical clinical manifestations are not present and where microfilariae are not found in the blood but may be found in the tissues. TPE is the classical example of occult filariasis. TPE associated with high eosinophil counts in the peripheral blood is an occult manifestation of both *W. bancrofti* and *B. malayi* filariasis. This syndrome is characterized by severe cough and wheezing (especially at night), diffuse mottled pulmonary interstitial infiltrate, peripheral blood eosinophilia > 2500 cells/µl, extreme elevation of immunoglobin (IgE), extreme elevation of anti-filarial antibodies and dramatic clinical improvement in response to specific antifilarial chemotherapy with diethylcarbamazine (DEC).

7. MAJOR PROBLEMS AND CHALLENGES FOR THE DISEASE CONTROL OF FILARIASIS

Though effective on larval stages, Mass Drug Administration (MDA) is fairly ineffective at killing adult worms and provides only partial benefit to infected patients. Efforts to alleviate suffering and disability of infected patients focus on hygiene aimed at decreasing secondary bacterial and fungal infection. In 2000, control efforts were formalized as the Global Program to eliminate Lymphatic Filariasis (GPELF) by the year 2020 through the distribution of drugs (Molyneux and Zagaria, 2002; WHO, 2012). The aims of this global program are to eliminate transmission of the disease and prevent morbidity in affected individuals through the use of antifilarial drug in endemic populations (WHO, 2012). The treatments for larval stages are fairly ineffective to kill adult worms and provide only partial benefit to infected patients. The strategies underlying the global programme are well documented and recently reviewed by Ottesen *et al.* (1997) and Gyapong *et al.* (2005). Data show that 336.6 million people were treated. In 2009, the total number of people treated in the region was 396 million. In 2010, people in all endemic countries except Brazil received combination therapy comprising diethylcarbamazine citrate (DEC) plus albendazole, or ivermectin plus albendazole. In the countries where the 2-medicine combination was distributed, about 130 million children aged 2–14 years received treatment through GPELF. These recommended strategies were (1) MDA with single dose of Albendazole and ivermectin tablets in areas where LF is co-endemic with onchocerciasis for 4-6 years annual, (2) In non onchocerciasis co-endemic areas single dose annual albendazole and DEC for 4-6 years, (3) Use of DEC tablets or DEC fortified salt for 1-2 years (4) vector control
measures, (5) home based management of lymphoedema and elephantiasis for affected individuals, (6) improved access to surgical intervention for men with hydrocele. All the currently used drugs are effective only on larval stages of parasite while are fairly ineffective at killing adult worms and provide partial benefit to infected patients. Efforts to alleviate suffering and disability for infected patients focus on hygiene aimed at decreasing secondary bacterial and fungal infection. Despite these admirable global efforts, eradication of filarial diseases will be extremely challenging with current technology. Current drugs can effectively eliminate the worm’s larval stages, but their broad use also increases the likelihood of accelerated drug resistance. The requirement for ongoing annual dosing to prevent the build-up of new larvae from the surviving adult worm is a significant operational challenge in endemic regions subject to poverty and civil unrest.

Early diagnosis and treatment play important role to interrupt transmission of infectious diseases and preventing the development of long-term complications. The useful diagnostic methods must be accurate, simple and affordable for the population where they are intended. Despite their importance, diagnostics have been an undervalued component of disease control and prevention. An ideal diagnostic test for filariasis should have the following characteristics: high sensitivity and specificity across a wide range of parasite prevalence levels and parasite loads; availability both in a field-user-friendly format and for use in laboratory-based batch testing; availability as a standardized quality assured kit; and cost effective and affordable to those countries where it will be used.

8. DIAGNOSIS OF LYMPHATIC FILARIASIS
In heavy *W. bancrofti* infection, the night blood examination is the definitive diagnosis method to detect microfilariae in the thin blood smear stained by Giemsa. In lighter infections, methods include filtering blood with a 0.45µm pore size nucleopore filter, and the stained with Giemsa. In the case of very light infection, 1ml of blood is preserved in 9ml of 1% formaline and then concentrated by centrifugation. The pellet contains RBC (red blood cell) and microfilariae can be used to examine microscopically to detect microfilariae by staining smear. Because of the nocturnal periodicity of *W. bancrofti*, it is best to draw blood during the customary hours of sleep.
(usually between 22:00 and 02:00 hours). It is also possible, to take blood sample in day time using 1mg of DEC per kg of body weight, known as ‘DEC provocation test’. In this method migration of microfilariae provoked by administering 1mg of diethylcarbamazine to an adult patient and collecting blood sample 45-60 minutes later. In the serological test, circulatory *W. bancrofti* antigen detection have made their way into clinical environment. Generally, monoclonal antibodies are used for detection of filarial antigens in the blood sample. On the basis of various literatures, filarial infection can be diagnosed by following ways-

1. Demonstration of microfilariae.
   a. Direct techniques, and
   b. Concentration technique
2. Detection of filarial antigen
3. Detection of filarial antibodies
4. Skin tests with filarial antigen
5. Detection of adult worms
6. Detection of parasite DNA by DNA probes or the PCR

**8.1. Demonstration of microfilariae**

**8.1.1. Direct Detection of Microfilariae**

Diagnosis of LF is dependent upon the detection of microfilariae in blood collected around midnight in areas where microfilariae exhibit nocturnal periodicity and around midday where periodicity is diurnal. Simonsen *et al.* (1997) have devised a method to adjust the effect of sampling time on microfilariae density which helps to predict the microfilariae density at midnight in blood collected at 22:00 hours. Thick blood film of capillary blood stained with Giemsa stain is one of the simplest and old method (Khamboonruang *et al.*, 1987; Schultz, 1988; Sabry, 1992), but still widely used method is that of Knott (1935). In this method 1 milliliter of blood is added to 9 ml of a 1% formalin solution in normal saline. After red cell lysis, the mixture is centrifuged and the deposit is examined for microfilariae. As the formalin preserves the microfilariae, Knott's tests can be set up in the field. Melrose *et al.* (2000) improved the Knott's method by adding a small amount of Triton X-100 to the diluent which
dissolves most of the proteinous deposit and enhances the visibility of the microfilariae. However, detection of microfilariae in night blood examination by thick blood smear has some disadvantages; first, it underestimates the prevalence of microfilariaemia at low density (Panicker et al., 1991; Turner et al., 1993) and the second disadvantage is loss of microfilariae from the film during processing especially if anti-coagulated blood is used. Loss of microfilariae in the thick blood smear methods is reported up to 51% (Southgate, 1973) and 31% Denham et al. (1971). This loss of microfilariae is minimized if non-anticoagulated blood is used and the films are dried overnight at room temperature (Goldsmid et al., 1976 a,b; Partono and Idris, 1977). Youssef et al. (1995) have applied a thin film of agar to the thick film before staining which greatly reduces the loss of microfilariae. The thick blood film does lack sensitivity when microfilariae density is low but is still a useful and cheap technique for survey work were other more sensitive techniques are too expensive (Moulia-Pelat et al., 1992) or when it is difficult for cultural or other reasons to obtain venous blood. Eberhard et al. (1988) has suggested that more microfilariae are present in capillary blood which may be an advantage to detect microfilariae at low density. The acridine orange staining and fluorescence microscopy has been used as an alternative to Giemsa staining (Goldsmid et al., 1976a,b) and various combinations of stains have been used to demonstrate the internal structure of microfilariae (Laurence and Simpson, 1969).

8.1.2. Concentration methods for microfilariae

The concentration techniques can be used where direct techniques generally fail to identify patients with low parasite density (Weller and Wheeldon, 1982). Counting chamber techniques using various diluents have been used for counting microfilariae by several investigators (Denham et al., 1971; Southgate 1973; Sucharit and Vutikes, 1975). Membrane filter technique is another widely used concentration method, in this 1-5 ml of blood which has been diluted in water is passed through a filter fitted with a polycarbonate membrane which traps the microfilariae on the filter (Nathan et al., 1982; Moulia-Pelat et al., 1992). Denis et al. (1976) suggested that, the volume of blood may increased from 1ml to 5ml to increase the sensitivity of the technique to allow day time blood for screening of microfilariae in areas where there is nocturnal periodicity alleviating the necessity of night surveys.
8.1.3. The diethylcarbamazine (DEC) provocation test

DEC can be helpful to detect microfilaria in the day time. 1mg of DEC per kg of body weight of the subject can provoke microfilariae to leave the lungs and entering the peripheral circulation where they can be detected by any of the above techniques (WHO, 1987). The use of this test was discouraged due to its low sensitivity than night blood examination method (WHO, 1987). The other antifilarial drugs ivermectin and albendazole do not induce microfilariae to leave the lungs and enter in the blood circulation during day time (Dunyo et al., 1996).

8.2. Filarial Antigen Detection

Filarial antigenicity is associated with active filarial infection (Hamilton, 1985). Several investigators have developed assay for detection of filarial antigens using both polyclonal and monoclonal antibodies raised against various antigens such as in *L. carinii*, *D. immitis*, *B. malayi*, *S. digitata* (Dasgupta et al., 1984; Hamilton, 1985; Harinath, 1986) and *W. bancrofti* (Weil and Lifits, 1987). Anti rabbit *B. malayi* adult worm antigens can be used for the detection of *W. bancrofti* microfilariae antigen (Hamilton and Scott, 1984). In the earlier reports, polyclonal antibody of *B. malayi* adult soluble antigen was useful for the detection of filarial antigen in 90-93% of microfilariaemic and 30% of clinical filarial sera (Cheirmaraj et al., 1992). Zheng and coworker (1987) showed higher level of filarial antigen detection (>95%) in microfilariaemic and clinical filarial (60%) sera of bancroftian and brugian filariasis using polyclonal antibodies along with a monoclonal antibody raised against *B. malayi* antigen.

Diagnosis of *W. bancrofti* infection has been revolutionized in recent years by the introduction of various filarial antigen detection tests. Double sandwich ELISA (Trop Bio Og4C3 Antigen Test, produced by Trop Bio Pty Ltd. Townsville, Queensland, Australia) based on the assay developed by More and Copeman (1991) is a good marker of active filarial infection with adult worms (Chanteau et al., 1994) and its antigenicity showed no significant nocturnal or diurnal variation, which suggested that blood can be taken at any time of day or night for detection of filarial antigens (Moulia-Pelat et al., 1993). Blood sample taken onto filter paper strips can be used for Og4C3 assay. The sensitivity of the Og4C3 assay may vary on the basis of amount of blood (from 20µl to 1ml) used for detection of microfilariae. The sensitivity of Og4C3 may
reduce at very low density of microfilariae (Rocha et al., 1996). At microfilarial densities of <1, 1 to 30 and >30 the sensitivity was found 72.2, 97.6 and 100% respectively. The majority of the field studies have shown the sensitivity of the Og4C3 ELISA to be of 94-100% (Freedman, 1998). ICT Diagnostics (Balgowlah, New South Wales, Australia) devised the ICT filarial antigen card test (Binax), a rapid immunochromatographic technique using specific monoclonal and polyclonal antibodies attached to colloidal gold, is used as W. bancrofti detection kit. It utilizes capillary or venous blood and is simple enough for field use by people with a minimum amount of training (Weil et al., 1997). The efficacy has been revived with compare to ICT and thick blood film and membrane filtration (Phantana et al., 1999) and obtained the following results: Sensitivity 100%, specificity 96.3%, predictive value positive 70.7%, predictive value negative 100%.

Detection of filarial antigen in body fluids other than blood

Needham et al., (1996) showed a novel approach for potential filarial diagnosis through the detection of antibodies in saliva. The saliva sample can be used for detection of filarial antigens with similar method used for blood, but this would be a much less invasive test than blood collection and would not expose health workers to the danger of blood-borne pathogens such as HIV and hepatitis B and C. Das et al. (1987) reported the use of urine sample for detection of potential filarial antigen. This anomaly is thought to be caused by the formation of antigen-antibody complexes in the circulation (Das et al., 1987), which should not interfere with detection of filarial antigen by Trop Bio test however as that assay includes a procedure to disassociate antigen-antibody complexes (More and Copeman, 1991).

8.3. Filarial Antibody Assays

Various methods are used to detect filarial antibody: Complement fixation, indirect haemagglutination, gel diffusion, immunoelectrophoresis, counter current immunoelectrophoresis, indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA) (Ambroise-Thomas, 1980). Fortunately, there is marked cross reactivity between filaroid species (Tandon et al., 1981) and wide range of other crude filarial parasites antigens have been utilized for filarial antibody detection. Many investigators have reported that cross reactivity of Dirofilaria immitis crude antigen with W. bancrofti (Ambroise-Thomas, 1980; Turner et al., 1993) and B. malayi serum
(Riyong et al., 2005). Dissanabake and Ismail (1980) reported that antigenic cross reactivity of Setaria digitata with surface antigen of W. bancrofti microfilariae and W. bancrofti infected serum antibody. There are several reports showing that Litmosoides carinii (Tandon et al., 1981; Rajasekariah et al., 1986) and Setaria cervi (Tandon et al., 1981; Almeida et al., 1990a,b; Sharma and Rathaur, 1999) cross reacts with human filarial infected individuals sera. Excretory secretory product and surface antigen of D. immitis used for the detection of W. bancrofti infection by ELISA (Dekumyoy et al., 2000).

