DEVELOPMENT OF A NAÏVE HUMAN FAB ANTIBODY LIBRARY TO GENERATE MONOCLONAL ANTIBODIES AGAINST B*m*SXP RECOMBINANT ANTIGEN

NOORSHARMIMI BINTI OMAR

UNIVERSITI SAINS MALAYSIA
2015

DEVELOPMENT OF A NAÏVE HUMAN FAB ANTIBODY LIBRARY TO GENERATE MONOCLONAL ANTIBODIES AGAINST B*m*SXP RECOMBINANT ANTIGEN

by

NOORSHARMIMI BINTI OMAR

Thesis submitted in Fulfillment of the Requirements
For the Master of Science

SEPTEMBER 2015

ACKNOWLEDGEMENTS

In the name of Allah S.W.T, the most Gracious and the most Merciful.

Thank you Allah! For the strength given throughout my master research life. I would like to address my appreciation to Dr Lim Theam Soon as my supervisor for his helping hand, guidance, support and patience throughout my research work, also many thanks to Professor Rahmah Noordin and Dr Choong Yee Siew from Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia. My acknowledgment also goes to all the administration and laboratory staffs in INFORMM for their cooperation.

For my laboratory mates (Hidayah, Bee Nar, Siang Tean , Qiuting , Chai Fung, Anizah, Faezee, Soo Khim and Hamizah) sincere thanks for the knowledge shared, assistance and best moments together. Plenty of thanks and love to my parents, husband, siblings and in-laws for giving spiritual support, encouragements and prayers during my study. Lastly, my lovely son you are my courage to continue the success in life.

I would like to acknowledge the contribution of Pusat Sejahtera Universiti Sains Malaysia, Penang during the blood collection process and My Brain Scholarship awarded to me from Ministry of Higher Education (MOHE) in order to complete this research.

Finally, this project was funded by the Malaysian Ministry of Higher Education, Centre of Excellence Program (311/CIPPM/44001005).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Absorbance
ABTS	2,2’-azino-bis(3-ethylbenzenethiazoline-6-sulphonic acid)
bp	Base pair
C	Carboxyl
cDNA	Complementary deoxyribonucleic acid
CDR	Complement determining region
CH1	Constant heavy chain 1
CH2	Constant heavy chain 2
CH3	Constant heavy chain 3
CH4	Constant heavy chain 4
CL	Constant light chain
D	Diversity/Diameter
DNA	Deoxyribonucleic acid
dH20	Distilled water
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
eGFP	Enhanced green fluorescence protein
ELISA	enzyme-linked immuno assay.
EtBr	Ethidium bromide
EtOH	Ethanol
Fab	Fragment antigen binding
Fr	Framework

Fv	Fragment variable
HC	Heavy chain
His	Histidine
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IgG4	Immunoglobulin G4
IPTG	Isopropyl-beta-D-thiogalactopyranoside
J	Junction
KF	King Fisher
kDa	kiloDalton
lacZ	β-galactosidase gene
LC	Light chain
N	Amino
N1	Amino 1
N2	Amino 2
OD	Optical density
pI-pXI	Phage coat protein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline containing Tween 20
PTM	Milk powder in PBST
RBS	Ribosome binding site

RE	Restriction endonuclese
RIN	RNA integrity number
RNA	Ribonucleic acid
U	Unit
V-gene	Variable gene
V	Variable/Voltan
VH	Variable heavy chain
VL	Variable light chain

PEMBANGUNAN PERPUSTAKAAN FAB ANTIBODI NAIF MANUSIA UNTUK MENGHASILKAN ANTIBODI MONOKLONAL TERHADAP ANTIGEN REKOMBINAN B*m*SXP

ABSTRAK

Antibodi adalah glykoprotein yang dihasilkan oleh sel B terhadap jasad molekul asing. Penyelidikan ini bertujuan menghasilkan antibodi Fab (fragmen ikatan antigen) manusia yang specifik terhadap antigen. Fragmen Fab terbahagi kepada satu domain malar dan domain manipulasi yang terletak pada rantai berat dan rantai ringan. Protokol optimum telah dicapai bagi menghasilkan perpustakaan Fab antibodi rekombinan daripada penderma tidak berimunisasi yang terdiri daripada tiga kumpulan etnik majoriti (Melayu, Cina dan India) di Malaysia. Kami menggunakan seluruh RNA dari limfosit periferal darah daripada 90 penderma bagi mendapatkan gen-gen manipulasi cDNA yang spesifik. Fragmen antibodi Fab yang mengekod bagi kelas IgM diamplifikasi secara individu melalui proses PCR menggunakan kepelbagaian set-set primer yang merujuk kepada bahagian manipulasi rantai berat (μ) dan rantai ringan (κ,λ). Kepelbagaian perpustakaan antibodi dicapai melalui gabungan-gabungan campuran semua rantai berat dan rantai ringan secara rawak. Kepelbagian perpustakaan antibodi naif yang tinggi adalah sangat berguna untuk saringan antibodi terhadap beberapa panel antigen. Saringan faj dilakukan terhadap antigen B*m*SXP meggunakan dua strategi 'panning' yang berbeza untuk memahami keberkesanan strategi 'panning'. *BmSXP* adalah antigen rekombinan bagi *Wuchereria Bancrofti* yang spesifik untuk penyakit limfatik Bancroftian filaria dan ia digunakan untuk kegunaan diagnostik bagi jangkitan limfatik filaria. Persembahan kedua-dua strategi 'panning' menunjukkan kesukaran 'panning' perpustakaan Fab secara proses automatik manakala cara 'panning' konvensional berjaya

mengasingkan gabungan-gabungan positif. Dua Fab antibodi monoklonal terhadap antigen B*m*SXP telah dikesan dan disahkan. Walaupun penghasilan perpustakaan Fab naif adalah sukar, namun ia berguna untuk penghasilan Fab antibodi monoklonal untuk aplikasi-aplikasi hiliran seperti diagnostik. 'Panning' perpustakaan Fab naif secara paparan faj boleh menghasilkan antibodi-antibodi monoklonal terhdadap pelbagai target antigen dengan berkesan. Oleh demikian, ini menyediakan penyelesaian yang menarik untuk penghasilan antibodi-antibodi manusia rekombinan.

