CHARACTERIZATION OF THE HOMOLOGS OF A DIAGNOSTICALLY SIGNIFICANT *BRUGIA MALAYI* GENE (*Bm17DIII*) IN *WUCHERERIA BANCROFTI*, *LOA LOA* AND *ONCHOCERCA VOLVULUS*

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UNIVERSITI SAINS MALAYSIA

2006

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by

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

June 2006

DEDICATIONS

My father, mother, brothers and sisters

ACKNOWLEDGEMENTS

First, I would like to express my utmost appreciation to my supervisor, Professor (Dr.) Rahmah Noordin for her constant guidance, critical discussion, concern and patience throughout the study period.

Gratitude is extended to all the staff and students of the School of Medical Sciences, School of Health Science, Institute for Research in Molecular Medicine (INFORMM), Institute of Post-Graduate Studies, ***Pusat Bahasa*** and library for the advice, moral support and technical assistance.

I really appreciate the cooperation and advice that I received from the following teachers (and the students) namely Prof. Asma Ismail, Prof. Mohd Zaki Salleh, Assoc. Prof. Rosli Ismail, Prof. Zainul Fadziruddin, and Cikgu Mahani. Thank to Dr. Peter Fischer (Benhard NOcht Institute for Tropical Medicine, Hamburg, Germany) and Dr. Dr. B. Ravindran, (Division of Immunology, Regional Medical Research, Indian Council of Medical Research, Bhubaneswar, India), Prof. J. Ashraful Haq (Department of Microbiology, Bangladesh Institute of Research & Rehabilitation in Diabetes, Endocrine & Metabolic Disorders, Daka, Bangladesh) and Filarial Genome Research Center, Smith College, Northampton, MA, USA for providing the parasite materials, sera samples and helpful comments.

Personally I would like to thank Dr. Lim Boon Huat for his exhaustive effort, assistance, moral support and technical advice in helping me finishing up this study. I am thankful to my dear friends for the guidance and knowledge sharing especially on the molecular

aspects of this thesis namely kak Noor A'Shikin, kak Rohana, kak Azni, kak Suharni, Hafiz, Muhammad Sarhan, Atif Ali, kak Kiren, kak Aziah, kak Halisa, Roziawati, Norhayati, Norhaida and also my grateful to the following sisters for their assistance namely kak Suriati, kak Ida, kak Suzanne, kak Mah and kak Anum.

I would like to express my personal gratitude to my parents, Abdul Aziz Ismail and Rosni Muhammad for their eternal love and inspiration. Special thanks to Yusshalizaa Harun for his true love, understanding, great patience and unconditional support.

The financial support from Prof. (Dr.) Rahmah Noordin's research grants. There were Malaysian Government's IRPA grant, No. 06-02-05-1007PR0016/06-05 and European Commission (EC) grant, No. ICA4-CT-2001-10081.

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CHAPTER TWO
MATERIALS AND METHODS

LIST OF ABBREVIATIONS

No.	Abbreviations	Full words
1	BLAST	Basic Local Alignment Search Tool
2	BR	***BRUGIArapid***
3	conc	Concentration
4	COV	Cut off value
5	ddH_20	Double distilled water
6	dH_20	Deionised water
7	g	Gram
8	***g***	Gradient
9	hr	Hour
10	L1	Larva stage 1
11	L2	Larva stage 2
12	L3	Larva stage 3
13	L4	Larva stage 4
14	LF	Lymphatic filariasis
15	mf	Microfilaria
16	min	Minute
17	NC	Negative control
18	nr	Non-redundant
19	OD	Optical density
20	p	Page
21	PC	Positive control
22	pfu	Plaque forming unit
23	rpm	Revolution per minute
24	RT	Room temperature
25	sec	Second
26	T	Time
27	λ	Lambda
28	+	Positive
29	-	Negative/ minus
30	µ	Micro

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PENCIRIAN HOMOLOG BAGI GEN *BRUGIA MALAYI* (*Bm17DIII*) YANG SIGNIFIKAN SECARA DIAGNOSTIK DI DALAM *WUCHERERIA BANCROFTI*, *LOA LOA* DAN *ONCHOCERCA VOLVULUS*

ABSTRAK

Satu ujian pantas yang dikenali sebagai ***BRUGIArapid*** (BR) yang mengesan antibodi IgG_4 terhadap antigen rekombinan ***B. malayi*** (***BmR1***) adalah sangat berguna dalam pemetaan dan pengawasan kawasan yang endemik bagi filariasis brugia. Kajian penilaian lapangan yang telah dilakukan menunjukkan bahawa ***BmR1*** adalah sangat sensitif dan spesifik dalam mengesan individu bermikrofilaria***.*** Corak reaktiviti IgG_4 terhadap ***BmR1*** daripada sampel individu yang terjangkit dengan ***W. bancrofti*** adalah berbeza-beza mengikut kawasan, manakala reaktiviti ***BmR1*** terhadap sampel serum daripada individu yang dijangkiti ***O. volvulus*** dan ***L. loa*** adalah sangat minima.

Memandangkan antigen rekombinan ***BmR1*** adalah signifikan secara diagnostik, maka adalah penting untuk mencirikan ***BmR1*** secara lebih mendalam. Perbezaan reaktiviti ***BmR1*** terhadap sampel serum daripada pesakit yang dijangkiti parasit filarial yang lain (selain ***B. malayi*** & ***B. timori***) mencetuskan persoalan samada antigen tersebut mempunyai gen homolog di dalam ***W. bancrofti*** (***Wb-BmR1***)***, O. volvulus*** (***Ov-BmR1***) dan ***L. loa*** (***Ll-BmR1***). Sehubungan dengan itu, dalam kajian ini, jujukan DNA dan/atau cDNA bagi ***W. bancrofti, O. volvulus*** dan ***L. loa*** dikenalpasti, proteinnya diekspresi dan reaktiviti terhadap sampel serum pesakit diuji.

Pencarian homolog berasaskan web bagi ***Bm17DIII*** di dalam ***W. bancrofti***, ***O. volvulus*** dan ***L. loa*** menggunakan beberapa pangkalan data DNA melalui ***server*** BLASTN menunjukkan tiada sebarang persamaan dengan jujukan ***W. bancrofti*** dan ***L. loa***, manakala bagi ***O. volvulus***, terdapat persamaan dengan dua jujukan L3 dengan nilai E-value yang rendah. Pencarian homolog melalui BLASTP (pangkalan data protein) menunjukkan protein ***BmR1*** tidak mempunyai persamaan dengan jujukan protein lain.

Kaedah PCR digunakan untuk pemencilan dan identifikasi jujukan cDNA daripada perpustakaan cDNA dan/atau DNA genomik daripada ***W. bancrofti***, ***O. volvulus*** dan ***L. loa*** menggunakan primer RNR dan RNF. Produk PCR bersaiz 618 bp (iaitu ***Wb-BmR1***, ***Ov-BmR1*** dan ***Ll-BmR1***) kemudiannya diklon ke dalam vector TOPO, dijujuk dan dianalisis menggunakan program Vector NTI dan ***server*** BLAST. ***Wb17DIII*** didapati 100% menyerupai jujukan ***BmR1***, manakala ***Ov17DIII*** dan ***Ll17DIII*** didapati mirip terhadap satu sama lain dan berkongsi homologi sebanyak 99.7% dengan ***Bm17DIII***. Oleh itu keputusan pencarian gen homolog ***Ov17DIII*** melalui web tidak selaras dengan keputusan yang diperolehi dalam makmal. Kajian ini juga menunjukkan bahawa keseluruhan gen ***Wb17DIII*** tidak mengandungi sebarang intron; dengan itu berbeza daripada gen ***Bm17DIII*** yang mempunyai satu intron dan dua ekson.

