

**Development of a Dusky kob scFv gene phage display library for
the discovery of antibodies to Brome mosaic virus - a proxy for a
novel, emerging fish pathogen**

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**Thesis Presented for the Degree of
DOCTOR OF PHILOSOPHY**



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DECLARATION

I hereby declare that this thesis, submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular and Cell Biology at the University of Cape Town, is the result of my own investigations, apart from the referenced work of others.

Signed by candidate

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Fish farming is rapidly becoming the world's fastest growing production sector, achieving an annual growth rate of approximately 8.9% since the early 1970s. However, high stocking densities result in elevated stress levels in farmed fish, leading to increased susceptibility to infection by opportunistic pathogens and parasites. Antibody phage display is a method that allows foreign peptides or proteins to be expressed on the phage surface through translational fusion with phage coat proteins. Consequently, antibodies expressed by a diverse repertoire of genes coding for the single chain variable fragment (scFv) of immunoglobulin M can be isolated and screened for affinity to a specific infectious agent or parasite. In this study, a phage display library displaying scFvs derived from combination pairings of Dusky kob (*Argyrosomus japonicas*) variable heavy and light chain fragments, sourced from the splenic B cells of healthy Dusky kob, was constructed. The library was subjected to two rounds of biopanning against brome mosaic virus (BMV), a grass virus to which Dusky kob would have no prior exposure that served as a proxy for an emerging fish pathogen. Five clones were identified as having high affinity and specificity to BMV, as determined by phage enzyme-linked immunosorbent assay (ELISA) and phage western blot analysis, respectively. To validate the diagnostic and therapeutic potential of antibody fragments isolated from this phage display library, the gene encoding the antibody fragment of the clone displaying the highest affinity to BMV was selected and expressed using a yeast surface display system. ELISA analysis of serum sampled from Dusky kob exposed to BMV by injection demonstrated that the yeast displayed anti-BMV antibody could successfully detect BMV in the blood serum of BMV-infected Dusky kob with similar sensitivity to a commercially available counterpart. Similarly, this study demonstrated the neutralising effect of yeast displayed anti-BMV antibodies which were found to successfully reduce BMV infection in barley. Overall, these findings demonstrate the feasibility of a Dusky kob phage display library as a source of diagnostically and therapeutically important antibodies against emerging fish pathogens or parasites that threaten the fish farming industry of South Africa.

List of abbreviations

α	alpha
β	beta
μg	microgram (s)
μL	microliter (s)
μM	micromolar
$^{\circ}\text{C}$	degrees Celsius
%	percentage
®	trademark
A	
aa	amino acid
Ag85	antigen 85
AMPS	ammonium persulfate
ANOVA	analysis of variance
B	
BLAST	basic local alignment search tool
BMV	brome mosaic virus
bp	base pair (s)
BSA	bovine serum albumin
BSAPBS-T	bovine serum albumin supplemented with phosphate-buffered saline and Tween 20
BTV	blue tongue virus
C	
CAA	casamino acids
cDNA	complementary DNA
CDR	complementarity-determining region
CFU	colony-forming units
C _H	heavy chain constant domain
C _L	light chain constant domain
CP	coat protein
cPCR	colony polymerase chain reaction

CW	cell wall
D	
dAb	single domain antibody
DEFF	department of environment, forestry, and fisheries
dH ₂ O	distilled water
E	
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ER	Endoplasmic reticulum
et al.	et alia
EtBr	ethidium bromide
F	
FAB	antigen-binding fragment
FACS	fluorescence-activated cell sorting
FAO	Food and Agriculture Organisation
Fc	fragment crystallisable
FDA	food and drug administration
Fv	fragment variable region
FW	framework region
G	
GAL	galactose inducible
GRAS	generally regarded as safe
H	
h	hour (s)
HCl	hydrochloric acid
His	histidine
H ₂ O	water
HGPRT	hypoxanthin-guanin-phosphoribosyl-transferase

I

Ig	immunoglobulin
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IHNV	infectious haematopoietic necrosis virus
IgM	immunoglobulin M
IPTG	isopropyl- β -D-thiogalactoside
ISAA	infectious salmon anaemia virus
IgT	immunoglobulin T

K

kbp	kilo base pair (s)
kDa	kilo dalton

L

L	litre
LA	Luria agar
LB	Luria broth

M

mAb	monoclonal antibody
MCS	multiple cloning site
min	minute (s)
mL	millilitre
M	molar
mM	millimolar
μ M	micromolar
mRNA	messenger RNA
MSA	multiple sequence alignment
mtDNA	mitochondria DNA

N

ng	nanogram (s)
nm	nanometre (s)
nM	nanomolar

O

OD	optical density
OIE	The World Organisation for Animal Health
ORF	open-reading frame

P

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline supplemented with Tween 20
PCR	polymerase chain reaction
p.f.u	plaque forming unit (s)
pH	potential of hydrogen
P.I	post induction
pNPP	p-nitrophenyl phosphate
PTM	post-translational modifications

R

RPM	revolutions per minute
RSIV	red sea bream iridovirus
RT	room temperature

S

SAB	sample application buffer
scFv	single chain variable fragment
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SRBCs	sheep red blood cells
ssDNA	single stranded DNA

T

TEM	transmission electron microscope
TEMED	N,N,N',N'-Tetramethylethylenediamine
™	trademark
TMV	tobacco mosaic virus
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
TY	tryptone yeast

V

V	volt
V _H	heavy chain variable
V _L	light chain variable
VHSV	viral haemorrhagic septicaemia virus

W

WSSV	white spot syndrome virus
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Y

YNB	yeast nitrogen base
YPD	yeast extract peptone and dextrose

Chapter 1

Literature review

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1.1 Introduction

Over the past 50 years, the annual consumption of seafood per capita has more than doubled, from an average of 9.9 kg in 1960 to just over 22 kg as of 2017 (FAO, 2018; Guillen *et al.*, 2019). Despite the continued global increase in seafood consumption, growth in developing and low-income countries has been low, with an increase from 3.5 kg to 7.6 kg over the same time period (Subasinghe, 2017). In Africa, despite having an annual seafood consumption rate of less than 10 kg per capita, seafood products contribute to approximately 20% of the total animal protein intake by African people, highlighting its importance in food security in poorer nations (Chan *et al.*, 2019). It is expected that by 2030 the global population size will rise to approximately 9 billion, and that over half of that growth is likely to occur in Africa (Chan *et al.*, 2019). To meet the rising demand, fish imports have grown substantially over the last decade. Regardless, capture fisheries still dominate Africa's domestic seafood supply, which currently produce about 10% of global fish catches (FAO, 2018). However, with the worldwide decline and collapse of global fisheries, there has been a dire need and push for rapid growth and development in the farming of fish and shellfish to meet the growing demand for seafood products (Naylor *et al.*, 2000). As of 2014, aquaculture contributed more to the supply of fish for human consumption than that of capture fisheries, and to date, it is the fastest growing food production industry worldwide. Despite its clear socio-economic importance, only one-sixth of all fish products produced in Africa are from aquaculture, with the continent contributing only 2.3% to the global supply of aquaculture products (FAO, 2017; Chan *et al.*, 2019). This fish supply deficit in Africa presents significant prospects for the development of aquaculture facilities that would aid in important socio-economic growth, especially in rural communities, which would contribute to food security and improved livelihoods (Bondad-Reantaso *et al.*, 2005).

In South Africa, marine aquaculture is a fast-developing sector, which, in 2015, contributed approximately 3600 tons to the total production of seafood in South Africa, with the mollusc sub-sector contributing to 98% of total production and finfish contributing the remaining 2%. Despite being a well-established industry globally, the finfish sub-sector of the aquaculture industry in South Africa remains an emerging industry. Over the years, several marine finfish species have been evaluated to determine their farming feasibility and marketability. However, to date, the only commercial marine fish species currently farmed in South Africa is the Dusky kob (*Argyrosomus japonicas*), with only four operational farms in existence as of 2015.

1.1.1 *A. japonicas* aquaculture

Dusky kob is a widely distributed, sciaenid species endemic to the coastal waters of Australia, Mozambique, and South Africa. Due to its large size and mild flavoured white flesh, Dusky kob is an important food species among commercial and recreational fishermen. However, because of its popularity, extensive fishing pressure and poor stock management have resulted in a near collapse in Dusky kob fish stocks in South Africa, leading to the establishment of several commercial Dusky kob farms in the country. Due to its rapid growth and good food conversion ratio, with the additional benefit of its tolerance of a range of water temperatures and salinities, Dusky kob are ideal candidates for aquaculture. Yet, despite its economic importance, the production of farmed Dusky kob contributes as little as 2% of the total production of all farmed seafood in South Africa and is an under-utilised resource in the region. As with most animal husbandry sectors, disease management remains a major challenge to the commercial success of these farms. In 2017, the Department of Agriculture Forestry and Fisheries reported that only 3 out of 10,000 juvenile Dusky kob reach market size, attributing disease management as the single greatest threat to the finfish farming industry in South Africa (The Department of Agriculture Forestry and Fisheries (DAFF), 2017a, 2017b).