Wide range of crude filarial parasites antigens have been utilized for filarial antibody detection: D. viteae (Rajasekariah et al., 1986), L. carinii (Rajasekariah et al., 1986), S. cervi (Almeida et al., 1990a,b), D. immitis (Turner et al., 1993) and B. malayi (Terhell et al., 1996). Other antigens under evaluation for filarial antibody detection include a chitinase-like recombinant antigen from W. bancrofti (Dissanayake et al., 1995), a cloned antigen with IgG4 specificity (Dissanayake et al., 1992), recombinant B. malayi antigen (Ramzy et al., 1995), a highly specific antigen from W. bancrofti third stage larvae (Burkot et al., 1996), fractionated urinary antigen (Ramaprasad and Harinath, 1995), fractionated circulating filarial antigen (Cheirmaraj et al., 1992). Antibodies to recombinant paramyosin from B. malayi can be used as a marker for adult worms of that species (Langy et al., 1998).

However, currently almost all filarial antibody studies utilize ELISA. A wide range of helminthes like Ascaris suum (Willenbucher et al., 1993), Ascaris lumbricoides (Kagan, 1963; Paranjape et al., 1985; Gam et al., 1987; Murthy et al., 1993; Rahmah et al., 1994), Trichuris trichiura (Rahmah et al., 1994), hook worm (Gem et al., 1987), Strongyloides (Gem et al., 1987; Rahmah et al., 1994), Fasciola hepatica and Schistosoma mansoni (Rahmah et al., 1994) and Trichinella spiralis (Kagan, 1963) cross reacted with anti-filarial IgG antibody due to shared phosphocholine antigens (Ambroise-Thomas, 1974; Chanteau et al., 1995). The degree of cross-reactivity between filaroid and other parasites vary between different species of filaroids like L. carinii and Ancylostoma, Ascaris, Taenia and amoeba did not showed any cross reactivity (Chanteau et al., 1991). The cross reactivity limits the usefulness of IgG antibody in filarial diagnosis (Chanteau et al., 1991). The assay of filarial IgG4 antibodies greatly increases specificity and enhances the diagnostic ability of the test (Kwan-Lim et al., 1990; Haarbrink et al., 1995; Chanteau et al., 1995;
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Terhell et al., 1996). Cross reactivity limits the usefulness of IgG antibody in filariasis diagnosis but they may be of some value in communities where parasites other than W. bancrofti are absent or rare (Chanteau et al., 1994) and have been used to diagnose adult filariasis in Indian children (Chaturvedi et al., 1995). Because humans are not able to synthesise anti-phosphocholine or anti-carbohydrate antigen IgG4 (Maizels et al., 1987; Scott et al., 1987; Lal et al., 1991; Maizels et al., 1995) the assay of filarial IgG4 antibodies greatly increases specificity and enhances the diagnostic ability of the test (kwan-Lim et al., 1990; Rahmah et al., 1994; Haarbrink et al., 1995; Chanteau et al., 1995; Terhell et al., 1996). Mahanty et al. (1994) have shown that antifilarial IgG4 is a good index of the intensity and duration of filarial exposure in endemic populations. Recently filter paper collection technique has been used for detection of filarial IgG4 antibody (Chanteau et al., 1994). In some cases anti-filarial IgM antibody (Ata et al., 1993), IgA antibody (Chanteau et al., 1992) was used for filarial diagnosis, but the specificity with IgG was higher.

Anti-filarial IgG4 antibody is produced in abundance during filarial infections especially in asymptomatic microfilaraemic carriers and unlike broad-spectrum IgG antibody shows little cross-reaction with non-filaroid helminths (Lal and Ottesen, 1988; Turner et al., 1993; Terhell et al., 1996). An antibody detection dipstick test, named Brugia Rapid (BR), that detects IgG4 antibodies reactive to a recombinant B. malayi antigen (BmR1), has been recently developed and appears to be a promising tool for mapping and monitoring areas where brugian filariasis is endemic (Rahmah et al., 2001; Rahmah et al., 2003). The same test can be used to detect IgG4 antibodies against the rarer B. timori, in human sera (Supali et al., 2004).

8.4. Filarial-Specific Enzymes Assay

Filarial-specific enzymes viz., acetylcholinesterase (Misra et al., 1993), glutathione binding proteins, glutathione S-transferase, proteases and superoxide dismutase (Beuria et al., 1995; Bal and Das, 1995; Bal and Das, 1996) have been characterized and show diagnostic potential either as antigens for antibody assays or by the detection of the enzyme itself.

Filarial glutathione binding protein, glutathione-s-transferase, proteinases and superoxide dismutase have been detected in the serum of filarial-infected cattle and humans (Beuria et al., 1995) and the latter has been shown to be strongly antigenic.
8.5. Adult Worms Detection

Noninvasive techniques like ultrasonography is also been used to detect adult worms in the scrotum and breast (the ‘filarial dance sign’) and has detected viable worms in children (Dreyer et al., 1996; Dreyer et al., 1999). This noninvasive technique shows great promise in detecting macrofilaricidal activity of candidate filaricidal agents (Mand et al., 2003). Ultrasonographical examinations of onchocercomas where living adult filariae can be displayed may serve as a new tool for the longitudinal observation in vivo of patients with onchocerciasis undergoing treatment and as an adjunct to histological evaluation (Mand et al., 2005). However, most of these techniques are specific for W. bancrofti, therefore, macrofilaricidal effects of any drug used against brugian infections is based on indirect evidences. Parallel animal studies are therefore necessary to evaluate the actual results of drugs administered against adult brugian parasites.

8.6. Detection of parasite DNA by DNA probes or the polymerase chain reaction (PCR)

8.6.1. Detection of Parasitic DNA by DNA Probes

In the late 1980s and early 1990s, many attempts were made to establish the potential capabilities of techniques based on DNA probes for the detection and diagnosis of infectious agents including those causing human lymphatic filariasis. Poole and Williams (1990), using a $^{32}$P-labelled probe, described an assay for species-specific detection and quantification of filarial parasites of the genus Brugia in human blood samples. The DNA assay showed comparable sensitivity and reliability to the mf concentration/staining method used as the control in their study. While several studies reported species-specific DNA probes for Brugian filariasis (McReynolds et al., 1986; Williams et al., 1988; Poole and Williams, 1990; Williams et al., 1993), few studies have been carried out with species-specific DNA probes for W. bancrofti (Dissanayake and Piessens, 1990; Siridewa et al., 1994; Ramzy, 2002).

8.6.2. Demonstration of parasite DNA by PCR

The PCR method has been successfully used for the diagnosis of filarial infection using W. bancrofti and B. malayi DNA. W. bancrofti DNA used in sputum (Abbasi et al., 1996; Abbasi et al., 1999), blood, plasma and urine (McCarthy et al., 1996). The B. malayi DNA in blood used for diagnosis of its active infection (Lizotte et
PCR methods have been successfully used for the detection of filarial DNA in humans and mosquito vectors (Bockarie et al., 2000; Fischer et al., 2002; Fischer et al., 2003), *W. bancrofti* DNA in blood, plasma, paraffin-embedded tissue sections (McCarthy et al., 1996) and sputum (Abbasi et al., 1999), and *B. malayi* DNA in blood (Rahmah et al., 1998) and urine (Lucena et al., 1998). PCR is also used for detecting *W. bancrofti* larvae in mosquitoes (Furtado et al., 1997). PCR based molecular techniques have been used to detect *W. bancrofti* in mosquito (Farid et al., 2001). Kluber et al. (2001) have reported the use of polymerase chain reaction-enzyme-linked immunosorbent assay (PCR-ELISA) for the detection of *B. malayi* DNA from blood spot by DNA detection test strips. A combination of PCR-ELISA, for detection of filarial DNA, has been devised by Fischer et al. (1999). While Ganesh et al. (2001) reported the application of dot-blot assay using the *B. malayi* microfilarial protein antigen for the diagnosis of bancroftian filarial infection in the endemic area. The semi-nested PCR is a specific, sensitive, and suitable technique for detection of the disease carriers. This technique was used for detection of *W. bancrofti* infected patients' blood samples with a long-term storage; the data revealed that all samples were positive (Kanjanavas et al., 2005).

For controlling vector-borne parasitic diseases several approaches have been utilized like: vector control, breaking of vector-host contact by use of repellents and bed nets, vaccination, and chemotherapy. Vector control does have an important part to play in filariasis control and can be very successful in situations where malaria and filariasis have common vectors (Webber, 1991). However, development of insecticide resistance in vectors makes these campaigns less effective (Feng et al., 2002). Despite some promising research, a vaccine for any filarial parasite is not yet a reality (McCarthy et al., 1996). The identification of sensitive molecular targets can provide a more rational approach for the chemotherapy of the parasitic diseases.

**9. CHEMOTHERAPEUTIC APPROACH OF FILARIASIS**

The adult worm is generally considered responsible for the pathogenesis of lymphatic filariasis and considerable effort has gone into the search for a safe and
effective macrofilaricidal agent (Ginger, 1986). The most common antifilarial drugs are diethylcarbamazine (DEC), ivermectin and albendazole (Figure 1.6).

9.1. Diethylcarbamazine

The currently recommended 12-day 72 mg/kg of body weight course of DEC treatment have standard for many years; however, recent data indicate that single-dose treatment with 6 mg/kg of DEC has comparable macrofilaricidal and long-term microfilaricidal efficacy. The 12 day course provides more rapid short-term microfilarial suppression, but when the other factors are considered, including cost, convenience and patient compliance, it seems reasonable to recommend single-dose treatment for individual patient with *W. bancrofti* or *B. malayi* infection. Single-dose treatment can be repeated every 6-12 months for person who remains infected. The half-life of DEC in the blood is only 10-12 hours (Ottesen, 1985).

The mechanism of action of DEC is not well understood. In addition to its antifilarial properties, the drug appeared to have complex and somewhat paradoxical effect on the immune system, cellular adherence, complement activation and arachidonic acid metabolism (Maizels and Denham, 1992). It is unknown whether any of these properties of DEC are related to its antifilarial action (Hawking, 1979; Ottesen, 1985; Maizels and Denham, 1992).

**Side effects and adverse reaction**

Side effects, i.e, signs and symptoms associated with DEC administration regardless of filarial infection status are mild or absent when the drug is given in daily doses of 6 mg/kg (Sasa, 1976). Symptoms of drowsiness, nausea and gastrointestinal upset are observed more frequently as the dosage of the drug is increased (Sasa, 1976; Ottesen, 1985; Dreyer *et al*., 1994a).

Adverse reactions are triggered by DEC in persons with filarial infections, can be either localized (associated with death of the adult worm) or systemic (associated with death of microfilariae). Local adverse reactions usually begin 2-4 days after the first dose of DEC (Dreyer *et al*., 1994b). It may include localized pain and inflammation, tender nodules, adenitis and retrograde lymphangitis (Kenny and Hewitt, 1949; Dreyer *et al*., 1994a). In a small percentage of patients, these reactions are accompanied by acute lymphoedema or hydrocoele. Most, but not all, of the hydrocoele are transient, some may require surgery (Dreyer *et al*., 1995). Biopsies of
the nodules reveal degenerating adult worms surrounded by intense inflammatory infiltrates rich in eosinophils (Dreyer et al., 1994a; Figueredo-Silva et al., 1996). Both the sensitivity of the adult worm to DEC and the severity of local adverse reactions appear to be greater for persons infected with *B. malayi* than for those with *W. bancrofti* infection (Sasa, 1976; Sutanto et al., 1985; WHO, 1992). Supportive therapy for local adverse reactions includes analgesics, cool compresses and rest.

Systemic adverse reactions following treatment with DEC include fever, headache, malaise, myalgias and haematuria (Kenny and Hewitt, 1949; Ottesen, 1985; Dreyer et al., 1994b). These reactions generally begin within 48 hours after beginning the treatment with DEC (Dreyer et al., 1994a) and last 1-3 days. The relationship between the severity of signs and symptoms and the number of circulating microfilaria has been extensively documented (Sasa et al., 1963, Ottesen, 1985; Sutanto et al., 1985).

Sometimes antihistamines have been shown to be ineffective in reducing the severity of systemic adverse reactions (Dreyer et al., 1994b). As with treatment of onchocerciasis, corticosteroids reduce the severity of these symptoms but they also reduce the clearance of microfilaria from the blood (Schofield and Rowley, 1961; Stingl et al., 1988). Because the inflammatory reaction around dying microfilariae can change ocular manifestations in persons with *Onchocerca volvulus* infection, DEC is advised in onchocerciasis infected persons also (Bird et al., 1980; Aziz, 1986). DEC may also cause severe adverse reactions, including encephalitis, in patients with loiasis who have high level of microfilariae (Carme et al., 1991). For these reasons, an attempt should be made to exclude *loa loa* and *O. volvulus* infection before treating persons for lymphatic filariasis if they also may have been exposed to these parasites.

### 9.2. Ivermectin

Ivermectin, a macrolide antibiotic, is the drug of choice for treatment and control of onchocerciasis. A potent microfilaricidal agent, Ivermectin in a single 200-400 mg/kg dose profoundly suppresses the concentration of *W. bancrofti* and *B. malayi* microfilariae in the peripheral blood for periods of 6-24 months (Eberhard and Lammie, 1991; Richards et al., 1991; Addiss et al., 1993; Kazura et al., 1993; Dreyer et al., 1995). Some authors have hypothesized that ivermectin exerts a macrofilaricidal effect (Ismail et al., 1996), but it is clear from recent ultrasound studies that the adult worms
are not killed, even at total doses of 400 µg/kg over a period of 6 months (Dreyer et al., 1995; Dreyer et al., 1996). For this reason, ivermectin is not the drug of choice for treatment of individual patients with *W. bancrofti* or *B. malayi* infection, however, the drug may play a very important role in community-based control programmes, where lack of local adverse reactions associated with death of the adult worm may enhance community acceptance (Sutanto et al., 1985; Ottesen and Campbell, 1994; Moulia-Pelat et al., 1995).

