DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST ANCYLOSTOMA CANINUM ANCYLOSTOMA-SECRETED PROTEIN 5 (ASP5) BY PHAGE DISPLAY

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by

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LIST OF SYMBOLS

α	Alpha
°C	Degree Celsius
g	Gram
h	Hour
kDa	Kilo Dalton
kV	Kilo Volt
%	Percent
µg	Microgram
µL	Microliter
µM	Micromolar
mg	Milligram
mL	Milliliter
ms	Millisecond
min	Minute
M	Molar
mM	Millimolar
rpm	Revolutions per minute
s	Second
×g	Gravity force
v/v	Volume / volume
V	Volt
w/v	Weight / volume

LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
APS	Ammonium Persulfate
ASP	Ancylostoma-secreted protein
β-ME	Beta-Mercaptoethanol
bp	Base pair
BSA	Bovine Serum Albumin
CDR	Complementarity-determining region
CV	Column volume
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
D genes	Diversity genes
E. coli	*Escherichia coli*
EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen-binding fragment
Fc	Crystallizable fragment
Fv	Variable fragment
Ig	Immunoglobulin
IMAC	Immobilized metal affinity chromatography
J genes	Joining genes
mAb	Monoclonal antibody
N nucleotide	Non-template encoded nucleotides
RE	Restriction Enzyme
scFv	Single chain variable fragment
ssDNA	Single-stranded deoxyribonucleic acid
TEMED	Tetramethyl Ethylenediamine
VH	Heavy chain variable region
VL	Light chain variable region

V genes Variable genes

LIST OF APPENDICES

PENGHASILAN DAN PENCIRIAN ANTIBODI MONOKLONAL TERHADAP ANCYLOSTOMA CANINUM ANCYLOSTOMA-SECRETED PROTEIN 5 (ASP5) DENGAN PAPARAN FAJ

ABSTRAK

Cacing nematoda parasit seperti *Ancylostoma caninum* terlibat dalam kitaran hidup yang kompleks dan telah memberi kesan terhadap hos dalam pelbagai peringkat perkembangan dan jangkitan. Semasa peringkat jangkitan, *Ancylostoma caninum* telah mengeluarkan sejumlah protein yang berlainan untuk meninggirendahkan gerak balas imun hos, bagi memastikan kelangsungan hidup mereka di persekitaran dalam hos. Antara protein tersebut, Ancylostoma-secreted protein 5 terlibat secara kritikal dalam interaksi antara hos dan parasit seperti imunomodulasi, penyerangan tisu dan mempermudah proses penghisapan darah, justeru menjadikan protein ini sebagai sasaran yang meyakinkan untuk strategi intervensi yang bertujuan mengawal jangkitan cacing tambang dalam kalangan anjing. Dengan memanfaatkan teknologi paparan faj, kajian ini bertujuan menghasilkan antibodi monoklonal yang mensasarkan Ancylostoma-secreted protein 5, dengan penggunaan perpustakaan scFv antibodi naif manusia melalui bio panning in vitro. Perpustakaan tersebut telah melalui sebanyak tiga pusingan pemilihan terhadap 10 µg antigen Ancylostoma-secreted protein 5 dengan syarat cucian yang ditingkatkan keketatannya dalam setiap pusingan (10, 20 dan 30 cucian masing-masing) untuk mengasingkan klon. Kajian ini meneroka Ancylostoma-secreted protein 5 sebagai calon antigenik untuk menghasilkan antibodi, iaitu antigen tersebut telah diekspresikan menggunakan strain bakteria BL21(DE3) dan ditulenkan dengan cara penulenan kromatografi keafinitian logam terimobil yang menggunakan resin afiniti bercas nikel. Kajian ini telah menerangkan ciri-ciri antibodi

yang terhasil dalam aspek interaksi antara antibodi dan antigen, termasuklah reaktiviti silang dan sifat pengikatan melalui asai immunoserap terangkai ensim. Melalui pencirian tersebut, antibodi telah menunjukkan sifat pengikatan kuat terhadap Ancylostoma-secreted protein 5 dengan reaktiviti silang yang rendah terhadap protein lain seperti Bovine Serum Albumin (BSA), Enhanced Green Fluorescent Protein (EGFP), 16kD dan ubiquitin. Ujian titrasi antibodi juga telah menunjukkan bahawa 10 µg antibody adalah mencukupi untuk mengesan 10 µg Ancylostoma-secreted protein 5, memberikan nisbah syarat kepada bunyi sebanyak 4.8. Penemuan ini telah menunjukkan bahawa antibodi monoklonal yang terhasil mempunyai kekhususan dan fungsi yang menyakinkan secara in vitro, memperlihatkan sifat pengikatan yang kuat terhadap antigen sasaran. Antibodi ini berpotensi untuk berfungsi sebagai komponen asas dalam pembangunan strategi diagnostik terhadap jangkitan cacing tambang, seterusnya membuka jalan untuk penyelidikan lanjut ke atas potensi diagnostik dan kegunaannya untuk memerangi jangkitan cacing tambang. Dengan itu, kajian ini telah ditekankan kepentingannya dalam mengatasi cabaran yang dihadapi oleh jangkitan parasit dalam populasi anjing dan juga manusia.

DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST ANCYLOSTOMA CANINUM ANCYLOSTOMA-SECRETED PROTEIN 5 (ASP5) BY PHAGE DISPLAY

ABSTRACT

Parasitic nematodes such as *Ancylostoma caninum* engages in a complex life cycle, in which it can impact the hosts at its various stages of development and infection. During infective stage, *Ancylostoma caninum* releases a multitude of proteins to modulate the host's immune response, which ensuring its survival within the host environment. Among these proteins, Ancylostoma-secreted protein 5 (ASP5) is critically involved in the parasite-host interaction such as the immunomodulation, tissue invasion and facilitating the blood feeding process, hence making it a promising target for intervention strategies aimed at controlling hookworm infections among canines. By harnessing phage display technology, this study aims to develop monoclonal antibodies targeting Ancylostoma-secreted protein 5 (ASP5), by employing the human naïve scFv antibody library through in vitro biopanning. The library was subjected to a total of three rounds of panning against 10 µg of ASP5 antigen, with progressively stringent washing conditions (10, 20 and 30 washes respectively) to isolate the clones. This study delves into Ancylostoma-secreted protein 5 as an antigenic candidate for monoclonal antibody development in which it was expressed using the bacterial strain of BL21(DE3) and purified with Immobilized Metal Affinity Chromatography (IMAC) purification method using the nickel-charged affinity resin. The developed antibody was characterized on its antibody-antigen interactions, including cross-reactivity and binding strength via Enzyme-linked immunosorbent assay (ELISA). The monoclonal antibody demonstrates robust

binding to Ancylostoma-secreted protein 5 with minimal cross reactivity to other proteins such as Bovine Serum Albumin (BSA), Enhanced Green Fluorescent Protein (EGFP), 16kD and ubiquitin. The antibody titration assay indicates that 10 µg of the antibody is sufficient to detect 10 µg of Ancylostoma-secreted protein 5, yielding a signal-to-noise ratio of 4.8. Hence, the findings indicate that the resulting monoclonal antibody shows promising specificity and functionality in vitro, demonstrating effective binding towards its target antigen. This antibody could possibly serve as a fundamental component for diagnostic strategies against hookworm infection and potentially pave the way for further exploration into its diagnostic potential and utilization in combating the hookworm-related morbidities. This highlights the significance of the study in addressing challenges posed by parasitic infections in canine and human populations.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Hookworm infection is a common occurrence in canines and occasionally affects felines. The rising population of stray dogs raises concerns as they are competent reservoir hosts for various zoonotic pathogens and serve as a readily available nutritional source for many blood-feeding nematodes (Traub et al., 2021). Dogs, which are popular pets in many households, can also act as the carrier of zoonotic pathogens, posing a potential risk for human disease. *Ancylostoma caninum*, a predominant canine hookworm species poses a serious threat to dogs, particularly leading to anaemia and fatalities especially in puppies (Morante et al., 2017). Beyond canine health, these hookworms can transmit disease like cutaneous larva migrans to human, with transmission occurring through larvae penetrating human skin from contaminated soil or sand (Feldmeier & Schuster, 2012).

The development of monoclonal antibodies targeting Ancylostoma-secreted proteins emerges as a promising avenue to address the challenges associated with the hookworm infection. The infective larvae of *A. caninum* reportedly release proteins, specifically Ancylostoma-secreted protein (ASP), which are highly associated with the hookworm pathogenicity (Hawdon et al., 1999). By targeting these specific proteins, monoclonal antibodies facilitate reliable diagnostic test, enabling early detection of infection in dogs and potentially preventing transmission to humans. Monoclonal antibodies and their derivatives have made remarkable strides in the field of medicine, finding utility in both therapeutic and diagnostic applications (Ch'ng et al., 2016; Zahavi & Weiner, 2020). As versatile biomacromolecules, they exhibit high specificity in binding to a diverse range of proteins and non-protein targets including small

molecules like haptens and toxins, carbohydrates, lipids, nucleic acids and inorganic molecules such as metal ions and nanoparticles (Dengl et al., 2016; Kappler & Hennet, 2020; Wang et al., 2020). Additionally, their capability for engineering and adaptation into various formats enhance their versatility and effectiveness (Basu et al., 2019). This study utilizes smaller recombinant antibody format, which is the single-chain fragment variable (scFv), as smaller fragments are more particularly suitable for bacterial expression compared to full antibodies (Gupta & Shukla, 2017).