1.1.2 Disease in finfish aquaculture

Disease in aquatic animals is common and has been well documented over the years, with cases typically being either non-infectious or caused by common endemic pathogens that are well established in the farming sector (Walker and Mohan, 2009). However, due to rapid expansion and intensification of the aquaculture industry, there has been an increase in host stress, and together with increased global movement of aquatic animals, there has been an emergence and spread of new diseases (Kibenge, 2019). The emergence of novel disease-causing pathogens has resulted in a substantial decline in fish populations, leading to extensive economic losses for commercial fish farms (Walker and Winton, 2010). Disease outbreaks are regarded as a key constraint on the growth and development of the aquaculture industry, especially in Africa. In the salmonid aquaculture industry alone, annual losses due to sea lice and related infections are estimated to be in the excess of US\$100 million. Overall, it has been estimated that disease is responsible for up to 10% of on-site losses, with an annual loss of several billion US dollars to the aquaculture industry (Defoirdt, Sorgeloos and Bossier, 2011). Additionally, Walker and Winton (2010) cautioned that the impact of disease on the industry is often intensified by other issues typically encountered on fish farms, such as the limited availability of diagnostic tools, allowing for the rapid and unintentional spread of pathogens. Furthermore, limited knowledge

regarding epidemiological factors and host range are fundamental factors that aid in the uncontrollable spread of emerging pathogens. For instance, up until the late 1980s, it was believed that the geographical range of the viral haemorrhagic septicaemia virus (VHSV) was limited to the waters of western Europe and assumed to be endemic among native freshwater fish species such as freshwater rainbow trout (*Oncorhynchus mykiss*). However, the presence of VHSV was soon discovered in chinook salmon (*Oncorhynchus tshawytscha*) farms off the coast of the United States of America (Crane and Hyatt, 2011). A study by Escobar *et al.* (2018) reported that VHSV was found in over 140 different fish species in marine and freshwater ecosystems. Furthermore, the authors reported that fish species belonging to the order Perciformes, Salmoniformes, and Gadiformes were likely to be infected with VHSV in previously unidentified areas (Escobar, Escobar-Dodero and Phelps, 2018). To date, VHSV is considered one of the most pathogenic viral finfish diseases due to its wide geographic and host range, and it is reported that VHSV infections have a mortality rate as high as 90% (Kim *et al.*, 2016; Escobar, Escobar-Dodero and Phelps, 2018). It is believed that the true host range of this virus may be so large that it will never be fully known, which highlights the threat that emerging and re-emerging pathogens have on global food security and economic stability.

In South Africa, as with all aquaculture farms, the farming of Dusky kob is burdened by disease. Unfortunately, as Dusky kob farming remains an emerging industry, there is limited knowledge regarding disease-causing pathogens in this sector. In Australia, the main health concern in farming Dusky kob are infections with ectoparasitic protozoans and monogenean trematodes, which have been reported to cause mass mortalities in juvenile and adult Dusky kob (Zilberg *et al.*, 2012). Currently, only the gill fluke *Diplectanum oliveri* is well-documented and known to cause extensive mortality in South African fish farms. Although bacterial and viral infections are similarly problematic, limited knowledge and diagnostic capabilities means that disease-causing organisms often go undetected. As the Dusky kob farming sector continues to develop in South Africa, an increase in the number of disease-related issues and the occurrence of emerging pathogens is likely.

1.1.3 Disease management in finfish farms

As with all farming sectors, disease prevention and management are of primary concern to ensure sufficient productivity, and thus, a number of different strategies are typically implemented to ensure adequate disease prevention. Ideally, the implementation of improved animal husbandry practises through the moderation and control of certain stress factors, such as handling, water quality, and stocking densities, would aid in the prevention and control of

disease causing pathogens (Ina-Salwany *et al.*, 2019). However, the incorporation of such practises in the farming sector is often unrealistic and not economically viable. To stay profitable, farms must focus on sufficient prevention and treatment measures. In contrast to mammalian therapeutics, the use of pharmacological therapeutics is severely limited in fish, which is restricted to basic anaesthetic agents and anti-infective agents for the control of microbial and parasitic infections (Burka *et al.*, 1997). In the aquaculture industry, viral infections are more difficult to control than bacterial infections due to a lack of effective anti-viral strategies (Shefat, 2018). Additionally, the use of antibiotics is ineffective against viral and parasitic infections. For many years, the control and prevention of bacterial infections has been through the extensive use of antibiotics. However, this has contributed to the selection, persistence, and spread of antibiotic-resistant bacteria (Santos and Ramos, 2018). In the United Kingdom alone, it is estimated that antibiotic-resistant bacteria are responsible for up to 700,000 deaths annually (Pérez-Sánchez, Mora-Sánchez and Balcázar, 2018). Due to increasing public health concerns, the use of antibiotics is under strict control, with some countries, including Japan and certain European Union territories, prohibiting the import of food products associated with antibiotic use (Ina-Salwany *et al.*, 2019). To reduce the use of antibiotics in aquaculture and provide effective treatment against viral and parasitic infections, alternative methods of disease treatment and prevention have been developed (Assefa and Abunna, 2018).

Consequently, interest in the development of vaccines as an alternative approach to disease prevention in farmed fish is steadily increasing and vaccination has been used successfully in a number of aquaculture sectors, including the salmon and trout farming industries (Bravo and Midtlyng, 2007; Gudding and Van Muiswinkel, 2013). To date, there is a total of 24 commercial vaccines against bacterial and viral infections in fish available for use in aquaculture (Shefat, 2018; Adams, 2019). However, the number of effective vaccines available for protection against viral pathogens remains limited. Furthermore, parasitic infections represent a significant threat to the Atlantic salmon industry, and there is currently no commercial vaccine available for these kinds of infections (Adams, 2019). Additionally, vaccine development is reliant on a thorough understanding of the host's immune system as well as a sound understanding of the pathogen in question. This is challenging due to the vast number of pathogens that infect commercially important fish species. Due to the relatively recent emergence of the industry, particularly in developing countries, there is a clear deficit of knowledge in this area. In cases of emerging disease, where the causative agent is often un- or

mis-characterised, vaccines are ineffective. Therefore, other means of disease prevention and possible treatments are required.

1.2 Adaptive immune system

A fundamental mechanism shared by all vertebrates is the presence of a host immune system, which functions to defend the host from foreign materials or organisms. The vertebrate immune system is typically divided into two separate systems, namely the innate and the adaptive immune response. The innate immune system serves as the first line of inducible defence in response to an infecting pathogen, and includes mechanisms such as physical and chemical barriers that are constantly present, as well as inducible factors such as inflammatory mediator proteins and acute phase proteins, which are activated in response to an invading pathogen (Watts, Munday and Burke, 2001). Innate immune responses are generally very rapid and non-specific to a particular pathogen. However, if a pathogen persists despite the innate immune system, it is the role of the adaptive immune system to engage and eliminate the pathogen. The adaptive immune system is composed of specialised cells called lymphocytes, which are comprised of B and T cells. T cells are important components of cell-mediated immunity, while B cells are responsible for the production of antibodies, which are key features of the humoral immune system.

In mammals, the bone marrow and thymus constitute the central lymphoid organs, and the lymph nodes, spleen, and mucosal-associated lymphoid tissues make up the peripheral organs (Mutoloki, Jørgensen and Evensen, 2014). Both B and T cells are produced in the bone marrow of mammals, however, upon production T progenitor cells migrate to the thymus, undergoing positive selection and maturation to become T cells that express T cell receptors. B cells remain in the bone marrow, where upon maturation, they migrate into the lymphatic system and circulate throughout the body (Workenhe *et al.*, 2010). However, as bony fish lack both bone marrow and lymph nodes as primary and secondary lymphoid organs, respectively, the production and maturation of lymphocytic cells is the role of other lymphoid organs such as the head kidney and spleen (Mutoloki, Jørgensen and Evensen, 2014). It is believed that both B and T cells originate in the head kidney of bony fish and that B cells remain in the head kidney for maturation while T cells migrate and mature in the thymus, similar to that of mammals (Soulliere and Dixon, 2017). Although the production and maturation of lymphocytes in fish species differs to that of mammals, it has been reported that the major

mechanisms involved in the fish immune response to pathogenic infections is similar (Workenhe *et al.*, 2010).