### 9.3. Albendazole

According to Shenoy et al. (1999), this antihelmintic drug is shown to destroy the adult filarial worms when given the doses of 400 mg twice a day for two weeks. The death of the adult worm induces severe scrotal reactions in bancroftian filariasis since this is the common site where they are lodged (Jayakody et al., 1993). Albendazole has no direct action against the microfilaria and does not immediately lower the microfilaria counts. When given in single dose of 400 mg in association with DEC or ivermectin, the destruction of microfilaria by these drugs becomes more pronounced. Albendazole combined with DEC or ivermectin is recommended in the global filariasis elimination programme of combination therapy. The strategy that appears most suitable for the elimination of filariasis in India is the administration of a single annual dose of albendazole 400mg along with DEC 6 mg/kg of body weight.

**Figure 1.6 : Structure of Antifilarial Drugs.**
9.4. Other Drug and Drug Combinations

Several recent studies have explored the effectiveness of co-administration of two drugs for lymphatic filariasis. When given sequentially or in combination, DEC and ivermectin tend to produce more prolonged suppression of microfilariemia than given alone (Addiss et al., 1993; Chodakewitz, 1995; Dreyer et al., 1995; Moulia-Pelat et al., 1995) however, ultrasonography and clinical findings suggest that the macrofilaricidal efficacy of DEC may be reduced when the drug is given simultaneously with ivermectin (Dreyer et al., 1998). Jayakody and colleagues have reported that high-dose albendazole may have a macrofilaricidal effect against *W. bancrofti* (Jayakody et al., 1993). Single dose of albendazole appear to have no significant short-term effect on microfilariemia (Addiss et al., 1997), but they may result in long-term reduction of microfilariemia (Ismail et al., 1998). Simultaneous administration of albendazole (400mg/kg) and ivermectin (200-400mg/kg) appears to result in greater microfilarial suppression than dose ivermectin alone (Addiss et al., 1997; Ismail et al., 1998).

10. BIOCHEMICAL TARGETS IN FILARIAL PARASITES

Current filariasis control strategies are not entirely successful and filarial infections are on the rise. In the absence of availability of antifilarial vaccines, chemotherapy remains the mainstay for treatment of the diseases caused by filarial nematodes. The pathways which have been identified as biochemical targets in filarial parasites are listed below.

10.1. Carbohydrate Metabolism

Filarial parasites have active glycogenic and glycolytic pathways with a somewhat submissive tricarboxylic acid cycle (TCA) (Saz, 1981; Barrett, 1983). This is in contrast to mammals, which have active TCA and electron transport systems. Most adult filarial parasites use the glycolytic breakdown of carbohydrate to lactate as a preferred route to supply their energy requirements (Saz, 1981; Barrett, 1983; Barrett et al., 1986; Dunn et al., 1988; Kohler, 1991). Microfilariae (Mf) exhibit an aerobic carbohydrate catabolism, requiring oxygen for motility. There are also minor catabolic routes, similar to those present in adults that result in acetate and succinate formation by Mf (Srivastava et al., 1988). High levels of enzymes involved in glycolysis has been
demonstrated in *Chandlerella hawkingi*, *D. immitis*, *L. carinii*, *B. pahangi*, *A. viteae* and *O. volvulus* (Srivastava et al., 1970; Srivastava and Ghatak, 1971; Saz and Dunbar, 1975; Anwar et al., 1977; Middleton and Saz, 1979; Walter and Schulz-Key, 1980a,b). Enzymes of the TCA cycle have also been demonstrated in adult *B. pahangi*, *D. immitis*, *C. hawkingi*, *D. viteae*, *L. carinii*, *O. volvulus*, and *S. cervi* (Srivastava and Ghatak, 1971; Anwar et al., 1977; Middleton and Saz, 1979; Walter and Shulz-Key, 1980a,b; Agarwal et al., 1986; Dunn et al., 1988). However, the TCA cycle does not play significant role in energy production (Wang and Saz, 1974; Walter and Van den Bossche, 1980; Dunn et al., 1988).

10.2. Respiratory Metabolism

Since the pathways of respiratory metabolism in the parasites differ from their hosts, this promises to be an important antifilarial target (Behm and Bryant, 1979). Saz and Dunbar (1975) demonstrated that phosphofructokinase as a drug target in *L. carinii*, *D. viteae* and *B. pahangi*. Walter and Schulz-Key (1980a) have demonstrated lactate dehydrogenase (LDH) as a drug target, however complete validation of LDH as a viable chemotherapeutic target could not be achieved due to lack of a suitably specific inhibitor of LDH, effective at low concentrations. Fructose 1,6-bisphosphate aldolase has been characterized in various filarial species (Barrett et al., 1986; Dunn et al., 1988). McCarthy et al. (2002) and its immunogenic component is quite distinct as compared to mammalian counterparts, thus it was identified as a potential vaccine target, but the extent to which it is significant as an antifilarial chemotherapeutic target is still an area of active research.

10.3. Isoprenoid Metabolism

Nematodes do not synthesize the sterol ring, *de novo*, but the early stages of the pathway necessary to provide the polyisoprenoid precursors of sterols are present (Comley and Jaffe, 1981). The filarial isoprenoid biosynthetic pathway leads to the formation of quinones, dolichols, geranyl geraniol, juvenile hormones and purine derivatives of isopentenyl pyrophosphate (Comley, 1985). Quinones have their role in filarial electron transport (Comley et al., 1981), dolichols are involved in glycoprotein synthesis (Comley et al., 1982; Walter et al., 1985), juvenile hormones are regulators of larval development (Mendis et al., 1983) and isopentenyl adenosine is known as the
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constituent of tRNA (Brown and Goldstein, 1980). Comley and Lancaster (1983) had demonstrated HMG-CoA reductase as a target in *B. pahangii*.

10.4. Amino Acid and Protein Metabolism

Amino acid metabolism has received little attention in filarial parasites. Data are restricted mainly to amino acid composition, uptake and incorporation into proteins (Barrett, 1983). Decarboxylation and transamination reactions have also been examined but only limited attempts have been made to elucidate pathways of amino acid catabolism (McManus, 1986). Parasitic helminths are able to take up amino acids from their surroundings through their tegument (Asch and Read, 1975). The functional importance of the free amino acid pool has been indicated in intracellular osmoregulation and protein synthesis (Kurelec and Rijavec, 1966). The incorporation of amino acids into proteins has been demonstrated in a number of filarial worms such as adults of *L. carinii*, *S. cervi* and Mf of *D. immitis* (Jaffe and Doremus, 1970; Anwar *et al*., 1978; Akinwande and Akinrimisi, 1980). Serine hydroxymethyl transferase, an enzyme responsible for the conversion of serine into glycine and vice versa has also been demonstrated in adult *D. immitis* and *B. pahangi* (Jaffe and Chrin, 1981). Sulphur amino acid metabolism of filarial parasites has attracted attention. The identification of a novel, non-mammalian form of cystathionine β-synthase in nematodes may facilitate the selective inhibition of a parasite-specific enzyme (Walker and Barrett, 1997).

10.5. Nucleic Acid Metabolism

Nucleic acids are essential components of all living organisms but relatively little work had been done on nucleotide and nucleic acid synthesis in parasitic filariids. Filarial worms possess both *de novo* and salvage pathways for purines and pyrimidines. Synthetic pathways are usually under tight metabolic control (Barrett, 1983). Incorporation of uridine and uracil into nucleic acids has been reported in adult *D. immitis* (Jaffe *et al*., 1972), whilst the Mf of *D. immitis* incorporates uridine, uracil, adenine and adenosine into RNA (Jaffe and Doremus, 1970). Incubation of adult *B. pahangi* with 5-14C methyltetrahydrofolate resulted in 14C-labelled adenosine and guanine ribonucleotides as well as 14C-labelled inosine monophosphate (Jaffe and Chrin, 1981). The incorporation of label from glycine into DNA and RNA and of inorganic phosphate into RNA has also been reported in adult *L. carinii* (Akinwande and Akinrimisi, 1980).
10.5.1. DNA Topoisomerase II

DNA topoisomerase II is an essential enzyme, which has an important role in DNA replication, repair and transcription, has been identified as a target for the development of antifilarial compounds (Pandya et al., 1997; Tripathi et al., 2001). The 7-O-acetamidyl-4-alkyl-2H-1-benzopyran-2-ones, glycosylated beta-amino acid derivatives, Ru (II) poly-pyridyl hydridocarbonyl complexes, 4-Methyl-7-(tetradecanoyl)-2H-1-benzopyran-2-one; 4-amino-5-cyano-2, 6-disubstituted pyrimidines; 2,4,6-trisubstituted pyrimidine derivatives and unnatural nucleosides were prepared and screened against DNA topoisomerase II of filarial parasite S. cervi (Tripathi et al., 2001; Katiyar et al., 2003; Chandra et al., 2004; Misra-Bhattacharya et al., 2004; Katiyar et al., 2005; Mishra et al., 2005; Singh et al., 2007; Kumar et al., 2008). A novel inhibitor of filarial topoisomerase II was isolated from the culture filtrate of native isolate Micrococcus luteus (Sivasamy et al., 2011).

10.5.2. Folate Metabolism

Folate derivatives are concerned with the transport and interconversion of one carbon units for synthetic reactions. The high synthetic capacities of parasites and the differential sensitivities of certain folate metabolizing enzymes to inhibitors lead folate metabolism a potential area for chemotherapy (Jaffe, 1980). Adult filariae possess an array of enzymes involved in the interconversion of folate analogues (Jaffe and Chrin, 1981; Jaffe et al., 1980; Comley et al., 1981). Dihydrofolate reductase has been demonstrated in a number of adult filarial worms of D. immitis, L. carinii, D. viteae and O. volvulus, and the sensitivity of this enzyme to inhibitors has been investigated (Jaffe, 1972; Jaffe et al., 1972). In contrast to the adults, no dihydrofolate reductase activity was detected in the Mf of B. pahangi or D. immitis (Jaffe, 1972; Jaffe et al., 1972; Jaffe et al., 1977). The enzyme 5,10 methylene FH₄ reductase catalyses the irreversible formation of N⁵-methylene FH₄ from N⁵,N¹⁰-methylene FH₄ in almost all vertebrates. However, in filarial parasites, this enzyme operates preferentially in the reverse direction, favouring the formation of N⁵,N¹⁰-methylene FH₄ (Jaffe, 1980; Comley et al., 1981). Another enzyme of interest is serine hydroxymethyltransferase, which catalyses the synthesis of N⁵,N¹⁰-methylene FH₄ from tetrahydrofolate and requires pyridoxal phosphate (Subrahmanyam, 1987).
10.6. Polyamine Metabolism

The polyamines putrescine, spermidine and spermine are found in all living organisms and are involved in growth, differentiation and macromolecular synthesis (Tabor and Tabor, 1984; Pegg et al., 1988; Svensson et al., 1993). Measurements of polyamine in *O. volvulus*, *D. immitis*, *B. patei*, *S. cervi* and *L. carinii* worms have demonstrated that these parasites contain high levels of spermidine and spermine but low levels of putrescine and N-acetylated polyamines (Srivastava et al., 1980; Wittich et al., 1987). The enzymes of polyamine biosynthesisviz., ornithine decarboxylase (ODC), S-adenosyl methionine decarboxylase (SAMDC) and arginine decarboxylase (ADC) were detected at very low levels or absent in filarial parasites (Wittich et al., 1987; Walter, 1988). Moreover, uptake of polyamines from the incubation medium as well as interconversion and excretion of putrescine and N1-acetylputrescine have been detected in filariids (Wittich et al., 1987; Singh et al., 1989). S-adenosyl-methionine decarboxylase (SAMDC), a key regulatory enzyme in polyamine biosynthesis, is considered as a potentially important antifilarial target. Various inhibitors of SAMDC, such as berenil and aromatic methylglyoxal bis (guanylhydrazone) analogues, might have potential as drug candidates against filarial worms (Dadara et al., 1998).

10.7. Glutathione Metabolism

Glutathione (GSH) is of major importance in filarial species because it has been proposed to constitute the antioxidant system responsible for the long term existence of filarial worms in mammalian hosts by protecting them from the reactive oxygen species produced by normal metabolism and by immune cells of the host (Callahan et al., 1988; Brophy and Pritchard, 1992). Therefore, it seems useful to develop drugs that could selectively deplete or distort glutathione stores in these parasites. The GSH either acts in solo or as a substrate to various enzymes like glutathione peroxidase (GPX), phospholipids hydroperoxide GPX and glutathione-S-transferase (GST) (Zhang et al., 1989; Lomaestro and Malone, 1995) to quench the free radicals. After GSH has been oxidized to GSSG, the enzyme glutathione reductase (GR) accomplishes the recycling of GSSG back to GSH (Carlberg and Mannervik, 1985; Schirmer et al., 1987). The group of six enzymes that are responsible for the synthesis and breakdown of GSH constitutes the γ-glutamyl cycle. At present only two enzymes of the γ-glutamyl cycle viz., glutamate-cysteine ligase (GCL) and γ-glutamyl transpeptidase (γ-GT) have been characterized from filarial species (Singh et al., 1996; Luersen et al., 2000). The whole
of this cycle needs to be characterized in filarial worms, although, it has been well characterized in mammalian systems (Orlowski and Meister, 1973; Werf et al., 1975; Oppenheimer et al., 1979; Kozak and Tate, 1982; Seelig and Meister, 1982). The sensitivity of filarial γ-glutamyl cycle enzymes and their counterparts from mammalian sources to known inhibitors needs to be compared. If marked differences in sensitivity exist this may provide targets for antifilarial drug development.

