CONSTRUCTION OF HUMAN LYMPHATIC FILARIASIS ANTIBODY PHAGE DISPLAY LIBRARY AND PRODUCTION OF RECOMBINANT MONOCLONAL ANTIBODIES AGAINST B*m*R1 AND B*m*SXP FILARIAL ANTIGENS

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by

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DEDICATIONS

This thesis is dedicated to my mother, Patumutu Ibrahim, my late father Rahumatullah Othman and my lovely sisters, Rafizah and Rumaisyah who has been a great source of inspiration and motivation.

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LIST OF ABBREVIATIONS

ABTS	2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
APC	Antigen presenting cell
APS	Ammonium persulfate
βME	Beta-mercaptoethanol
bp	Base pair
B. malayi	*Brugia malayi*
BSA	Bovine serum albumin
B. timori	*Brugia timori*
cDNA	Complementary deoxyribonucleic acid
CDR	Complementary determining region
CFU	Colony forming unit
CH	Constant heavy chain
CNBr	Cyanogen bromide
CV	Column volume
DEC	Diethylcarbamazine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
Fab	Fragment antigen-binding
GFP	Green fluorescence protein

GPELF	Global Programme to Eliminate Lymphatic Filariasis
HC	Heavy chain
His-tag	Histidine tag
HRP	Horseradish peroxidase
Ig	Imunoglobulin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LB	Luria bertani
LC	Light chain
LF	Lymphatic filariasis
NTD	Neglected tropical diseases
OD	Optical density
PBS	Phosphate buffered saline
PBST	Phosphate buffer saline with Tween 20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PTM	Milk powder in PBST (blocking buffer)
RE	Restriction site
RIN	RNA integrity number
RNA	Ribonucleic acid
scFv	Single chain fragment variable
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
W. bancrofti	*Wuchereria bancrofti*
WHO	World Health Organization

LIST OF SYMBOLS

°C	Degree Celsius
g	Gram
g	Gravity
l	Liter
µg	Microgram
µl	Microliter
mg	Milligram
ml	Milliliter
ng	nanogram
nm	nanometer
rpm	Revolution per minute
U	Unit of enzyme
v/v	Volume/volume
w/v	Weight/volume

PEMBANGUNAN PERPUSTAKAAN ANTIBODI PAPARAN FAJ FILARIASIS LIMFATIK MANUSIA DAN PENGHASILAN REKOMBINAN MONOKLONAL ANTIBODI TERHADAP ANTIGEN FILARIAL B*m*R1 DAN B*m*SXP

ABSTRAK

Program Penghapusan Filariasis Limfatik secara Global (GPELF) telah dilancarkan oleh WHO pada tahun 2000 untuk menghapuskan penyakit ini sebagai masalah kesihatan awam menjelang tahun 2020. Brugia Rapid dan PanLF Rapid merupakan diagnostik pantas yang digunakan dalam program ini di kawasan endemik filariasis brugian. Kedua-dua ujian ini berdasarkan pengesanan antibodi terhadap protein rekombinan B*m*R1 dan B*m*SXP. Memandangkan GPELF sedang berkembang ke arah fasa pengakhiran yang melibatkan pengawasan pasca-MDA dan pasca-pengesahan, ujian pantas dengan kepekaan diagnostik yang lebih tinggi daripada yang ada sekarang adalah diperlukan. Dalam hal ini, pendekatan yang diambil adalah dengan menggunakan antigen rekombinan yang berketulen tinggi untuk membolehkan penggunaan jumlah antigen yang meningkat tanpa mengurangkan spesifikasi ujian. Ketersediaan monoklonal antibodi rekombinan terhadap dua protein rekombinan ini adalah sangat berguna dalam menghasilkan antigen berketulenan tinggi. Peringkat awal kajian ini melibatkan pembangunan perpustakaan scFv filariasis limfatik manusia menggunakan teknologi paparan faj. Perpustakaan dihasilkan menggunakan sistem berasaskan perantara TA untuk pengklonan, dan kepelbagaian perpustakaan yang dicapai adalah 10^8. Kemudian antibodi monoklonal diasingkan melalui proses "biopanning" menggunakan perpustakaan imun yang baru dibina dan perpustakaan naif yang dihasilkan dalam makmal. Bagi perpustakaan imun, enam dan dua antibodi

monoclonal, masing-masing kepada B*m*SXP dan B*m*R1 telah diasingkan. Bagi perpustakaan naif pula, dua antibodi monoklonal untuk setiap protein rekombinan telah diasingkan. Semua antibodi monoklonal dicirikan berdasarkan penggunaan V-gen mereka, pasangan gen, panjang CDR, taburan asid amino, arah kutuban dan kedudukan. Keputusan menunjukkan bahawa perpustakaan imun mempunyai perwakilan gen keluarga dari kappa dan lambda, manakala hanya klon keluarga lambda yang berjaya diasingkan daripada perpustakaan naif. Keputusan ujian kompetitif ELISA menunjukkan bahawa terdapat klon yang bersaing untuk tapak ikatan yang sama, dengan itu ia melekat pada tapak epitope yang sama pada antigen. Selanjutnya klon-klon antibodi yang digunakan untuk titrasi kepekatan antibodi oleh ELISA adalah 5B, 4F, 3A dan 4, 20 untuk antigen B*m*SXP dan B*m*R1 bagi perpustakaan imun; manakala klon-klon antibodi yang terpilih dari perpustakaan naif adalah XP_D5, XP_B6 dan H4R1, GIR1 masing-masing untuk antigen B*m*SXP dan B*m*R1. Kesemua klon antibodi menunjukkan had pengikatan yang berbeza, namun antibodi monoklonal 5B dan 4 menunjukkan keupayaan pengikatan yang tinggi pada kepekatan antigen yang rendah iaitu 1 pg dan 100 pg, masing-masing untuk B*m*SXP dan B*m*R1. Akhirnya, klon antibodi 5B, 3A dan 4, 20 dipilih, masing-masing untuk pembangunan kolum afiniti bagi penulenan B*m*SXP dan B*m*R1. Bagi penulenan antigen B*m*SXP, kolum antibodi campuran didapati lebih baik daripada kolum antibodi tunggal. Manakala bagi penulenan antigen B*m*R1 pula, kolum antibodi tunggal memberi hasil yang lebih baik. Antibodi monoklonal 5B telah dikonjugasikan dengan nanopartikel emas dan ia menunjukkan reaktiviti yang baik dengan ujian dipstik aliran sisi antigen B*m*SXP, serta didapati tidak reaktif apabila diuji dengan ujian dipstik aliran sisi B*m*R1. Kesimpulannya, kajian ini telah berjaya menghasilkan sebuah perpustakaan filariasis limfatik manusia yang baru dan antibodi monoklonal

terhadap antigen B*m*SXP dan B*m*R1. Seterusnya, kolum antibodi afiniti untuk penulenan B*m*SXP dan B*m*R1 juga telah dibangunkan dan dinilai serta antibodi monoklonal terhadap antigen B*m*SXP telah berjaya dikonjugasikan kepada emas dan diaplikasikan dalam ujian dipstik aliran sisi.