Antibody phage display, the in vitro selection technology has been widely employed to discover high-affinity antibodies specific to various antigens. In 1985, George P. Smith pioneered this technology by demonstrating the ability of filamentous phages to display peptide of interest on their surfaces through the insertion of foreign DNA fragment into the phage coat protein gene (Smith, 1985). Subsequently, Parmly and Smith introduced a selection and affinity enrichment process known as biopanning for the isolation of peptide-phage fusion (Parmley & Smith, 1988). Expanding this work, McCafferty and Winter further utilized the same technology to discover antibodies, creating combinatorial antibody libraries aimed at generating antigen-specific monoclonal antibodies (McCafferty et al., 1990; Winter & Milstein, 1991). The benefit of this technology is that in vitro selection method like antibody phage display circumvent the dependence on the vivo immune response thus enable the discovery of a broader range of epitopes that may be otherwise be suppressed by the immune system (Frenzel et al., 2016). In this study, phage display is employed to develop monoclonal antibodies by isolating scFv clone from a human naïve antibody library. The study's outcome can serve as a potential diagnostic tool for canine hookworm infection caused by a specific secreted protein. The application of phage display-derived antibodies for diagnostics offers flexibility in designing various forms

of diagnostic tools (Anand et al., 2021). The primary advantage of using recombinant antibodies such as scFv lies in the ability to modify them. This study primarily focuses on expressing the ASP5, one of the secreted proteins by *A. caninum*, and characterizing the soluble form of isolated monoclonal antibodies against this particular ASP in terms of specificity and binding strength.

1.2 Rationale of the study

The parasitic nematode *Ancylostoma caninum* poses a significant threat to both animal and human health (Conlan et al., 2011). During the transition from the infective L3 larvae stage to the parasitic adult hookworm, certain proteins are released as the excretory/secretory products and ASPs are one of them (Abuzeid et al., 2020). Studies revealed that ASP exhibit properties related to angiogenesis and share amino acid homology with allergens in vespid venom (Mulvenna et al., 2009; Zhang et al., 2022). This suggested that ASP could be playing crucial role in pathogenesis of *A. caninum*, contributing to the parasite's survival and successful invasion of the host. To date, a total of six ASPs have been identified with ASP5 being one of them, which localized at the intestinal brush border membrane (Zhan et al., 2003). Therefore, targeting ASP5 could facilitate the development of monoclonal antibodies specific to *A. caninum* infection. However, the absence of specific antibodies against such ASP protein creates a substantial barrier to in-depth investigation of the particular infection. Previous efforts relied on polyclonal antibodies such as egg yolk polyclonal IgY and IgE/IgG antibodies reveal excellent specificity and sensitivity (Adam et al., 2023; Souza et al., 2020). However, despite high diagnostic value, they exhibit cross-reactivity with antigens from closely related diseases such as the hookworm infection caused by various Ancylostoma species including *Ancylostoma duodenale* and *Ancylostoma ceylanicum*, which mainly affect humans, alongside with the canine hookworm infection caused by *Ancylostoma caninum*. This issue might complicate the accurate distinction between human and canine hookworm infections, leading to false diagnoses of the target infections. Thus, the development of monoclonal antibodies could offer a more precise and targeted intervention. Given the ability of monoclonal antibodies to recognize and bind unique epitopes on the same or different antigen

surfaces, recombinant monoclonal antibodies offer unparalleled precision in targeting specific protein thereby preventing cross-reaction issues (Guliy et al., 2023).

There are major types of recombinant antibody fragments expressed in bacterial cell namely Fragment variable (Fv) and Fragment antigen-binding (Fab). This study utilizes smaller recombinant antibody format which is the single chain fragment variable (scFv) format mainly due to its practicality for expression in bacteria, providing a more feasible approach than full antibodies (Gupta & Shukla, 2017). This format, comprising of only the variable domains of heavy and light chains (VH and VL) with a short peptide linker, is the smallest units that retain binding properties to a given antigen (Asaadi et al., 2021).

Various technology platform, including hybridoma technology and recombinant methods such as phage display, yeast display and ribosomal display can be employed in the manufacturing of monoclonal antibodies, with each approach possesses distinct advantages and limitations (Parray, Shukla, et al., 2020; Pedrioli & Oxenius, 2021; Schwimmer et al., 2013). Phage display emerges as an ideal platform for challenging targets like ASP5 due to its robust nature and high stability of phage that allow the selection of high-affinity antibodies from vast libraries (Nagano & Tsutsumi, 2021). This platform offers precise control over the selection process, ensuring structural integrity and early definition of antibody properties. Additionally, it offers cost-effective and scalability through the *E. coli* expression system, in which the periplasmic space in bacteria facilitates VH and VL pairing, akin to lymphocyte endoplasmic reticulum conditions, yielding fully functional antibodies (Sandomenico et al., 2020).

In conclusion, this study aims to develop and characterize monoclonal antibodies against *A. caninum* ASP5 using phage display technology. This study may pioneer a path towards more targeted and effective strategies in combatting hookworm infections. Additionally, the study will address the critical gap of available monoclonal antibodies against ASP5 that has potential diagnostic value for future use.

1.3 Objectives of study

1.3.1 General objective

To develop and characterize monoclonal antibodies against *Ancylostoma caninum* Ancylostoma-secreted protein 5 with phage display technology.

1.3.2 Specific objectives

1. To express and purify *Ancylostoma caninum* Ancylostoma-secreted protein (ASP5) antigen.
2. To isolate single-chain fragment variable (scFv) clones from human naïve antibody library by phage display panning.
3. To express and characterize soluble scFv monoclonal antibodies.