1.2.1 Antibodies

Antibodies are a group of proteins called immunoglobulins (Ig) and are a common feature shared by all jawed vertebrates. Antibodies are one of the prime effector molecules produced by the plasma cells of the vertebrate immune system in response to the presence of foreign organisms (Milstein, 1980). Antibodies can recognise the conformations and unique sites on the surface of a foreign antigen, allowing them to effectively bind to and neutralise the target antigen. Antibodies are multichain glycoproteins consisting of one or more basic units containing four polypeptides; two identical heavy (H) chains and two identical light (L) chains, which are joined together by disulphide bridges and noncovalent bonds to form a Y shaped molecule (Mestecky, 1972). Every heavy chain is encoded for by variable (V_H), joining (J_H), diversity (D), and constant (C_H) genetic segments, with each light chain consisting of V_L , J_L , and C_L genetic segments (Fig. 1.1) (Azzazy and Highsmith Jr., 2002). Each variable region consists of conserved alternating framework (FW) regions, and the more diverse hyper-variable or complementary-determining regions (CDRs). The heavy and light chains each include three hyper-variable regions, and together, contribute to the majority of the antigen interaction and binding properties (Lipman *et al.*, 2005). Through different combinations of the V_H and V_L domains, an immense number of different antibodies can be generated by the adaptive immune system, with each antibody having a unique antigen-binding site (paratope). Once an antibody has recognised a particular antigen epitope, the tail region of the antibody, known as the fragment crystallisable (Fc), can then be recognised by specific immune cells, such as phagocytic cells, and trigger an immune response.

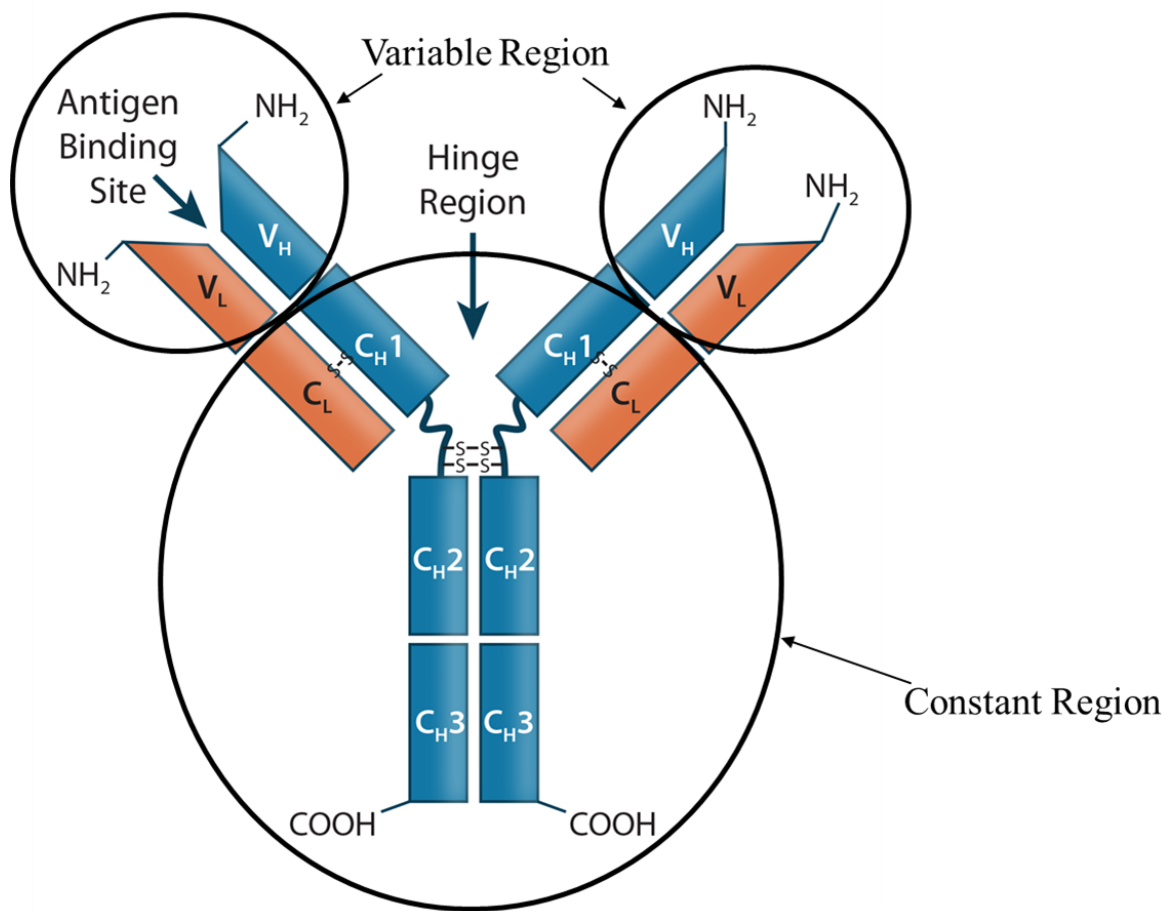


Figure 1.1 Basic structure of an antibody, which is comprised of two identical heavy chains (H) that consist of a heavy variable domain (V_H) and three constant domains (C_H), and two identical light chains (L), which include a light variable domain (V_L) and a single constant domain (C_L). Figure adapted from Bratt *et al.*, (2018).

Although the basic structure of all antibodies are similar and consist of a variable and constant region, antibodies may differ based on the structure of their C_H region, and therefore can be further categorised into different classes or isotypes based on differences within these structures (Spiegelberg, 1989). The change in antibody class is the result of a process called isotype switching, which occurs between tandemly repeated sequences, known as switch regions, located upstream of each of the C_H genes (Stavnezer, 1996). Isotype switching allows for different functional classes of antibodies to be produced, which retain the original specificity to the target antigen, and the particular isotype determines the effector function of the antibody (Spiegelberg, 1989; Stavnezer, 1996).

1.2.1.1 Antibody isotypes

In mammals, a total of five distinct antibody isotypes have been described based on their characteristic structures and effector functions, and are as follows: IgM, IgD, IgA, IgE, and IgG. IgM is the first isotype that is expressed on the surface of B cells, is the primary isotype secreted by B cells during initial pathogenic infection, and is responsible for the activation of the complement system (Brezski and Georgiou, 2016). The secreted form of IgM is mostly present as a pentamer. However, a monomeric isoform exists as surface antigen receptors of naïve B cells, similar to IgD. IgD is the second isotype that is expressed on the surface of naïve B cells, and its unique hinge region renders it responsive to polyvalent antigens but not monovalent antigens (Brezski and Georgiou, 2016). IgA exists in a dimeric form, and found throughout the mucosal surfaces of the nose, lungs, and intestines, and plays an essential role in mucosal immunity. Upon pathogen detection, before tissue infection, IgA binds to the pathogen and forms mass aggregates, isolating pathogenic organisms in secretions that are then expelled, preventing the pathogen from infecting the tissues (Spiegelberg, 1989). IgE is a monomeric antibody isotype that is present in minute amounts in the blood serum of mammals. The main function of IgE is in protection against parasites. However, in regions with low parasitic infectivity, it is believed that the primary role of IgE is in stimulating allergic reactions. It is thus believed that an overproduction of IgE can lead to severe allergic disorders (Spiegelberg, 1989). The most predominant antibody isotype found in mammals is IgG, which is secreted by plasma cells in response to a secondary immune response. IgG is primarily involved in eliciting antibody-dependent, cell-mediated cytotoxicity and antibody-dependent cellular phagocytosis, which is responsible for the opsonisation of pathogens for phagocytosis (Mashoof and Criscitiello, 2016).