CONSTRUCTION OF HUMAN LYMPHATIC FILARIASIS ANTIBODY PHAGE DISPLAY LIBRARY AND PRODUCTION OF RECOMBINANT MONOCLONAL ANTIBODIES AGAINST *Bm*R1 AND *Bm*SXP FILARIAL ANTIGENS

ABSTRACT

A Global Programme for Elimination of Lymphatic Filariasis aims to eliminate the disease as a public health problem by the year 2020. Brugia Rapid and PanLF Rapid are rapid diagnostic tools that are being used in this programme. They are based on the detection of recombinant proteins B*m*R1 and B*m*SXP. The quality of these tests should be well-maintained at all time or even improved as the GPELF progresses and a sensitive and specific test may be needed at the end of the programme and for surveillance post-certification. To address this need, the availability of recombinant monoclonal antibodies to the two recombinant proteins would be very useful. The first objective of this study involved the construction of a human lymphatic filariasis scFv library using phage display technology. The library was generated using a TA based intermediate shuttle system for cloning and the diversity of the library was 10^8. Then monoclonal antibodies were isolated via biopanning using the newly constructed immune library and an in-house produced naïve library. From the immune library six and two monoclonal antibodies to B*m*SXP and B*m*R1, respectively were isolated. From the naïve library two monoclonal antibodies to each recombinant protein were isolated. All the monoclonal antibodies were characterized based on their V-gene usage, gene pairing, CDR length, amino acid distribution, polarity and position. The results showed that the immune library has both kappa and lambda family gene representation, however only lambda family clones were isolated from the naïve

library. The result of cross-reactivity showed that there were clones competing for the same binding site, thus had bound to the same epitope of the antigen. Subsequently the clones used for titrations of the antibody concentrations by ELISA were 5B, 4F, 3A and 4, 20 for B*m*SXP and B*m*R1 antigens for immune library; while the selected antibody clones from naïve library were XP_D5, XP_B6 and H4R1, GIR1 for B*m*SXP and B*m*R1 antigens respectively. All the antibody clones showed different binding limits, however monoclonal antibody 5B and 4 showed high ability to bind at low antigen concentration: 1 pg and 100 pg for B*m*SXP and B*m*R1, respectively. Finally, antibody clones 5B, 3A and 4, 20 were selected for development of affinity columns for purification of B*m*SXP and B*m*R1 antigens. For purification of the B*m*SXP antigen, the 'mixture antibody column' performed better than 'single antibody column'. Meanwhile, single antibody column worked better for purification of the B*m*R1 antigen. Monoclonal antibody 5B was conjugated with gold nanoparticles and it showed good reactivity with a dipstick test lined with B*m*SXP antigen and not reactive with a dipstick lined with *Bm*R1. In conclusion, this study has successfully produced a novel human lymphatic filariasis library and monoclonal antibodies against B*m*SXP and B*m*R1 antigens. Affinity antibody columns for B*m*SXP and *Bm*R1 purifications were developed and gold conjugated monoclonal antibody against *Bm*SXP antigen was successfully applied in a lateral flow dipstick test.

CHAPTER 1

INTRODUCTION

1.1 Lymphatic filariasis: An overview

Lymphatic filariasis (LF), or more commonly known as elephantiasis is a mosquito-borne parasitic disease caused by three species of tissue dwelling filaroid nematodes that live in the human lymphatic system. This disease is widely prevalent in populations living in tropical and sub-tropical regions of the world, such as Asia, Africa, Central and South America. Latest report from the World Health Organization (WHO) stated that around 947 million people in 54 countries worldwide are threatened by LF and they need immediate treatments to stop the spread of the infection (http://www.who.int/mediacentre/factsheets/fs102/en/). The infection usually acquired in early childhood and in charge for considerable morbidity, causing social stigma among children, women and men (Pandey et al., 2011).

WHO has listed LF as one of the neglected tropical diseases (NTDs) among 16 other diseases (http://www.who.int/neglected_diseases/diseases/en/). These diseases have been ranked low on national and international agenda because most of the developed parts of the world successfully wiped out the infection. Meanwhile, these diseases only persist in the poorest, most marginalised communities. The outcome of the disease causes tremendous but hidden suffering due to their disfiguring, debilitating and sometimes fatal impact. There is little incentive for the industry to develop products for diseases linked with poverty. Thus, despite the need for better treatment and prevention is huge, the affected populations have limited access to them.

In 2000, WHO initiated a global programme for elimination of the disease known as the 'Global Programme to Eliminate Lymphatic Filariasis' (GPELF) and has identified it to be one of the six infectious diseases that has the potential to be eliminated as a public health problem (WHO, 1998). Two major aims of this programme is to interrupt transmission of the parasite and morbidity control by providing care for those who suffer the devastating clinical manifestations of the disease (Addiss and Brady, 2007). The choice of diagnostic tool plays an important role in GPELF because it affects the decision of treatment. Thus, various types of diagnostic tolls have been suggested at different phases of GPELF, such as parasitological diagnosis, rapid antigen and antibody tests and molecular diagnostics (Weil and Ramzy, 2007). However, the outmost pivotal diagnostic tool is a field-applicable tool that produces rapid results and allows for timely programmatic decisions to be made. Besides, diagnostic assays based on antibody and antigen have been proven to be very useful in the early identification of filarial infections (Pandey et al., 2011). Examples of commercial tests used are the ICT Filariasis NOW^{TM}, Og4C3-ELISA, Brugia Rapid and Pan LF Rapid.

Although much emphasis and improvement in the diagnosis of filariasis had been highlighted over more than a decade ago, the evaluation of current research and challenges in overcoming helminth diseases found major deficiencies in control tools, diagnostics, fundamental knowledge and biology of helminth (WHO, 2012). Thus, challenges to eliminate LF still exist.

1.2 History of discovery: Lymphatic filariasis and microfilariae

There is no clear written record on historical evidence of LF. Ancient artifacts showing the symptoms of LF infection such as a statue of Pharaoh Mentuhotep II depicts

swollen limbs and the Nok civilization in West Africa showing scrotal swelling suggest that the disease may have been present as early as 2000 BC. (https://web.stanford.edu/class/humbio103/ParaSites2006/Lymphatic_filariasis/). Microfilariae was associated with elephanthiasis by Jean Nicolas Demarquay in 1863, when he recorded microfilaria in the milky fluid extracts from hydrocele of a Cuban patient in Paris. This was followed by Otto Henry Wucherer in 1866 who found microfilaria in urine, and subsequently Timothy Lewis found microfilaria in blood in 1872 (Otsuji, 2011).