10.7.1 Glutamate-Cysteine Ligase (GCL)

Glutamate-cysteine ligase is the rate limiting enzyme of GSH biosynthesis which catalyses the ligation of the L-glutamate and L-cysteine to form L-γ-glutamyl-L-cysteine (Plummer et al., 1981). This enzyme has been well characterized in *O. volvulus* and *S. cervi* (Luersen et al., 2000; Tiwari et al., 2003). The *Kᵢ* values of *O. volvulus* GCL for buthionine sulfoximine (BSO) and cystamine were found to be lower than those of the corresponding mammalian enzyme (Luersen et al., 2000). Hence, this enzyme represents a potential target for antifilarial drug development.

10.7.2 Glutathione Reductase (GR)

Glutathione reductase (GR) is also utilized as a drug target. The mode of action of macrofilaricidal arsenicals is principally through the depletion of filarial GSH (Worthington and Rosemeyer, 1974; Krohne-Ehrich et al., 1977; Bhargava et al., 1983). *L. carinii* GR was found to be much more vulnerable to inhibition with arsenical melarsen oxide than the enzyme isolated from human erythrocytes (Bhargava et al., 1983). Similarly, studies on the inhibition of GR by melarsen oxide revealed that human erythrocyte GR is less susceptible to inhibition by the arsenical than the enzyme from two cattle filariae viz. *S. digitata* and *O. gutturosa* along with differences in mechanisms of inhibition (Muller et al., 1995). These differences between host and parasite enzyme might reflect differences in the primary and secondary structures of the proteins that might be exploitable for the design of new specific filaricidal drugs.

10.7.3 Glutathione-S-Transferase (GST)

Glutathione-S-transferases are one of the major detoxification systems ubiquitous among eukaryotes and have been found in a wide range of parasitic helminths (Brophy and Barrett, 1990a). In filarial parasites glutathione-S-transferase (GST) is also considered as a target (Rathaur et al., 2008). Helminths lack the cytochrome P-450 dependent detoxification system (Saxena et al., 1988), enhancing the
requirement for GST activity. They perform functions ranging from catalysing the detoxification of electrophilic compounds to protecting against peroxidative damage (Armstrong, 1991). The compounds that can be detoxified by GSTs include the cytotoxic products of lipid peroxidation, such as lipid hydroperoxides and reactive carbonyls. Which in the absence of GSTs, may cause membrane damage. GSTs are therefore postulated to protect the parasite against host-mediated lipid peroxidation of the membrane (Brophy and Barrett, 1990b; Brophy and Pritchard, 1994). The inhibition of parasite GST(s) thus deprives the parasite of its major defence against oxidative stress and impairs its ability to survive. Filarial GST(s) is therefore important target for antifilarial drug design.

10.7.4. Glutathione Peroxidase (GPX)

The GPX family is well characterized in some filariids. The major soluble cuticular protein of adult *B. pahangi*, gp29 (Maizels et al., 1989), was identified as a secreted variant of a GPX (Cookson et al., 1992). Recombinant *B. pahangi* gp29 was shown to reduce fatty acid and phospholipid hydroperoxides but was not active with $\text{H}_2\text{O}_2$ as the substrate (Tang et al., 1996). The *D. immitis* homologue of *Brugia* gp29, called Di29 on the other hand possessed significant activity with $\text{H}_2\text{O}_2$ (Tripp et al., 1998). The nucleotide sequences encoding homologues of this enzyme were also found in *B. malayi* and *W. bancrofti*. These enzymes belong to the group of selenium-independent GPXs. Selenium containing GPXs, a major family of enzymes in mammals appear to be absent from filarial species (Cookson et al., 1993; Tripp et al., 1998). GPXs protect the filarial worms from oxidative damage, and are thus important targets for novel chemotherapy (Singh et al., 2010).

11. BRUGIA MALAYI GENOME SEQUENCE

Current filariasis control strategies are not entirely successful and filarial infections are on the rise. In the absence of availability of antifilarial vaccines, chemotherapy remains the mainstay for treatment of the diseases caused by filarial nematodes. However, the precise primary effects of different chemical class of compounds currently used as antifilarials are still unclear. Therefore, there is an utmost need to identify new targets for drug development to make it possible to have a repertoire of approaches in combating the disease.
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Genome sequencing of the filarial parasite *B. malayi* has been completed (Ghedin *et al.*, 2007) hence comparative genomics would undoubtedly lead to a greater understanding of filarial evolution, host parasite interaction and biochemical processes of parasite, especially related to those that are crucial in drug and immunological response.

Parasitic nematodes that cause elephantiasis and river blindness threaten hundreds of millions of people in the developing world. Most filarial nematodes, including *B. malayi*, carry three genomes: nuclear, mitochondrial (available at GenBank, accession no. AF538716) and that of an alphaproteobacterial endosymbiont, *Wolbachia*. The ~90 megabase (Mb) genome of the human filarial parasite *B. malayi* was predicted to have ~11,500 protein coding genes in 71 Mb of robustly assembled sequence. The genome of the filarial nematode *B. malayi* is estimated to be comprised of approximately ~19,000 genes (Sim *et al.*, 1987; Blaxter, 2003) and is organized as five pairs of chromosomes; four autosomal and one XY sex determination pair. The filarial genome is highly AT rich (75%), but protein-coding genes tend to be of lower AT content (~65%) than intronic or intergenic regions (~80%). The number of protein coding genes in *C. elegans* is also estimated to be about 19,000 based on genetic and sequence data. In addition to the nuclear genome, *B. malayi* has a mitochondrial genome of about 14 Kb and 1-2 Mb genome of bacterial endosymbiont *Wolbachia*. Comparative analysis with the free-living, model nematode *C. elegans* revealed that, despite these genes have maintained little conservation of local synteny during ~350 million years of evolution; they largely remain in linkage on chromosomal units. More than 100 conserved operons were identified. Analysis of the predicted proteome provides evidence for adaptations of *B. malayi* to niches in its human and vector hosts and insights into the molecular basis of a mutualistic relationship with its *Wolbachia* endosymbiont (Taylor *et al.*, 2000).
1. PENTOSE PHOSPHATE PATHWAY (PPP)

1.1 Principal properties and functions

In most organisms glucose is metabolized through two major pathways: the glycolytic, or Embden-Meyerhof pathway, and the Pentose Phosphate Pathway. The PPP (phosphogluconate pathway or hexose monophosphate shunt) metabolises glucose to generate five carbon sugars and reducing equivalents. The principal function of this pathway is to generate reducing potential in the form of reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) for biosynthesis of fatty acid, steroid, and ribose-5-phosphate (R5P) for the synthesis of the nucleotides and nucleic acids. In erythrocytes, the PPP is the only source of NADPH, which is essential for protecting the cells against oxidative damage. This detoxification effect is achieved through maintaining a high level of reduced glutathione (GSH) and the action of catalase, both of which are dependent on the availability of NADPH.

The reactions of the PPP operate exclusively in the cytoplasm (Figure 1.7). PPP has both an oxidative and a non-oxidative arm. The oxidation steps, the first committed reaction, which is catalysed by glucose 6-phosphate dehydrogenase (G6PD, EC1.1.1.49), converts glucose-6-phosphate (G6P) to 6-phosphogluconolactone with the concomitantly releasing two electrons that reduce one molecule of NADP\(^+\) to NADPH. The ensuing when 6-phosphoglucuronate is decarboxylated to D-ribulose-5-phosphate by 6-phosphogluconate dehydrogenase (6PGDH), releases two additional electrons, which reduce a second molecule of NADP\(^+\). A five-carbon sugar, D-ribulose-5-phosphate, is produced in the reaction. By isomerization, D-ribulose-5-phosphate is transformed into D-ribose-5-phosphate (R5P). To be used in nucleic acid synthesis, R5P is transformed into 5-Phosphoribosyl 1-pyrophosphoric acid (PRPP) by ribose-phosphate diphosphokinase.

The non-oxidative reactions of the PPP are primarily designed to generate R5P. Equally important reactions of the PPP are to convert dietary 5 carbon sugars or D-ribose-1-phosphate generated in the salvage of purines (that can be slowly converted to R5P by phosphoglucomutase) into both 6 (fructose-6-phosphate) and 3 (glyceraldehyde-3-phosphate) carbon sugars which can then be utilized by the pathways of glycolysis. In the first reaction, R5P will accept two carbon atoms from xylulose-5-phosphate (obtained by epimerization of ribulose-5-P), yielding sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate (catalyzed by transketolase). Sedoheptulose-7-phosphate
transfers three carbons to glyceraldehyde-3-phosphate (catalyzed by transaldolase), yielding fructose-6-phosphate (F6P) and erythrose-4-phosphate. Erythrose-4-phosphate then accepts two carbon atoms from a second molecule of xylulose-5-phosphate (catalyzed again by transketolase), yielding a second molecule of F6P and a glyceraldehyde-3-P (GAP) molecule, both of which form a reversible link between PPP and glycolysis and eventually produce ATP. The ribose 5-phosphate is required for nucleotide synthesis and erythrose 4-phosphate a precursor of aromatic amino acids and vitamins (Wood, 1986). The excess D-ribose 5-phosphate can then be recycled to glucose 6-phosphate. Interestingly, this pathway produces free ribitol that is excreted (Panagides and Rothstein, 1973). The advantage to the animals of ribitol production and excretion is not clear. One possible explanation is that it serves as a regulatory mechanism of NADPH production (although ribitol was not excreted by C. briggsae).

Figure 1.7: Pentose Phosphate Pathway (Hexose Monophosphate Shunt)
1.2 Regulation

It is generally believed that the first reaction of a metabolic pathway is rate limiting, and this is also the case regarding G6PD in the PPP. Two mechanisms, the “coarse” control and “fine” control seem to operate in order to regulate the PPP (Levy, 1979).

The “coarse” control is found to be adaptive and tissue-specific (Krebs and Eggleston, 1974; Kletzien et al., 1994). The adaptive regulation in hepatic cells was first reported when rats were fed with excess carbohydrate after being starved (Greenbaum et al., 1971). These tissue-specific differences include variations in the rate of transcription, post-transcriptional processing, mRNA stability, the rate of translation, and the rate of proteolytic degradation (Tepperman and Tepperman, 1963; Piomelli et al., 1968; Persico et al., 1982; Stumpo et al., 1984; Battistuzzi et al., 1985; Fritz et al., 1986; Kletzien et al., 1986). In addition, hormones, nutrients and some growth factors could enhance G6PD expression in adipose tissue and other tissues (Louie et al., 1990; Stabile et al., 1996; Hodge and Salati, 1997). Under oxidative stress, an elevated G6PD activity was found in various human cell lines and primary rat hepatocytes. This observation was mainly due to the increased rate of transcription (Cramer et al., 1995; Ursini et al., 1997). However, this means of control is slow and may not be sufficiently sensitive for human G6PD to respond in time of a sudden increase in metabolic demand.

Gumaa et al. (1971) first identified the “fine” control when the cofactor NADP⁺ was limiting, while the intracellular concentration of the substrate G6P was usually present in large amounts. In 1974, Krebs and Eggleston showed that NADPH was a strong inhibitor of G6PD. The activity of G6PD in rat liver cells was found to be largely dependent on the cytosolic [NADP⁺]/[NADPH] ratio. The inhibition was complete when the [NADP⁺]/[NADPH] ratio reached about 1:9. Under physiological conditions, it appears that the reaction catalysed by G6PD is almost completely inhibited. The actual intracellular G6PD activity was found to be less than two percent of its theoretical activity (Yoshida and Lin, 1973; Kirkman and Gaetani, 1986). Since the total intracellular amount of NADP⁺ + NADPH is relatively fixed, whenever NADPH is oxidised to NADP⁺, this simultaneously provides the substrate NADP⁺ for the G6PD reaction and decreases the inhibition by product NADPH. Considering the usually low basal activity, it is possible for G6PD activity to increase significantly in response to oxidative stress without any change in the number of G6PD molecules. This regulatory control works effectively in response to sudden metabolic demand, particularly in a
highly specialized cell such as the erythrocyte (Levy, 1979). Under physiological concentration of ATP, it inhibited the activity of human G6PD. Several studies showed that oxidised glutathione (GSSG), cooperating with a “cofactor”, could counteract the inhibition in rat liver, kidney and adipose tissues (Krebs and Eggleston, 1974; Nogueira et al., 1986; Garcia et al., 1989). The NADPH is used by the cell as a reducing agent in a variety of biosynthetic processes as well as for the defense against reactive oxygen species by keeping glutathione in its reduced state (Rosemeyer, 1987). Glutathione is the major endogenous cellular antioxidant, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced, active forms.

2. GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD)

Glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate: NADP\(^+\) 1-oxidoreductase; EC 1.1.1.49) was first described by Warburg and Christian in 1931a,b. It catalyzes the transformation of glucose-6-phosphate to 6-phosphogluconolactone concomitant with conversion of NADP to NADPH (Figure 1.7). This enzyme has aroused considerable attention because of its important role in pentose phosphate pathway, its involvement in various haemolytic disorders, its activity variation under different nutritional and hormonal conditions (Tepperman and Tepperman, 1958; Kletzien and Berdanier, 1993; Kletzien et al., 1994; Hodge and Salati, 1997; Farhud and Yazdanpanah, 2008) and its potential as a regulator for the availability of the reduced NADPH required for various biosynthetic processes (Chung and Langdon, 1963). The reducing power produced is necessary for the reductive biosynthesis of fatty acids, isoprenoids and aromatic amino acids in the dark and for nitrogen assimilation in heterotrophic tissues (Turner and Turner, 1980; Copeland and Turner, 1987; Bowsher et al., 1992; Graeve et al., 1994; Hauschild and Schaewen, 2003). The NADPH and pentose phosphates produced also serves as the route of entry of 3-5 carbon sugars to the glycolytic pathway (Danisan et al., 2004). NADPH also plays important functions in the protection of the cell against oxidative agents by transferring its reductive power to glutathione disulphide (GSSG) via glutathione disulphide reductase (Levy, 1979; Debnam et al., 2004; Farhud and Yazdanpanah, 2008; Machida et al., 2010). The tightly controlled concentrations of reactive oxygen species (ROS) and fluctuations in redox potential are important mediators of signaling processes, stress response, development and aging (Schulz et al., 2007; Saxena et al., 1996; Van Raamsdonk and Hekimi, 2010;
Pandolfi et al., 1995). G6PD is a critical cytosolic antioxidant enzyme, maintains the NADPH/NADP⁺ and GSH/GSSG ratio and plays a crucial role in the protection from redox stress induced apoptosis and in cell growth and death. Therefore decrease in G6PD activity and as a result NADPH level will impair the entire antioxidant system (Fico et al., 2004; Zhang et al., 2010; Ciftci et al., 2003; Tian et al., 1999; Filosa et al., 2003). The enzyme is widely distributed and has been isolated from microorganisms, plants and various mammalian tissues (Danisan et al., 2004; Ulusu et al., 2005; Ibraheem et al., 2005; Igoillo-Esteve and Cazzulo, 2006; Demir et al., 2009; Cardi et al., 2011). G6PD is a housekeeping enzyme critical in the redox metabolism of all aerobic cells but it is easily inducible in different conditions (Kletzien et al., 1994; Luzzatto, 2006). Although most organisms have other means to produce cellular NADPH, notably by the enzymes isocitrate dehydrogenase and malic enzyme, the generally important role of G6PD in it is illustrated by the severity of G6PD deficiency in humans. The deficiency may lead to nonimmune hemolytic anemia triggered by oxidative stress as a result of infections or exposure to chemicals such as present in medication or certain foods (Ronquist and Theodorsson, 2007). G6PD deficiency is the most common human enzyme defect, occurring in more than 400 million people worldwide (Cappellini and Fiorelli, 2008). However, all the individuals with G6PD deficiency are not anaemic but it can cause several other disorders such as neonatal jaundice, mild haemolytic anaemia to chronic non-spherocytic haemolytic anaemia with attacks of severe anaemia induced by infections, specific drugs or consumption of fava beans (Yan et al., 2006).

3. IMPORTANCE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Hydrogen peroxides, hydroxyl radicals and super oxides, known as reactive oxygen species (ROS) cause oxidative stress and damage cell membranes (Shihabi et al., 2002; Leopold and Loscalzo, 2005). Decreased antioxidative enzyme activity plays an important role in oxidative injuries of different organs, tissues and cells including vascular cells, heart, brain, (Leopold and Loscalzo, 2005) and causes Alzheimer’s and Parkinson’s disease and also contribute to the aging process (Halliwell, 1992; Tsun-Yee Chiu and Liu, 1997; Savitha et al., 2005; Farhud and Yazdanpanah, 2008). Antioxidative enzymes like glutathione reductase, superoxide dismutase, catalase, peroxidase and G6PD protects the cells against oxidative damage. G6PD is said to be an
essential modulator in the body’s antioxidative defence system that plays a very important role in all cells especially in red blood cells (Leopold and Loscalzo, 2005). G6PD is a key enzyme for maintenance of redox potential in cells (Farhud and Yazdanpanah, 2008). G6PD leads to NADPH production through PPP, which is important as a central reductant and regulator of redox potential (Tian et al., 1999). It also acts as a cofactor for other anti-oxidant enzymes like glutathione reductance (Fico et al., 2004; Leopold and Loscalzo, 2005; Farhud and Yazdanpanah, 2008). Reduced glutathione acts as a cofactor for the glutathione peroxidase and is crucial for neutralization of peroxides and also protects protein sulphhydryl groups against oxidation (Beutler, 1994). Regeneration of reduced glutathione from its oxidized form requires the NADPH produced in the G6PD reaction. NADPH is critical for conversion of inactive form of catalase into active form (Beutler, 1994). Researchers have shown that stimulation of cell growth is associated with increased G6PD activity. It has been found that epidermal growth factor and insulin stimulated cell growth in rat liver cell culture is closely associated with G6PD activity (Yoshimoto et al., 1983; Burdon et al., 1995). Growth hormone induced cell growth, cancer and cultured tumor cells also increased G6PD activity (Sulis, 1972; Schaffer, 1985; Weber, 1987; Kletzien et al., 1994). Cell growth can be stimulated by over expression of G6PD (Tian et al., 1998). Necrosis and apoptosis are the two described patterns of cell death. Necrosis is associated with inflammation whereas the regulated apoptosis have association with nuclear fragmentation and chromatin condensation (Orrenius, 1995). Deletion of G6PD gene leads to death of cells exposed to oxidative stress (Filosa et al., 2003). Inhibition of G6PD activity leads to hydrogen peroxide induced cell death, significant increase in apoptosis, loss of protein thiols and changes in mitogen activated protein kinase phosphorylation (Tian et al., 1999). G6PD plays a crucial role in the protection from redox stress induced apoptosis (Fico et al., 2004). Hence, G6PD plays an important role in cell death by affecting the redox potential (Figure 1.8). G6PD deficiency is associated with a large number of clinical manifestations like acute haemolytic anaemia (Mehta et al., 2000; Prchal and Gregg, 2005), neonatal jaundice (Dennery et al., 2001), malaria (Luzzatto and Bienzle, 1979; Luzzatto 1979; Ruwende and Hill, 1998), etc.
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Figure 1.8: Glucose 6-phosphate dehydrogenase effect on different metabolic pathways of living organisms

4. REGULATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

4.1 Regulation of G6PD by nutritional and hormonal factors

For the production of NADPH and for control of carbon flow through PPP, G6PD is required in each cell and NADPH for fatty acid biosynthesis whereas high levels of fatty acids are present in filarial parasites, G6PD has long been considered as a member of lipogenic enzymes family (Kletzien and Berdanier, 1993; Kletzien et al., 1994). Animal tissues with high levels of fatty acid biosynthesis control the oxidative pentose phosphate pathway at the level of G6PD regulation. In hepatocytes, G6PD is induced in response to excess dietary carbohydrate and is repressed in response to starvation (Levy, 1979; Iritani, 1992). The molecular basis of the elevated activity is not well understood but seems to be dependent upon the presence of insulin, glucocorticoids and a high carbohydrate; low fat diet (Kletzien and Berdanier, 1993; Kletzien et al., 1994). The activity of the enzyme is also affected by the type of dietary carbohydrate.
Higher enzyme activity was found when the rats are fed on a diet with higher percentage of glucose to starch and induction by fructose is higher as compared to glucose (Kastrouni et al., 1984). G6PD activity is inhibited by a high fat, low carbohydrate diet both in intact animal and in primary hepatocytes in culture (Salati et al., 1988; Stabile et al., 1996; Stabile et al., 1998). Polyunsaturated fatty acids are potent inhibitors of lipogenic gene expression in liver. The inhibition of G6PD gene expression is induced by presence of polyunsaturated fatty acid in diet. The inhibition of expression is through a posttranscriptional mechanism which involves decrease in the efficiency of splicing of the pre-mRNA (Tao et al., 2002). The insulin is the primary inducer of G6PD expression, and fatty acids such as arachidonic acid are the primary inhibitors of G6PD expression; this regulation is independent of other hormonal requirements. The insulin stimulation of G6PD involves the phosphoinositide 3-kinase (PI 3-kinase) pathway. The signalling pathway that causes increased expression of G6PD involves insulin activation of phosphatidylinositol 3-kinase pathway resulting in accumulation of G6PD mRNA (TaluKDAR et al., 2005). Polyunsaturated fatty acids such as arachidonic acid inhibits accumulation of glucose-6-phosphatde dehydrogenase mRNA by inhibiting insulin signalling through the activation of p38 mitogen activated protein kinase (p38 MAPK) and the subsequent Ser307 phosphorylation of insulin receptor substrate-1 (IRS-1) (TaluKDAR et al., 2005). In rat hepatocytes, arachidonic acid activation of AMP activated protein kinase (AMPK) is involved in the G6PD expression (Kohan et al., 2009). First activation of AMPK inhibits the insulin induction of G6PD mRNA whereas second activation of AMPK by AICAR (aminoimidazole carboxamide ribonucleotide) activates p38MAPK resulting in Ser307 phosphorylation of insulin receptor substrate-1 (IRS-1) and inhibits AKT phosphorylation similar to the action of arachidonic acid (Kohan et al., 2009). Inhibition of p38 MAPK blocks the effect of AICAR on accumulation of G6PD mRNA (Kohan et al., 2009). The inhibition of G6PD gene expression is mainly due to polyunsaturated fatty acids and not due to saturated or monounsaturated fatty acids.

Regulation of G6PD by hormonal factors has been observed in hepatocytes in primary culture. In hepatocytes primary culture role of insulin and glucocorticoids has been examined for the expression of G6PD (Kletzien and Berdanier, 1993; Kletzien et al., 1994). Role of insulin in induction of G6PD activity in hepatocytes primary culture was given by Kurts and Wells in 1981. A 3-4 fold induction in G6PD activity is seen on incubation of hepatocytes with insulin which is also accompanied with changes in rate
of synthesis and mRNA abundance (Manos et al., 1991). Several other studies have led to the conclusion that insulin is a primary hormone in upregulating G6PD expression at the level of transcription (Manos et al., 1991; Fukuda et al., 1992). However, the molecular mechanism of insulin action on transcription of G6PD gene and other insulin regulated genes is not well understood. Glucocorticoids are also positive regulators of G6PD activity in rat hepatocytes.

The increase G6PD mRNA in the presence of insulin (Manos et al., 1991; Fukuda et al., 1992) without affecting the relative rate of enzyme synthesis, indicates that it may be due to translational control which can be activated by insulin but not by the glucocorticoids (Kletzien et al., 1994). However, maximum increase in G6PD mRNA was found in the presence of insulin and dexamethasone (a corticosteroid, is a potent synthetic member of the glucocorticoid class of steroid drugs. It acts as an anti-inflammatory and immunosuppressant.), suggesting that both hormones are capable of increasing transcription (Manos et al., 1991; Fukuda et al., 1992; Kletzien et al., 1994).

G6PD expression by hormones, nutrients and some growth factors has also been observed in tissues other than liver. Carbohydrate refeeding has shown a 9 fold increase in G6PD mRNA in rat epididymal fat pads (Louie et al., 1990). Increase in G6PD mRNA is also observed as 3T3-L1 cells differentiate into adipocytes or upon insulin treatment of confluent, non proliferating brown adipose cells (Kletzien et al., 1992; Valverde et al., 1992). Estrogen has been shown to increase G6PD in uterus (Smith and Barker, 1974). Epidermal growth factor has also been shown to induce G6PD mRNA and relative rate of enzyme synthesis indicating that growth stimulation of cells is linked to induction of G6PD gene (Yoshimoto et al., 1983). Additional examples of endocrine regulation of G6PD include norepinephrine induction of mRNA and activity in heart (Zimmer et al., 1992), elevation of activity due to vitamin D3 in gut epithelium (Nasr et al., 1989). The above discussion provides evidence that variety of tissues exhibit the capacity for adaptive regulation of G6PD.

4.2 Regulation of glucose-6-phosphate dehydrogenase by oxidative stress

G6PD induction by a number of non hormonal agents indicates that the gene acts as a guard for oxidant stress and shows quick response to the need of NADPH for maintenance of redox state in the cells. G6PD plays an important role in the cellular response to the oxidant stress in E. coli (Greenberg et al., 1990). Agents responsible for production of oxygen radicals induce G6PD through soxR regulon, which regulates
G6PD and eight other genes required to protect cells from free radical damage (Greenberg et al., 1990). In *Saccharomyces cerevisiae*, a mutation (Par1) that renders the cells which are sensitive to oxidant stress leads to decreased expression of G6PD as well as other enzymes required for oxygen detoxification (Kletzien et al., 1994; Schnell et al., 1992). In mammalian lung, exposure of alveolar type II cells from neonatal rats to hyperoxia in vitro resulted in increased G6PD activity (Kennedy et al., 1989). The adaptive response of the G6PD gene in several tissues makes up a part of the coordinated cellular response to oxidant stress. Hepatic cells are highly exposed to a number of oxidants from dietary, environmental and pharmaceuticals sources.

Hepatic G6PD is induced by chemicals such as diquat (Cramer et al., 1993), ethanol (Stumpo and Kletzien, 1985). Studies have revealed that ethanol induces G6PD activity, relative rate of G6PD enzyme synthesis and mRNA in primary cultures of rat hepatocytes (Stumpo and Kletzien, 1985). The oxidative stress experiments in *Trypanosoma cruzi* showed that metacyclic trypomastigotes and amastigotes have high G6PD activity (Igoillo-Esteve and Cazzulo, 2006). The mechanism of enzyme induction is similar to that of reported in *S. cerevisiae*, *E. coli* and mammalian cells (Igoillo-Esteve and Cazzulo, 2006). However, in plants oxidative stress does not affect the cytosolic G6PD activity (Hauschild and Schaewen, 2003).