DEVELOPMENT OF A NAÏVE HUMAN FAB ANTIBODY LIBRARY TO GENERATE MONOCLONAL ANTIBODIES AGAINST B*m*SXP RECOMBINANT ANTIGEN

ABSTRACT

Antibodies are glycoproteins produced by B-cells against molecules foreign to the body. This study focused on the production of antigen specific human The Fab region consists of one constant and one variable domain which are both located at the heavy chain and the light chain. An optimized protocol for the production of a recombinant Fab library of unimmunized donors from 3 major ethnic groups (Chinese, Malay and Indian) in Malaysia was carried out. We used peripheral blood lymphocytes total ribonucleic acids (RNA) from 90 donors to obtain variable genes (V-gene) specific cDNA. The Fab-encoding regions of IgM repertoire were individually amplified by polymerase chain reaction (PCR) using a diverse set of primers corresponding to the different variable region of the heavy (μ) and the light chains (κ,λ). The diversity of the library is created via random combinatorial mixing of all heavy and light V-genes. A highly diverse naïve library is useful for the selection of antibodies against a panel of antigens. Phage selection was carried out against B*m*SXP using two different panning strategies to understand the efficiency of Fab library panning with different strategies. B*m*SXP is a recombinant antigen of *Wuchereria Bancrofti* which is specific for *Bancroftian* lymphatic filarial disease and is used for diagnostic of lymphatic filarial infection. The performance of both panning strategies suggests the difficulty for Fab library panning with an automated process as only the conventional panning method was able to isolate positive binders. Two monoclonal Fab antibodies against B*m*SXP were identified and confirmed. Although difficult, the production of a Fab naïve library is useful for the generation

of monoclonal Fab antibodies for downstream applications such as diagnostics. Phage display panning of a naïve Fab library is able to select monoclonal antibodies against the target antigens efficiently. This provides an attractive solution for the generation of recombinant human antibodies.

CHAPTER 1

INTRODUCTION

1.1 The Immune system

The human immune system is a complex protection mechanism that involves several different cellular mechanisms. There are two natural mechanisms involved in fighting off infection and diseases in the human body, which are the innate and adaptive immune response (Kindt et al,. 2007). A major feature of the innate immunity is the ability to block the infiltration of infections by providing a physical barrier to wade off pathogens and ensure they never gain access into the host. This mechanism does not lead to any memory recognition of the antigens. On the other hand, the adaptive immune response is capable of specifically killing foreign microorganisms and differentiate self from non-self antigens. Unfortunately, pathogens use several strategies to escape elimination from the adaptive system for example by reducing its antigenicity, mimicking the host cell surfaces, selectively suppress the immune responses, and continual variation of antigen surfaces. The adaptive immunity provides a line of defense that eliminates a specific pathogen and generates a memory towards it whenever the body is invaded by the similar pathogen again. The natural cooperation between the innate and adaptive immune response in human provides a critical defense against organisms causing diseases, but such responses are not sufficient to completely protect a person from widespread infectious diseases (Janeway et al., 1999).

1.2 Antibody development and its diversity.

The human adaptive immune system has the capability to produce a highly diverse collection of antibody specificities due to the immunoglobulin genes encoded in the

germline. The maturation process is carried out by a gene assembly process that produces a collection of new genes through site-specific recombination. Variable region gene of the antibody consists of multiple germline segments that are classified as variable (V), diversity (D) and junction (J) segments.

Human antibody variable heavy gene contains 48 VH, 23 D and 6 JH. The immunoglobulin (Ig) κ gene contains 41Vκ and 5 Jκ while Igλ contains 34 Vλ and 5 Jλ (Kindt et al., 2007). These different segments are joined by recombination to produce a full antibody VDJ segment whereas light chain genes are able to undergo similar recombination excluding the D segment gene as outlined in figure 1.1. The random gene rearrangement of V-J and V-D-J combination contributes to the generation of a vast diversity of antibody repertoire (Lewis, 1994).

A part of the antibody diversity also involves the pairing association between heavy and light chains. The combination of the associated heavy and light chain will lead to a higher complexity resulting in higher diversification of antibodies. Apart from that, antibody diversity is also associated by junctional flexibility caused by changes of amino acid at CDR3 of heavy and light chain. It is a process where nucleotides can either be lost or added (P / N –addition) during V-D-J and V-J rearrangements (Parslow, 2001). Antibody diversity could be generated in rearranged variable region gene units called somatic hypermutation after stimulation of B-lymphocyte by antigen encounter. As a result, nucleotides in VJ or VDJ units are substituted with alternatives and thus altering the specificity of the encoded antibodies further (Raaphorst et al., 1997).