Then in 1877, Joseph Bancroft documented adult worm while examining the fluid extracted from an abscess on a patient's arm and it was called as *Filaria bancrofti*. The most important discovery was made by Patrick Manson in 1877, where he found microfilariae in the stomach of blood sucking mosquito and pin-pointed mosquito as the intermediate host. This discovery was considered as the birth of medical entomology and later was applied to other tropical diseases such as malaria. It was only in 1921 that the name *Wuchereria bancrofti* (*W. bancrofti*) was accepted. New species of microfilariae was discovered in parts of Indonesia by Lichtenstein in 1927. The new species was noticed to be different than the earlier found worm and in 1960 Buckley suggested to name it as *Brugia malayi (B. malayi)*. Later new species of Brugia was found Timor Island and it is named as *Brugia timori* (*B. timori*).

The life cycle of LF was initially wrongly hypothesized by Manson that the disease is transmitted to human after the ingestion of contaminated water or direct skin penetration with water in which the mosquitoes had laid eggs. Only in 1900, the actual mechanism of transmission was revealed by George Carmichael Low when he discovered microfilariae in the proboscis of the mosquito vector.

1.3 The filaria organism

1.3.1 Taxanomy

Filarial worms are classified under the family of Filarioidea of nematodes and the genus of Brugia. There are eight important species of filarial worms which use humans as the host and they are divided into three groups. Lymphatic filariasis is caused by the *W. bancrofti*, *B. malayi*, and *B. timori*. Cutaneous filariasis is caused by *Loa loa* also called as the African eye worm, *Mansonella streptocerca* and *Onchocerca volvulus*. Body cavity filariasis is caused by *Mansonella perstans* and *Mansonella ozzardi*. Most cases of filariasis in humans worldwide are caused by *W. bancrofti* (https://www.cdc.gov/parasites/lymphaticfilariasis/).

1.3.2 Morphology of lymphatic filarial worms

Differences among the three LF worms reside in vectors, epidemiology, reservoirs, symptoms, and mainly species morphology. *W. bancrofti* has the most significant difference in morphology compared to other species and it is also the most well documented species. It exhibits sexual dimorphism and it is hard to be removed from tissues due to their delicate body. The adult worm is long, cylindrical, slender, and smooth with rounded ends. The worm appears in white in colour and almost transparent. The sheathed microfilariae (larvae) of *W. bancrofti* measures 245 to 300 µm. and it take several months to sexually mature. The male worm measures 40 mm in length and by 100 µm in width. In contrast, the female worm is three times larger in diameter than the male worm with 60 mm to 100 mm in length and 300 µm width. One end of the round body is blunt, while the other is pointed (Figure 1.1a).

On the other hand, the *B. malayi* male worm measures 13 to 23 mm in length by 70 to 80 µm in width and the female worm measures 43 to 55 mm in length by 130

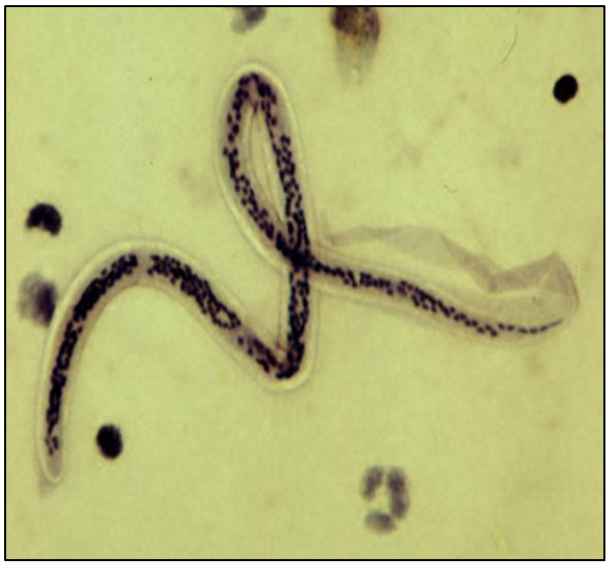
to 170 µm in width. Adult worms produce microfilariae, measuring 177 to 230 µm in length and 5 to 7 µm in width (Figure 1.1b) (https://web.stanford.edu/class /humbio 103/ParaSites2006/Lymphaticfilariasis/).

The nuclei of *B. malayi* extends nearly to the tip of the tail while for *W. bancrofti*, the nuclei do not appear at the end of the tail. This is a major difference between these two species. Furthermore, both species lack a digestive system, instead absorbing nutrients from their hosts. The adult male and female worms inhabit primarily the lumen of lymphatics whereby the microfilariae usually migrate from the lymphatics into the blood stream (Nanduri and Kazura, 1989).

1.3.3 Transmission and life cycle of lymphatic filaria

The vector-borne disease is transmitted to human by the bite of more than 70 species and subspecies of infected mosquitoes mainly *Anopheles*, *Aedes*, *Culex* and *Mansonia*; (Stone et al., 1959; Nanduri and Kazura, 1989). All the three lymphatic filariae have similar biphasic life cycle where larval development takes place in mosquitoes (intermediate host) and adult development takes place in the human (definitive host).

a



b

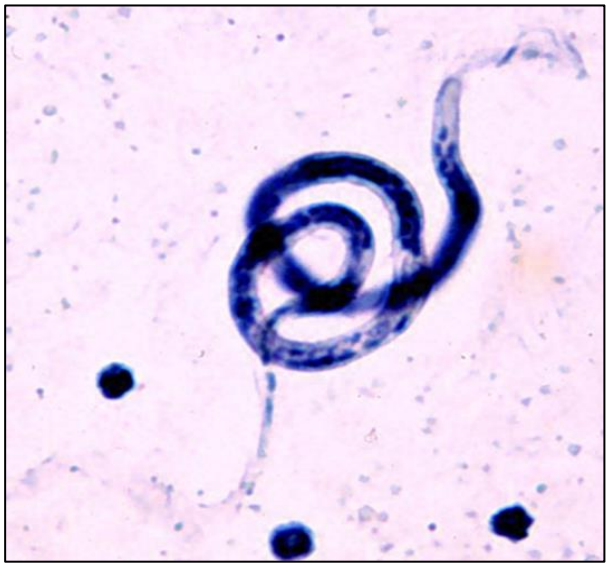


Figure 1.1 Lymphatic filariasis microfilariae worms. (a) *Wuchereria bancrofti*. (b) *Brugia malayi*.