Gen rekombinan ***Ov17DIII*** or ***Ll17DIII*** kemudiannya diekspresi di dalam pPROEX^{TM} HTa/TOP10F. Molekul ***Ov-BmR1*** or ***Ll-BmR1*** didapati bersaiz ~25 kDa dan analisis secara blot Western menunjukkan ia reaktif terhadap sampel serum daripada pesakit ***L. loa*** dan ***O. volvulus*** yang bermikrofilaria dan tidak reaktif dengan sampel serum daripada penderma darah yang sihat. Dengan menggunakan kaedah IgG_4-ELISA, corak pengenalan antibodi IgG_4 dalam semua sampel serum didapati sama terhadap

BmR1 dan ***Ov-BmR1*** or ***Ll-BmR1.*** ini termasuklah reaktiviti antibodi IgG_4 yang lemah yang dipamerkan oleh sampel serum daripada pesakit yang dijangkiti ***O. volvulus*** dan ***L. loa***. Kajian tentang reaktiviti terhadap subkelas IgG yang lain menunjukkan bahawa sampel serum daripada pesakit yang dijangkiti ***O. volvulus*** dan ***L. loa*** memberikan keputusan positif (bila diuji dengan ***Ov-BmR1*** or ***Ll-BmR1 atau BmR1)*** hanya dengan IgG_1 dan tidak dengan subkelas IgG_2 atau IgG_3. Begitu juga reaktiviti ***BmR1*** terhadap sampel serum daripada individu yang terjangkit dengan ***B. malayi*** dan ***W. bancrofti*** (jangkitan aktif dan kronik) turut memberi reaktiviti positif terhadap IgG_1 dan negatif terhadap subkelas antibodi IgG_2 atau IgG_3. Namun begitu, sampel serum daripada individu normal dan yang dijangkiti cacing bawaan tanah juga menunjukkan corak reaktiviti yang serupa (iaitu positif dengan IgG_1 dan negatif dengan IgG_2 & IgG_3).

Kajian ini menunjukkan bahawa homolog bagi antigen rekombinan ***BmR1*** wujud di dalam ***W. bancrofti***, ***O. volvulus*** dan ***L. loa*** dengan konservasi yang tinggi. Pengecaman antigen (***BmR1, Wb-BmR1, dan Ov-BmR1*** or ***Ll-BmR1***) oleh sampel serum pesakit adalah sama terhadap IgG_1, IgG_2 atau IgG_3, tetapi berbeza bagi antibodi IgG_4. Kesimpulannya ialah, walaupun antigen ***BmR1*** adalah sesuai digunakan untuk pengesanan antibodi IgG_4 terhadap jangkitan filariasis brugia, bagaimanapun, protein homologinya (***Wb-BmR1***, ***Ov-BmR1*** dan ***Ll-BmR1***) tidak sesuai digunakan bagi tujuan pengesanan jangkitan penyakit filaria yang lain.

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ABSTRACT

An antibody-detection rapid test, ***BRUGIArapid***, that detects IgG_4 antibodies reactive to a recombinant ***B. malayi*** antigen (***BmR1***), is a promising tool for mapping and monitoring the areas where brugian filariasis is endemic. Field trials have revealed that ***BmR1*** is highly sensitive and specific in detecting microfilariaemic individuals. In sera of individuals infected with ***Wuchereria bancrofti*** the IgG_4 reactivity to ***BmR1*** is variable, and cross-reactivity of sera from individuals infected with ***O. volvulus*** or ***L. loa*** was observed only in single cases.

Due to its diagnostic significance, it is therefore important to characterize the ***BmR1*** antigen more closely. The varying degree of ***BmR1*** recognition in other filarial infections (other than ***B. malayi*** & ***B. timori***) raises the question whether the homologous antigen is also present in ***W. bancrofti*** (***Wb-BmR1)***, ***O. volvulus*** (***Ov-BmR1***) and ***L. loa*** (***Ll-BmR1***). In this study, the respective cDNA sequences were identified, the protein expressed and the antibody reactivities of patients' sera to the homologous recombinant antigens was studied.

Web-based homology searches for homologs of ***Bm17DIII*** in ***W. bancrofti***, ***O. volvulus*** and ***L. loa via*** BLASTN server of several DNA databases resulted in no similarity to any sequence of ***W. bancrofti*** and ***L. loa***, while for ***O. volvulus***, there were two L_3

sequences which had a low E-value. Homology searches ***via*** BLASTP (protein databases) revealed that ***BmR1*** protein did not have any similarity with other protein sequence.

PCR was used to isolate the cDNA sequences from cDNA libraries and/or genomic DNA of ***W. bancrofti***, ***O. volvulus*** and ***L. loa*** based on RNR & RNF primers. The 618 bp PCR products (namely ***Wb17DIII***, ***Ov17DIII*** and ***Ll17DIII***) was then cloned into TOPO vector, sequenced and analysed using Vector NTI software and BLAST server. ***Wb17DIII*** was found to be 100% identical to ***Bm17DIII***, while ***Ov17DIII*** and ***Ll17DIII*** were found to be identical to each other and shared 99.7% homology with ***Bm17DIII***. Thus the results of the web-based search for ***Ov17DIII*** were not in agreement with the laboratory results. This study also revealed that, unlike the complete ***Bm17DIII*** gene which contains an intron (and two exons), the complete ***Wb17DIII*** gene did not possess any intron.

The ***Ov17DIII*** or ***Ll17DIII*** recombinant gene was then expressed in pPROEX^{TM} HTa/TOP10F. The MW of ***Ov-BmR1*** or ***Ll-BmR1*** was ~25 kDa and analysis by Western blot showed reactivity with sera from ***L. loa*** and ***O. volvolus*** mf+ patients and no reactivity with serum from healthy blood donor. By employing IgG_4-ELISA, the pattern of IgG_4 recognition of all serum samples to ***Ov-BmR1*** or ***Ll-BmR1*** and ***BmR1*** was found to be identical. This included weak IgG_4 reactivities demonstrated by sera from ***L. loa-*** and ***O. volvulus-***infected patients. With respect to reactivities to other IgG subclasses, sera from ***O. volvulus-*** and ***L. loa-*** infected patients showed positive reactions (when tested with ***Ov-BmR1*** or ***Ll-BmR1*** or ***BmR1***) only with IgG_1; and no reactivity was observed with IgG_2 or with IgG_3. Similarly, sera from individuals infected

with ***B. malayi*** or ***W. bancrofti*** (active and chronically-infected patients) were positive with ***BmR1*** only for IgG1 and were negative when tested with IgG_2 and IgG_3 subclasses. However, it is also noted that sera from non-endemic normals and soil-transmitted helminth infections also showed similar reactivities ***i.e.*** IgG_1 positive and IgG_2 and IgG_3 negative.

This study demonstrated that ***Bm17DII gene and its homologs*** in ***W. bancrofti***, ***O. volvulus*** and ***L. loa*** are highly conserved. Recognition of the recombinant gene products (***BmR1*** or ***Wb-BmR1*** and ***Ov-BmR1*** or ***Ll-BmR1***) by patients' sera are similar with regard to IgG_1, IgG_2 and IgG_3, but different for IgG_4 antibodies. Thus this study demonstrated that although IgG_4 antibodies to ***BmR1*** are a good infection marker for brugian filariasis, its homologs are not of diagnostic value.