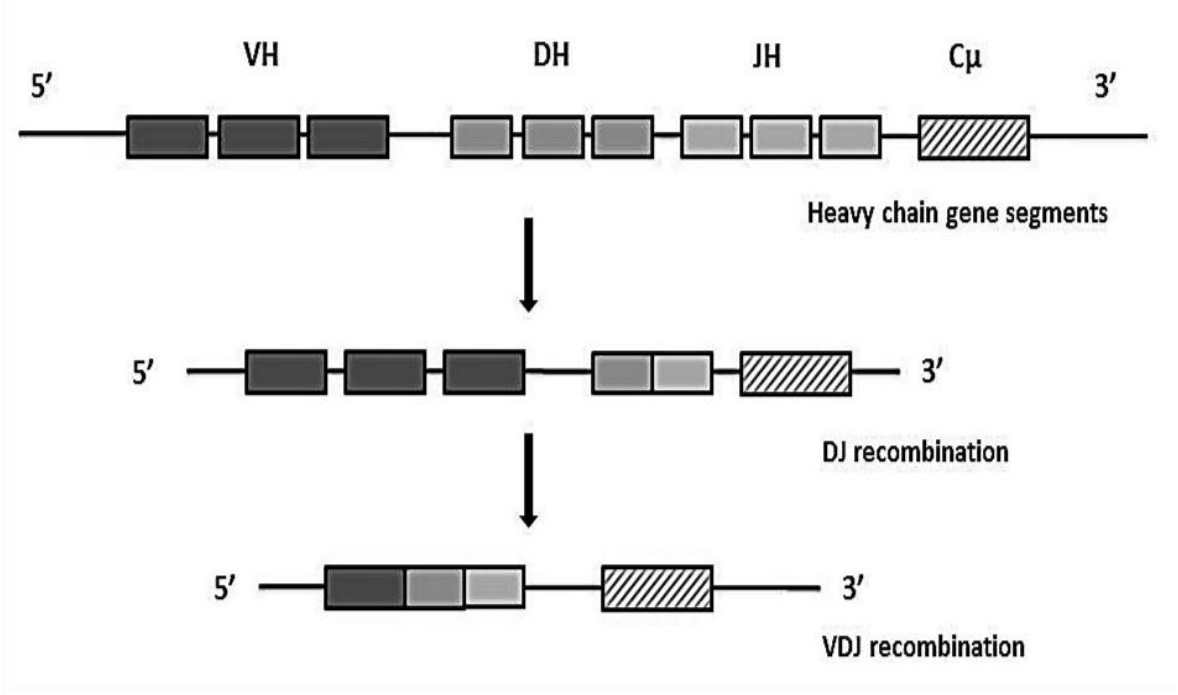

Figure 1.1 Organization of immunoglobulin heavy chain gene segments rearrangement.

1.3 Antibody structure

Antibodies are produced through secretion by plasma cells to act as the front line defense mechanism of our immune system. The common structure of an immunoglobulin molecule consists of four subunits that is made up of two heavy chain polypeptide chains held together by disulphide links and non-covalent interactions (Davies et al., 1990). Antibodies are heterodimers composed of 2 heavy chains and 2 light chains. There are two different light chains known as kappa (κ) and lambda (λ), either one of these will be associated with the heavy chain as one antibody, never both. The light chain consists of only a variable domain (VL) and a constant domain (CL).

The heavy chain is made up of a variable domain, either three or four constant domains juxtaposed to its variable domain (CH1, CH2, CH3 or CH4) and a hinge region depending on the antibody class (Kindt et al., 2007). The classes of the antibody are IgM, IgG, IgA, IgD, IgE or IgG where the heavy chain of each class will have either a κ or λ light chains. For IgG, IgA and IgD isotypes the constant domains involved are CH1, hinge, CH2 and CH3 while for IgM and IgE the domains are CH1, CH2, CH3 and CH4. Therefore, each class of antibody is determined by the proportion of heavy chain constant domain (Parslow, 2001).

There are two significant parts of an antibody that is the antigen binding and effector functions located at the amino (N) - terminal and carboxyl (C) - terminal respectively Janeway et al. (1999). The general Y-structure is the same for all antibodies, however only the heavy and the light chain are responsible for recognizing antigens. The variability calculations (number of different amino acids at particular position / frequency of the most common amino acid at particular position), is able to show the

variation at the formation of the loop at the β-strand called the hypervariable region mostly known as complementary determining regions (CDRs) (Kindt et al., 2007). The remaining region with the least amount of variation is the framework (Fr) region. The variations among antibodies are located at the three CDRs that make up the binding site of the antigen epitope. It is this particular diversification that a lot of the antibodies are produced that can distinguish any protein target. These CDRs are separated by four relatively constant gene sequences as the framework (Janeway et al., 1999). Figure 1.2 shows the antibody Y-shaped structure and its ribbon drawing structure presentation.

1.4 Phage display technology

Antibodies are in high demand as they are suitable for diagnostic applications. Conventional methods for monoclonal antibody generation are lengthy and tedious (Li et al., 2006, Cambrosio and Keating, 1988). The introduction of phage display allowed for rapid generation of monoclonal antibodies (Willats, 2002). The method is based on a bacteriophage that is harmless to humans but only infects bacteria as a reproduction host. The viral genomic material is able to enter the bacteria host cell and directs the production of progeny phage. The progeny phage would infect other bacteria cells and secrete a new phage in the cytoplasm of the cell. However, a filamentous phage assembles its progeny on the cell surface by a special secretory mechanism and leaves the cell viable for the next round of infection (Marvin, 1998). It has been reported that the insertion of a foreign DNA into the phage genome is capable of yielding a phage particle with the foreign peptide sequence displayed on the phage coat protein (Smith, 1985). By exploiting the morphology and secretory mechanism of the filamentous phage, it is possible to physically link genotype and phenotype together (Barbas et al., 2004). Therefore, DNA sequence encoding