**4.3 Regulation of glucose-6-phosphate dehydrogenase by other factors**

Glucose-6-phosphate dehydrogenase regulation in higher plants occurs at the transcription and translation level. In alfalfa, induction in G6PD transcription and activity in response to fungal elicitors in roots and nodules has been reported (Fahrendorf et al., 1995). Induction in plastidic G6PD expression has been observed in response to illumination of light deprived potato shoots (von Schaewen et al., 1995). Role of ferredoxin/thioredoxin system has been examined under light dependent inactivation of G6PD. The ferredoxin/thioredoxin system consists of ferredoxin, thioredoxin and ferredoxin-thioredoxin reductase (Buchanan et al., 1994). Ferredoxin is the terminal electron acceptor of photosynthetic electron transport chain. Excitation of Photosystem-I by light, transfers the electrons to ferredoxin and then to ferredoxin dependent enzymes and NADP⁺ which are reduced. Electrons are then passed to ferredoxin-thioredoxin reductase reducing a disulphide bond to its dithiol form. Reduced ferredoxin-thioredoxin reductase then reduces thioredoxin which regulates the activity of variety of enzymes by changing the redox status. A dark activation and light
inactivation cycle as a way of activity regulation has been observed in isolated spinach chloroplasts (Scheibe et al., 1989). G6PD is inhibited by dithiothreitol (DTT), a sulphhydryl reagent that imitates the effect of thioredoxin, however, inactivation by DTT and light may not be achieved through same mechanism (Scheibe et al., 1989). All chloroplast G6PD homologues are inactivated by redox modification of the ferredoxin-thioredoxin system (Buchanan, 1991). G6PD is kept in reduced state in light and becomes active only in its oxidized state in the dark. The molecular basis of this regulation is disulphide-dithiol interchange of certain regulatory cysteine residues in the target enzymes. Transfer of algal cultures to nitrogen free media is reported to induce G6PD activity (Huppe and Turpin, 1996). Elevated enzyme activity may be linked with assimilation of inorganic nitrogen by the unicellular green algae.

However, the information regarding G6PD isoform regulation is limited. Several studies describing conditions that stimulate G6PD activity did not distinguish the different isoenzymes nor analyze possible mechanisms involved (Daniel et al., 1990). In potato, regulations of cytosolic and chloroplastic G6PD are governed by distinct mechanisms. Transcription of cytosolic G6PD gene via sugar-mediated signalling to the nucleus is triggered by high sugar levels in the cytosol (Hauschild and von Schaewen, 2003). Plastidic G6PD can be stimulated by low NADPH to NADP ratio, most probably via dephosphorylation of the existing enzyme pool (Hauschild and von Schaewen, 2003).

5. DISTRIBUTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Glucose-6-phosphate dehydrogenase is widely distributed among microorganisms (Banerjee and Fraenkel, 1972; Vander Wyk and Lessie, 1974; Reuter et al., 1990; Jeffery et al., 1993; Ragunathan and Levy, 1994; Anderson and Anderson, 1995; Moritz et al., 2000; Hansen et al., 2002; Ibraheem et al., 2005; Igoillo-Esteve and Cazzulo, 2006), G6PD has been reported in a number of bacteria such as Zymomonas mobilis (Scopes et al., 1985), Pseudomonas multivorans (Vander Wyk and Lessie, 1974), Pseudomonas W6 (Reuter et al., 1990), Escherichia coli (Banerjee and Fraenkel, 1972), Acetobacter xylinum (Ragunathan and Levy, 1994), Azotobacter vinelandii (Anderson and Anderson, 1995), Corynebacterium glutamicum (Moritz et al., 2000), Thermotoga maritima (Hansen et al., 2002), plants (Graeve et al., 1994; Espoito et al., 2001; Espoito et al., 2003; Demir et al., 2004; 2009; Cardi et al., 2011) and in different
tissues of animals (Holten, 1972; Lee et al., 1979; Ozer et al., 2001; Ozer et al., 2002; Ciftci et al., 2003; Danisan et al., 2004; Ulusu et al., 2005). Mammalian form of G6PD was isolated from a number of species (Kanji et al., 1976; Lee et al., 1979; Pittalis et al., 1992; Ozer et al., 2001; Ozer et al., 2002; Beydemir et al., 2002; Beydemir et al., 2003; Yilmaz et al., 2003; Ciftci et al., 2003; Ciftci et al., 2004; Danisan et al., 2004; Ulusu et al., 2005). Studies about regulation of G6PD has been carried out in plants, eukaryotic algae and cyanobacteria but G6PD encoding gene has been isolated from only few of these sources (Srivastava and Anderson, 1983; Fickenscher and Scheibe, 1986; Graeve et al., 1994; Gleason, 1996). Two isoforms (cytosolic and chloroplastic isoforms) of G6PD has been reported in green algae *Chlorella vulgaris* C-27 (Honjoh et al., 2003). Several isoforms of G6PD have been reported in cytosol, plastidic stroma, and peroxisomes of higher plants (Corpas et al., 1998, Schnarrenberger et al., 1973, von Schaewen et al., 1995, Knight et al., 2001, Nemoto and Sasakuma, 2000).

6. GLUCOSE-6-PHOSPHATE DEHYDROGENASE PROPERTIES

6.1 Extraction, purification and characterization of G6PD from different sources

G6PD has been extracted and purified from various animals, plants, bacteria and fungi utilizing various methods of purification. The pH optima of maximal activity appear to depend on the nature of buffer and source of enzyme. Studies on determination of optimum temperature have not been so practicable in the case of G6PD. Very few reports on optimum temperature are available. A wide range of optimum temperature was reported in different sources. The range varies in between 30 - 92°C. The most thermoactive G6PD was reported from hyperthermophilic bacterium *Thermotoga maritima* whose temperature optimum was reported to be 92°C (Hansen et al., 2002). There have been several attempts to determine the molecular weight using various techniques. G6PD has been reported to exist as monomer, dimer, tetramer and hexamer, however only dimeric and tetrameric form was found to be enzymatically active.

The extraction and purification of brewers’ yeast G6PD enzyme by ammonium sulphate precipitation, calcium phosphate gel adsorption and ethyl alcohol fractionation and shows maximal activity at pH 8.5 (Glaser and Brown, 1955).

Human erythrocyte G6PD has been purified 47 fold using several steps of ammonium sulphate fractionation and DEAE-Cellulose fractionation (Chung and Langdon, 1963). A rapid purification of human erythrocyte G6PD has been carried out by combined affinity and anion-exchange chromatography (Pittalis et al., 1992).
rapid method allows a high recovery of enzyme activity from even a small amount of blood. The percentage yield after first purification step is about 90 and 70% at the end of the procedure (Pittalis et al., 1992). The molecular weight of human erythrocyte G6PD dimer was reported to be 105 KDa (Kirkman and Hendrickson, 1962). In 1963, Chung and Langdon made an attempt for the calculation of human erythrocyte G6PD from the diffusion and sedimentation constants of the enzyme by Svedberg equation and the molecular weight calculated was 190 KDa. Purification and characterization studies of leukocyte G6PD in Sardinian Mutants has been carried out by three step ammonium sulphate fractionation along with Calcium phosphate gel adsorption after the first ammonium sulphate fractionation (Bonsignore et al., 1966).

An eight step purification of wild type Neurospora crassa G6PD has been carried out by ammonium sulphate fractionation, calcium phosphate gel adsorption and column chromatography. The columns used were DEAE-cellulose, hydroxylapatite column and bio-gel A (Scott and Tatum, 1971). N. crassa G6PD was found to exhibit maximal activity in Tris-HCl buffer. The pH of the enzyme in Tris-HCl is broad, ranging from about pH 7.4 to pH 8.2. However, in malate-NaOH buffer, activity was 75% of that observed in Tris-HCl buffer at comparable pH values (Scott and Tatum, 1971). A 2400-fold purification was achieved with a 10% yield and an average specific activity of 470 (Scott and Tatum, 1971).

G6PD was purified from Thiobacillus ferrooxidans by ammonium sulphate precipitation and followed by Sephadex G-200 column chromatography. The enzyme possesses dual nucleotide specificity for either NADP or NAD and has a molecular weight of 110,000 as determined by gel electrophoresis (Tabita and Lundgren, 1971). The pH optima of maximal catalytic activity appear to depend on the nature of the buffer and source of enzyme. In Thiobacillus ferrooxidans, when the enzyme was assayed with NADP at different pH values of Tris buffer, a relatively sharp optimum was reported at pH 7.8 to 7.9, whereas, enzyme activity with NAD as coenzyme differed in that no activity was obtained at pH values below 7.5; a relatively broad pH optimum was observed from pH 8.0 to 8.5 (Tabita and Lundgren, 1971).

A highly NADP$^+$ and glucose-6-phosphate specific G6PD has been purified from pig liver to 1000 fold by employing acid denaturation, Triton X-100 treatment, Sephadex G-200 and DEAE-cellulose ion exchange chromatography (Kanji et al., 1976). Kanji et al. (1976) have studied the effect of pH on activity in pig liver. They performed their studies in glycine/NaOH and Tris/Malate/NaOH buffers and observed a
slow rise in activity with increasing pH to a maximum at pH 8.5. G6PD from pig liver occurs as dimer with molecular weight of 133 KDa with monomeric unit having a molecular weight of 67.5 KDa (Kanji et al., 1976).

The enzyme was also purified from a basidiomyceteous yeast Cryptococcus neoformans which is an opportunistic pathogen of AIDS patients with a specific activity of 50 U/mg (Niehaus and Mallett, 1994). G6PD had a subunit molecular weight of 50 KDa in Cryptococcus neoformans (Niehaus and Mallett, 1994).

Azotobacter vinelandii G6PD isolated from cell sonicates was purified 81 fold to electrophoretic homogeneity and a specific activity of 73 units/mg protein using ion-exchange and Matrex Dye chromatography (Anderson and Anderson, 1995). G6PD from A. vinelandii exhibit a pH optimum of 8.5 (Anderson and Anderson, 1995). The sodium dodecyl sulphate polyacrylamide gel electrophoresis and molecular exclusion chromatography indicated the presence of a tetrameric enzyme from A. vinelandii with subunit molecular weight of 52 KDa (Anderson and Anderson, 1995).

Some methods have been given for studying the G6PD activity in brain areas (Ninfali et al., 1997a) and higher G6PD activity was reported in the olfactory lobe (Ninfali et al., 1997b) but no attempts has been made for the extraction and purification studies of G6PD from brain.

A 250 fold purified enzyme was obtained from Schizosaccharomyces pombe (Tsai and Chen, 1998). A 112 KDa dimeric G6PD was also reported from Schizosaccharomyces pombe (Tsai and Chen, 1998).

G6PD was also purified from dog liver with a specific activity of 130 U mg−1 and a yield of 18% and dog liver pH optimum of 7.8. PAGE showed two bands on protein staining; only the slower moving band had G6PD activity (Ozer et al., 2002). Monomeric enzyme has been reported from dog liver with a molecular weight of 52.5 KDa (Ozer et al., 2002).

Sheep erythrocyte G6PD has been purified with a yield of 37.1% and specific activity of 4.64 U/mg proteins by using ammonium sulphate fractionation and 2′, 5′-ADP Sepharose 4B affinity chromatography (Beydemir et al., 2002).

G6PD was purified from buffalo (Bubalus bubalis) erythrocytes in two steps: hemolysate preparation and 2′, 5′-ADP-Sepharose 4B affinity gel chromatography. The enzyme from buffalo erythrocyte shows optimum pH of 8.0 using 1 M Tris-HCl. However, the stable pH of the enzyme was reported to be 9.0 in Tris-HCl. (Ciftci et al., 2003).
two consecutive processes gives 650 fold purified enzyme having a specific activity of 69.7 EU/mg proteins with a yield of 31% (Ciftci et al., 2003).

Goose erythrocyte G6PD has been purified to 3892 fold with a specific activity of 36.2 EU/mg protein and 68.79% yield by using ammonium sulphate precipitation and 2’, 5’-ADP Sepharose 4B affinity gel chromatography (Beydemir et al., 2003). The optimal pH of goose erythrocyte G6PD was found to be 7.0 using Tris-HCl buffer; however, the stable pH of the enzyme was reported to be 9.0 in the same buffer (Beydemir et al., 2003). Goose erythrocyte G6PD shows highest activity point at 50°C (Beydemir et al., 2003). Similar reports were found for rat liver and kidney-cortex (Corpas et al., 1995) and bovine lens (Ulusu et al., 1999).

Two isoforms of G6PD from cells of a freezing tolerant strain of Chlorella vulgaris C-27, by sequential steps of chromatography on five kinds of columns, including a Hi Trap Blue column which showed excellent separation of the isoforms from each other (Honjoh et al., 2003). The two isoforms were purified up to 109 and 197 fold respectively with specific activity of 14.4 and 26 U/mg proteins, respectively (Honjoh et al., 2003). Two isoforms of G6PD were reported from a free tolerant strain of C. vulgaris C-27 with an apparent molecular weight of 200 KDa and a subunit molecular weight of about 58 KDa (Honjoh et al., 2003).

G6PD was purified from turkey erythrocytes by ammonium sulphate precipitation and followed by ADP Sepharose affinity gel chromatography. The yield was 49.71% and specific activity of the enzyme was found to be 44.16 EU/mg protein. The optimum pH of G6PD from turkey erythrocytes has been found to be 9.0, whereas, the enzyme is stable at pH 8.0 (Yilmaz et al., 2003). The optimum temperature of turkey erythrocyte was found to be 50°C (Yilmaz et al., 2003). The molecular weight of G6PD from turkey erythrocyte was determined by Rf –logM.W graph and by gel filtration method and was found 73.117 and 75.46 KDa respectively. Interestingly, the enzyme was found to be active in its monomeric form (Yilmaz et al., 2003).