In lower vertebrates, a smaller number of antibody isotypes exist. In teleost fish, only three different antibody isotypes have been identified, namely IgM, IgD, and IgT. Furthermore, it has been reported that teleost fish potentially contain two or more major subsets of B cells. In catfish, three subsets of B cells have been described, including $\text{IgM}^+/\text{IgD}^-$ (IgM^+), $\text{IgM}^+/\text{IgD}^+$, and $\text{IgM}^-/\text{IgD}^+$ (IgD^+) (Edholm *et al.*, 2010). However, in other species, such as rainbow trout, only two subsets have been described, including $\text{IgM}^+/\text{IgD}^+/\text{IgT}^-$ (IgM^+ B cells) and $\text{IgM}^-/\text{IgD}^-/\text{IgT}^+$ (IgT^+ B cells) (Zhang, Salinas and Oriol Sunyer, 2011). In contrast to mammals, class switching does not occur in teleost fish, and therefore it is suggested that an alternative mechanism for generating B cell isotype diversity exists (Mutoloki, Jørgensen and Evensen, 2014). Edholm *et al.* (2010) showed in catfish that the expression of $\text{IgM}^-/\text{IgD}^+$ B cells was the

result of alternative mRNA splicing, which gave rise to the IgM and IgD isotypes; however, this did not occur for IgT (Edholm *et al.*, 2010). IgT rearrangements have been shown to be independent of IgM and IgD. It has been demonstrated that IgM and IgT are never co-expressed by the same B cell, thus indicating the existence of two mutually exclusive lineages of B cells in fish (Zhang, Salinas and Oriol Sunyer, 2011; Castro *et al.*, 2013). IgM⁺ B cells are the predominant B cell population in the circulatory system of teleost fish, while IgT⁺ B cells comprise a small subpopulation, and are found predominately in the gut, skin, and gill mucosa (Mutoloki, Jørgensen and Evensen, 2014).

IgM was the first antibody isotype to be identified in fish and is the predominant antibody in teleost fish (Uribe *et al.*, 2011). Similar to mammals, IgM can be expressed either on the surface of B cells or secreted as an antibody, and is responsible for the activation of the complement system (Workenhe *et al.*, 2010). Both monomeric and tetrameric IgM structures have been identified in teleost fish and have been shown to have similar binding affinities. However, due to structural differences in the Fc portion of the tetrameric IgM, these molecules are more effective in activating the complement system than monomeric IgM (Uribe *et al.*, 2011). The second antibody isotype to be identified was IgD, which was first found in catfish. Similar to IgM, teleost fish contain both membrane bound and secreted variants of IgD but at much lower concentrations, which are localised in the spleen, gills, and kidneys (Mashoof and Criscitiello, 2016). Teleost IgD has been shown to be a potential homolog of mammalian IgD due to sequence similarities, the location of the gene immediately downstream of the IgM locus, and its co-expression with IgM in teleost fish B cell lines (Stenvik and Jørgensen, 2000; Edholm, Bengten and Wilson, 2011). The third antibody isotype, IgT, is predominantly found in the gut mucosa of teleost fish and is believed to be the homolog of mammalian IgA (Mutoloki, Jørgensen and Evensen, 2014). Although this isotype is still poorly understood, recent studies have shown that fish surviving parasitic infection exhibited increased numbers of IgT⁺ B cells, with IgM⁺ B cells remaining unchanged, indicating the importance of IgT as a dedicated mucosal antibody isotype and its importance in mucosal immunity (Zhang, Salinas and Oriol Sunyer, 2011; Buonocore *et al.*, 2017).

1.3 Therapeutic antibodies

Antibodies are thought to be ideal molecules for use as therapeutic agents due to their overall stability and ability to bind with high specificity and affinity to a wide variety of antigens (Chames *et al.*, 2009). Although the use of antibodies as therapeutics is often thought of as a recent development, the use of animal antisera for protection against diphtheria dates back to

the mid-1890s (Winau, Westphal and Winau, 2004). Traditionally, antibodies were produced by injecting a host animal with a target antigen, and the resulting antibodies subsequently isolated from blood serum of the host animal (Bayer, 2019). This process involves the activation of multiple B cells that secrete antibodies that recognise multiple epitopes of the target antigen, resulting in a collection of serum containing multiple antibody clones, termed polyclonal antibodies (Hanly, Artwohl and Bennett, 1995). While the creation of polyclonal antibodies is straight forward and cost effective, the recovered antibodies have low specificity and recognise a range of epitopes of the target antigen (Stills, 2012). Furthermore, as these molecules are produced in animals, their use as therapeutics in other hosts such as humans often results in the occurrence of immune reactions, such as acute anaphylaxis or serum sickness, due to the presence of foreign antibodies (Hansel *et al.*, 2010; Posner *et al.*, 2019).

In contrast to polyclonal antibodies, monoclonal antibodies (mAbs) are monospecific and homogeneous, and therefore ideal for the development of effective therapeutics and diagnostics (Yamada, 2011). The concept of mAbs dates to the mid-1930s, when McMaster and Hudack (1935) successfully isolated agglutinins from lymph nodes. Yet, it was not until the late 1960s that the origin of mAbs was confirmed (McMaster and Hudack, 1935; Bayer, 2019). The first laboratory-generated mAbs was described by Askonas *et al.* in 1970, who published a technique for isolating a single plasma cell clone that generated a homogenous antibody and was propagated by repeated passage of spleen cells into irradiated genetically identical mice (Askonas, Williamson and Wright, 1970; Kaunitz, 2017). However, the major breakthrough in mAb development came in 1975, when César Milstein and Georges Köhler described a method for fusing antibody-producing mouse plasma cells with a myeloma cell line (Köhler and Milstein, 1975; Kaunitz, 2017; Posner *et al.*, 2019). This fusion was termed “hybridoma” and enabled the generation of large quantities of mAbs with a single predefined specificity. This jump-started an interest in the research and development of therapeutic antibodies (Suzuki, Kato and Kato, 2015). By 1986, the first therapeutic mAb, muromonab-CD3, was approved by the United States Food and Drug Administration (FDA) for the treatment of kidney transplant rejection (Ecker, Jones and Levine, 2015). To date, approximately 570 therapeutic mAbs have been studied at various phases of clinical trials, with a total of 79 therapeutic mAbs with market approval by the FDA or the European Medicines Agency, of which 30 mAbs are used to treat various types of cancer (Kaplon and Reichert, 2019; Kaplon *et al.*, 2020; Lu *et al.*, 2020). Over the years, several methods have been developed for the generation and isolation of mAbs from

immune and non-immune sources using hybridomas, transgenic animals, and various recombinant and display methods.

1.3.1 Hybridoma technology

The hybridoma technique developed by César Milstein and Georges Köhler in 1975 was the first method used for the derivation of therapeutic mAbs. They demonstrated that by isolating and fusing murine B lymphocytes, which had been immunised with sheep red blood cells (SRBCs), with immortal myeloma cells, they could generate long-living anti-SRBC antibody secreting cells (Hanack, Messerschmidt and Listek, 2015). By plating the fused cells on SRBC-containing agar plates, the cells secreting anti-SRBC antibodies were observed to create large zones of clearance as a result of the secreted antibodies lysing the SRBCs in the agar (Rajewsky, 2019). Since its discovery, hybridoma technology has been regarded as the gold standard for the development of homogeneous mAbs due to reproducible, hybrid cell lines that can be stored indefinitely (Benjouad, 2009). Modern-day development of mAbs by hybridoma involves multiple steps. Initially, B lymphocytes are isolated from an immunised animal host and fused to myeloma cells, resulting in hybridoma cells. The myeloma cells used specially for hybridoma fusion are deficient in hypoxanthin-guanin-phosphoribosyl-transferase (HGPRT), which is a key enzyme in the biosynthesis of nucleotides. Therefore, by growing fused cells in a selective media such as hypoxanthine aminopterin thymidine, it is possible to select for hybridoma cells only, as myeloma cells that are HGPRT deficient cannot divide and unfused B lymphocytes can only survive for approximately seven days, allowing for the selection of hybrid cells after the seven day period (Benjouad, 2009; Hanack, Messerschmidt and Listek, 2015). After approximately fourteen days, the media is replaced and the resulting hybridoma clones are identified by microscopy, and then screened weekly for growth and specific antibody production until the most efficient antibody producing cells are identified and stabilised (Hanack, Messerschmidt and Listek, 2015).

Hybridoma technology is regarded as the most primitive, yet most successful technology for the development and isolation of mAbs, and remains the go-to method for mAb discovery for *in vivo* application (Parray *et al.*, 2020). Of the 79-market approved therapeutic mAbs, more than 90% are generated by traditional hybridoma technology. Initially, the use of hybridoma was limited to murine derived lymphocytes and antigens, but over the years it has become a well-established approach for the development of mAbs against a variety of antigens and from different animal species (Hanack, Messerschmidt and Listek, 2015; Parray *et al.*, 2020). Although the discovery of hybridomas paved the way for the rapid development of therapeutic

mAbs, the technology is not without its shortcomings. Most notable in a clinical setting was the development of a human anti-mouse antibody response following the administration of murine mAbs as therapeutics in human patients. Repeated administration lead to a reduced antibody half-life and severe anaphylactic shock (Chames *et al.*, 2009). The murine nature of the antibodies rendered them ineffective in interacting with crucial components of the human immune system, thus restricting their biological efficacy and therapeutic application (Chames *et al.*, 2009; Shim, 2017). Regardless of this limitation, hybridoma technology is appropriate for the isolation of murine antibodies (Unkauf *et al.*, 2016).