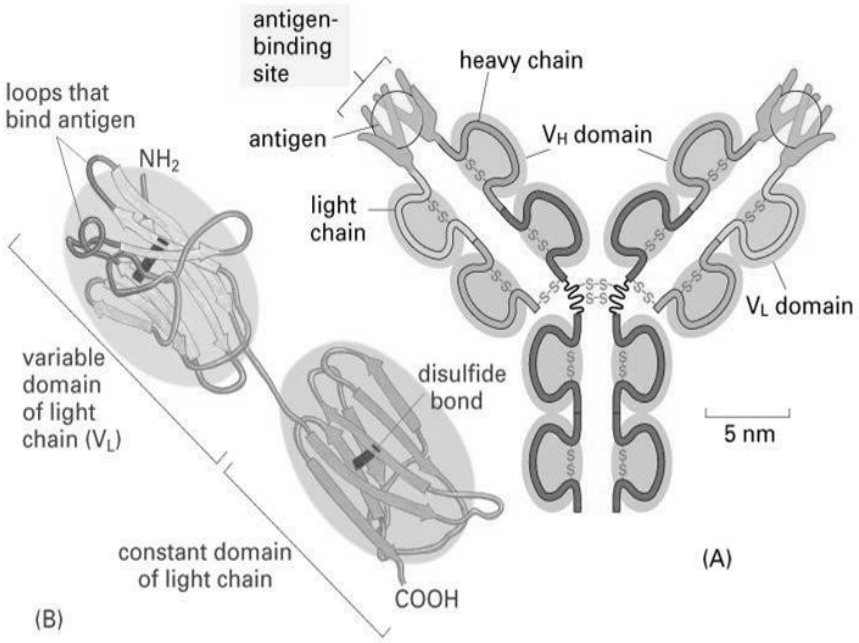

Figure 1.2 Typical structure of an antibody molecule. **A)** Antibody consists of two heavy chains and two light chains which are linked together by disulphide bonds. One bond linked together VH and VL, two bonds linked two VHs and several bonds linking antibody subdomains. **B)** Finger-like loops of a single light chain whereby binding of antigen occur. Figure adapted from (Alberts et al., 2013).

the foreign molecules can be retrieved through DNA sequencing as first reported by George Smith in 1985 leading to the innovation of phage display technology.

1.4.1 Structure of bacteriophage

Filamentous bacteriophage belongs to a type of related viruses that contains single-stranded viral DNA that infects only gram-negative bacteria. It is 2000 nm in length and 6-10 nm in diameter enclosing all viral DNA genome consisting of 11 genes (Zimmermann et al., 1986). The functions of the 11 genes are: p (III, VI – IX) for coat proteins, p (I , IV and XI) for phage assembly, p (II and X) for DNA replication and lastly pV for binding ssDNA. The viral mass consists mainly of about 2700 copies of the pVIII major coat protein, about 3-5 copies of pVII and pIX at one side which are assembled first and 3-5 copies of pIII and pVI on the other side are for phage particle stability and infection (Russel et al., 1997). Figure 1.3 shows the basic filamentous phage particle consisting of its major coat proteins.

1.4.2 Infection mechanism

Filamentous phage is dependent on the F-pilus for infection and it does not kill its host (Marvin, 1998). The pIII minor coat protein consists of three domains which are carboxyl terminal (C), amino terminal 2 (N2) and amino terminal 1(N1) which are separated by a glycine-rich linker region (Deng et al., 1999). Initially, the N2 domain of the phage particle binds strongly with the F-pilus of *E.coli* and mediate the binding of the N1 domain to the bacteria protein TolA (Click and Webster, 1997). By natural forces, it pulls the phage particle closer to the bacteria. Consequently, the N1 domain is released from the N1N2 complex of the pIII after the F-pilus binding. Next, free N1 domain will interact with TolA receptor (Karlsson et al., 2003). TolA is a membrane bound protein which was believed to incorporate other membrane

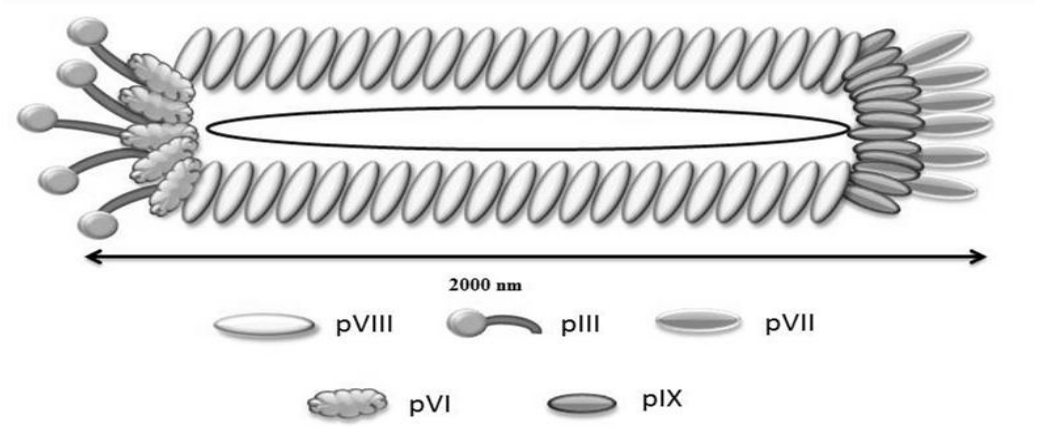

Figure 1.3 A representation of filamentous phage particle. The major coat proteins of the filamentous phage such as M13KO7 ; pIII, pVI, pVII, pVIII and pIX.

proteins, TolQ and TolR to form a complex (Click and Webster, 1997). This protein complex facilitates the pIII and pVIII into the cytoplasmic region of the bacteria. Finally, anchored TolA at the cytoplasm will enter the periplasm where phage infection occurrs. At this point, the viral ssDNA is secreted into the cytoplasm and gets replicated into dsDNA format; making it ready for the expression of the coat proteins. The new phage particles are assembled and will be secreted into the medium (Rakonjac et al., 1999). Figure 1.4 shows the infection mechanism of the filamentous phage.