CHAPTER 2

LITERATURE REVIEW

2.1 Canine hookworm and diagnostic methods of the infection

Canine hookworm infection is endemic in Southeast Asian countries, with prevalence rates spanning from 70% to 100% (Conlan et al., 2011; George et al., 2016; Ng-Nguyen et al., 2015; R. P. Singh et al., 2022; Thompson & Conlan, 2011). Despite being pervasive, the associated public health risks, particularly in terms of zoonotic transmission, are frequently overlooked and underestimated. In Malaysia, the overall prevalence is reported to be 48% among dogs residing in rural and urban settings, where stray dogs recorded the highest prevalence of 71% in rural areas followed by 48% in urban areas and lastly 28.7% exhibited by those in shelters (Mahdy et al., 2012). Notably, this remains the most recent data available on the subject. The observed higher prevalence among stray dogs can be primarily attributed to their unrestricted access to scavenging rubbish and their proclivity for defecating in public spaces. This behavioural pattern intensifies the risk of environmental contamination and human exposure which in consequence amplify the potential of zoonotic transmission (Tun et al., 2015).

The causative agents of canine hookworm infection comprise of *Ancylostoma caninum*, *Ancylostoma braziliense*, *Ancylostoma ceylanicum* and *Uncinaria stenocephala* (Traub et al., 2021). Among these, the former three are ubiquitous in tropical regions whereas the distribution of the *U. stenocephala* is confined to cool and temperate climates (Massetti et al., 2022). These hookworm species are of paramount veterinary importance due to their ability to cause anaemia and hypoproteinaemia in dogs (Campos et al., 2017; C. S. Palmer et al., 2007). The most significant difference between these species can be distinguished from the morbidity standpoint in which *A.*

caninum causes far greater blood loss in the host per worm (0.08-0.2 ml/day) than *A. ceylanicum* (0.033 ml/day), A. *braziliense* (0.002 ml/day) and *U. stenocephala* (0.0003 ml/day) (Clements & Addis Alene, 2022; Seguel & Gottdenker, 2017). This distinction in morbidity underscores the varying pathogenic potentials of these hookworm species, with *A. caninum* posing a comparatively higher threat to the haematological well-being of infected dogs.

It has been documented that *A. caninum* which predominates as the species responsible for hookworm disease in dogs has been identified to be present in humans, which justifies its zoonotic characteristics (Furtado et al., 2020; Loukas et al., 1992; Nezami et al., 2023). *A. caninum* is also a well-recognized etiological agent in eosinophilic enteritis and has been implicated in cutaneous larva migrans (CLM) that contributes to the less frequently encountered follicular dermatitis (Bowman et al., 2010). Hookworm related cutaneous larva migrans, often referred to as creeping eruption, is a self-limited, parasitic skin diseases that can be characterized by erythematous and highly pruritic serpiginous track on skin (Caumes & Danis, 2004; Feldmeier, 2023; Hochedez & Caumes, 2007; Shrestha et al., 2023). The linear tracks are mainly caused by the penetration and migration of hookworm infective larva (Rodriguez-Morales et al., 2021).

Diagnostic measures within the definitive host and environment detection are key to prevent dissemination and attaining control over this infection (Zibaei et al., 2020). Owing to the zoonotic potential inherent in *A. caninum* which capable of not only cause skin damage, eosinophilic enteritis, or patent infection in humans, it is imperative to establish diagnostic tools to monitor infection in dogs as the primary reservoir hosts for human infection particularly due to the role of free-roaming domestic

animals serving as the primary source of contamination by hookworm and other nematodes (Inpankaew et al., 2014; Shepherd et al., 2018).

The identification of hookworm infection instigates the development of reliable diagnostic assays tailored to detect canine hookworm infection. In general, there are several methods employed to diagnose hookworm infection, depending on the diagnosis purpose and the characteristics of the species. These methods involve coprological methods, molecular methods, immunological methods, and clinical observation on the symptoms exhibited by the infected (Miswan et al., 2022). The coprological method involves the use of faecal samples of the host to detect parasites by observation through microscope, therefore it is less sensitive to detect low-level infection (Cringoli et al., 2011; Inês et al., 2011; Nikolay et al., 2014). Meanwhile, molecular methods such as Polymerase Chain Reaction (PCR) involve amplifying and detecting DNA of a specific hookworm. This method allows species-specific identification and has higher sensitivity even for light infections (Kotze et al., 2020; Massetti et al., 2020; O'Connell & Nutman, 2016). Additionally, there is immunological method that involves the utilization of assay like Enzyme-linked Immunosorbent Assay (ELISA), Lateral Flow Assay, Western Blotting which require the development of antibodies to detect the specific hookworm antigen (Adam et al., 2023). This method offers rapid results coupled with high sensitivity, thus making it suitable for mass screening. ELISA is often applied for cases with limitations such as low parasitic burden, intermittent shedding of eggs to overcome sensitivity problems such as the traditional method of faecal examination (Elsemore et al., 2017; Papaiakovou et al., 2019). To date, polyclonal antibodies have been utilized to detect hookworm infection (Avila et al., 2021; Souza et al., 2020). The study by Souza et al. (2020) applied egg yolk polyclonal immunoglobulin (IgY) antibodies to detect immune complexes in the serum from