CHAPTER ONE

INTRODUCTION

1.1 Human Filariasis

Filariasis is caused by roundworms that inhabit the lymphatic and subcutaneous tissues and cause some of the most debilitating diseases, including elephantiasis and river blindness (WHO, 2002a,b; 2003). Human filarial infections are endemic in tropical regions (Figure 1.1) and affect an estimated 200 million people, and exposing another billion to the risk of infection (Ottesen ***et al***., 1997). The parasites are transmitted by blood-feeding insects, which act as vectors. Filarial nematodes enter the human body at their third larval stage by escaping from the mouthparts of their vector arthropod as they bend during biting and enter through the bite wound in the skin.

Eight main species infect human namely ***Wuchereria bancrofti***, ***Brugia malayi*** and ***Brugia timori*** which causes lymphatic filariasis; ***Loa loa*** which causes loaisis; ***Onchocerca volvulus*** which causes onchocerciasis; and three species of ***Mansonella***.

Filariasis is rarely fatal, it is the second leading cause of permanent and long-term disability in the world. The World Health Organization (WHO) has named filariasis one of only six “potentially eradicable” infectious diseases and has embarked upon a 20-year campaign to eradicate the disease. The immunologic hallmark of infections by filarial parasites is induction of allergic type responses (Bundy ***et al***., 1991, King, 2000). Typically this produces peripheral eosinophilia and elevated levels of polyclonal and parasite specific IgE. Filarial specific IgG subclasses are also present, with IgG_4 subclass most prominently elevated (Ottesen ***et al***., 1985).

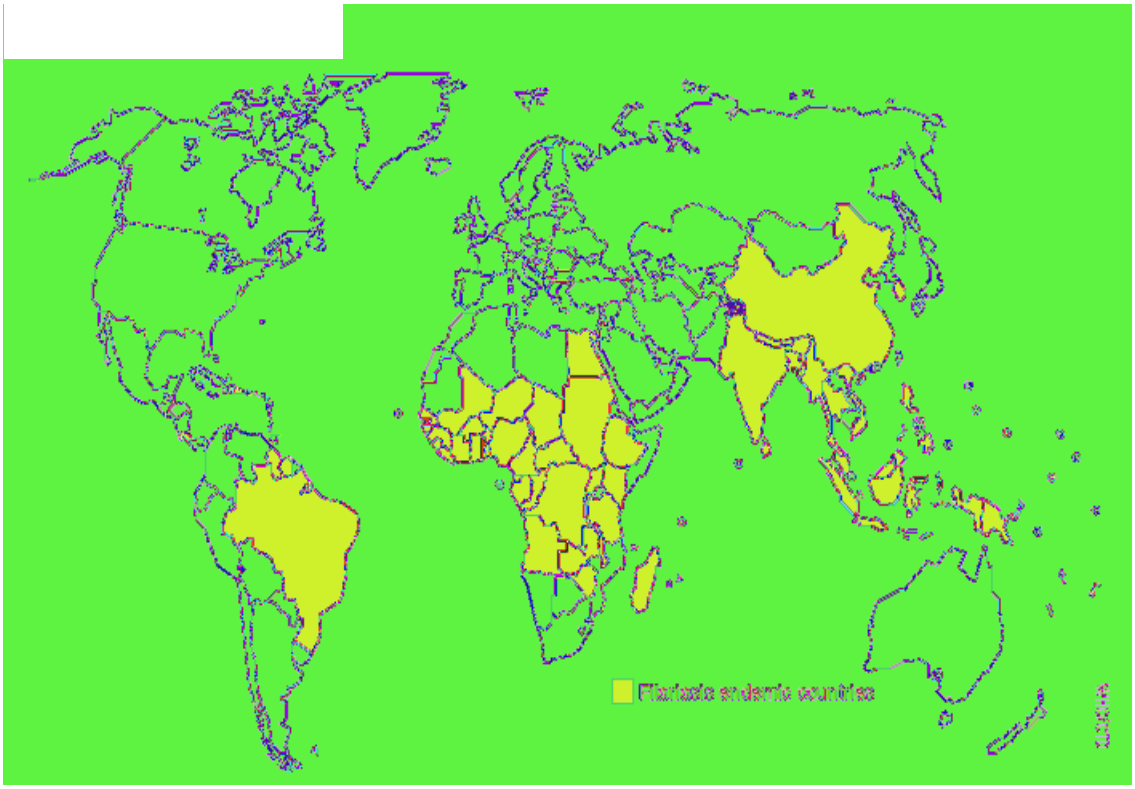

Figure 1.1 A map showing filariasis endemic countries (WHO, 1997).

1.2 Lymphatic Filariasis

Lymphatic filariasis (LF) also known as elephantiasis is a disabling and disfiguring infection caused by ***W. bancrofti***, ***B. malayi*** or ***B. timori*** (Michael and Bundy, 1997). It is estimated that 120 million people or 2% of the world's population are infected in around 80 countries throughout the tropics and subtropics (WHO, 2000; WHO, 2002; Molyneux, 2003).

It is a major cause of acute and chronic morbidity affecting persons of all ages and both sexes (Ottesen, ***et al***., 1997) and prevails in those of low socioeconomic level (Dreyer, ***et al***., 2000). LF is a major burden on health and hospital resources (Gyapong, ***et al***., 1996) includes high medical expenses, loss of productivity, diminished social function and reduced quality of life (Evans, ***et al***., 1993). Approximately 44 million people demonstrate signs of elephantiasis, lymphoedema and genital pathology (Ottesen, ***et al***., 1997). Persons with chronic manifestations of the disease are often unable to work or marry, become dependent for care and financial support and consequently lead to lost self-confidence. Thus, LF has been identified by the World Health Organization (WHO) as the second leading causes of permanent and long-term disability world-wide (WHO, 1997).

These filarial infections affect individuals from all age groups and races. Men are more susceptible compared to woman and children (Kazura, 1999). This phenomenon may be explained by the greater exposure of men to mosquito vectors at work. In endemic areas, children are usually exposed to this infection early in their life and as many as one third of the children were infected before the age of five (Witt & Ottesen, 2001).

The total global burden of LF is not known and mapping of its endemicity and prevalence is on-going. LF is the most prevalent filarial infection of humans, most of which is caused by ***W. bancrofti***, while the closely related species, ***B. malayi*** and ***B. timori*** cause the remaining infections (Taylor, 2003). Of the three parasites causing LF,

W. bancrofti accounts for over 90% of the global burden (WHO, 2002a,b). It is endemic in India, Africa, South America, Indonesia, Burma, Vietnam, Egypt and China. ***B. malayi*** is limited in distribution to Asia (India, China, Indonesia, Philippines, Thailand, Vietnam and Malaysia) and ***B. timori*** to a few islands in Indonesia (WHO, 2003).