1.4.3 Display formats

There are two commonly used coat protein for phage display which is pVIII and pIII (Smith and Petrenko, 1997). The major coat protein, pVIII exist in thousands of copies that make up the long cylindrical phage particle while pIII consists of three functional domains (Webster, 1996). The presentation of any foreign sequence is usually inserted at the C - terminus of the pVIII and pIII coding sequences while the linker sequences are inserted at the N - terminus on a phagemid vector. Protein pVIII only allows short peptides (6 - 8 residues) to be displayed on it as it could interrupt the interactions of the pVIII molecules during assembly (Qi et al., 2012). However, there are reports on the fusion of antibody fragments on pVIII (Kang et al, McCafferty et al). On the other hand, pIII coat proteins are able to tolerate large foreign molecules on it and its infectivity still remained active. This makes pIII the ideal coat protein for the presentation of foreign proteins for display.

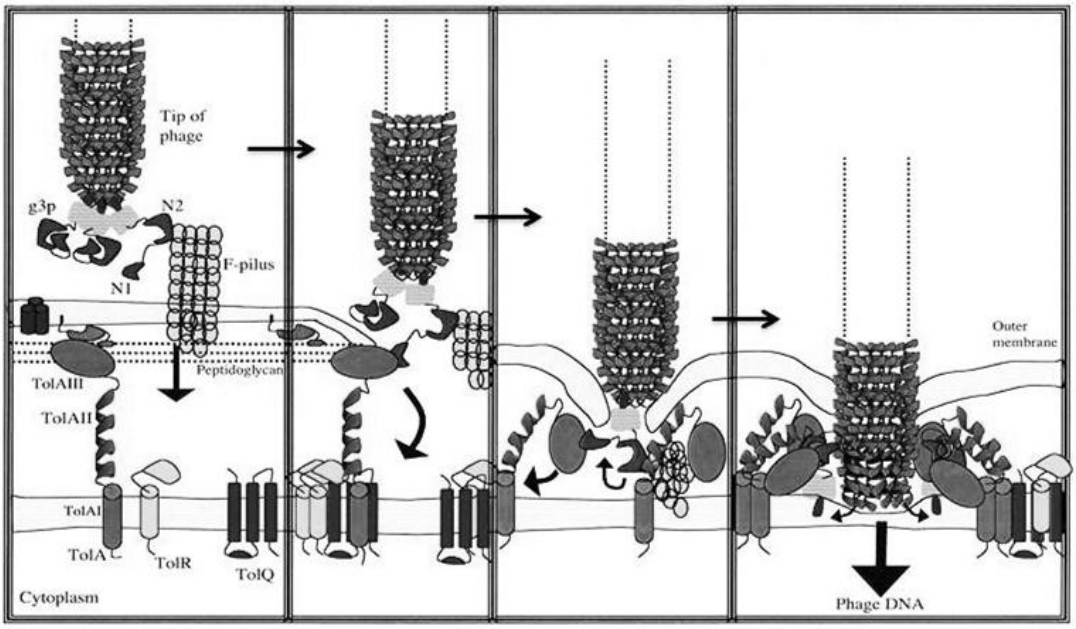

Figure 1.4 The mechanism of bacterial infection by filamentous phage. First, the pIII coat protein N2 domain interacts with the bacterial F-pilus followed by recognition of N1 domain with TolA. Next, retraction of the F-pilus brings the phage closer and opening of the phage head to release phage DNA. Figure adapted from (Karlsson et al., 2003)

1.4.4 Antibody libraries by phage display

Phage display has become a powerful technology that allows for *in vitro* mimicking of the natural human production of antibodies *in vivo*. Fully human monoclonal antibodies (mAbs) production is efficient for downstream application for humans due to the highe risk associated with the use of murine derived mAbs (Vaughan et al., 1998). Phage display is a robust scientific research and development for antibody selection.

1.4.4.1 Recombinant antibody libraries

There are four types of antibody libraries that can be generated for scientific applications depending on the source of the antibody genes obtained; naïve (non-immunized), immunized, semi-synthetic and fully synthetic library (Ponsel et al., 2011, Marks and Marks, 1996). Libraries derived from immunized and non-immunized individuals are directly influenced by the natural human antibody gene diversity while semi-synthetic and synthetically design libraries are based on the designed gene to exhibit similar diversities found in natural human antibody repertoires.

Naïve libraries can be built using peripheral blood or bone marrow of healthy individuals. These libraries are used to harvest the natural binding sites of the antibodies (Løset et al., 2005). The antibody repertoire is constructed from natural rearranged V-genes of the B-cells representing *in vivo* IgM antibodies that are commonly produced by developing B-cells that do not show any preference to any particular antigen that provides higher percentage of success when isolating new binders.

Immunized antibody libraries are made using genetic materials from individuals that have contracted or have been exposed to a particular disease (de Carvalho Nicacio et al., 2002). This will skew the antibody producing cells to generate antibodies against immunogenic antigens related to the disease. This can prove to be useful to generate high affinity and specific antibodies against antigens of that particular disease. Therefore the preferred antibody gene isotype for immunized libraries is usually IgG.

The synthetic antibody libraries are made up of antibody gene variation that can be designed and generated through degenerate oligonucleotides to produce fully synthetic heavy and light chains with different properties. The entire gene fragment of a synthetic antibody is chemically synthesized *in vitro* (Knappik et al., 2000). Such libraries allow massive customization in terms of framework preferences and even CDR designs. As the randomization in such libraries generates highly diverse repertoires, the library will have the tendency to exhibit unskewed repertoire much like those of the naïve libraries. Semi-synthetic libraries are a fusion of the naïve and synthetic library designs (De Kruif et al., 1995). This is because in semi-synthetic libraries, the genetic information can be obtained from donors and the randomized regions or framework regions can be synthesized. This allows semi-synthetic antibody libraries to present a naïve repertoire too. Therefore semi- and fully synthetic libraries can function to generate antibodies against any antigen target. Figure 1.5 shows the four different types of antibody libraries can be generated for phage display.