Source: http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Filariasis_il.htm

The life cycle of lymphatic filariae consists of four different stages (L1-L4) as depicted in **Figure 1.2**. Mosquito ingests microfilariae during their feeding on an infected person (microfilaria carrier) as part of its blood meal. The microfilariae penetrate the mosquito's gut wall and within a few hours they migrate to the flight muscles. There the microfilariae develop into three stages under optimum conditions of temperature and humidity. The L1 and L2 molt occur in 6 to 10 days then after several days, the parasite molt to the L3 parasite. The L3 then migrates from the flight muscles to the mouth parts of the vector, where they are positioned to be passed on to the host during a subsequent blood meal (Scott, 2000).

The deposition of the third stage larvae (L3) on the skin of human (definitive host) following a bite by an infective mosquito initiates the infection. In human, the larval undergo an additional molt to the fourth larval stage (L4) between 9 to 14 days of post-infection as they mature into the lymphatic-dwelling adult male and female worms to complete the life cycle. The adult worms survive for around 5-8 years or sometimes up to 15 years or more. The lymphatic-dwelling filariae are diecious and undergo ovoviviparous reproduction resulting in the release of fully formed, sheathed first stage larvae (L1) from the female. Then, the microfilariae enter the peripheral circulation of the human host where they are available to be ingested by the vector during a blood meal (Scott, 2000). In brugian filariasis, besides humans, other mammals such as domestic cat, Presbytis monkeys and wild mammals are the potential definitive host for the parasite (Mak, 1987).

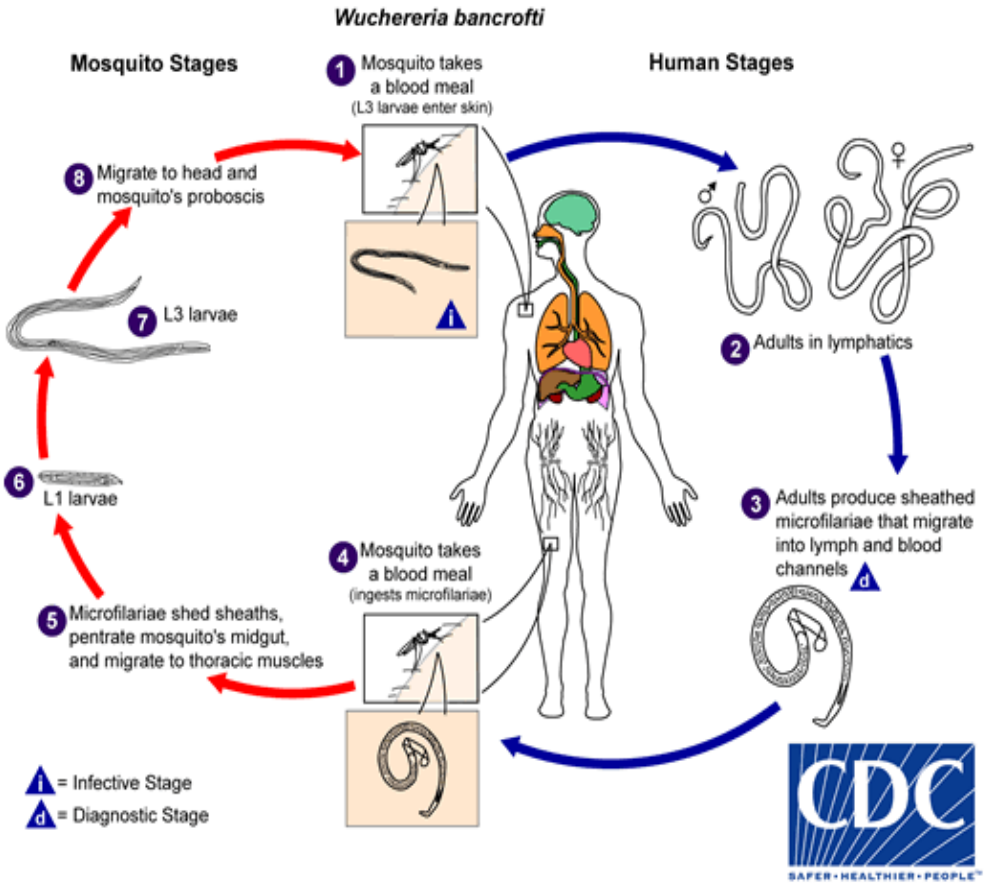


Figure 1.2 The life cycle of *Wuchereria bancrofti*.

Source: www.dpd.cdc.gov/dpdx

Microfilaria stage of the parasites show a unique characteristic which is they have “periodicity” that restricts their appearance in the blood to only certain periods of the day. They can be divided in two types of strains namely noctural periodic or as nocturnaly subperiodic. Nocturnally periodic strain microfilaria virtually disappears from the peripheral circulation and is found predominantly in the blood vessels of lungs and deep tissues during the daytime. Conversely, during the night time (especially between 10 pm until 2 am), it can be found in the peripheral blood. Meanwhile, for the nocturnally subperiodic strain, the microfilaria is mostly found at night and tend to lose their sheath in the process of dying on microscope slides (Bowman et al., 2002). In addition, there is also another microfilaria strain that is transmitted by day-biting mosquitoes of genus Aedes. It is called as non-periodic or diurnally subperiodic strain of microfilaria, but is limited to the South Pacific (Nanduri and Kazura, 1989).

The periodicity characteristic in lymphatic filarial worms is due to the difference in the oxygen tension between the arterial and venous blood in the lungs (Hawking and Gammage, 1968; Burren, 1972; Nanduri and Kazura, 1989). However, it was suggested that these parasites have adapted their periodicity to the vector feeding behavior, possibly to facilitate their transmission (Nanduri and Kazura, 1989).

1.3.4 Clinical manifestation

Majority of the infected individuals who lives in LF endemic area fall into the following categories; asymptomatic amicrofilaraemia, symptomatic microfilaraemia, asymptomatic microfilaraemia, acute infection with filarial fever and adenolymphangitis (inflammation of the lymphatics and lymph nodes), chronic elephantiasis and occult filariasis. Asymptomatic amicrofilaraemia patients are the

infected individuals that do not exhibit any symptom and have no circulating microfilaria. Microfilaraemic patients are those with microfilaria circulating in their blood and these individuals may have symptoms (symptomatic) or have no physical symptoms (asymptomatic). In some cases, the asymptomatic individual may have hidden damage to their lymphatics, kidneys, and altered body's immune system. Individual with acute symptoms may suffer from recurrent attacks of fever with painful inflammation and swelling of the lymph glands or lymph channels. Chronic manifestations often affect the arms and legs and usually give rise to a condition of lymphoedema (tissue swelling) or elephantiasis (skin/tissue thickening). However, the clinical manifestations of LF varies geographically and species of the parasite. For example, in India subcontinent and Brazil where bancroftian filariasis is prevalent, the most common clinical form of the disease is tropical pulmonary eosinophilia while in parts of Africa is hydrocoele. Meanwhile, it is recognized that inguinal lymphadenitis and lymphangitis are more common in brugian filariasis with the exceptions of genital disease (hydrocoele and filarial scrotum) (Kumaraswami, 2000).