In an attempt to develop therapeutic mAbs more suitable for human application, antibodies were modified to create chimeric antibodies. The variable domains of murine antibodies, isolated by the hybridoma approach, were fused with human constant domains (Neuberger *et al.*, 1985; Chames *et al.*, 2009). This modification resulted in the creation of antibodies that were approximately 70% human and possessed a full human Fc portion, allowing for the chimeric antibodies to be considerably less immunogenic in human patients and to interact fully with the human immune system (Lu *et al.*, 2020). As antibody engineering technology advanced, it became possible to humanise antibodies further by replacing the hypervariable loops of full human antibodies with the hypervariable loops of murine hybridoma-derived antibodies (Jones *et al.*, 1986). This allowed for the creation of humanised antibodies that were between 85-90% human, which were significantly less immunogenic than previous chimeric antibodies (Suzuki, Kato and Kato, 2015). However, while these chimeric, humanised mAbs are less immunogenic and better tolerated than the original mAbs, they are still immunogenic and result in adverse effects such as anaphylaxis, serum sickness, and antibody neutralisation due to rapid clearance of the mAbs (Posner *et al.*, 2019).

1.3.2 Phage display technology

Hybridoma technology has been pivotal in the development of therapeutic mAbs against various human related pathogens. However, despite its advantages, hybridoma technology has numerous drawbacks with regard to antibody development, such as the inability to generate antibodies against toxins or non-immunogenic regions, high production costs, labour intensive cell screening processes, the requirement of animal hosts, and adverse immune responses due to murine origin (Hairul Bahara *et al.*, 2013; Omidfar and Daneshpour, 2015; Unkauf *et al.*, 2016; Rahbarnia *et al.*, 2017; Rami *et al.*, 2017a). Furthermore, the development of mAbs by hybridoma is limited to rodents, with very little success in humans and no application to other species such as bovines, sheep, chickens, and fish (Kumar *et al.*, 2019).

Phage display, an alternative technology for the isolation of mAbs, was first described by George P. Smith in 1985 as an effective molecular technique in which the DNA of non-lytic filamentous phages can be genetically modified to express foreign proteins on the phage surface (Sioud, 2019). Since its discovery, the use of phage display as a means of generating diagnostic and therapeutic antibodies has become increasingly popular as it enables *in vitro* production of target specific antibodies. Furthermore, phage display-derived antibodies are independent of any immune system, thus circumventing any limitations of antibody production systems that rely on the immune system (Unkauf *et al.*, 2016; Shim, 2017). To date, over 80 antibodies isolated from phage display libraries have gone through clinical trials (Kumar *et al.*, 2019).

1.3.2.1 Filamentous phage

An integral component of phage display technology is the biology of the bacteriophage used to display proteins. Although various bacteriophages, such as T4 and lambda, have been successfully used for phage display, the majority of phage display systems primarily utilise the filamentous bacteriophage M13 (Ledsgaard *et al.*, 2018). The M13 bacteriophage belongs to a group of filamentous phages commonly referred to as Ff phages (Rakonjac *et al.*, 2011). The genome of Ff phages is comprised of a 6407 bp single-stranded DNA (ssDNA) that includes eleven genes, five of which code for coat proteins (g3p, g6p, g7p, g8p, and g9p) and the remaining six are involved in replication and phage assembly (Azzazy and Highsmith Jr., 2002; Carmen, 2006; Ledsgaard *et al.*, 2018). Of the five expressed coat proteins, the g8p major capsid protein is the most abundant, with approximately 2700 copies, and is responsible for the encapsulation of the phage DNA (Fig. 1.2). Five copies of coat proteins g7p and g9p are located at the distal end of the M13 capsid, while five copies of coat proteins g3p and g6p occur at the proximal end of the filamentous phage capsid (Fig. 1.2) (Carmen, 2006).

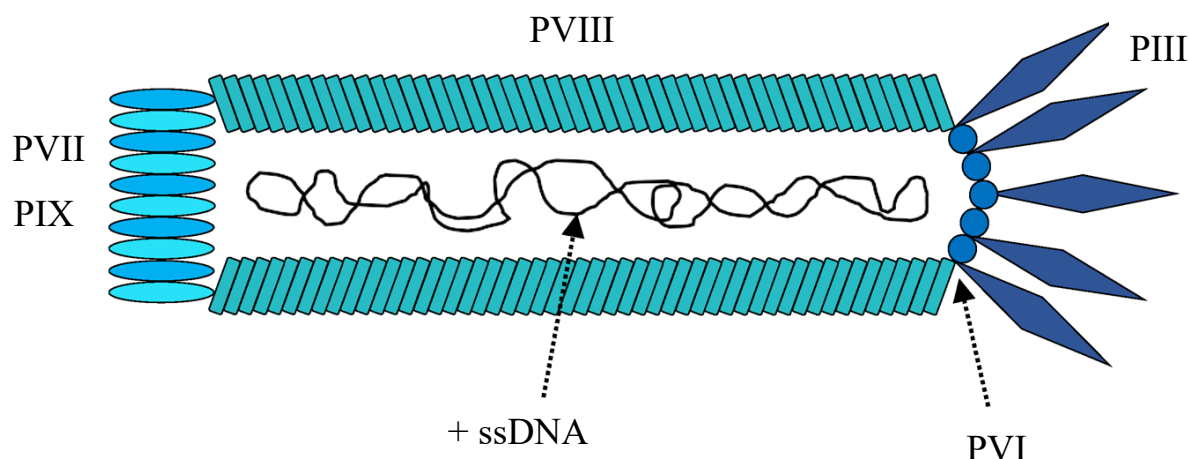


Figure 1.2 Schematic of an M13 filamentous bacteriophage with its single-stranded DNA (ssDNA) chromosome. The genome contains eleven genes, five of which encode the major and minor coat proteins (PIII, PVI, PVII, PVIII, and PIX). Figure adapted from Ledsgaard *et al.* (2018).

M13 filamentous phages are unique in that they are neither temperate nor lytic in nature; they do not lyse the infected host cell, in contrast to other phages such as T4, which kill their host (Paschke, 2006). Instead, filamentous phages establish a chronic infection within the host cell that allows for the continuous release of new phages while the host cell continues to grow and divide (Carmen, 2006; Ledsgaard *et al.*, 2018). M13 filamentous phage infection of *Escherichia coli* strains is initiated through attachment of the phage minor coat protein, pIII, to the F pilus of the bacterium (Nilsson, Malmberg and Borrebaeck, 2000). Since the F pilus is critical for the adsorption of the phage, filamentous phages can only infect *E. coli* strains that actively express the F pilus. The pIII is made up of two amino-terminal domains (N1 and N2) and a carboxyl-terminal domain (CT), which are essential for attachment to and infection of host cells (Smith, 1985). Phage attachment to the host cell is facilitated by the N2 domain binding to the *E. coli* F pilus, which brings the N1 domain and TolA into close proximity, facilitating their interaction and subsequent phage infection (Hoogenboom *et al.*, 1998; Carmen, 2006). Once a host cell has been infected and the ssDNA has been injected, the host DNA replication machinery will initiate the synthesis of a DNA strand complementary to the ssDNA strand of the phage (Rakonjac *et al.*, 2011). The two strands then form a double-stranded plasmid-like replication form (RF) that is necessary for the replication of the M13 chromosome, which undergoes rolling circle replication to synthesize more ssDNA. The RF also serves as the template for the production of the pIII and pVIII encoded coat proteins (Azzazy and Highsmith Jr., 2002). The synthesized ssDNA is packaged into the coat proteins, allowing for

the assembly of new phages, which are then extruded through the bacterial membrane into the surrounding medium. Once bacterial cells have been infected and the production of additional phages commences, the infected cells are no longer able to be infected by other phages (Carmen, 2006). While all five coat proteins have been exploited as fusion proteins for the display of various antibodies, PIII and PVIII are the most extensively used phage proteins for phage display (Shim, 2017). To date, display of PIII fusion proteins is more commonly used as it tolerates larger fusion proteins compared to PVIII, the function of which is typically negatively affected when displaying large proteins (Sioud, 2019).

1.3.2.2 Display of antibody fragments

Due to their large size and complexity, full-length antibodies cannot be sufficiently expressed and displayed in phage display systems. In 1990, McCafferty *et al.* demonstrated that phages carrying V genes encoding an anti-lysozyme antibody could be displayed on the surfaces of fd bacteriophages, and that the phages bound specifically to the target antigen with an affinity similar to that of the parental antibody (McCafferty *et al.*, 1990). Since then, various antibody formats have been introduced, such as the single-chain variable fragment (scFv), the antigen-binding fragment (Fab), and single domain antibodies (dAbs) (Hairul Bahara *et al.*, 2013). These antibody fragments retain the antigen-binding activity of the parental antibody, as the antigen-binding activity is determined by the CDRs of the variable domain of an antibody (Hentrich *et al.*, 2018). These antibody fragments can be obtained from natural B cells sourced from either an immunised or healthy patient or animal, or the fragments can be generated synthetically to allow for the construction of highly specific and highly diverse antibody libraries (Shim, 2017).