1.5 Selection process (Bio-panning)

A heterogeneous mixture of the phage clones or phage library is produced with standard molecular biology techniques involving the exploitation of DNA vectors to accommodate V-gene inserts and accommodate packaging to mature phage particles

by appropriate *E.coli* hosts (Galanis et al., 1997) . In order for the antibody to be presented on the phage particle, the V-genes need to be tethered to the phage coat protein so that the presentation will be a fusion of both proteins. The integrity of the antibody presented is maintained as it is physically placed on the surface of the phage particle. This physical interaction between the antibody and phage particle allows for easy in-vitro selection of specific antibodies against immobilized target antigens (Romanov, 2003). This selection process is commonly known as bio-panning. The selection process is a collection of repetitive rounds that allows antibody displaying phages to bind immobilized antigens on solid phases. The antigens can be immobilized onto (1) plastic tubes (Marks et al., 1991), (2) columns with target-activated matrix (McCafferty et al., 1990), (3) biotinylated antigens selection (Hawkins et al., 1992), (4) an automated selection whereby the antigens are coated on the magnetic particles (Konthur and Walter, 2002), (5) immunopins (Lou et al., 2001) and (6) microtitre plate (Krebs et al., 2001). Each solid phase provides a different set of advantages and disadvantages. The choice of the best suitable solid phase is mainly dependent on the target antigen and selection conditions. During the panning process, a repetitive cycle of physical binding and removal of unwanted phage particles is carried out. The initial binding step allows for specific binders to capture the target antigens and interact with it in a suitable environment. The wash steps are introduced to remove unbound phages and contaminants (Goodchild et al., 2005). The panning process can be customized to allow for different stringency. This will affect the end product of the panning whereby antibody selectivity can be tuned with different competing conditions. The antibody bound to the target can be eluted using several different techniques such as acid/basic elution (Hoogenboom et al., 1998, Marks et al., 1991), enzymatic cleavage (Ward et al., 1996) or reducing agents

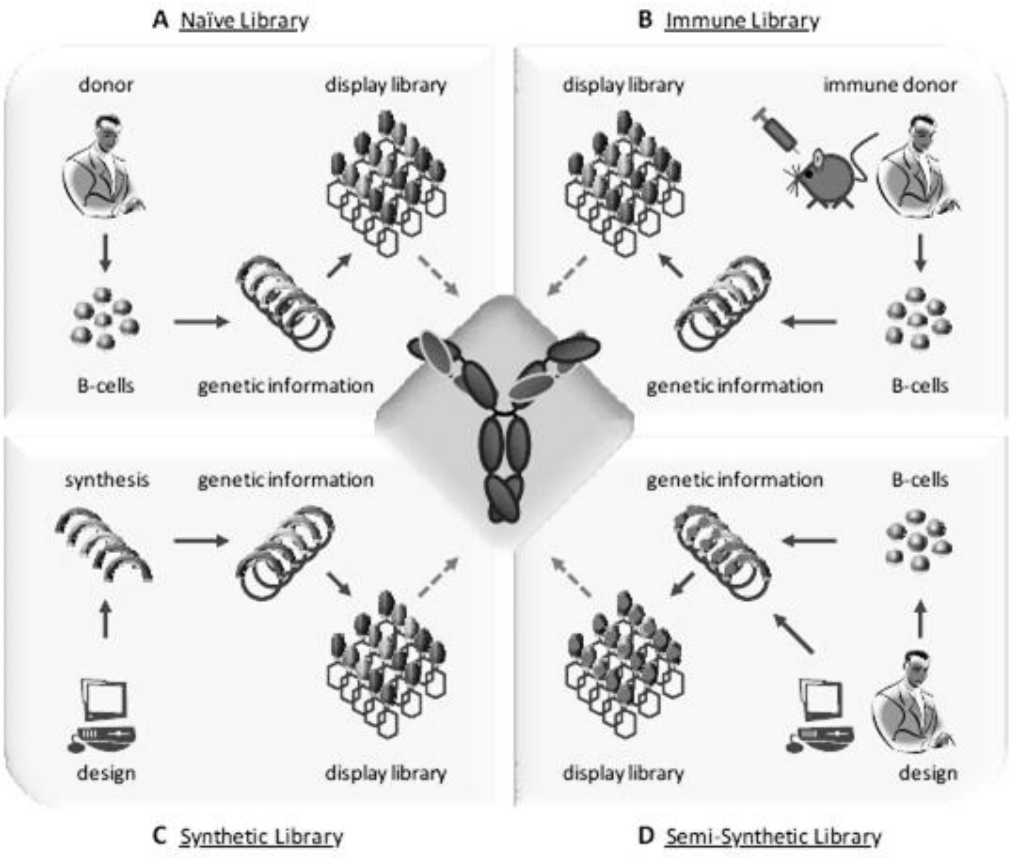

Figure 1.5 Antibody libraries type **A)** Generation of naïve libraries are from the B-cells isolation of non-immunized donor. **B)** Generation of immunized libraries are from the B-cells isolation of non-immunized donor. **C)** Generation of synthetic libraries are fully based on the computational design of the gene sequence **D)** Generation of semi- synthetic libraries are combination of pre-designed segments and natural source of gene sequence. Figure adapted from (Ponsel et al., 2011).

(Kretzschmar and von Rüden, 2002). The rescued phage clones are then infected with *E.coli* allowing the selected phage to be amplified as input phage for the next round of panning (Goodchild et al., 2005). To identify monoclonal binders, single clones have to be picked from a pool of enriched binders and analyzed individually. Figure 1.6 shows the bio-panning process by phage display technology.