Basically, three groups of people will be found in filarial-endemic areas (Ottesen, 1992, 1993; Ottesen, and Campbell, 1994). There are those who are exposed to the infection but display no evidence of disease, these are the so-called as endemic normals; the second group are those who are clinically asymptomatic but demonstrate presence of the larvae (asymptomatic microfilaraemics). The third groups are those with chronic disease such as chronic lymphoedema, hydrocele and elephantiasis. Infected people suffer episodes of acute filarial disease such as acute filarial lymphangitis. This is believed to be partly due to the result of an immunological reaction to dead or dying adult worms which have either been killed by the immune system or by chemotherapy (Dreyer ***et al***., 1999a, b). The compromised lymphatic function coupled with accumulation of protein-rich fluid in the tissue predisposes the limbs to bacterial secondary infection, and is an important risk factor for development of elephantiasis (Dissanayake ***et al***., 1995)

Lymphatic filariasis can be transmitted by more than 70 species and subspecies of mosquitoes. However, the principal mosquito species that are efficient vectors are found in the following four genuses: ***Anopheles*** (***W. bancrofti***, ***B. malayi*** and ***B. timori***), Aedes (***W. bancrofti*** and ***B. malayi***), Culex (***W. bancrofti***) and ***Mansonia*** (***W. bancrofti*** and ***B. malayi***) (Scott, 2000). Rural-urban migration and uncontrolled urbanization often lead to over burdening of sewerage and waste-water systems. The resulting pools of stagnant, polluted water provide an ideal breeding ground for ***Culex quinquefasciatus***, a major vector of filariasis (Ottesen, ***et al***., 1997; Mak, 1987). In contrast to

Anophelines, Culicines can efficiently transmit filariasis in situations where the microfilaria (mf) density is low (Webber, 1991).

1.3 W*uchereria bancrofti*

W. bancrofti is a filarial nematode which lives in lymphatic channels and lymph nodes of humans. The adult worm is elongated and slender (30 to 100 mm long by 100 to 300 µm wide); and males are about half the size of females. The width of the microfilaria (mf) is the diameter of a red blood cell and the length is 250 to 300 µm. Adults produce mf measuring 244 to 296 µm by 7.5 to 10 µm, which are sheathed and have nocturnal periodicity, except for the South Pacific strain which have the absence of marked periodicity (Cross, 2003). ***W. bancrofti*** belongs to the class of Secernentea, subclass of Spiruria, a family of Filariidae and super family of Filarioidea.

Elephantiasis has been written about since the time of the early Greeks and Romans. The larval mf was first seen in hydrocele fluid by the French surgeon Jean-Nicolas Demarquay in 1863 and in urine by Otto Henry Wucherer in Brazil in 1866. The adult worm was described by Joseph Bancroft in 1876 and named Filarial bancrofti in his honor by the British helminthologist, Thomas Spencer Cobbold. The discovery of the life cycle by Patrick Manson in 1877 is regarded as one of the most significant discoveries in tropical medicine (Cross, 2003).

The life cycle of ***W. bancrofti*** is shown in figure 1.2. When an infected mosquito bites a person who has lymphatic filariasis, the mf circulating in the person's blood enter and infect the mosquito. The mf passes from the mosquito through skin, and travel to lymph vessels. In the human lymph vessels they grow into adults and these adult worm lives for about 5–10 years. When matured, the adult worms mate and release millions of mf into their host blood system; these in turn are picked up by mosquitoes during their blood meal.

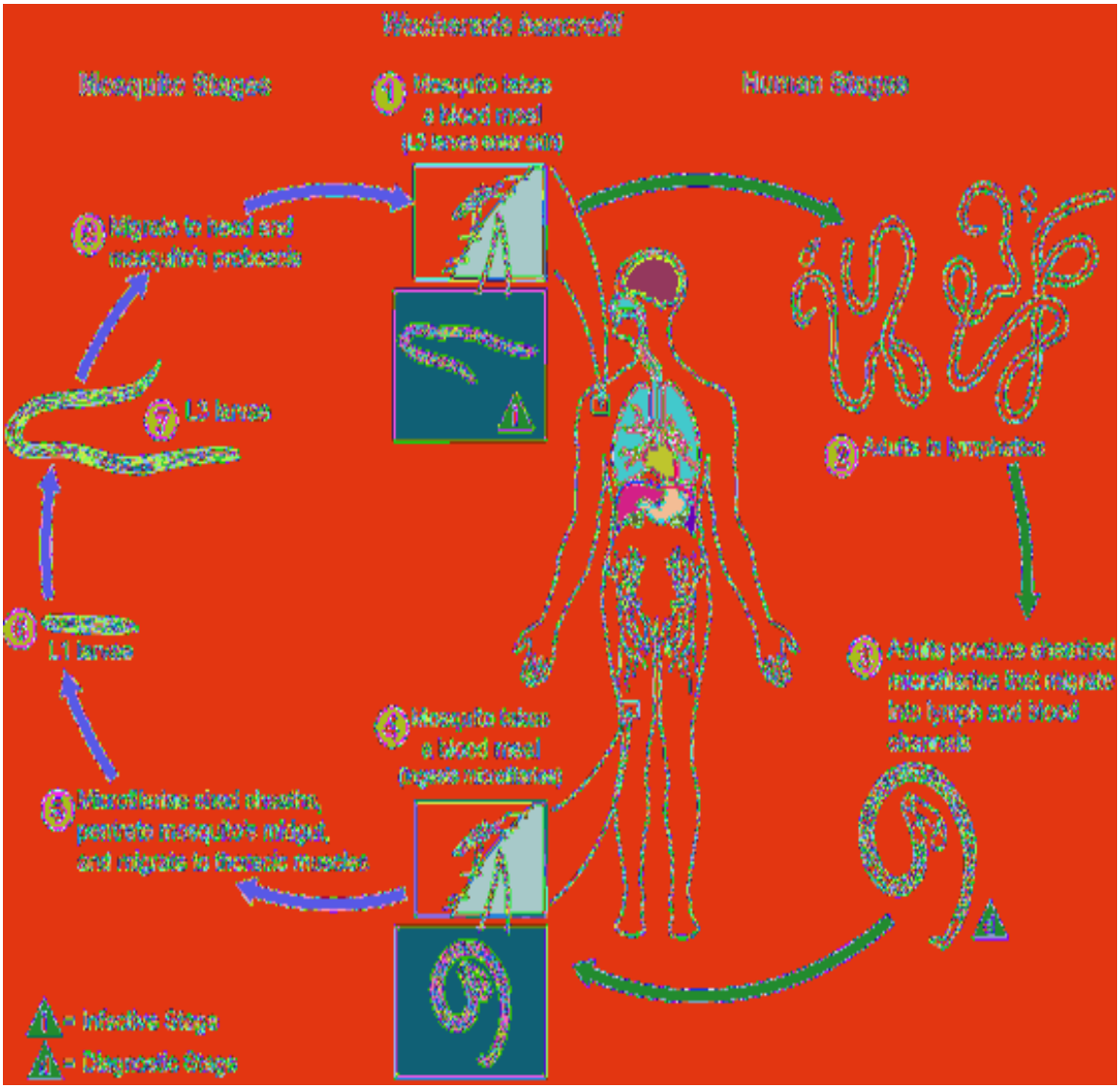

Figure 1.2 The schematic life cycle of ***W. bancrofti*** (CDC Image Library, 2004).

1.3.1 Pathology and Clinical Manifestation

LF can present a wide variety of clinical manifestations, ranging from apparently asymptomatic cases to severe disfigurement of the limbs and genitalia. Following infection with third stage larvae there is usually a period of vigorous immune response to the invading larvae (Dreyer ***et al***., 2000). The incubation phase from the point of infection to the time of appearance of first mf seems to be symptomless, but in some cases transient lymphatic inflammation occurred. Then the second stage, acute or inflammatory phase follows. This is the time when females reach maturity and releases the mf causing intense lymphatic inflammation, chills, fever, swollen lymph nodes, hydrocele and lymphadenitis to the infected human (Dreyer ***et al***., 2000).