Initially, antibodies were displayed using phage vectors based on the genome of a wild-type filamentous, male-specific bacteriophage which conveyed tetracycline resistance (fd-tet) and its pIII as a fusion partner (Zacher *et al.*, 1980; McCafferty *et al.*, 1990; Hoogenboom *et al.*, 1998). These phage vectors were utilised as they incorporated all the genetic information necessary to complete the phage life cycle (Hoogenboom *et al.*, 1998). However, these systems were limited by the variety of restriction enzyme sites available for insertion of foreign DNA, genetic instability, and their capacity to tolerate large DNA fragments (Qi *et al.*, 2012).

Phagemid vectors are based on small plasmids which are an ideal alternative to phage vectors (Hoogenboom *et al.*, 1998). Unlike phage vectors, which incorporate all the genes required for phage assembly, replication, and synthesis of coat proteins, phagemid vectors only include

genes that encode for the phage coat protein, a selectable marker, and the origin of replication of the M13 phage genome (Ledsgaard *et al.*, 2018; Sioud, 2019). The antibody gene fragment is cloned upstream of the coat protein gene sequence, either pIII or pVIII, and its expression is controlled by the use of a promoter such as *lacZ* (Carmen, 2006; Zhao *et al.*, 2016). Due to their small size, phagemid vectors allow cloning of larger gene products, have a higher transformation efficiency, and allow the construction of libraries with a larger antibody repertoire compared to standard phage vectors (Qi *et al.*, 2012).

Phagemid vectors lack all other structural and non-structural genes, such as *PVI*, *PVII*, *PVIII*, *PIX*, and those required for replication and assembly, necessary for producing complete phages. Consequently, *E. coli* cells harbouring the phagemid vector are infected with a helper phage in a process called “phage rescue”, in order to produce fully functional phage particles that display the pIII fusion protein (Azzazy and Highsmith Jr., 2002; Ledsgaard *et al.*, 2018). Helper phages are M13 phages, such as M13K07, that contain a slightly defective origin of replication, and other important proteins necessary for capsid production, phage assembly, chromosome replication, and budding (Ledsgaard *et al.*, 2018). Upon infection of recombinant *E. coli* cells, the helper phage supplies all necessary proteins for producing fully functional phages, and the resulting phage particles incorporate either the pIII derived from the helper phage, or the antibody-pIII fusion protein derived from the phagemid vector (Azzazy and Highsmith Jr., 2002). This competition for the display of wild-type pIII or the antibody-pIII fusion protein usually results in phage populations where approximately only 10% of the phage displayed proteins are the fusion protein and the vast majority that display the fusion protein will only display a single copy (Hoogenboom *et al.*, 1998; Shim, 2017; Ledsgaard *et al.*, 2018). In order to improve the chances of finding a clone of interest and to improve the overall effective size of the phage library, Rondot *et al.* (2001) developed a modified helper phage, known as a hyper phage, that displays the g3p phenotype but contains a genome that lacks the *pIII* gene sequence (Rondot *et al.*, 2001; Breitling *et al.*, 2010). Therefore, upon infection of *E. coli* cells harbouring a phagemid vector, the only pIII that is produced during phage production is the antibody fusion protein provided from the phagemid vector (Carmen, 2006). The use of hyper phages allows for the multi-valent display of antibody fragments and an increased overall level of display (Shim, 2017).

1.3.2.3 Advantages of phage display

For the past three decades, phage display has seen significant growth and development, becoming a pivotal technology for a variety of applications including vaccine development

(Aghebati-Maleki *et al.*, 2016), epitope mapping of antigens (Bottger and Bottger, 2009; Wu *et al.*, 2016), and affinity maturation of antibodies (Hoogenboom, 2002; Thie *et al.*, 2009; Rahbarnia *et al.*, 2017). Additionally, phage display is slowly becoming the gold standard technology for the isolation of mAbs due to the robust nature of the phages and their high stability, allowing for unique advantages over other isolation technologies (Kumar *et al.*, 2019). Phages are unique in the fact that they are able to withstand extreme conditions, such as high temperatures, variations in pH, and exposure to ultraviolet radiation and proteolytic enzymes. This makes the isolation of various antibody classes that are more tolerant to harsh environmental conditions possible (Wörn and Plückthun, 2001; Kumar *et al.*, 2019). To date, more than 80 antibodies at various stages of clinical trials are derived from phage display, accounting for more than 30% of the total number of mAbs currently in clinical trials (Omidfar and Daneshpour, 2015; Kumar *et al.*, 2019).

As the use of phage display is independent of any immune system, phage display allows the identification and development of antibodies against all types of antigens, whether they are immunogenic or non-immunogenic (Azzazy and Highsmith Jr., 2002; Kuhn *et al.*, 2016). This is a fundamental advantage of phage display over traditional methods such as hybridoma, which is reliant on the host immune system for the development of antibodies and thus is limited to the production of antibodies targeting immunogenic antigens only (Rahbarnia *et al.*, 2017). Furthermore, phage display can identify antibodies against various toxins, such as botulism toxin from *Clostridium botulinum* (Chahboun *et al.*, 2011), as well as venom from different animal species, such as snakes, scorpions, and spiders (Khanongnoi *et al.*, 2018; Ledsgaard *et al.*, 2018; Riaño-Umbarila *et al.*, 2019).

Two of the key limitations of phage display are that the antibodies derived from phage display libraries, particularly naïve libraries, often have a lower affinity compared to antibodies from other technologies, and that full length antibodies are not able to be presented on phage particles (Azzazy and Highsmith Jr., 2002; Carmen and Jermutus, 2002; Silva de Almeida *et al.*, 2011; Frenzel *et al.*, 2017; Kumar *et al.*, 2019). Technologies, such as hybridoma, rely on the host's immune system for the production of antibodies, and therefore tend to have a high affinity as these antibodies have been naturally produced by B cells that have undergone an affinity maturation process due to somatic hypermutation of the CDR genes of the Ig (Hairul Bahara *et al.*, 2013). This affinity maturation is absent in phage display, resulting in antibodies with lower affinity; however, this limitation can be easily overcome. An immunised library can be developed by using B cells from donors who have had prior exposure to the target antigen. This

allows for the isolation of high affinity antibodies that have already undergone affinity maturation from the phage library for a particular antigen (Carmen and Jermutus, 2002; Ledsgaard *et al.*, 2018). As the gene encoding the selected antibody is easily accessible, another approach would be to increase the affinity of the recombinant antibodies using *in vitro* affinity maturation methods, such as site-directed mutagenesis, as demonstrated by Thie *et al.* (2009). Here, the authors describe a method whereby the affinity of a recombinant scFv antibody is improved by random mutagenesis using error-prone polymerase chain reaction (PCR) (Thie *et al.*, 2009). Additionally, as the genetic information encoding the displayed protein is directly available, the isolated antibody can be easily upscaled in bacterial expression systems or produced in other formats, such as IgG or as an scFv-Fc fusion protein, to improve the half-life of the recombinant protein and the therapeutic capabilities of the antibody (Frenzel *et al.*, 2017; Wang *et al.*, 2019). Such a method was demonstrated by Wang *et al.* (2019), who genetically fused an anti-*Staphylococcus aureus* scFv antibody to the Fc fragment of a bovine antibody and expressed the recombinant antibody in a bacterial system; this antibody had an 82% treatment success rate in *S. aureus*-induced bovine mastitis subjects (Wang *et al.*, 2019).

It is clear that phage display is a powerful, inexpensive, and user-friendly technology, suitable for the discovery and production of antibodies for numerous applications. Phage display is largely superior to traditional antibody production technologies such as hybridoma, allowing for the faster development of antibodies, which are suitable to any target and without requiring immunised animals. Since the early 1990s, antibody phage display has generated recombinant antibodies from various animals, including bovines, chickens, camels, macaques, sheep and sharks, thus allowing for the expansion of antibody research beyond human application.