hookworm infected patients using sandwich ELISA. The result demonstrates high diagnostic value with a sensitivity of 90%. ELISA is one reliable method for diagnostic purposes, and it can be used in conjunction with histological examination and clinical features. However, the use of polyclonal antibodies for diagnosis may exhibit cross-reaction among cases with closely related infections (Ou et al., 2020; Rodriguez-Quijada et al., 2020). Clinical symptoms of the hookworm infection can be misleading due to overlap with other infections and nutritional deficiencies such as the common symptom of iron-deficiency anaemia, along with loss of appetite and weight, and abdominal discomfort (Raza et al., 2018). Some dogs can have hookworm infestations without exhibiting obvious sign. In cases of zoonotic *A. caninum* infection for which humans are incompatible hosts, the larvae can only penetrate and live at the skin, which cause creeping eruption (Shrestha et al., 2023). This condition is presented as erythema, itchy papules and linear serpiginous ridges on the skin, usually on the extremities. Additionally, it can induce eosinophilic enteritis, marked by abdominal pain and peripheral blood eosinophilia (Furtado et al., 2020).

2.2 *Ancylostoma caninum* and its secreted protein

The *Ancylostoma caninum* hookworm, a parasitic nematode, engages in a complex life cycle with a profound impact on both animal and human hosts. In general, the life cycle of nematodes begins with the hatching of hookworm larvae from eggs present in the host's feces (Datu et al., 2008). These larvae develop into an infective stage in soil and enter the definitive host through skin penetration. Alternatively, infections occur from larvae ingestion (Sommer & Streit, 2011).

Dogs and occasionally cats serve as the normal definitive hosts for *A. caninum*, with a life cycle akin to that in humans. The life cycle initiates with the excretion of eggs in the host's stool, and under favorable conditions, larvae hatch in 1-2 days. The larvae grow in feces or soil and become infective filariform larvae within the span of 5-10 days (Moser et al., 2005). The larvae can survive for 3-4 weeks in conducive environmental conditions. At this period, the larvae are very infective and ready for infection upon contact with a suitable host through skin penetration. Then, they travel through the blood vessels, to reach the heart and lungs, before progressing to the bronchial tree and ultimately the pharynx, where they are ingested. Within the small intestine, larvae mature into adults and attach to the intestinal wall to feed on the blood of the host. Some larvae are arrested in the tissue to serve as a source of infection for pups through transmammary and potential transplacental pathways (Stone & Smith, 1973).

A. caninum hookworm species is known to release various protein that represent range of activities such as lectins (MCGREAL et al., 2004), lysozymes, proteases (Hawdon et al., 1995), hyaluronidase (Hotez et al., 1992), but the most highly represented protein family is the activation-associated protein ASP with the vital role

in parasite-host interactions in relation to its pathogenicity function (Hawdon et al., 1996).

ASP is a group of cysteine-rich proteins that belong to the sperm-coating protein (SCP)-like extracellular proteins or SCP/TAPS family, which can be characterized by the presence of a single or double SCP-like extracellular domain (Osman et al., 2012). A single domain represents ASP with a single signal peptide, an SCP domain and a cysteine-rich tail. Meanwhile, double domain consists of two SCP domains with two cysteine-rich tails that C-termina the domains. These SCP-domain ASPs are secreted during the transition of *A. caninum* from free-living to parasitic L3 and eventually the adult stage, in response to the host-specific signals during infection process (Bethony et al., 2005; Gibbs et al., 2008). To date, there have been at least six ASPs identified in adult *A. caninum*, namely Ac-ASP1, Ac-ASP2, Ac-ASP3, Ac-ASP4, Ac-ASP5, Ac-ASP6, which each located at distinct parts of the hookworm (Zhan et al., 2003). ASP has multifaceted functions in host-parasite interactions and plays a crucial role in the infection process. Primarily it is involved in immunomodulation which dampens the host's immune response to evade detection and elimination (Schmid-Hempel, 2009). This protein also contributes to tissue invasion and migration, aiding the hookworm to navigate through the host tissue to establish infection. Additionally, ASP facilitates successful feeding on the host's blood by modulating the physiological response (Brooker et al., 2004). Table 2.1 summarizes the information on ASP and differentiates each ASP secreted by the *A. caninum* (Mulvenna et al., 2009). Although the detailed function of ASPs remains limited, their crystallographic structure on radiograph suggests possible roles in proteolysis and host immunomodulation (Zhan et al., 2002).

Table 2.1 List of Ancylostoma-secreted protein by *A. caninum* hookworm and the details of the protein.