Rat small intestine G6PD was purified with a yield of 19.2% and specific activity of 53.8 U/mg proteins and pH optimum of the enzyme from rat small intestine was found to be 8.1 (Danisan et al., 2004). G6PD from rat liver has been found to have pH optima of 7.6 in glycylglycine buffer whereas in veronal buffer the pH optimum was 8.5 (Glock and McLean, 1953). Rat small intestine G6PD shows an optimum temperature of 38°C (Danisan et al., 2004).
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G6PD from rainbow trout (Oncorhynchus mykiss) erythrocytes was purified, using a simple and rapid method which involves hemolysate preparation, ammonium sulphate precipitation and 2’, 5’-ADP Sepharose 4B affinity gel chromatography. G6PD from rainbow trout was more stable at pH 8.9 than at other pH values for the duration of 48 h (Ciftci et al., 2004). The rainbow trout enzyme was found to show the highest activity at 45°C after tried between 10-80°C (Ciftci et al., 2004). The enzyme was 1271.19 fold purified with a specific activity of 14.51 EU/mg proteins and 70.40% yield (Ciftci et al., 2004). The molecular weight of the G6PD from rainbow trout was estimated to be 64.26 KDa by using SDS-PAGE and 66.22 KDa by gel filtration (Ciftci et al., 2004). An interesting fact about the rainbow trout enzyme is that the enzyme is active in its monomeric form whereas the active forms reported were only dimers or tetramers (Ciftci et al., 2004).

G6PD from goat erythrocyte was purified by using DEAE-cellulose as anion and CM-Sephadex as cation exchange column chromatography and ammonium sulphate precipitation with 45000 fold in a yield of 23%. The optimum pH was found to be 8.0. Goat erythrocyte contains a tetrameric enzyme with subunit molecular weight of 52 KDa (Bayazit et al., 2005).

The enzyme was also purified from a filamentous fungus, Aspergillus aculeatus previously isolated from infected tongue of a patient (Ibraheem et al., 2005). The purified enzyme was apparently homogeneous with a specific activity of 220 U/mg proteins (Ibraheem et al., 2005). The apparently homogeneous enzyme from A. aculeatus had a molecular weight of 105± 5 KDa with a subunit molecular weight of 52±1.1 KDa (Ibraheem et al., 2005), whereas the enzyme from Aspergillus parasiticus had a molecular weight of 180 KDa and was composed of four subunits of apparently equal size (Niehaus and Dilts, 1984).

Two isoforms of glucose-6-phosphate dehydrogenase was also purified from an extraordinarily UV-resistant bacterium Dienococcus radiophilus (Sung and Lee, 2007). The two isoforms were purified 122 and 44 fold respectively by using DEAE-Cellulose column chromatography and 2’, 5’-ADP Sepharose 4B affinity chromatography and shows optimum activity at pH 8.0 (Sung and Lee, 2007). The two isoforms of G6PD from D. radiophilus show optimum activity at 30°C (Sung and Lee, 2007). The activities of both the enzymes decreases above 40°C, but at 70°C, the activity of G6PD-I decreases more profoundly than the activity of G6PD-II. This indicates that G6PD-I was more sensitive than G6PD-II (Sung and Lee, 2007). Two isoforms of G6PD has
been isolated from *D. radiophilus* with subunit molecular weight of 35 KDa (tetramer) and 60 KDa (dimer) respectively (Sung and Lee, 2007).

The enzyme has also been purified to homogeneity from the soluble fraction of larval *Taenia crassiceps* by salt fractionation, ion exchange and affinity chromatography (Rendon *et al*., 2008). The enzyme from *Taenia crassiceps* shows maximal activity between pH 6.7 and 7.8 (Rendon *et al*., 2008).

### 6.2 Substrate and coenzyme specificity

G6PD was earlier regarded to be specific for glucose-6-phosphate. However, later on it has been claimed that other phosphorylated monosaccharides were also oxidized by G6PD. Scott and Tatum (1971) have examined various phosphorylated and non-phosphorylated monosaccharides for the oxidation by *N. crassa* G6PD and they found that in addition to glucose-6-phosphate only galactose-6-phosphate and 2-deoxyglucose-6-phosphate were oxidized by the enzyme (Scott and Tatum, 1971). However, the relative rates of oxidation were low as compared to glucose-6-phosphate. Fructose-6-phosphate was also oxidized but its effectiveness as substrate was speculative, since it was known to be contaminated with glucose-6-phosphate. However, no activity was observed with glucose-1-phosphate, glucose, glucosamine-6-phosphate, 6-phosphogluconic acid, mannose-6-phosphate and fructose. Similar observations were observed in case of G6PD from other sources also. The coenzyme NADP⁺ shows more affinity towards the enzyme as compared to the substrate, glucose-6-phosphate. The substrate binding enhances the affinity of the enzyme for coenzyme (Ibraheem *et al*., 2005). G6PD isolated so far from different sources can be divided on the basis of their nucleotide specificity. One group exemplified by the enzymes from brewers’ yeast (Warburg and Christian, 1931a,b), *E. coli* (Banerjee and Fraenkel, 1972), *N. crassa* (Scott and Tatum, 1971), spinach leaf (Schnarrenberger *et al*., 1973) *Anabaena* sp. (Schaeffer and Stanier, 1978; Gleason, 1996), goose erythrocyte (Beydemir *et al*., 2003), *A. aculeatus* (Ibraheem *et al*., 2005), reacts exclusively with NADP. The second group, which appears to include animal G6PD, in general reacts with NADP but also gives weak activity with NAD. The third group of the enzyme can react approximately equally well with NAD and NADP. This class includes the enzyme from a number of bacterial strains such as *Leuconostoc mesenteroides* (Demoss *et al*., 1953; Olive *et al*., 1971), *Pseudomonas aeruginosa* (Ju-Fang *et al*., 1998), *Pseudomonas W6* (Reuter *et al*., 1990), *Streptomyces aureofaciens* (Haghighi *et al*.,
In dog liver G6PD NADP was replaced by d-NADP (Deamino NADP). The latter exhibit more affinity as well as
catalytic efficiency than NADP (Ozer et al., 2002). The human placental G6PD oxidizes
a variety of substrates other than glucose-6-phosphate, such as galactose-6-phosphate
and deoxyglucose-6-phosphate. The affinity of the enzyme for glucose-6-phosphate and
galactose-6-phosphate were nearly equal and Deamino- NADP was also found to act as
coenzyme for the human placental enzyme (Ozer et al., 2001). The rate of G6PD
catalyzed reaction varies with the condition of measurement, method of assay, nature of
buffer and its pH. Therefore, wide variations in Km and Vmax values have been
reported by various workers. The Km value for glucose-6-phosphate is found to be more
than NADP$^+$ except in some cases indicating that G6PD shows more affinity towards
NADP$^+$. Km values and kinetic properties in some of the sources are given in Table 1.3.
Table 1.3: km values and kinetic properties of *Brugia malayi* G6PD and other parasites.

<table>
<thead>
<tr>
<th>Species</th>
<th>Km for G6P (Mm)</th>
<th>Km for NADP (3 mM)</th>
<th>Specific activity (U/mg 40 sec-1)</th>
<th>Kcat (µM)</th>
<th>Ki for NADPH (17.1±3.2 µM)</th>
<th>Ki for ATP (6.5 mM)</th>
<th>Inhibition with DHEA (µM)</th>
<th>Oligomerization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brugia malayi</em></td>
<td>0.245±0.008 Mm</td>
<td>0.014±0.000 Mm</td>
<td>0.535</td>
<td>40 sec-1</td>
<td>Competitive</td>
<td>Noncompetitive</td>
<td>NI</td>
<td>Tetramer 75000 Da</td>
<td>Current Study</td>
</tr>
<tr>
<td>HUMAN</td>
<td>40 ±8 µM</td>
<td>20 ±10 µM</td>
<td></td>
<td></td>
<td>Competitive</td>
<td>8.9 ± 0.3 µM G6P</td>
<td>Dimer, tetramer</td>
<td>Ozer et al. (2001)</td>
<td>Gordon et al. (1995)</td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>27 µM</td>
<td>4.5 µM</td>
<td>4.3 µM</td>
<td></td>
<td></td>
<td></td>
<td>Homodimer 62 000 Da</td>
<td></td>
<td>Kurdi-haidar and Luzzatto (1990)</td>
</tr>
<tr>
<td><em>Trypanosoma brucei</em></td>
<td>138 µM</td>
<td>5.3 µM</td>
<td>14 µmol. (min mg of protein)_1.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Heise and Opperdoes (1999)</td>
</tr>
<tr>
<td><em>Trypanosoma brucei</em> (recombinant)</td>
<td>57.8 ± 2.4 µM</td>
<td>9.4 ± 0.4 µM</td>
<td>16.4 ± 0.6 sec-1</td>
<td></td>
<td></td>
<td>1.70 ± 0.1 µM G6P</td>
<td></td>
<td></td>
<td>Cordeiro et al. (2009)</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>300 mM</td>
<td>87 mM</td>
<td></td>
<td></td>
<td>Competitive</td>
<td>0.76 µM</td>
<td></td>
<td></td>
<td>Igoillo-Esteve and Cazzulo (2006)</td>
</tr>
<tr>
<td><em>Leishmania maxiana</em></td>
<td>74.5 ± 3.0 µM</td>
<td>12.1 ± 0.5 µM</td>
<td>22.2 ± 0.9 sec-1</td>
<td></td>
<td></td>
<td></td>
<td>NI</td>
<td></td>
<td>Ciftci et al. (2003)</td>
</tr>
<tr>
<td>Buffalo (Bubalus bubalis)</td>
<td>0.106 mM</td>
<td>0.87 mM</td>
<td>69.7 EU/mg</td>
<td></td>
<td>Competitive</td>
<td>0.53 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat small intestine</td>
<td>70.1 ± 20.8 µM</td>
<td>23.2 ± 7.6 µM</td>
<td>53.8 U/mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat liver</td>
<td>329 µM</td>
<td>100 µM</td>
<td>327.1 µM/mg</td>
<td></td>
<td>Competitive</td>
<td>10 µM</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rat kidney-cortex</td>
<td>206 µM</td>
<td>25 µM</td>
<td>76.55 µM/mg</td>
<td></td>
<td>Competitive</td>
<td>4 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em></td>
<td>81 µM</td>
<td>5.69 µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baker’s yeast</td>
<td>51 µM</td>
<td>19 µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig liver</td>
<td>36 ±3 µM</td>
<td>4.8 ±0.5 µM</td>
<td></td>
<td></td>
<td>non competitive</td>
<td></td>
<td></td>
<td>Dimer 67500 Da</td>
<td></td>
</tr>
</tbody>
</table>

References:
- Ozer et al. (2001)
- Gordon et al. (1995)
- Heise and Opperdoes (1999)
- Cordeiro et al. (2009)
- Igoillo-Esteve and Cazzulo (2006)
- Ciftci et al. (2003)
- Corpus et al. (1995)
- Olive et al. (1971)
- Gould and Goheer (1976)
- Kanji et al. (1976)
### CHAPTER 1. REVIEW OF LITERATURE

<table>
<thead>
<tr>
<th>Species/Sample</th>
<th>Concentration (µM)</th>
<th>Activity (U/mg)</th>
<th>Inhibition Type</th>
<th>Tetramer MW (Da)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>50 µM</td>
<td>10 µM</td>
<td>Competitive 18 µM</td>
<td>Inhibited</td>
<td>Lee et al. (1979)</td>
</tr>
<tr>
<td>Dog liver</td>
<td>122±18 µM</td>
<td>10±1 µM</td>
<td>Competitive 12.0±7.0 µM</td>
<td>Monomer 52500 Da</td>
<td>Ozer et al. (2002)</td>
</tr>
<tr>
<td>Goose Erythrocyte</td>
<td>24.3 µM</td>
<td>7.4 µM</td>
<td>Noncompetitive 0.144 mM</td>
<td>Dimer 73000 Da</td>
<td>Beydemir et al. (2003)</td>
</tr>
<tr>
<td>Turkey erythrocytes</td>
<td>49.7 µM</td>
<td>17.1 µM</td>
<td>Competitive 0.1609 mM</td>
<td>Noncompetitive 16.6mM</td>
<td>Yilmaz et al. (2003)</td>
</tr>
<tr>
<td>Aspergillus aculeatus</td>
<td>75±6 µM</td>
<td>6±1 µM</td>
<td>220 U/mg</td>
<td>Monomer 75000 Da</td>
<td>Ibraheem et al. (2005)</td>
</tr>
<tr>
<td>Goat erythrocyte</td>
<td>334 µM</td>
<td>135 U/mg</td>
<td>Inhibited</td>
<td>10-4 M conc. Inhibited all</td>
<td>Bayazit et al. (1995)</td>
</tr>
<tr>
<td><em>Taenia crassiceps</em> (cysterceri)</td>
<td>14±1.7 µM</td>
<td>1.3±0.4 µM</td>
<td>33.8±2.1 U mg−1</td>
<td>Dimer 61.000±1,700 Da</td>
<td>Rendon et al. (2008)</td>
</tr>
</tbody>
</table>
7. INHIBITION

7.1 Product inhibition

Product inhibition studies of G6PD were conducted with NADPH (Table 1.3). NADPH is a potent inhibitor of NAD-linked G6PD and a competitive inhibitor of NADP-linked G6PD (Levy et al., 1966). In case of L. mesenteroides when NADP⁺ was the varied substrate, linear competitive inhibition was obtained, whereas with glucose-6-phosphate as varied substrate the inhibition was linear non competitive (Olive et al., 1971). For NAD⁺ linked reaction, the inhibition was linear non competitive when NAD⁺ was used as varied substrate and glucose-6-phosphate was non saturating and when glucose-6-phosphate was used as varied substrate the inhibition by NADH was non-competitive without any correlation with NAD⁺ being saturating or non saturating (Olive et al., 1971). The enzyme isolated from obligate methylotrophic bacterium Pseudomonas W6 is not inhibited by NADPH but by NADH (Miethe and Babel, 1976), however the purified enzyme from Methylomonas M15 was reported to be inhibited by both NADPH and NADH (Steinbach et al., 1978). Grossman and McGowan (1975) reported that NADPH inhibits the activity of cyanobacterial G6PD, which was later on confirmed by Pelroy et al. (1976). Inhibition of G6PD in the cyanobacterium Anabaena sp. has also been reported (Schaeffer and Stanier, 1978). Human placental G6PD was also reported to be competitively inhibited by NADPH (Ozer et al., 2001). Several other reports have been shown that G6PD from yeast (Gould and Goheer, 1976; Niehaus and Mallett, 1994; Tsai and Chen, 1998), bacteria (Raghunathan and Levy, 1994; Hansen et al., 2002), fungi (Ibraheem et al., 2005), animals (Ozer et al., 2002) and plants (Demir et al., 2004) are strongly and competitively inhibited by NADPH. S. aureofaciens G6PD was inhibited by NADH and NADPH (Haghighi et al., 2005). Both NADH and NADPH inhibited the enzyme competitively and noncompetitively with respect to the corresponding oxidized coenzymes and glucose 6-phosphate, respectively (Haghighi et al., 2005).