1.3.2.4 Phage display in aquaculture

Despite the numerous advantages of this technology, phage display in the aquaculture industry is significantly underutilised and related studies are limited. The use of phage display has mainly focused on the identification of anti-viral peptides. This includes the identification of anti-viral peptides against infectious salmon anaemia virus (ISAA) isolated from a heptapeptide phage display library (Ojeda *et al.*, 2016), and a peptide against white spot syndrome virus (WSSV) which was isolated from a 10-mer phage display peptide library (Yi *et al.*, 2003). Phage display has also been successfully used to map and identify the epitope to which the anti-red sea bream iridovirus (RSIV) mAb M10 binds (Takano *et al.*, 2019).

In the development of mAbs against aquatic pathogens using phage display libraries, only three studies have been reported to date (Dai *et al.*, 2003; Yuan *et al.*, 2006; Liu *et al.*, 2014). These studies isolated scFv recombinant antibodies against WSSV (Yuan *et al.*, 2006) and infectious haematopoietic necrosis virus (IHNV) (Liu *et al.*, 2014), using a murine scFv phage display library developed by Dai *et al.* (2003). These studies demonstrated that the antibodies could recognise and neutralise the target antigen, preventing viral attachment and infection of host cells. These studies demonstrate the significant potential of phage display as a technology for the identification and development of novel therapeutic and diagnostic antibodies for disease management in aquaculture. These findings imply that it could be highly beneficial to develop an antibody phage display library for the isolation of diagnostic and therapeutic antibodies that would aid in disease management of farmed Dusky kob (*A. japonicas*) in South Africa.

1.4 Objectives of this study

Aquaculture in South Africa is still a relatively new enterprise and faces many challenges regarding the detection, treatment, and prevention of disease outbreaks. As with all aquaculture sectors, the occurrence and reoccurrence of pathogens and disease outbreaks in fish farms is a significant constraint to the industry, resulting in major financial losses (Burka *et al.*, 1997). Currently, *A. japonicas* is one of South Africa's more important, commercially farmed fish species. However, very little is known about the defence mechanisms of this species and the various pathogens that infect it (The Department of Agriculture Forestry and Fisheries (DAFF), 2017b). Thus, there are limited options available for the diagnosis and treatment of disease-causing pathogens. Currently, infections propagated by disease-causing pathogens are regulated by the use of either antibiotics or various chemical strategies. In the worst-case scenario, entire stocks of fish are sacrificed in order to prevent the spread of pathogens, resulting in significant economic loss and negative socio-economic impacts.

The use of antibodies for the detection and neutralisation of various pathogens has been successfully reported over the years for various human-related diseases (Kaplon and Reichert, 2019; Kaplon *et al.*, 2020; Lu *et al.*, 2020). Previously, the development of antibodies relied on hybridoma technology, which significantly limited the application of these antibodies to human-related diseases. However, with the advent of phage display, therapeutic and diagnostic antibodies can be developed outside the scope of human application. Recent developments in phage display technology have made it possible to construct antibody libraries for the isolation of antibodies against various pathogens, without requiring prior knowledge of the target antigen. Phage display is a powerful tool for the isolation of antibodies against emerging

pathogens threatening various food producing sectors, such as in the aquaculture sector, where disease management is of significant concern.

The present study will focus on the development of a naïve phage display library displaying scFvs derived from combination pairings of *A. japonicas* V_H and V_L chains, amplified from mRNA isolated from splenic B cells of healthy *A. japonicas*. The ability to utilise the resulting library as a resource from which antibodies against a pathogen to which *A. japonicas* has had no prior exposure can be isolated will be investigated. It was decided that the plant virus brome mosaic virus (BMV) would be a suitable proxy of an emerging pathogen of *A. japonicas*. The diagnostic and therapeutic potential of an anti-BMV antibody isolated from a phage display library of *A. japonicas* scFvs will be assessed through challenge trails incorporating *A. japonicas* and *Hordeum vulgare* (Barley plant). Ultimately, this study will develop a strategy to rapidly identify, isolate, and produce antibodies with diagnostic and therapeutic potential that can be used for the early detection and treatment of emerging pathogens of farmed *A. japonicas*. The results of this study will contribute to the overall improvement of disease management in *A. japonicas* and other fish species farmed in South Africa. The aims of the study are as follows:

- i. Construct a naïve phage display library displaying scFvs derived from the combination pairings of *A. japonicas* V_H and V_L chains, to identify and isolate recombinant scFv antibodies against the plant virus BMV, which serves as a proxy of an emergent pathogen.
- ii. Establish an effective means of expressing and producing the antibodies, identified in the phage display library, to effectively produce sufficient amounts of the recombinant protein for downstream applications.
- iii. Validate the diagnostic and therapeutic potential of the identified antibody which in turn will demonstrate the antibody phage display library constructed in this study as a source of diagnostic and therapeutic antibodies against emerging pathogens of farmed *A. japonicas*.

Chapter 2

The development of an *A. japonicus* naïve phage display antibody library

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2.1. Introduction

Phage display is a molecular technique, first described by George Smith in 1985, which involves the presentation or display of various peptides on the surface of lysogenic bacteriophages (Smith, 1985). Phage display differs from other expression systems in that displayed proteins and the DNA encoding them are directly linked, allowing for direct access to the gene encoding the displayed protein. The linkage between the phenotype of the displayed protein and the gene encoding that molecule permits screening and amplification of specific clones from a vast pool of potential target proteins (Azzazy and Highsmith Jr., 2002; Sioud, 2019). As phage display libraries contain billions of variants that can be constructed simultaneously, it is considered advantageous over traditional expression systems because specific proteins no longer have to be engineered and purified individually (Azzazy and Highsmith Jr., 2002).

In the early 1990s, McCafferty *et al.* used the technique to express the fused V_H and V_L chain variable domains of an anti-lysozyme antibody on the surface of a filamentous phage (McCafferty *et al.*, 1990; Almagro *et al.*, 2019). McCafferty *et al.* demonstrated that display of the anti-lysozyme antibody as a scFv fusion protein not only bound to lysozyme with high affinity, but bound the antigen with a similar affinity to that of the parent antibody (McCafferty *et al.*, 1990). This discovery encouraged the development of mAbs using phage display over traditional methods, such as animal immunisation and hybridoma, owing to the numerous advantages that phage display had over these methods. By utilising this technology, mAbs could now be developed without immunisation, thus reducing the risk of host immunogenicity (Almagro *et al.*, 2019; Sioud, 2019). Additionally, antibodies to a particular antigen can be generated within a couple of weeks (Carmen, 2006).

Over the years, this technology has been extensively used for the isolation and development of numerous antibodies, and today phage display is considered the gold standard for the isolation of recombinant mAbs (Kumar *et al.*, 2019). Expanding on the work by McCafferty *et al.* (1990), various antibody formats have been exploited and successfully displayed on the surface of filamentous phages. Of these, the display of scFvs is the favoured format due to its relatively small size and because the majority of the displayed antibody is comprised of the antigen binding sites. scFvs are composed of only a V_H and V_L region linked by a 15 amino acid glycine-serine linker sequence, making them a smaller (25-35 kDa) recombinant version of the Fv region. The inclusion of the linker allows the fragment to be expressed as a single

polypeptide chain which forms the antigen binding site. scFv antibody fragments have been successfully expressed on the surface of bacteriophages, with no apparent loss of the antibody's specificity and affinity, and thus are suitable for pathogen neutralisation.

A study by Ward *et al.* (1989) reported that a repertoire of V_H domains isolated from mouse spleens retained good binding affinity to lysozyme. This was the first study to demonstrate the effective use of an antibody fragment comprising a single antibody domain that retained its affinity to its target antigen (Ward *et al.*, 1989; Holt *et al.*, 2003). This fragment type, termed a single domain antibody (dAb), is twice as small as a scFv antibody. This makes it the smallest functionally effective antibody fragment with a molecular weight of 12-15 kDa – approximately one-tenth the size of a full-sized antibody (Ward *et al.*, 1989). A dAb is a variable domain of either the V_H or V_L domain of an antibody, with each domain containing three of the six CDRs of a full antibody. Typically, the CDRs of both chains contribute to antigen binding, however, significantly less binding has been observed with isolated V_L domains compared to isolated V_H domains. This may be due to the CDR2 and CDR3 of the V_H domain contributing to most of the antigen binding, as the CDR2 and CDR3 domains are responsible for the majority of the surface contact and molecular interaction with the target antigen (Reiter *et al.*, 1999). The discovery of dAbs has allowed the development of smaller antibodies with improved biophysical properties that are able to identify cryptic epitopes of antigens normally unavailable to larger antibody fragments (Holt *et al.*, 2003; de Marco, 2011; Hassanzadeh-Ghassabeh *et al.*, 2013; Barrera *et al.*, 2015; Liu *et al.*, 2018).