1.6 Applications of recombinant antibody

The main characteristic for the antibodies to be useful for various applications is dependent on the high affinity and specificity towards an antigen. In 1975, Köhler and Milstein (Köhler and Milstein, 1975) reported the revolutionary method to generate antibodies by fusing murine antibody-producing cells with myelomas from bone marrow to produce hybridomas. Hybridoma technology has been applied successfully to produce monoclonal antibodies against many antigens. The method involves the isolation of hybridomas producing antibodies against the selected antigen by screening each individual hyridoma (Cambrosio and Keating, 1992). One major complication associated with hyridoma-based antibodies is the potential allergic reaction accustomed to the application of animal derived antibodies in humans. The allergic reaction is a result of the human immune system reacting against the foreign animal derived antibody as a potential threat (Khazaeli et al., 1994). Therefore, antibodies derived using hybridoma technology was not able to achieve its intended benefits. Therefore, to circumvent this issue various modifications have been introduced. The modifications lead to the introduction of chimeric, humanized and ultimately fully human antibodies. Another major consideration for recombinant antibodies is the ability to increase production with microbial expression systems. For example, *Escherichia coli* (*E.coli*) as an expression host has become a widely used technique that is able to produce

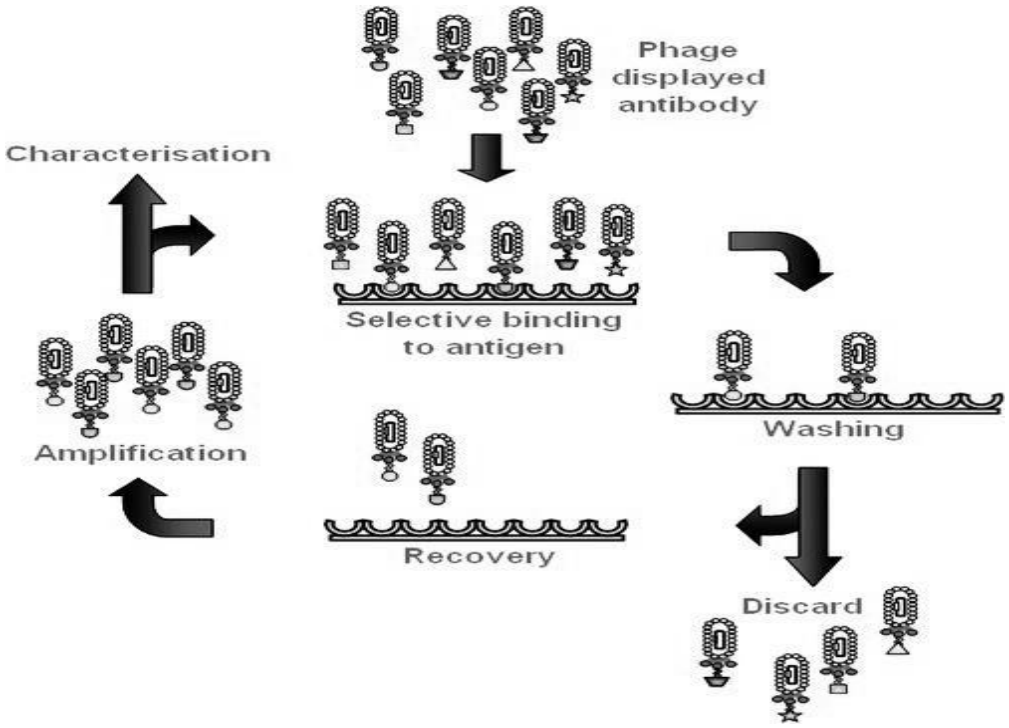

Figure 1.6 A scheme of bio-panning strategy by phage display. In phage display panning where antibodies are presented on the phage particles and add into immobilized target antigens for binders. This is followed by several wash steps to remove unbound phages and elution and propagation of the bounded phages. Figure adapted from (Goodchild et al., 2005).

monoclonal antibodies due to its fast growth rate, the ability to accept a vector carrying specific genetic material and a high transformation rate (Plückthun, 1992). The production of full-length antibody is relatively difficult by expression in the *E.coli* system if compared to the expression of smaller antibody fragments (Simmons et al., 2002). For instance, the first antibody produced is the fragment variable (Fv) domain antibodies which comprises of either the VH or VL domains expressed in the periplasmic environment of the bacterial cells (Skerra and Pluckthun, 1988). As the binding characteristics of an antibody is associated to the ability of both the heavy and light chain domains to work in tandem, a modified fragment namely the single chain Fragment variable (scFv) was introduced. scFv fragments consist of both variable domains to be expressed as one fragment with the help of a peptide linker (Bird et al., 1988). Even so, variations in the length of the linker sequence have been reported to promote aggregation during expression (Whitlow et al., 1993).

On the other hand, the stabilization of Fab molecules is generally associated to the disulphide bond between the variable heavy and the light chain. The production of Fab molecules requires the transportation of the Fab to the periplasmic space for disulphide bond formation. However, expression levels of Fab are difficult due to the need for both fragments to be independently expressed and form a disulfide bond in the periplasmic space (Skerra and Plückthun, 1991). However, Fab molecules are important fragments for antibody engineering. This is because Fab fragments are suitable for direct transfiguration to the larger full immunoglobulin molecule without the loss of binding efficiency (Rader, 2009). This would make full antibody molecule production easier and more efficient. Figure 1.7 shows the different recombinant antibody formats for phage display.

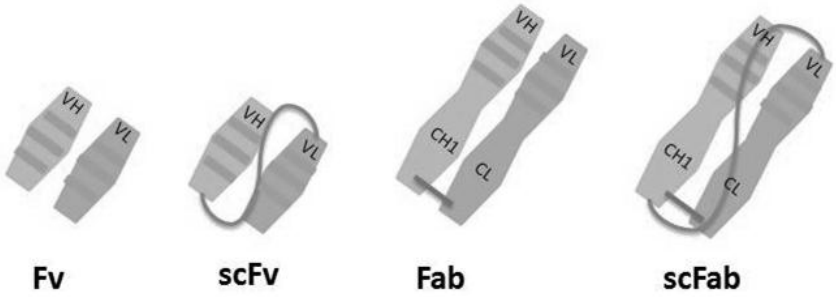

Figure 1.7 Schematic representation of different antibody formats. Fv (fragment variable) comprises of VH and VL. scFV (single chain fragment variable) is included of VH and VL connected by a linker. Fab (fragment antigen binding) comprises of VH and VL with a constant region (CH1 and CL respectively) connected by a disulphide linkage. scFab (single chain fragment antigen binding) consists of Fab but with a linker connection.