7.2 Inhibition by various inhibitors

Several classes of G6PD inhibitors are reported. G6PD from brewers’ yeast was reported to be competitively inhibited by D-glucosamine-6-phosphate with a Ki value of 7.2 X 10⁻⁴ M (Glaser and Brown, 1955). Dehydroisoandrosterone, pregnenolone, and certain related steroids, at concentrations of 10⁻⁶ M or less, inhibit mammalian G6PD
activity (Table 1.3). However, these steroids do not inhibit spinach or yeast G6PD (Marks and Banks, 1960). G6PD was found to be highly sensitive to inhibitory effect of long chain acyl coenzyme A (Eger-Neufeldt et al., 1965). Inhibition of partially purified \textit{Leuconostoc} G6PD from iodoacetate has been reported (Demoss et al., 1953) but later studies on \textit{Leuconostoc} G6PD inhibition shows no inhibition by iodoacetate, iodoacetamide and p-hydroxymercuribenzoate (Olive et al., 1971). A pH and concentration dependent inhibition of G6PD by pyridoxal 5’-phosphate was also reported (Olive et al., 1971). Pyridoxal 5’-phosphate inhibits the enzyme at 0.1mM and 7.66 and 6.32 pH values (Olive et al., 1971). Pyridoxal 5’-phosphate behaves as competitive inhibitor when the glucose-6-phosphate was varied at a saturating concentration of NAD+ and behaves as non competitive inhibitor when NAD+ was varied at a saturating concentration of glucose-6-phosphate (Olive et al., 1971). The activity of G6PD in leaf extract of barley and spinach was reported to be 20-35% inhibited by dithiothreitol (Johnson, 1972). However, the inhibition is reversible. Cyanate inhibition of erythrocyte G6PD has been reported by Glader and Conrad (1972). Palmitoyl coenzyme A is reported to reversibly inhibit the yeast G6PD (Kawaguchi and Bloch, 1974). Phosphoenol pyruvate inhibits the G6PD activities of different bacteria, in contrast the enzyme from methanol grown \textit{Methylomonas} M15 was not inhibited by this metabolite but ATP inhibits the enzyme non-competitively (Table 1.3) with respect to NAD, NADP and glucose-6-phosphate (Steinbach et al., 1978). Zinc ion was reported to be a powerful inhibitor of G6PD, inhibition being competitive with respect to glucose-6-phosphate with Ki about 2.5 μM (Niehaus and Dilts, 1984; Niehaus and Mallett, 1994; Ibraheem et al., 2005). Human erythrocyte G6PD was reported to be inhibited by some antibiotics (Ciftci et al., 2000). Sodium cefoperazone, Gentamicin sulphate and Netilmicin sulphate were found to be competitive inhibitor of human erythrocyte enzyme, whereas, Sodium ceftizoxime, Sodium ampicillin, Sodium cefuroxime, Sodium cefazolin and Streptomycin sulphate were found to be non-competitive inhibitor of the enzyme (Ciftci et al., 2000). Inhibition of human erythrocyte G6PD by aluminium, nickel and cadmium is also reported (Haghighi and Ilghari, 2004). Aluminium causes 60% inhibition, nickel causes 70% inhibition and cadmium cause 65% inhibition of the enzyme (Haghighi and Ilghari, 2004). It was reported that cadmium binds either to the enzyme or enzyme substrate complex producing binary and ternary complexes and hence resulting in conformational changes leading to enzyme inactivation, aluminium leads to a pure competitive inhibition with
respect to glucose-6-phosphate surprisingly, as the two molecules are not substrate analogues (Haghighi and Ilghari, 2004). The inhibition might be due to metal induced conformational change that prevents proper binding of substrate. Nickel shows a mixed type inhibition of the enzyme (Haghighi and Ilghari, 2004). Phosphoenol pyruvate, ATP, fructose-6-phosphate and cobalt ion was also reported to inhibit the enzyme (Ibraheem et al., 2005). ATP and oleic acid inhibits the two isoforms of enzyme from D. radiophilus (Sung and Lee, 2007). Oleic acid was found to be more profound inhibitor for G6PD-I than G6PD-II (Sung and Lee, 2007). The profound inhibition of both enzymes by β-naphthoquinone-4-sulphonic acid suggests that lysine is present at the active site of enzyme (Sung and Lee, 2007). Cu2+ was found to be a potent inhibitor of G6PD-II but a lesser degree to G6PD-I (Sung and Lee, 2007).

8. STRUCTURE AND FUNCTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Glucose-6-phosphate dehydrogenase is the first enzyme of pentose phosphate pathway and catalyzes the conversion of glucose into pentose sugar required for various biosynthetic reactions like nucleotide synthesis in both plants and animals. In addition, the pathway also provides reducing power in the form of NADPH by the action of G6PD and 6-phosphogluconate dehydrogenase which serves as an electron donor for many enzymatic reactions of biosynthetic pathways. NADPH produced plays an important role in protecting the cells from oxidative stress (Pandolfi et al., 1995). G6PD also helps in regeneration of reduced glutathione along with formation of a molecule of NADPH (Luzzatto, 1995; Tsai et al., 1998). Almost all the G6PD isoforms that have been studied so far are highly specific for their substrate, glucose-6-phosphate and NADP⁺, for this reason many of the properties of the enzyme have been evolutionary preserved (Levy, 1979). However, some of the bacterial isoforms are also reported for utilising NAD+ instead of NADP⁺ (Anderson et al., 1997). G6PDs reported so far have single subunit type with native form existing as dimer, tetramer or hexamer. However, the enzyme is active as dimer or tetramer (Luzzatto, 2006) in a pH dependent equilibrium. The single subunit of enzyme consists of 515 amino acids and molecular weight of 59096 Da (Luzzatto et al., 2001). The enzyme monomer consists of two domains which are linked by an α-helix, which contains conserved eight residue peptide that acts as substrate binding site (Mason, 1996; Naylor et al., 1996; Au et al.,
The structure of the enzyme reveals one NADP⁺ molecule in every subunit of tetramer, away from active site but close to dimer interface (Boyer and Graham, 1965). Complete primary structure of the human enzyme has been deduced from the sequence of a full length complementary DNA clone (Persico et al., 1986). Human G6PD enzyme has two NADP⁺ binding sites per subunit. One is the “catalytic” and other is “structural” site. However, the bound NADP⁺ at the structural site is not involved in enzyme catalysis but many enzyme deficiency mutations are located in the dimer interface and close to the “structural” NADP⁺ site (Au et al., 2000). The auxiliary cofactor is said to be crucial for the stability and integrity of the active form of enzyme (Wang et al., 2008). NADP⁺ is required for monomer hybridization and refolding (Beutler and Collins, 1965; Yoshida et al., 1967; Gomez-Gallego et al., 1996; Gomez-Gallego et al., 2000; Wang and Engel, 2009). Most of the characterized enzyme shows hyperbolic kinetics, however enzymes isolated from cyanobacteria shows sigmoidal kinetics (Schaeffer and Stanier, 1978).

9. GENETICS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Glucose-6-phosphate dehydrogenase encoding genes are located at the telomeric region of the distal long arm of X chromosome (Xq28) from base pairs 153,759,605 to 153,775,786; which also contains the gene for haemophilia A (Oberle et al., 1987), fragile X syndrome and colour vision (Filosa et al., 1993). G6PD gene consists of 13 exons and 12 introns and is 18.5 Kb long (Luzzatto, 2006; Peters and Van Noorden, 2009). The promoter of G6PD is embedded in the CpG island which is found to be conserved from mice to humans (Toniolo et al., 1991). The G6PD gene promoter contains a TATA like sequence (TTAAAT, but no CAAT element) and numerous stimulatory protein 1 elements (Toniolo et al., 1991; Philippe et al., 1994; Rank et al., 1994). Three DNase1 hypersensitivity sites (Hss-1, Hss-2 and Hss-3) have been localized in the 5” end of the G6PD gene. Hss-3 located in introns 2 is liver specific whereas the other two Hss-1 and Hss-2 are ubiquitously present in all tissues (Hodge et al., 1998). The translation start site is located in exon 2 and has been mapped in rats and humans (Rank et al., 1994; Franze et al., 1998). The gene is remarkably conserved through evolution. The regions flanking the G6PD coding sequences have been analyzed and compared between species. The 5’ untranslated flank is 71 bp in rodent and humans (Toniolo et al., 1991) and 3’ untranslated flank is 608 bp and contains
noncanonical polyadenylation site which is preserved in a processed pseudogene of rat genome (Kletzien and Berdanier, 1993). The entire gene sequence for G6PD has been reported (Chen et al., 1991). More than 450 G6PD variants have been differentiated on the basis of different parameters (Luzzatto and Battistuzzi, 1985; Chen et al., 1991; Peters and Van Noorden, 2009), out of which the World Health Organisation have acknowledged about 300 variants (WHO, 1967; Peters and Van Noorden, 2009). The variants have been categorized into approximately 30 mutant groups (Beutler, 1992; Vulliamy et al., 1992). The mutations are present all over the gene being found in all exons except 3 and 13 (Kletzien et al., 1994). All but one mutation are point mutations and small deletion which causes enzyme structural defects, with over 50% being C to G transitions (Kletzien et al., 1994; Peters and Van Noorden, 2009). DNA sequence of more than 140 mutations has been established (Beutler and Vulliamy 2002). Mutation leads to instability of the enzyme by decreasing its affinity towards its substrates (Luzzatto, 2006). cDNA library from potato leaves were also screened (Graeve et al., 1994) and the homology of the plant sequence with G6PD sequences from animals and yeast was found to be rather high (52%), whereas there was significantly lower homology with sequences of bacterial origin (37%). The 5” untranslated sequence comprises good “Kozak consensus” around the start codon. This region does not encode a transit peptide, since there is a UGA stop codon at position -9 upstream of the ATG whereas the 3”-untranslated trailer carries putative polyadylaion signals within 100-200 nt downstream of the termination codon (Graeve et al., 1994).
10. OBJECTIVES UNDERTAKEN FOR THE STUDY

The cloning, overexpression, purification and characterization the key enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD), from B. malayi was carried out. In addition, we have performed molecular homology modelling of BmG6PD protein.

To clone, express and purify glucose-6-phosphate dehydrogenase (G6PD), from B. malayi:

- Primer designing to amplify G6PD from B. malayi genome.
- To clone the amplified fragment in pGEMT Easy cloning vector and subclone the pTriEx-4 expression vector.
- Recombinant BmG6PD protein expressed in E. coli cells and purified by affinity chromatography.
- Determination of subunit and native molecular mass (Mr) of BmG6PD.

To study the recombinant BmG6PD biochemical properties:

- Estimation of BmG6PD activity in the over-expressed protein.
- Kinetic studies with its various parameters viz. \( K_m \), optimum pH and temperature and effect of metal ions.
- Inhibition studies of BmG6PD by NADP\(^+\), NADPH, regulatory ligands, effect of SH inhibitor, metal chelators and its specific inhibitors.

Biophysical characterization of recombinant BmG6PD:

- Fluorescence and CD spectra of purified BmG6PD.
- Determination of dissociation constant (Kd) of NADP-BmG6PD complex.
- The influence of the bound NADP cofactor on the protein structure.
- The effect of chemical denaturants such as urea and GdmCl on the activity, structure and stability.

Antibody generation and immunolocalization:

- Antibody generation against purified BmG6PD.
- Specificity of Anti-BmG6PD antibodies.
- Localization of G6PD in the parasite (B. malayi).
Molecular modeling and ligand docking study of recombinant BmG6PD:

- Predicted secondary structure of BmG6PD.
- Homology modelling of BmG6PD.
- NADP docking study of BmG6PD.