Ancylostoma-secreted protein (ASP)	Type of domain	Stage of life	Location in hookworm
ASP1	double	L3	-
ASP2	single	L3	Pharyngeal gland of activated L3
ASP3	single	Adult	Pharyngeal/Esophageal
ASP4	double	Adult	Cuticular surface
ASP5	double	Adult	Intestinal brush border membrane
ASP6	double	Adult	Cephalic and excretory gland

2.3 Phage display technology

Phage display technology was introduced decades ago when George P. Smith demonstrated that the bacteriophage virions were able to present short peptides on their surface in 1985 (Smith, 1985). The technology was further developed by McCafferty and colleagues in 1990 to produce recombinant antibodies (McCafferty et al., 1990). Since then, improvements have been made to the system in phage display to allow the display of various biomolecules including T-cell receptor, proteomes, secretomes, and protein scaffolds. This allows the employment of this method in numerous fields of biotechnology for various applications. Phage display is a molecular technique that involves genetically modifying phage DNA to facilitate the expression of peptides, antibody fragments or other protein on its surface (Sioud, 2019). This is achieved by incorporating exogenous DNA sequences into the phage genome that is responsible for encoding a phage coat protein. Then, the fusion of genes governing the fusion protein will be displayed on the surface of the phage (Domingo-Calap et al., 2016; Ledsgaard et al., 2018).

Phage display technology relies predominantly on bacteriophage, a virus that can infect Gram-negative bacteria, such as *Escherichia coli* which bear the F plasmid (Elois et al., 2023). Among the phages utilized, filamentous phage is extensively employed, which resembles the shape of an elongated rod, featuring capsid that enclosed the genetic material (Rakonjac et al., 2017). Filamentous phage includes phages *M13*, *f1* and *fd* but *M13* in particular, is the favored due to its ability to adapt long fragments of foreign DNA into its genome (Rakonjac et al., 2011; van Wezenbeek et al., 1980). The Ff group constitutes circular, single stranded DNA with size of approximately 6.4 kilo base pairs and is composed of 11 genes that encode for different functional group of proteins (listed in Table 2.2) (Rakonjac et al., 2023). Remarkably,

the insertion of bigger size of exogenous DNA does not compromise the functionality of the phage (Marvin et al., 2014). The structural integrity of the phage is chiefly governed by capsid proteins (pIII, pVI, pVII, pIX, and pVIII), whereas DNA replication is orchestrated by proteins denoted as pII, pV, and pX. Additionally, the assembly of new phage particles involves proteins designated as pI, pIV, and pXI. The phage capsid is composed of the major capsid proteins pVIII which polymerizes 2700 copies, along with four minor coat proteins - pIII, pVI, pVII, pIX, each present in 3-5 copies, with two proteins situated at each end of the elongated virion (Mai-Prochnow et al., 2015). Figure 2.1 shows the filamentous phage structure which is mainly composed of the major capsid protein and four minor coat proteins.

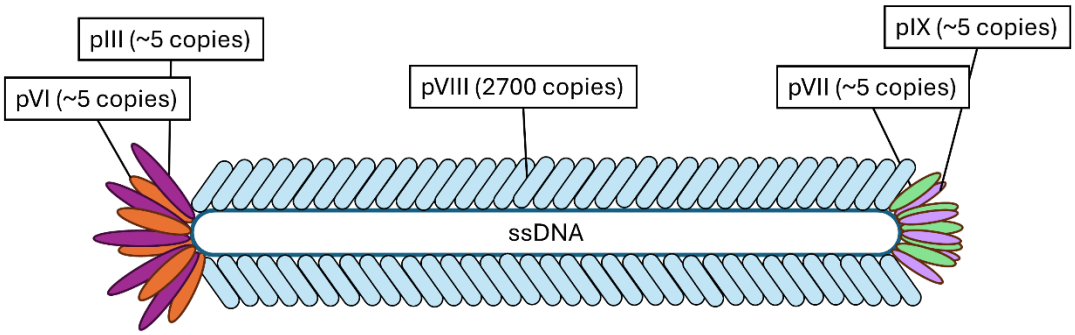

Figure 2.1 The schematic drawing of a filamentous phage structure.

The phage is made up of five structural protein that coat a single-stranded circular DNA of approximately 6.4 kilo base pairs.

Table 2.2 The list of protein encoded by 11 genes in Ff phage.

Protein Name (Abbreviation)	Size (kDa)	Function
Gene 1 protein (G1P)	39.6	Assembly
Gene 11 protein (G11P)	12.4	Assembly
Virion export protein (G4P)	45.9	Assembly and extrusion
Replication-associated protein (G2P)	46.2	Replication
DNA-binding protein (G5P)	9.7	Replication
Gene 10 protein (G10P)	12.7	Replication
Attachment protein (G3P)	44.7	Coat protein, adsorption, and extrusion
Head virion protein (G6P)	12.4	Coat protein, infection and budding
Tail virion protein (G7P)	3.6	Coat protein, assembly and budding
Tail virion protein (G9P)	3.7	Coat protein, assembly and budding
Capsid protein (G8P)	7.6	Coat protein

In the phage display system, there are two broad categories of vectors used: phage and phagemid. Phage vectors were initially utilized generally because of their inherent simplicity as they carry all requisite genes for infection, replication, assembly, and budding as well as the gene for the protein fusion (Henry et al., 2015). Despite that, the drawbacks such as the loss of pIII infectivity upon foreign protein fusion and the high risk of less affine binders' selection has prompted phagemid as the alternative vector. Phagemid is a hybrid of phage and plasmid vector designed to include the key elements: antibiotic marker, a gene that encodes G3P-antibody fusion protein, origin of replication for both M13 phage and *E. coli* plasmid (Qi et al., 2012). However, a phagemid vector is lacking in structural and non-structural genes that are required for folding a complete and functional phage particle, thus a helper phage which contains

the complete M13 genome is imperative to produce phage particles (Rondot et al., 2001; Soltes et al., 2003).