Lymphoedema is the worst clinical manifestations of lymphatic filariasis. An important contributor to this condition is the repeated adenolymphangitis and cellulitis caused by secondary bacterial infections (Dissanayake ***et al***., 1995). This can result in gross enlargement of the affected limbs and with ***W. bancrofti*** infections, these enlargements are usually unilateral (Figure 1.3 a, b, c, d). Besides being physically handicapped, patients' abilities to carry out their daily activities are greatly restricted. There are four different stages of lymphoedema. In the initial stage, the lymphoedema can spontaneously be reversible through elevation and resulted in no skin changes. In the final stage, the lymphoedema is non-reversible, skin thickens and accompanied by warts or papilloma (WHO, 1992).

The mf in the blood and lungs can also cause an IgE-mediated allergic response which can result in asthma-like symptoms (tropical pulmonary eosinophilia or TPE).

(a) (b) (c) (d)

Figure 1.3 Elephantiasis of the legs due to the lymphatic filariasis, (a) Elephantiasis of the legs due to lymphatic filariasis; (b) Elephantiasis of the leg in a young mother (c) Comparison of a normal and diseased leg of a young woman with elephantiasis; (d) Close-up of the legs of a 64-year-old coir factory worker with elephantiasis. She has been infected for 45 years (TDR Image Library, 2005).

1.3.2 Diagnosis of Bancroftian Filariasis

LF is diagnosed by a combination of the appropriate epidemiological history, physical findings and laboratory tests. Accurate and early diagnosis of filarial infection, followed by appropriate treatment can prevent sufferings due to the irreversible chronic elephantiasis. Signs of chronics filariasis are easily recognized, especially among individuals living in the endemic areas. However detection of adenolymphangitis (filarial fever) or TPE among suspected patients may not be as easy. Diagnosis is not only important to the infected individuals but is also a useful screening tool for the mapping of the endemic areas which is the first important step towards realizing the success of the lymphatic filariasis elimination program (WHO, 1997; 2002a,b).

1.3.2.1 Direct physical examination and survey

Information for the distribution of infection and disease can be assessed by rapid assessment through questionnaire among local residents. In areas where questionnaires cannot be administered or patients are not knowledgeable about the disease prevalence, physical examinations can be used as a rapid assessment tool (WHO, 2000) to look for lymphoedema of the limbs or hydrocele in males.

1.3.2.2 Thick blood film (TBF)

The accurate diagnosis of active infection can be made essentially only by detecting mf in the blood of infected individuals (Weil, 1987). The simplest method is a thick blood film of capillary blood stained with Giemsa stain (Khamboonruang ***et al.,*** 1987). For epidemiological screening, 60 µl of finger-prick blood can be dried on a slide, stained and examined under a microscope. Disadvantages of thick blood films include the need to collect blood at night since the mf in peripheral blood peak between 10 p.m. and 2 a.m. The sensitivity of this method is relatively low, leading to misdiagnoses of people with low-density infection, amicrofilaraemic stage of infection and single sex

infection. Consequently this allows them to progress to irreversible major lymphatic damages (Braga ***et al***., 2003).

1.3.2.3 Concentration techniques

Use of concentration techniques increases the sensitivity but amicrofilaraemic cases will still not be detected. An old but still widely used method is that Knott (1935). One milliliter of blood is added to 9 ml of a 2% formalin solution in water. After red cell lysis is complete, the mixture is centrifuged and the deposit is examined for mf. The theoretical detection limit is one mf per ml. The problem is when blood is processed from individuals with a large amount of plasma gamma globulin. The formalin precipitates the protein and makes the examination of the deposit difficult. The Knott's method has been improved by Melrose ***et al***. (2000) who add a small amount of Triton 100-X to the diluents which dissolves most of the proteingenous deposit and enhances the visibility of the mf. Another technique is membrane filter technique in which 1-5 ml of blood which has been diluted in water is passed through a filter fitted with a polycarbonate membrane which traps the mf. The membrane is removed and the mf is stained and counted (Moulia-Pelat ***et al***., 1992; McMahon ***et al***., 1979).

1.3.2.4 Ultrasonography

High-frequency ultrasonography can directly visualize adult worms in the dilated lymphatics. It has being used to detect adult worms in the scrotum and breast (Amaral ***et al***., 1994; Dreyer ***et al.***, 1996a,b; 1999c) and has detected viable worms in children (Dreyer ***et al***., 1999a).

1.3.2.5 Polymerase chain reaction

PCR methods have been successfully used for the detection of parasite-specific DNA of ***W. bancrofti*** in blood, plasma, paraffin-embedded tissues sections (McCarthy ***et al***., 1996) and sputum (Abbasi ***et al***., 1996, 1999). PCR assays offer rapidity, higher sensitivity and specificity over conventional dissection and microscopic technique but they are relatively expensive (Zhong ***et al***., 1996). This is mainly due to the involvement of costly chemicals and lengthy procedures of DNA extractions and facilities not often available in filarial-endemic areas. A highly sensitive and specific PCR assay, based on ***Ssp I*** repeats sequence, has been developed for detecting ***W. bancrofti*** in human blood and vectors (Zhong ***et al***., 1996). This ***Ssp I*** PCR assay was found to be highly species specific, as it did not detect the DNA of a closely related filarial parasite, ***B. malayi*** and also proved to be highly sensitive as it did detect as little as 0.04 pg of ***W. bancrofti*** DNA (Hoti ***et al***., 2001). Therefore, it has potential application in rapid assessment of transmission of filariasis.

1.3.2.6 Detection of circulating antigen

Two different groups of inventors independently produced monoclonal antibodies, ***Og4C3*** and ***AD12***, which recognized a protein moiety of a major phosphocholine-containing circulating antigen of ***W. bancrofti*** (More and Copeman, 1990; Weil, 1987). These antigen detection assays were found to be more convenient than the microfilarial detection technique since night blood sampling could be avoided as circulating antigens were present even in daytime blood samples (Pani ***et al.,*** 2000) collected on filter paper (Itoh ***et al.,*** 1998).

The murine monoclonal antibody, ***Og4C3*** directed against antigen of ***Onchocerca gibsoni*** was used successfully as the detection-antibody in a sandwich ELISA for the detection of circulating antigen of ***W. bancrofti***. However, this assay was not effective

in detecting antigens of ***B. malayi, B. timori, O. volvulus*** or ***Loa loa*** (More and Copeman, 1990).

Another commercially available antigen detection assay used the ***AD12*** monoclonal antibody in a rapid-format card test for the detection of bancroftian filariasis. The test is a rapid ICT technique using specific monoclonal and polyclonal antibodies which recognized the filarial antigen in the blood of infected humans. This kit (ICT Filariasis) that was initially manufactured by ICT Diagnostics, Balgowlah, New South Wales, Australia is currently marketed by Binax Now^{R}ICT, Portland, USA. It utilizes capillary or venous blood collected either the night or day and is very easy to handle, very fast to perform, can be used in the field by people with a minimum amount of training (Weil ***et al.***, 1997). It has previously been shown to be highly sensitive for infections with ***W. bancrofti*** and highly specific with respect to other filarial parasites including ***O. volvulus***, ***B. malayi***, ***L. loa*** and ***Mansonella Streptocerca*** (Weil ***et al***., 1997). Its reported sensitivity and specificity rates were 96-100% and 100% respectively. Thus, this test is useful for mapping of endemic areas in control programs for bancroftian filariasis.