1.3.5 Lymphatic filariasis in Malaysia

The detection of microfilariae of the parasite in Malaysia has shown a lot of reduction with about 1,000 cases in 1987 to less than 300 in 2003, 172 cases in 2006 and 156 cases in 2010. However, in 2011, about 387 microfilaria-positive cases were reported, with most new cases in Sabah and Sarawak (Malaysian Ministry of Health Annual Report, 2011).

B. malayi is the main species of parasite causing LF, and is mostly transmitted by mosquitoes belonging to the genus *Mansonia*, namely *M. bonneae*, *M. dives*, *M. uniforms* and *M. Indiana* (Chang et al., 1991; Kwa, 2008). However, in recent years the detection of *W. bancrofti* microfilaria positive cases has increased, and found mostly among migrant workers from Nepal, Myanmar, Bangladesh, Indonesia, India and Philippines (Malaysian Ministry of Health Annual Report, 2011). Thus, it is important to include the detection of bacroftian filariasis in an LF control or elimination programme in Malaysia.

1.3.6 Diagnosis of lymphatic filariasis

The diagnosis of LF can be divided into five methods as follows; microfilariae detection, filarial antigen detection, detection of specific antibodies, and radiological detection of adult worms.

1.3.6(a) Microfilariae detection

Microfilariae detection uses the thick blood smear technique. A drop of blood (50–60 µl) is taken from a fingerprick and spread on a clean microscope slide, dried, stained and examined under the microscope for the presence of microfilariae. This method is simple and provides the definitive diagnosis in which the microfilariae can be visualised under the microscope. However, this method will probably have missed individuals with low microfilariae counts and those with amicrofilaremic infections. These individuals have the potential to contribute to future transmission. In addition, the phenomenon of microfilariae periodicity, which requires night blood collection are troublesome to the staff, villagers and impractical in some endemic areas (Weil and Ramzy, 2006). Subsequently, skilled personnels are needed to examine the

morphological features of the different species of lymphatic filariae. Modified methods such as Knott technique, membrane filtration or counting chamber improve the sensitivity of this method but are not practical to be performed at a large scale (Goldsmid, 1972; Melrose et al., 2000).

1.3.6(b) Antigen detection

Filarial antigen tests gain its entry in 1980 since then it has revolutionised the diagnosis of bancroftian filariasis. These tests are sensitive and specific because they do not require the presence of microfilariae, blood can be taken at any time and able to detect both the microfilaraemic and amicrofilaraemic cases. It is available in two formats, rapid card test and ELISA formats. The Binax NOW® Filariasis Immunochromatographic Test (ICT) is a rapid card test that uses capillary blood and give immediate result. This test has become one of the choices in diagnosis and the elimination programme (Phantana et al., 1999; Weil et al., 1997) but careful adherence to reading times is required to get accurate results (Simonsen and Dunyo, 1999). Recently an improved antigen test called Alere Filariasis test strip (FTS) was developed and showed better stability and sensitivity compared with the previous ICT. The TropBio ELISA test also called as the Og4C3 test has been reported to be more sensitive and convenient for laboratory and field-based study (Chanteau et al., 1994; More and Copeman, 1990; Simonsen and Dunyo, 1999). The microtitre plate is coated with Og4C3 (anti-filarial monoclonal antibody) and concentration of the circulating filarial antigen is determined using a standard curve. However, this test requires laboratory facilities, equipment and skilled personnel which limit its use in control programmes. To date there is no good antigen detection test for brugian filariasis.

1.3.6(c) Antibody detection

Studies on antibody detection are mainly focused on IgG and IgG4 responses against the LF infection. Many studies have shown that anti-filarial IgG4 antibodies are able to detect active infection, particularly in children (Lal and Ottesen, 1988; Rahmah et al., 2001). Earlier report shows that the native antigen was useful for LF antibody detection but causes cross-reactivity with non-filaroid helminths. Therefore, recombinant antigens are preferred because they allow the production of a standardised assay and ensures the reproducibility of the test results. There are several recombinant antigens with good diagnostic values have been reported. The recombinant antigen test for brugian filariasis are B*m*R1 (Lammie et al., 2004; Rahmah et al., 2001) and B*m*14 (Weill et al., 2011) while for bancroftian filariasis are B*m*SXP (Noordin et al., 2007), WbSXP (Rao et al., 2000), Wb123 (Kubofcik, and Nutman, 2012) and Bm33 (Hamlin et al., 2012). Antibody testing appears to be more sensitive than antigen testing. There are two commercialized rapid tests available for detection of brugian LF which are based on detection of anti-filarial IgG4 antibody test namely Brugia Rapid and PanLF Rapid. Although there is an antigen detection test for bancroftian filariasis, antibody detection test is preferred for surveillance at the end of the LF elimination programme.

1.3.6(d) DNA detection

A number of molecular diagnostic assays such as Polymerase Chain Reaction (PCR), loop-mediated isothermal amplification (LAMP) and other techniques are being evaluated for LF detection but not yet available for routine use. These techniques are based on detection of specific DNA of lymphatic filarial species. There are several DNA targets have been used for LF detection, such as a highly conserved and repetitive 322 base pairs Hha1 DNA sequence. This is a common target for

conventional and real-time PCR assays and has been reported to be highly sensitive and specific for *B. malayi* detection (Fischer et al., 2000; Lizotte et al., 1994; Rahmah et al., 1998). The reported genes for *W. bancrofti* detection are Ssp1 repeat (Mishra et al., 2007; Zhong et al., 1996), LDR repeat (Rao et al., 2006; Rao et al., 2006) and ITSI (Nuchprayoon et al., 2005). In addition, a LAMP assay has been developed for *B. malayi* or *B. timori* detection which can amplify as low as 1 pg of *B. malayi* genomic DNA (Poole at al., 2012). A species specific 114 bp region of mitochondrial 12S rRNA genes has been used to differentiate between *B. malayi*, *B. pahangi* and *D. immitis (*Ky and Van Chap, 2000) However, the main obstacle with the molecular diagnosis methods is that it requires sophisticated laboratory equipment and trained personnels to perform the assay and analysis. Besides it also requires several hours to obtain the results and they not practical to be used with a large number of samples and for field screening.