Although phage vector exhibits polyvalent display which is beneficial in producing greater diversity of binders, phagemid vectors are more typically used in library construction because of higher transformation efficiency that can facilitate the construction of large libraries (O'connell et al., 2002). The major difference between phagemid and phage vector lies in the valency in which phagemid confer significant advantage by its monovalent display property, resulting in the presentation of only one copy of each protein per phage particle which can prevent avidity effects that caused the expression of many copies of the displayed protein and therefore enhancing the selection stringency (Chasteen et al., 2006). In brief, monovalent display is chosen for the selection of strong binders from complex libraries whereas polyvalent display is more suitable for low affinity or rare clones.

The life cycle of the Ff-phage (notably M13 bacteriophage) is best described in three main stages involving infection, replication, and assembly. As M13 is lysogenic in nature, it invades and co-exists within the hosts without harming the host cell (Rakonjac et al., 2017). The infection stage is initiated by a highly specific interaction between pIII protein of the Ff phage and the top of the F-pilus, which is a protein tube made up of pilin subunits. When the pilin subunits depolymerized, they cause the retraction of the pilus and thus exert a force that draws the Ff phage towards the bacteria cell. Upon successful penetration of the phage DNA into the cytoplasm of bacteria cell, the replication enzyme undertakes a transformative process which converts the phage DNA into a replicative plasmid-like molecule known as Replicative Form (RF) molecule. This molecule will serve as a template for transcription and translation of phage proteins. Therefore, as the number of RF-molecules increases, the production of

phage protein increases until a certain concentration pV protein is achieved, the newly synthesized single-stranded DNA forms a complex with DNA-pV for bacteriophage assembly. Priorly, the virion proteins are aligned along the inner membrane of the cell. The assembly process commences as the packaging signal interacts with two minor coat proteins, pVII and pIX, along with the inner membrane assembly complex via pI (Loh et al., 2019). Towards the end of assembly process, two additional minor coat proteins, pIII and pVI form a terminating cap to release the fully formed bacteriophage from the host cell.

2.4 Phage display libraries design

The first and foremost step in phage display experiment is the construction of libraries to isolate diverse antibodies. This process involves strategic cloning and fusion of antibody genes to generate diverse and functional antibody fragments, allowing the display on the surface of phage for selection (Almagro et al., 2019). For instance, Fab fragments can be generated by cloning of variable and constant domain genes of both light (VL, CL) and heavy chains (VH, CH1). Normally, one of these chains is fused to the phage coat protein, while the other is secreted into the periplasm. Within this oxidizing environment of periplasm, the assembly and folding of the Fab fragment are facilitated on the phage surface (Nelson & Valadon, 2017). A similar cloning strategy can lead to the production of single chain variable fragments by exclusively cloning the VL and VH genes connected with flexible linker peptide gene, and subsequently fusing them to the phage coat protein (Norbury et al., 2019). In brief, the genes encoding the V domains of the antibody undergo reverse transcription and PCR amplification from lymphocytes. They are then combined using various methods to produce unique antibody fragments (Reader et al., 2019).

The genetic information from mammalian V-genes that encode antibody variable domains provide the essential building blocks for phage antibody library construction which can be categorized based on the construction method – natural and artificial libraries (Kumar, Parray, et al., 2019). Within natural libraries, they are generated from natural human B cell repertoire and fall into two distinct classifications depending on the origin of the gene sequences.

Immune phage libraries are constructed mainly from mRNA of IgG genes while IgA and IgE are occasionally employed mucosal-, parasitic- and allergic-based libraries (Lai & Lim, 2020). Immune libraries are particularly enriched with antigen specific

antibodies as they are predisposed for recognition to certain targets. The rationale behind lies in the assumption of exposure to infections leads to the in vivo selection and affinity maturation of sequences with specificity for the antigen. Consequently, these libraries may be pre-biased towards a particular antigen and exhibit high affinities in the nanomolar range (Pan et al., 2021). The construction of immune libraries can involve various sources of B-cells such the infected patients, infection recovered individuals or from the peripheral blood mononuclear cells (PBMC), bone marrow, lymph nodes, spleen, or tonsil of vaccinated individuals (Bashir & Paeshuyse, 2020). A study by Garcia-Calvo et al. (2023) utilized immune library to develop novel high affinity antibodies against gluten. These antibodies were intended for use as the main component in an enzyme-linked immunosorbent assay (ELISA) test. To construct the immune library, mRNA was extracted from two celiac patients who exhibited strong immune response to gluten-related proteins. The resulting library comprising $1.1x10^7$ antibodies was then screened through phage display selection against gliadin. The advantages of immune library are evident in their ability to direct isolation of binders with high affinity and used directly as diagnostic or therapeutic applications (Hairul Bahara et al., 2013).