1.3.2.7 Detection of anti filarial IgG4 antibody

New and more sensitive and specific assays for diagnosis of LF have been developed (Dissanayake ***et al***., 1994; Chandrashekar ***et al***., 1994). One study on bancroftian filariasis reported that IgG_4 antibodies reacted well with recombinant ***W. bancrofti*** SXP-1 antigen (Engelbrecht ***et al***., 2003). A sensitivity of 100% was obtained in patients with patent ***W. bancrofti*** infections using the ***Wb***-SXP-1 antigen in IgG_4-ELISA (Rao ***et al.***, 2000). This recombinant antigen has now being developed into a rapid test for the identification of total IgG antibodies to ***Wb***-SXP-1. The test is a flow-through immunofiltration test that employed colloidal gold-protein A as the antibody capture reagent (Baskar ***et al***., 2004). Another two recombinant-antigens, namely Bm14 and ***BmR1,*** have been developed into IgG4 antibody detection tests and have been shown

to be sensitive and specific for determining LF infection/exposure. The Bm14 antigen was reported to be equally sensitive for ***Wuchereria*** and ***Brugia*** infection/exposure (Ramzy ***et al***., 1995; Weil ***et al***., 1999). This antigen has some cross-reactivity with sera from patients with other filarial infections (loiasis and onchocerciasis), but not with sera from people with non-filarial nematode infections (Ramzy ***et al***, 1995). Field studies in Egypt showed that prevalence rates of antibody to Bm14 prior to initiation of MDA were much higher than antigen or mf prevalence rates in young children (Weil ***et al***, 1999). The ***BmR1*** antigen which performed well in the detection of brugia infections, has limited sensitivity in detecting ***W. bancrofti*** infection (Lammie et al, 2004).

1.3.3 Treatment and prognosis

Individuals found to be mf-positive or filarial antigen-positive during the initial assessment period, monitoring, or on voluntary examination should be treated with diethylcarbamazine (DEC). DEC has been used to treat filariasis since 1947 (Santiago-Stevenson ***et al.,*** 1947) and still is the most widely used anti-filarial. Many countries use a 12-day course (***W. bancrofti***) or a 6-day course (***B. malayi***) of 6 mg/kg/day of DEC (WHO, 1992a,b). In some of the control programs in the Pacific and Papua New Guinea, colour-coded DEC tablets are used without weighing the patient. A 300mg tablet is given to adults and a 150mg tablet to children. DEC is very effective in killing microfilaria but only partly effective against adult filarial parasites. This is evidenced by the work by Weil ***et al.*** (1988) who demonstrated that filarial antigenaemia persists for up to 12 months after DEC therapy. Figueredo-Silva ***et al.*** (1996) removed nodules after DEC treatment and found that all contained degenerating adult worms, thus proving that DEC has a limited amount of macrofilaricidal activity.

A study showed that single dose albendazole (400mg) has similar efficacy in the clearance of mf as that of DEC (6 mg/kg) or the co-administration of the two drugs, i.e.

albendazole (400mg) plus DEC (6 mg/kg) (Pani ***et al.,*** 2002). This showed that albendazole can be used in mass single dose administration for the control of LF.

In areas co-endemic with onchocerciasis or loiasis, ivermectin has been used widely as microfilaricidal in LF cases to avoid the potentially severe allergic reaction with DEC. Ivermectin is a highly effective and generally well-tolerated drug for the treatment of LF (Brown ***et al***., 2000) and a series of single dose ranging studies from 20 to 200µg/kg for the treatment of bancroftian filariasis was effective in decreasing blood microfilaria density (Cartel ***et al.,*** 1990a,b,c,d; Kumaraswami ***et al***., 1988). Ivermectin with higher doses resulting in more sustained clearance of mf (Kar ***et al.,*** 1993a, b; Kumaraswami ***et al***., 1988). It is a very effective microfilaricide but how effective it is against adult worm is a contentious issue.

Simple hygiene measures, supplemented with antibiotics or antibacterial cream helps prevent damage tissues from worsening, stop secondary bacterial infections and help to reduce the limb enlargement caused by repeated filarial and bacterial infections (Ottesen, 1997). Effective hygiene measures include regular twice-daily washing of the affected parts with soap and water; raising the affected limb at night; keeping the nails clean; wearing shoes; and using local antiseptics or antibiotic creams to treat small wounds.

Anti filarial drugs alone will not be able to revert the fibrotic changes of the skin and connective tissue involved (Kazura, 1999). Now expertise is available for surgical therapy of genital manifestations of filariasis. Generally, the surgery involves removal of the excess fibrotic tissue. The most common surgery for hydrocele is complete excision of the sac. However, its effect is short lasting as the edema will recur. As an alternative, micro vascular surgery can be performed (Kazura, 1999) which involves anastomosis of the lymph vessels with the nearby veins (WHO, 2002a,b).

1.3.4 Global Programme to Eliminate Lymphatic Filariasis (GPELF)

The goal of the Global Programme to Eliminate Lymphatic Filariasis (GPELF) is defined as “The Elimination of Lymphatic Filariasis as a Public Health Problem by 2020” (WHO, 1997). The 50th World Health Assembly in 1997 had passed a resolution identifying the elimination of LF as a public health problem, a priority. This includes some strategic plans which are identified to have four major elements (WHO, 1999a,b) namely the interruption of transmission; the prevention of disability; the provision of additional technical support; and the implementation of operational research (up-scaling program). Interruption in transmission can be achieved in reducing and eliminating the reservoir of mf through treating the human population and by reducing contact between humans and mosquito vectors (Chaubal ***et al***., 2003).

The traditional method ***i.e.*** thick blood smear examination which was used before, have a sensitivity of only 25-40% (WHO, 1998). If it is the sole diagnostic method used in post intervention surveillance, many false negatives will occur; hence a low incidence will not actually indicate successful interruption of transmission. Therefore, there is a clear need for specific, sensitive and field applicable tests as to ensure the success of this global elimination program.

Mass drug administration (MDA) of at-risk population (Freeman ***et al.,*** 2001) is being used to interrupt transmission. This is based on the evidence of the effectiveness of a single dose of DEC (6mg/kg) in the clearance of mf and sustaining this over a period of at least one year (Farid ***et al***., 2003). Thus this reduces the number of mf in the blood to levels below which the mosquito vectors can no longer transmit infection. These comprise two approaches. First is the once-yearly treatment with single dose of two drugs given together i.e. albendazole plus either ivermectin or DEC for 4-6 years

(Molyneux, 2003). The second approach is the use of DEC-fortified table or cooking salt for 1-2 years (Lymphatic Filariasis Support Center, 2004).

GlaxoSmithKline has committed to provide the entire supply of albendazole, while Merck & Co. Inc., committed to supply ivermectin in those African countries with onchocerciasis and loaisis (WHO, 1997). By the end of 2001, nearly 26 million people in over 22 countries had been administered the combination drugs in MDA campaigns (WHO, 2002a,b). Vector control has been used as an important adjunct in the LF program in certain areas. The strategy to prevent disability is designed to encourage home-based self-care, i.e. regular skin care, exercise and appropriate footwear, and by trying to develop facilities in the health care system for disability control (WHO, 1997). The control of lymphoedema management is the prevention of acute ADL attacks through basic hygiene by using soap and water, and prevention and treatment of small skin lesions with application of topical antifungal or antibiotic cream (WHO, 2000). This basic hygiene can stop the acute attacks and improve the patient's condition.