1.6.1 Application of Fab fragments

The developments in antibody generation technology have enabled recombinant antibodies to be made fully of human fragment (Yan and Xu, 2006, Barbas et al., 2004). The most widely used recombinant antibodies with respect to the antigen binding fragments are the scFV and Fab. Both recombinants are biologically and physically different in properties. Fab in general has a better stability comparison. The interaction of the HC and LC by an interchain disulfide bond is the major contribution towards the stability of a Fab fragment (Röthlisberger et al., 2005) . Apart from that, a synergy between the VH, VL, CH1 and CL domains had shown significant stabilization effects within the interfaces by the intrachain disulfide bonding. These were reported by Shimba et al. which discussed Fab stability in comparison to Fv based on thermodynamics study (Shimba et al., 1995). Another study by Daniela Röthlisberger et al. discussed the stabilizing effects of Fab domain interactions and compared that with the scFv. The Fab domain showed several key advantages such as better mutual stabilization of both HC and LC, better stability due to the presence of the interchain disulfide bond producing and kinetic stabilization (Röthlisberger et al., 2005). As such the application of Fab monoclonal antibodies are still used due to the wide benefits it is able to provide in terms of format conversion even with the challenges posed in handling.

1.7 Model Target – B*m*SXP Recombinant Antigen

In this work, the model antigen selected was B*m*SXP antigen used for detection of bancroftian filariasis. It is a recombinant protein of *Wuchereria Bancrofti* - a filarial nematode that lives in the human lymphatic system. B*m*SXP is a highly specific recombinant antigen used for the detection of specific IgG4 antibodies for bancroftian worms (Khoo et al., 2010). B*m*SXP recombinant protein was an ORF of

SXP1 gene from *Brugia Malayi* cDNA library which its sensitivity is 95% in detection of bancroftian filariasis infection. The used of this recombinant antigen with B*m*R1 recombinant antigen (specific for brugian filariasis detection) was found to enhance the specificity and sensitivity of lymphatic filariasis for diagnostic purposes. (Ab Rahman, 2008) Therefore, there is a necessity to produce antibodies against it for future downstream applications, mainly for immunopurification. A common challenge in the production of the diagnostic kit is the purification of the antigen with high specificity. As the current diagnostic kit is based on antibody detection, the generation of monoclonal antibodies could also pave the way for the development of an antigen detection system.

1.8 Statement of problem and rationale of study

The antibody molecule is an integral part of many proteomics, diagnostic and therapeutic research due to its ability to specifically bind a target antigen. Production of human antibodies has been explored widely in a race to find unique binders. *In vitro* antibody production through phage display technology is an interesting alternative to conventional methods for antibody development. Phage display allows for a faster and easier approach to generate antibodies with the generation of an antibody library mimicking the natural antibody diversity. Antibody variable regions (variable heavy chain/variable light chain) are easily manipulated and displayed on the phage particle. In order to develop this technology in-house to overcome the complications of antibody production, a naïve Fab library was developed and used to identify monoclonal antibodies against B*m*SXP. Fab antibody still possess a lot of challenges as a tool for detection of diagnostic and therapeutic studies by development of phage display technology. Bypassing the use of animals, phage display is a well-established technology for antibody isolations of fully human small

antibody format such as scFV and Fab. Generation of Fab antibody by this technology was a challenge due to two significant reasons. First, Fab fragment is larger compare to scFv fragment to be expressed in *E.coli* system. Second, the presentations of the Fab on the phage particle was a synergy between two independent chains endeavoring for each other in *E.coli* periplasm which always leading to low yield of phage presenting full Fab fragment. Therefore, the generation of a highly diverse naive Fab library for phage display was indeed required to produce quality Fab antibodies.

1.9 Research Objectives

1. To generate a naïve fragment antigen- binding library (Fab fragment library)
2. To produce human monoclonal Fab antibodies by phage display against B*m*SXP recombinant antigen
3. To evaluate the specificity of the monoclonal antibodies generated

CHAPTER 2

MATERIALS AND METHODOLOGIES

2.1 Materials for blood collection and isolation of B – lymphocytes

2.1.1 Blood samples

90 donors (Malay, Chinese and Indian)

2.1.2 Related materials

K2 EDTA Blood collection tubes 3.0 mL	BD Vacutainer , USA
Ficoll – Paque ^{TM} PLUS	GE Healthcare Life Sciences , UK
β – Mercaptoethanol (β-ME)	Sigma Aldrich, USA

2.2 Materials for molecular biology work

2.2.1 Enzymes and corresponding buffers

NcoI	NEB3/BSA	New England Biolabs, USA
MluI	NEB3/BSA	New England Biolabs, USA
SalI HF	NEB4/BSA	New England Biolabs, USA
NheI HF	NEB4/BSA	New England Biolabs, USA
Pfu DNA polymerase	10X *Pfu* Buffer ($MgSO_4$)	Thermo Scientific, USA
Vent DNA Polymerase	10X ThermoPol buffer	New England Biolabs, USA
T4 DNA Ligase	T4 DNA ligase buffer	New England Biolabs, USA

2.2.2 Bacterial strains and phages

DH10B T1 Phage Resistant Cells	F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1*ara*D139Δ (*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G *ton*A	Invitrogen, Carlsbad, USA
TG1	supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 (rK–mK–) [F´ traD36 proAB lacIqZΔM15]	Agilent Technologies, USA
XL1 Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)]	Agilent Technologies, USA
M13KO7 helper phage	kn^r	New England Biolabs, USA