1.3.6(e) Radiological detection

Radiological or ultrasonography detection is based on 'filarial dance sign' which refers to live adult worm inside the lymphatic vessels (Amaral et al., 1994). Advancement in imaging technology such as technological refinement using colour and pulse wave Doppler allows the imaging of adult worms in deeper lymphatics (Mand et al., 2006; Shenoy et al., 2007). Another useful imaging technology called lymphoscintigraphy allows the observation of abnormal and dysfunctional lymphatics in infected individuals. However, this technique is not suitable for large scale studies.

1.3.7 Treatment of lymphatic filariasis

Basically, there are three different types of drugs available to treat filariasis namely, diethylcarbamazine (DEC), ivermectin and albendazole. These drugs kill microfilaria and/or adult worms. Diethylcarbamazine is found to be effective against microfilaria, but only partially effective against the adult worms and the drug does not act directly on the parasite but its action is mediated through the host immune system. Single annual administration of 6 mg/kg DEC has been reported to lower the blood microfilaria levels markedly and this effect is sustained even at the end of one year (Noroes et al., 1997). However, it is reported that a single dose of DEC kills the adult worms when they are sensitive to the drug but if they are not sensitive even repeated administrations of the drug do not have any effect on the adult worms (Freedman et al., 2001). The adverse effects of this drug are only experienced by patients with microfilaria and mainly due to their rapid destruction. The symptoms are fever, headache, myalgia, sore throat or cough that usually last for 24 to 48 hours. Direct adverse effects related to the drug are very rare.

Ivermectin acts directly on the microfilaria and effectively keeps the blood microfilaria counts at very low levels, even at the end of one year in single annual doses of 200–400 mg/kg. The adverse effects of this drug are similar to those produced by DEC but then milder due to the slower clearance of the parasitaemia. To date there is no evidence to prove ivermectin action against the adult parasite or in tropical eosinophilia (Shenoy et al., 1993). This drug is also found to be effective against many human intestinal helminths, human ectoparasites like head and body lice and scabies (Ottesen et al., 1997).

Another well-known drug which can destroy the adult filarial worms is albendazole. The recommended doses are 400 mg twice daily for 2 weeks (Ismail et al., 1998). This drug has no direct action against the microfilaria thus does not immediately lower the microfilaria counts. However, this drug becomes more pronounced in lowering of blood microfilaria levels when it is combined with DEC or ivermectin. Only a single dose of 400 mg combined drugs is sufficient to carry out the adult worm destructions. Combination of albendazole, either with DEC or ivermectin is recommended for the filariasis elimination programme (Ottesen et al., 1997).

Treatment using above drugs only effective at the early stages of the disease when there is an active filarial infection. Once the development of lymphoedema established, there is no permanent cure and treatment as DEC does not seem to reverse the existing lymphatic damage (Harinasuta, 1984). There are practices recommeneded to alleviate the lymphoedema and to prevent further progression of the swelling. These include applying elastocrepe bandage or tailor-made stockings while ambulant, keeping the limb elevated at night, after removing the bandage, regular exercising of the affected limb, regular light massage of the limb to stimulate the lymphatics and to promote flow of lymph towards proximal larger patent vessels; intermittent pneumatic compression of the affected limb using single or multicell jackets and heat therapy using either wet heat or hot ovens. There are also various surgical procedures available like lymph nodo-venous shunts, omentoplasty, excisional surgery and skin grafting but the care of the limb is continued for life to prevent recurrence of the swelling.

1.4 Global Programme for Elimination of Lymphatic Filariasis (GPELF)

WHO has reported that more than 20% of the world's populations are at risk of acquiring LF. It is estimated that over 120 million of people in at least 83 countries have already been infected and more than 40 million people are incapacitated by the disease [The Global Alliance to Eliminate Lymphatic Filariasis (GAELF), http://www.filariasis.org]. Over 25 million men suffered from the genital form of the disease, while more than 15 million of people suffered from lymphoedema or elephantiasis of the leg. Out of these, 90% of the cases are caused by *W. bancrofti* whereas *B. malayi* which accounts for about 10% (or 13 million) of the infected people, especially in South and Southeast Asia, South Korea, and parts of China. In addition, *Brugia timori* is restricted to Timor Leste and a few islands in Indonesia (Michael, 2000).

In year 1994, a consultative meeting was held at Universiti Sains Malaysia, Penang, to discuss about LF and its Global Control strategies. This was followed by the World Health Assembly resolution on elimination of LF as a public health problem in 1997. In year 2000, the GAELF was formed in order to support the GPELF. The targeted year for world-wide elimination of LF as a public health problem is 2020. The elimination strategies are being carried out on a global scale and the main method is by interrupting the transmission of infection through mass drug administration (MDA); and the second strategy is by alleviation of morbidity caused by the disease (http://www.filariasis.org/resources/globalalliance history.htm). The latter focuses on decreasing the secondary bacterial and fungal infection of limbs and genitals whose lymphatic function has already been compromised by filarial infection (Ottesen, 2000).

The endemic countries of LF and status of MDA are shown in **Figure 1.3**. A pictorial view of the main strategy of GPELF is shown in **Figure 1.4**. The first step is mapping of the endemic areas based on historical data and population testing using the then available diagnostic tools namely microscopic examination of thick smears of night blood for brugian filariasis and antigen detection test for bacroftian filariasis. This is followed by MDA to the entire 'at risk' population for at least five years. This reduces both infection prevalence and transmission rates to levels below those required for sustained transmission (Ottesen et al., 1997; Molyneux, 2001; Ottesen, 2006; Weil and Ramzy, 2006). In this way, the level of microfilariae in the population remains below that necessary for active transmission. The MDA treatment comprise a single dose of two drugs regimens i.e. albendazole 400 mg plus DEC 6 mg/kg or albendazole 400 mg plus ivermectin 200 μg/kg for a period of 4-6 years which correspond to the reproductive life span of the parasite (Ottesen, 2000). Once the infection level fell below 1% microfilaremia or 2% antigenemia, transmission assessment surveys (TAS) in children 6-7 years are performed to determine whether transmission has been interrupted. This is followed by post-MDA surveillance comprising TAS 2 and TAS 3. Upon passing TAS 3, a dossier is developed which summarizes the LF epidemiologic, programmatic, monitoring, evaluation, and findings for the country, before validation of the elimination is completed.