1.3.5 Lymphatic Filariasis in the Malaysian Context

The Filariasis Control Program in Malaysia was established more than 30 years ago; however the disease is still a public health problem in rural areas, especially in some areas in Sarawak, Pahang, Terengganu, Johor, Perak, Sabah, Kedah and Kelantan. More than 85% of the annual incidence rate of lymphatic filariasis in Malaysia is due to ***B. malayi*** (Ministry of Health, 1990-1999). The annual incidence of chronic elephantiasis is around 5 to 10, and about 2.9 million people are at risk of acquiring this disfiguring disease (Che Abdullah, 2004). In early 1960, the Vector Borne Disease Control Unit (RKPBV) of Ministry of Health initiated filariasis surveillance to detect and treat every filariasis cases. Later, three approaches were implemented in Malaysia, namely, mass treatment and treatment of index cases; eradication and control of the

vectors; and avoiding human-mosquito contact (Noorhayati, 1999). In June 2003, Malaysia started the National Program for Elimination of Lymphatic Filariasis and the aim is to eliminate lymphatic filariasis as a public health problem by 2013 (Che Abdullah, 2004).

The mosquito vectors belonging to the ***Anopheles*** and ***Mansonia*** genera are involved in the transmission of filariasis in Malaysia, the latter being the more important vector. ***Anopheles donaldi*** was found to be infected with infective larvae of ***B. malayi*** (Vythilingam ***et al***., 1996) where the peak biting time was around 11 pm to 12 am. Monkeys and domestic cats are the reservoir hosts for the subperiodic strain of ***B. malayi*** (Marzhuki ***et al***., 1993)***.*** The most common monkeys in Malaysia are the macaques (***Macaca spp***.) and leaf monkeys (***Presbytis spp***.) [WHO, 1984].

The historical record of filariasis in Malaysia can be categorized under three phases (Lim, 2005). The first phase (1908-1952) mainly focused on the statistics of microfilarial carriers which had been identified in Hospital Kuala Lumpur and Raub. The second phase (1953-1961) dealt with the mapping of endemic foci in East Pahang, Terengganu and Kelantan. At this phase it was found that ***Culex quinquefasciatus*** was implicated for spreading bancroftian filariasis. Successful mass chemotherapeutic trials were conducted in two villages of Pahang ***i.e.*** treatment regime of weekly and monthly doses of diethylcarbamazine (DEC) at 4-6 mg/kg body weight for 6 weeks and 6 months respectively. The activities of the third phase (1962-onwards) was mainly towards identifying the most suitable method for filariasis control.

In Malaysia mf densities in many infected individuals are too low to be detected by the traditional method of thick blood smear examination. In addition, thick blood smears do

not allow for the detection of individuals harbouring nonfertile worms, pre-patent infections and single sex infections (Turner ***et al***., 1992).

In the official report of the district of Pasir Mas in the state of Kelantan, one case in 2428 samples (0.04%) from the subdistrict of Gual Periok was detected in 1997; and in 2000, two cases were detected in 803 samples (0.25%) from the subdistricts of Gual Periok and Rantau Panjang (Pasir Mas Health Office 1997–2001). However, no cases were reported in 1998 (1385 samples), 1999 (985 samples) and 2001 (1100 samples). These figures probably do not reflect the true prevalence of the infection as it is based on the insensitive thick blood smear examination. Recently, Rahmah ***et al*** (2003a) conducted a filariasis survey on 5138 pupils schools located in the subdistricts (mukim) of Pasir Mas bordering Thailand border. Out of 2439 boys and 2699 girls screened. ***Brugia malayi*** infection was detected in 18 children, giving an overall prevalence of 0.35% (18 of 5138). The investigators employed a recombinant antigen (***BmR1***) based ELISA (***Brugia***-Elisa) that has been shown to highly specific and sensitive for detection of brugian filariasis (Supali ***et al.,*** 2004; Rahmah ***et al***., 2003b). In 2001, Lim ***et al*** had collected a total of 1,134 finger-pricked blood samples from residents of Setiu, Terengganu and the findings showed that 0.26% (3/1,134) were positive by thick blood smear examination, while 2.47% (28/1,134) were positive using ***Brugia***-Elisa. In another study conducted by Jamail ***et al*** (2005) among residents of seven endemic districts in the state of Sarawak, the overall prevalence of brugian filariasis as determined by a rapid test was 9.4% while that determined by microscopy was 0.90% thus the dipstick detected about 10 times more cases than microscopy. The test employed was ***BRUGIArapid***™ dipstick test, which is also based on the ***BmR1*** recombinant antigen. Equal percentages of adults and children were found to be positive by the dipstick whereas microscopy showed that the number of infected children was seven times less than infected adults. Thus the results of the above studies showed that the use of insensitive microscopic examination leads to many

untreated cases of infected people, which will become reservoir for the transmission of the infection. Due to its superior sensitivity, ***BRUGIArapid***™ is being employed by the Ministry of Health Malaysia to assist in the National Lymphatic Filariasis Elimination Program.

1.4 Onchocerciasis

Onchocerciasis or river blindness is a major public-health and socio-economic problem in many rural areas (Shah ***et al.,*** 1987). It is caused by the filarial worm ***Onchocerca volvulus***, vectored by the black fly, ***Simulium spp.*** (Cox, 2002; Greene, 1992) including ***S. damnosum*** (Shah ***et al***., 1987). It infects about 20 million people and is endemic in 28 countries in Africa, 6 countries in the Americas and in Yemen (WHO, 2002a,b). Onchocerciasis is one of the leading causes of infectious blindness worldwide (Klotz ***et al.,*** 2000; Duke, 1990). It has caused visual impairment in 500,000 and blindness in 270,000 people, rendering onchocerciasis the second most frequent cause of preventable blindness in sub-Saharan Africa (WHO, 1995). Rarely life-threatening, the disease cause chronic suffering and severe disability (Figure 1.5).

The most important signs are blindness and chronic skin disease such as scaly, itchy and unusual nodular skin (Cox, 2002; Pogonka ***et al.,*** 1997). Onchocerciasis used to be the major cause of blindness throughout sub-Saharan Africa, often affecting more than 50% of the inhabitants of towns and villages in endemic areas (Greene, 1992). In some small communities in Africa and Central America, most of the people of middle age and over are blind.

(a) (b) (c)

Figure 1.4 Two signs of onchocerciasis; (a) and (b) a 60-year-old male farmer scratching his legs which show the tell-tale depigmentation (leopard skin), the consequence of years of fenzied scratching. Having the incessant itching chronic skin disease, and (c) close up picture of an eye damaged (river blindness) as a result of infection with ***O. volvulus*** (TDR Image Library, 2005).

1.4.1 *Onchocerca volvulus*

Infection with ***O. volvulus*** is initiated by the feeding of an infected black fly on a human. There are no reservoir hosts for this parasite, but some experimental infections have been established in non-human models such as cattle (Yien-Ming and Bianco, 1995), chimpanzees and mice (Abraham ***et al***., 2001). It is a member of class of Secernentea, subclass of Spiruria, order Spirurida, belonged to the superfamily of Filarioidea and a family member of Onchocercidae.

The morphology is similar to that of ***W. bancrofti***. They are slender and blunt at both ends. Lips and a buccal capsule are absent, and 2 circles of four papillae each surround the mouth. Males are 19 to 42 cm long by 130 to 210 µm wide and the females are 33.5 to 50 cm long by 270 to 400 µm wide, while the mf are 300mm in length and 0.8mm in diameter, unsheathed with sharply pointed, curved tails.