On the other hand, there are naïve libraries, which are also called as 'single pot' or universal libraries because they contain diverse antibodies without prior exposure to specific antigen, therefore they could potentially recognize a wide array of antigens (Kügler et al., 2018). These libraries are generated from the IgM mRNA sourced from non-immunized and healthy donors of B cells found in peripheral blood lymphocytes, spleen and bone marrow cells (Chan et al., 2017). Since the genes of naïve B cells have not undergone an affinity maturation process, the antibodies isolated from these libraries typically exhibit low affinity ranging from 4 nM to 220 pM. The affinity of the

selected antibodies is primarily influenced by the size of the library which normally has the size from 10^7 to 10^{10}. Nevertheless, the affinity of the antibody can be further enhanced for both stability and affinity through subsequent refinement processes such as in vitro affinity maturation (Lim, Choong, et al., 2019). Increasing the diversity of the library can be achieved by isolating in vivo rearranged V genes from a broad spectrum of healthy donors and by amalgamating B cells from various origins. While theoretically, the diversification of the six CDRs could yield an infinite array of combinations, the practical repertoire of a phage display library is usually confined to 10^{10} –10^{11} due to the transformation efficiency of *E. coli* (Hu et al., 2015; Tabasinezhad et al., 2019). Despite this limitation, the large repertoire size allows the generation of antibodies against an infinite number of targets, highlighting its versatility in various applications. A lot of phage display naïve antibody libraries are available such as XFab1 and XscFv2 from XOMA (Schwimmer et al., 2013), HAL9/10 scFv library (Kügler et al., 2015), KNU-Fab library (Kim et al., 2017), a multi-ethnic naïve scFv library (Lim et al., 2016). As for artificial libraries, it encompasses synthetic and semi-synthetic libraries. They can be differentiated based on the composition of synthetic and natural sequences or fully synthetic sequences within the libraries (Huang et al., 2023). The construction of artificial libraries is conducted by chemical synthesis and in vitro assembly of genes, guided by bioinformatic analyses for the fundamental design (Fischman & Ofran, 2018). Normally, synthetic antibody libraries are engineered with scaffolds tailored for efficient expression in *E. coli*, ensuring proper folding for optimal solubility and antigen binding (Shim, 2015). The design principles of synthetic libraries are drawn upon the understanding of the key sequences within the complementarity-determining regions (CDRs) that shape the antigen binding site. The antibody specificity in recognizing antigens relies significantly on the CDR regions in heavy and

light chain genes and CDRH3 plays the pivotal role in antigen binding site formation and antigen recognition due to its diverse composition (10^{23} sequences) and variable length (6-24 amino acids) (Harel Inbar & Benhar, 2012; Sidhu et al., 2004).

A fully synthetic library involves insertion or grafting of randomized CDR region into fully synthetic framework sequences, whereas semi-synthetic library employs a more controlled approach, typically conducting randomization in CDR3 of the heavy chain (Tiller et al., 2017). CDR randomization can be accomplished through mutagenesis using degenerate codons, such as NNN, NNS, and NNK, where N denotes all four nucleotide bases, S represents guanine (G) and cytosine (C), K represents G or Thymine (T). However, the uncontrolled nature of this process may yield codons that encode unnatural amino acids or stop codons that can disrupt the folding and translation of antibody fragments (B.-K. Ko et al., 2015; Yang et al., 2009). Therefore, another randomization approach can be conducted alternatively by employing trinucleotide mutagenesis (TRIM) technology which utilizes trimers that encode 20 amino acids instead of random nucleotide combinations. This technique not only allows precise control over antibody library diversification but also minimizes the risk of stop codons and unfavorable amino acids distribution (Knappik et al., 2000; Yan et al., 2014). Meanwhile, semi-synthetic which focuses on CDRH3 can be constructed either from non-rearranged V genes of pre-B cells or by employing a single antibody framework, meaning CDRH3 undergoes random mutation using oligonucleotide-directed mutagenesis of PCR-based methods (Valadon et al., 2019; Zhang, 2023). Alternatively, all six CDR may be mutated and cloned into a defined antibody framework.

Synthetic and semi-synthetic antibody libraries offer the advantage of antibodies selection against self-antigens which cannot be done using natural libraries as the natural human immune system lacks naïve B cells that target such antigens (Pan et al.,

2021; Weber et al., 2014). This is mainly because these libraries are artificially constructed and thereby have not undergone depletion processes. Therefore, increased diversity facilitates the isolation of antibodies with specificities that may not have been naturally selection for in human immune repertoire. Consequently, the library provides valuable resources for generating antibodies against challenging target with high affinity (Chan et al., 2011; Parray et al., 2020).