Other than TAS, post-MDA surveillance may also include other activities to confirm that transmission interruption such as testing of military recruits, university students, blood donors, hospital patients. The status of having validated or certified the

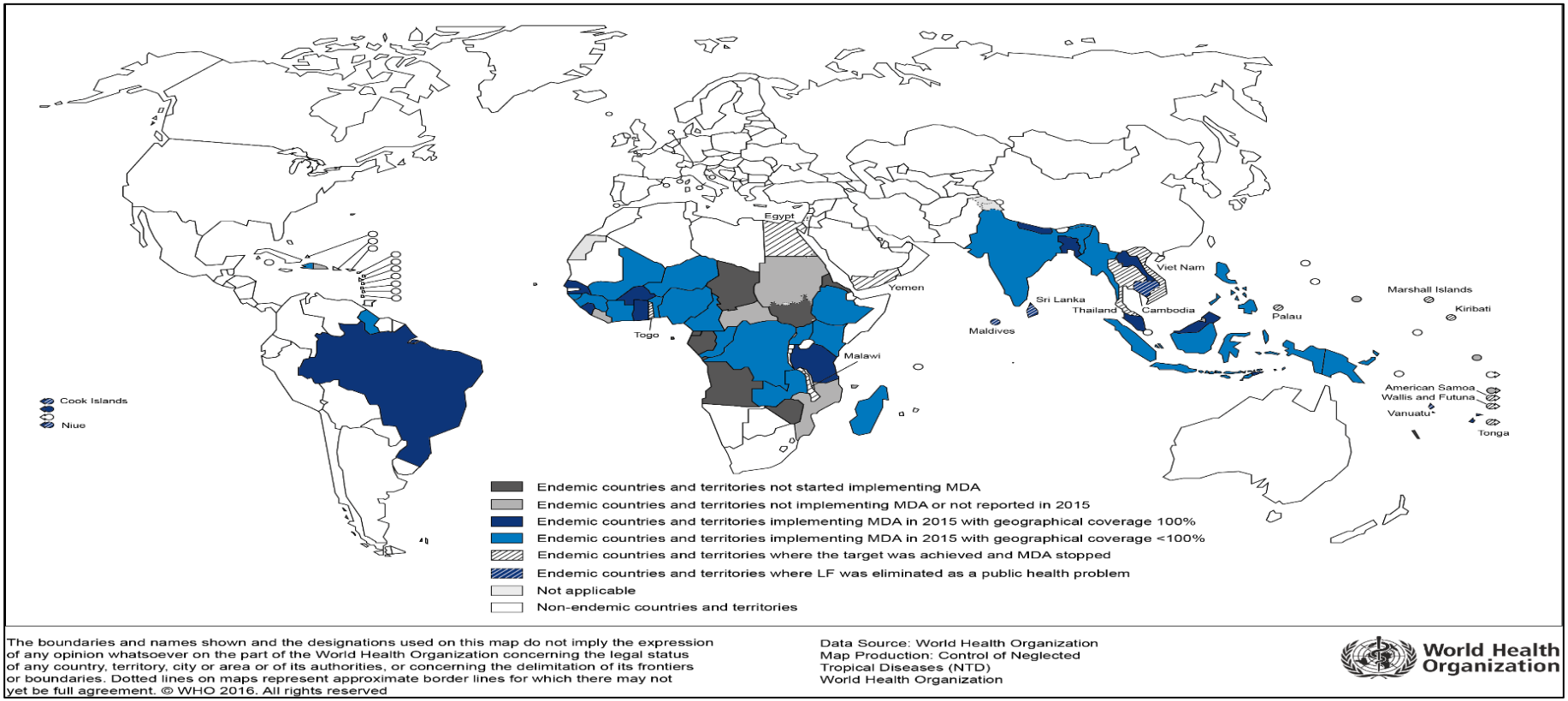


Figure 1.3 World endemic countries of lymphatic filariasis and status of mass drug administration (MDA) in these countries, 2015.

Source: http://gamapserver.who.int/mapLibrary/app/searchResults.aspx

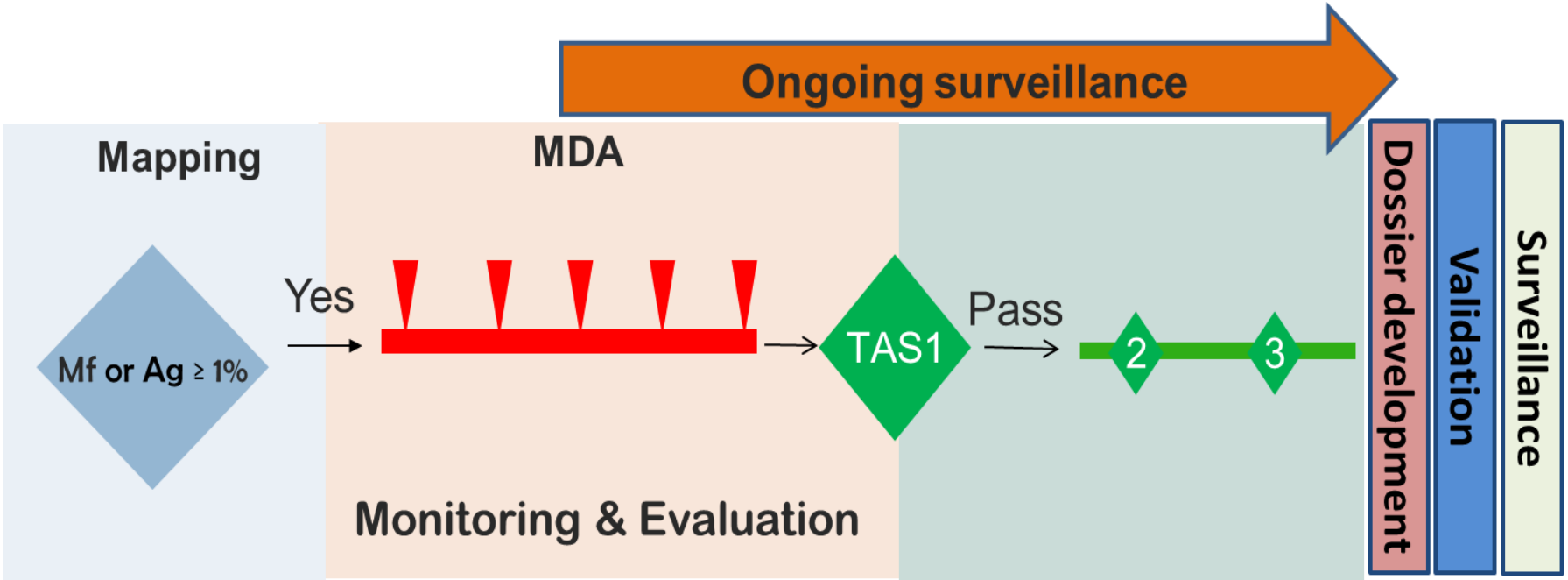


Figure 1.4 Strategy of the global programme to eliminate lymphatic filariasis. **Mf**: Microfilaremia, **Ag**: Antigen, **MDA**: Mass drug administration, **TAS**: Transmission assessment survey.