1.4.2 Life cycle of *Onchocerca volvulus*

Human onchocerciasis is caused by the filarial parasite ***O. volvulus*** whose life cycle occurs in two different hosts namely ***Simulium*** black flies and humans (Figure 1.6). ***Simulium*** vectors breed in fast flowing rivers. A female worm may produce 1000 mf per day which is shed in the tissues and blood of their human host (Klotz ***et al***., 2000). The life cycle begins when a parasitized female black fly takes a blood-meal. The host's skin is stretched by the fly's apical teeth and sliced by its mandibles. The larvae from the fly then move to the subcutaneous tissues (molt to L3, the infective stage for human) where they migrate, form and lodge in nodules, and slowly mature into adult worms (L4) in the subcutaneous tissue for years (Klotz ***et al.,*** 2000). The adult worm locates to a single niche in the subcutaneous tissue. New worms form new nodules or find existing nodules and cluster together. After mating, eggs from inside the female worm develop into mf and leave the worm one by one. The mf migrate throughout this tissue, inducing injury to a variety of anatomical sites contiguous with that tissue or

where they die after several years (Klotz ***et al.,*** 2000). When female black flies take a blood meal they ingest the mf that will then undergo transitions to L2 stage in the fly.

1.4.3 Pathology and clinical disease

Unlike other filarial infections, the problems of onchocerciasis are caused by mf rather than adult worms. An early sign of infection with Onchocerca is the raised nodules that can be seen under the skin. These are most often seen in areas over a bony prominence and may develop into a firm, non-tender nodule called as onchocercomata which contained adult worms (Klotz ***et al***., 2000). It has been suggested that this phenomenon occurs because the larvae are immobilized in these locations long enough for them to be trapped by the body's cellular defense mechanisms. This migration lead to intense pruritus manifesting as dermatitis, whereby the skin may become thickened, edematous, wrinkled and depigmented (Klotz ***et al***., 2000).

The mf can be found free in the fluid within the nodules and in the dermal layers of the skin, spreading away from the nodules containing the adults. Mf also can be found in the blood and eye during heavy infection (Klotz ***et al.***, 2000). Mf can be killed only during a limited period of their development, after which the larvae become resistant to attack by the immune response (Abraham ***et al.,*** 2001). Reactions to dead mf around these nodules can lead to several unpleasant conditions, including serious visual impairment and blindness, skin rashes, lesions, itching and depigmentation of the skin, lymphadenitis (lead to hanging groin) and general debilitation (Klotz ***et al***., 2000). In the skin, there is destruction of the elastic tissues and the formation of redundant folds.

Dead mf in the eye leads to an inflammatory immune response and the eventual formation of secondary cataracts and ocular lesions. Because of this, heavy infections often lead to progressive blindness. Much of the pathology associated with

onchocerciasis takes place during the immune responses against the mf that are found in the skin and ocular tissues (Kazura ***et al***., 1993).

The establishment of a chronic filarial infection in humans is accompanied by characteristic cell-mediated and humoral immune responses. The antifilarial humoral immune response is characterized by high levels of immunoglobulin E (IgE) (Kurniawan ***et al***., 1993), eosinophilia (Gbakima ***et al***., 1996). In areas of endemicity, most filarial infections are initiated during early childhood (Gbakima, 1996). The precise pathogenesis of onchocerciasis lesions is still unknown.

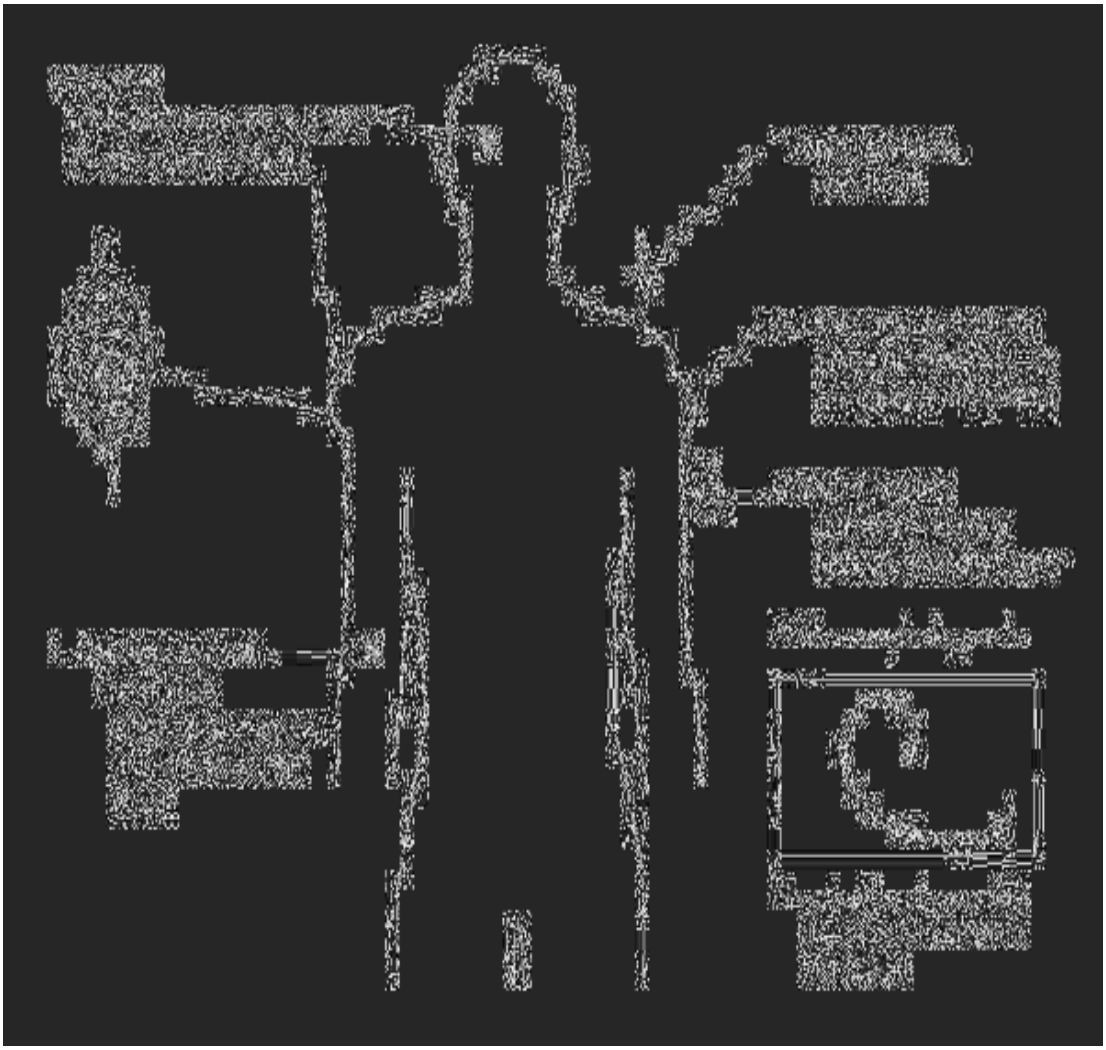

Figure 1.5 The incubation phase of filarial parasite which affective from the point of infection to the time of appearance of first mf and resulted in eye lesions, altered pigmentation and loss of elasticity of skin (Cross, 1992).