(Source: Adapted from Ichimori et al., 2014)

LF elimination is potentially reversible, thus surveillance activities should still be continued post-validation to ensure there is no resurgence or recrudescence of active transmission (WHO/HTM/NTD/2016.6). A sensitive and specific field-applicable diagnostic tools are required for the various phases of the programme. For TAS activities in brugian LF areas, Brugia Rapid and PanLF Rapid are being used. Meanwhile, for bancroftian filariasis, rapid antigen detection test (ICT) is used. These antibody and antigen rapid detection tests are robust, field-applicable, sensitive, specific, sample can be taken at any time of the day, and easy to use and interpret.

1.4.1 The use of Brugia Rapid and PanLF Rapid in GPELF

Brugia Rapid and PanLF Rapid are two important rapid tests to detect of LF. These tests are used by the WHO for transmission assessment surveys of the GPELF.

1.4.1(a) Brugia Rapid

Brugia Rapid is a rapid cassette test that utilized B*m*R1 recombinant antigen for the detection of brugian filariasis. The B*m*R1 recombinant antigen is expressed by the clone Bm17DIII/pROEX^{TM} HTa/TOP 10 carrying the *Brugia malayi* gene. It comprises 618 bp, coding for a total of 206 amino acid residues and was cloned into the multiple cloning site (MCS) of the pROEX^{TM} HT located at 407-1024 bp. The molecular mass of the translated B*m*R1 recombinant protein is approximately 30 kDa.

The rapid test is based on the fact that the level of IgG4 antibody to filarial antigens is significantly elevated in an active infection (Rahmah et al., 2001). Since it is an antibody-based test, it does not require any night blood sampling. In this test, the anti-filarial antibodies in patient sera will react with this antigen, followed by binding of this complex with monoclonal anti-human IgG4 conjugated to colloidal gold. Thus,

samples containing anti-filarial IgG4 antibodies that react specifically to the antigen will result in the appearance of a purple-reddish colour at the test line. In the initial stages, an ELISA format of the test was developed, known as Brugia-ELISA. Evaluation studies on the ELISA demonstrated diagnostic specificity rates of 95.6-100%, the sensitivity rates of 96-100%, positive predictive values of 75-100%, and the negative predictive values of 98.9-100% (Rahmah et al., 2001). However, since this test is more suitable for laboratory rather than field use, the test format was changed into a rapid immunochromatographic test, which was named as Brugia Rapid. This rapid test format is easy to perform, rapid and robust. It does not need any laboratory facility, thus useful for field-work in remote areas.

Evaluation studies of the rapid test have been performed to validate its diagnostic sensitivity and specificity. One of the studies showed 97% sensitivity, 99% specificity, 97% positive predictive value, and 99% negative predictive value (Rahmah et al., 2003).

1.4.1(b) PanLF Rapid

The PanLF Rapid test utilizes both B*m*R1 and B*m*SXP recombinant antigens for the detection of specific IgG4 antibody against LF parasites of both bancrotian and brugian filariasis. *Bm*SXP recombinant antigen was first derived from the clone isolated from a *B. malayi* adult male worm cDNA library with sera from bancroftian filariasis patients. The recombinant strain BmSXP/pROEX^{TM} HT/TOP 10 was constructed with the open reading frame (ORF) of SXP1 gene (462 bp). After subcloning into pROEX^{TM} HTa expression vector, the size of the gene is 585 bp, which transcribed and translated to the B*m*SXP recombinant protein that has a molecular mass of about 21.8 kDa.

The B*m*SXP gene was reported to successfully identify 83% (64/72) of bancroftian filariasis patients when tested with IgG4-ELISA (Chandrashekar et al., 1994). In another study, the same assay showed 100% detection (72/72) (Rao et al., 2000). In addition, a rapid flow-through IgG immunofiltration test utilizing the B*m*SXP gene was developed and recorded a sensitivity of 91% (30/33) in detecting *W. bancrofti* infection (Lammie et al., 2004).

The principle of PanLF Rapid test is similar to the Brugia Rapid but it consists of two test lines comprising B*m*SXP and B*m*R1 recombinant antigens. The evaluation studies of this test have been performed to validate the sensitivity and specificity of the rapid test. The average sensitivity and specificity of this rapid test were reported to be 96.5% and 99.6% (Rahmah et al., 2007).

The B*m*R1recombinant antigen showed high sensitivity in detecting *B. malayi* infection (98% and 84% respectively) compared to B*m*SXP recombinant antigen. On the other hand, the B*m*SXP recombinant antigen showed higher sensitivity (95%) in detecting *W. bancrofti* infection as compared to B*m*R1 (14%). Thus, this rapid test is useful for brugian filariasis areas where there are bancroftian filariasis cases.

1.5 Immune response in lymphatic filariasis

The immune responses to filarial infection encompass a complex network of innate and adaptive cells. Interactions of the parasite with these cells result in a spectrum of clinical manifestations. Asymptomatic condition may occur to an individual harbouring high parasite numbers, with stronger regulatory response while immunologically reactive patients with lower numbers of parasites can manifest chronic pathology.

Principally, innate immune system is the first arm of the immune defense that identify infectious agents (Kindt et al., 2007). Next is the adaptive immune system with its humoral and cellular responses which are able to respond and remember the exogenous encounters. Both these immune systems act in concert to eliminate and control infectious diseases. The components of innate immune response are lysozyme, interferons, complement and toll-like receptors while those for adaptive immune response are antigen presenting cells (APC) and lymphocytes. The main function of APCs (macrophages and dendritic cells) is to process and present the antigens to the antigen-specific receptors on T cells. Lymphocytes are made up of B lymphocytes also called as humoral immune system and T-lymphocytes known as cell-mediated immune system (McCullough and Summerfield, 2005). B lymphocytes are the essential cells of the immune system stimulated during an immune response to produce antibodies. B cells express receptors on their cell surface for antigen recognition and binding. However, pathogens can escape the immune systems in several ways such as reducing its antigenicity, mimicking the host cell surfaces, selectively suppress the immunity, and continual variation of antigen surfaces (Janeway, 2001).

B cells differentiate from pluripotent hematopoietic stem cells. During early embryonic development, the hematopoietic cells migrate into the fetal liver and they develop and mature into B cells (Melchers, 2015). B cells can be found in various lymphoid organs such as spleen, lymph nodes, tonsils, Peyer's patches, lungs, peritoneal cavity and blood. It typically produces antibody to fight pathogens but there is a small subset of B cells known as regulatory B cells that function to supress immune responses. These cells secrete a specific cytokine called interleukin-10 (B10) which are crucial for controlling inflammation, autoimmunity and limit the normal immune