Modern phage display antibody libraries are generally categorised based on the source of the antibody sequence that they originate from, with each library differing in terms of coverage and diversity (Lim *et al.*, 2016; Omar and Lim, 2018). Phage display libraries can be characterised as either natural or synthetic, with natural libraries being further characterised as either immune or naïve libraries. Immune and naïve libraries are constructed exclusively from naturally occurring sequences isolated from IgG or IgM mRNA of naturally occurring B cells. Here, the diversity of these libraries is a result of somatic hypermutation of the donor's own cells (Azzazy and Highsmith Jr., 2002; Kumar *et al.*, 2019). Synthetic libraries are phage libraries that are generated from synthetic sequences. These synthetic sequences are devoid of any natural immune maturation and thus lack any natural diversity. Diversity in these libraries is generated by the synthetic sequences themselves (Frenzel, Schirrmann and Hust, 2016; Rami *et al.*, 2017b).

Immune antibody phage libraries are relatively unique compared to naïve and synthetic libraries, as they are derived from immunised donors. The V-genes in these libraries are derived from IgG mRNA of donors, usually animals, immunised with a target antigen of interest (Bazan, Całkosiński and Gamian, 2012). These libraries are enriched in antigen-specific antibodies to what the donor was immunised with. An important advantage of this library type is that generated antibodies have undergone affinity maturation by the donor's immune system, resulting in the isolation of antibodies from the phage display library that have very high affinity to the target antigen, thus making them prime candidates for therapeutic applications (Bazan, Całkosiński and Gamian, 2012; Sioud, 2019). Selection and isolation of antibodies with high affinity is possible even from libraries of relatively small size (10^7 clones) due to these libraries undergoing natural enrichment in response to the target antigen, thus diminishing the requirement for large antibody repertoires (Chester *et al.*, 1994; Beerli and Rader, 2010). The isolation of antibodies from immune libraries has been reported to yield antibodies with higher affinity than those obtained by traditional methods, such as hybridoma (Chester *et al.*, 1994; Hoogenboom *et al.*, 1998). Chester *et al.* (1994) demonstrated that an immune phage display library not only produced an antibody to a carcinoembryonic antigen with a higher affinity than traditional antibodies, but also demonstrated that these antibodies showed better specificity than commercially available antibodies (Chester *et al.*, 1994). However, the development of an immune phage displayed library can be a double-edged sword as immune libraries are targeted libraries that are generated from an antibody repertoire that have undergone antigen driven *in vivo* selection (Azzazy and Highsmith Jr., 2002). Thus, a new antibody library must be constructed for each antigen of interest, which significantly increases the time needed for antibody development, while the unpredictability of the immune response necessitates continuous animal immunisation (Frenzel *et al.*, 2017; Kumar *et al.*, 2019). Consequently, it is not feasible to develop and construct new immune libraries for each new emerging pathogen, especially during outbreak events.

Naïve libraries are non-immune phage display libraries, also known as single-pot libraries. Naïve libraries are different to immune libraries in that the antibody V-gene sequences in naïve libraries are sourced from IgM mRNA extracted from donors not specifically pre-immunised to the target antigen (Rami *et al.*, 2017b). As naïve libraries are derived from healthy non-immune donors, they do not contain antibodies that have undergone affinity maturation for a specific antigen, and thus produce antibodies that can bind potentially any antigen (Norbury *et al.*, 2019). A single naïve library may be used to isolate antibodies against a variety of different

antigen types, including self-antigens, non-immunogenic, and toxic antigens (Kumar *et al.*, 2019). However, the lack of affinity maturation results in antibodies that generally have lower affinities than those antibodies derived from immune libraries (Ledsgaard *et al.*, 2018).

The affinity of antibodies isolated from naïve libraries is dependent on the size of the library itself. Libraries with very large repertoires have been shown to produce antibodies with higher affinity than those isolated from much smaller libraries (Azzazy and Highsmith Jr., 2002; Bobkov, Woning and Haard, 2018). The diversity of these libraries may be increased through the generation and combination of different V_H and V_L antibody genes from a large pool of donors, as well as utilising B cells from different sources. However, numerous techniques can be employed for *in vitro* affinity maturation of antibodies isolated from naïve libraries that can further increase the stability of the antibody as well as improve its overall affinity. Two such techniques for improving the binding affinity of isolated antibodies are through mutagenesis, either random or targeted, or through shuffling of the V_H and V_L chains. Improvement of antibody affinity through mutagenesis involves the introduction of mutations, either through random mutagenesis of the whole variable region of the antibody, or at specific sites within the CDRs of the variable regions of the antibody (Ledsgaard *et al.*, 2018). Alternatively, antibody affinity may be improved through a process called chain shuffling, whereby either the V_H or V_L chain from the original library is replaced with antibody fragments isolated from different rounds of selection. In this way, a new antibody library is generated that keeps either the heavy or light chain constant, while replacing the opposite chain after subsequent selection rounds. The premise of this method is to identify the best match between heavy and light chains, resulting in an increased affinity to the target antigen (Ledsgaard *et al.*, 2018; Sioud, 2019). Although the antibodies generated by naïve phage display libraries have lower binding affinities than those derived from immune libraries, there are numerous advantages that naïve libraries have over immune libraries. As previously mentioned, due to the emergence and re-emergence of new pathogens, especially with regard to sudden outbreaks, it is not possible or feasible to develop a new immune library for each emerging pathogen. Due to the nature of naïve libraries, a single library can be generated and utilised against any number of emerging pathogens, regardless of origin. This not only speeds up the development of specific antibodies, which is essential in outbreak scenarios, but also avoids the use of experimental animals for immunisation, making this library format the more ethical and cost-effective option (Azzazy and Highsmith Jr., 2002; Unkauf *et al.*, 2016; Lee *et al.*, 2018; Kumar *et al.*, 2019).

Equally important as library construction is the process of the selection of antibodies from the antibody phage display library (Lim *et al.*, 2014). Antibody phage display libraries are screened for antigen specific clones using a technique known as biopanning (Azzazy and Highsmith Jr., 2002). Biopanning involves the incubation of phages displaying scFvs with an antigen of interest. While multiple biopanning strategies are available, the most conventional strategy available involves the selection of antibody clones against a target antigen that has been immobilised to the surface of a solid phase plastic, such as an immunotube (Griffiths and Duncan, 1998). Phage libraries are selected by incubating in an immunotube with the immobilised target of interest. Only phages with high affinity to the target antigen are bound, while the phages that fail to bind are removed through rigorous wash steps, with variations in the wash steps resulting in the enrichment of clones with varying affinity and avidity (Lim *et al.*, 2014; Mimmi *et al.*, 2019). Bound phages are eluted from the target antigen, through simple enzymatic digestion or a shift in the pH, and are subsequently amplified by infecting *E. coli* (Azzazy and Highsmith Jr., 2002). In theory, only a single round of selection is required in order to isolate target specific clones, however, the binding of non-target clones limits the overall enrichment that can be achieved. Therefore, multiple rounds of selection are required, which ensures the enrichment of target specific clones (Griffiths and Duncan, 1998; Azzazy and Highsmith Jr., 2002). These positively selected clones can be easily evaluated by an ELISA, as well as easily sequenced to obtain the genetic information of the clone, allowing easy modifications to the antibody gene if necessary (Lim *et al.*, 2014).

It is evident that phage display is a useful and powerful technology for the generation and isolation of novel antibodies for a wide range of applications. Although phage display has been applied successfully in humans, very few studies have demonstrated its application in the aquaculture industry, specifically with regard to farmed fish. Phage display offers a potentially powerful resource for the development of novel antibodies that may assist in the mitigation of stock losses resulting from the emergence of novel pathogens.

2.1.1. Chapter aims

Currently, the aquaculture industry is the fastest-growing food-producing sector globally, with an annual growth rate of approximately 9% (Bondad-Reantaso *et al.*, 2005). However, due to its rapid growth, the aquaculture industry has been burdened with the emergence and spread of infectious diseases, partly due to stressful farming practises and the global movement of aquatic animals (Kibenge *et al.*, 2012). Due to the frequency at which disease causing pathogens can occur and spread in an aquaculture farm, disease outbreaks are regarded as a key constraint to

the growth and development of the aquaculture industry, especially in Africa (Walker and Winton, 2010). It has been reported that the annual losses of aquaculture products due to disease is estimated to be in excess of several billion US dollars (Defoirdt, Sorgeloos and Bossier, 2011).

In contrast to terrestrial farming, the choice of available pharmaceutical substances for the treatment of fish pathogens is limited to anaesthetic and anti-infectivity agents for the control of parasitic and bacterial diseases (Burka *et al.*, 1997). The use of broad-spectrum antibiotics has been applied for many years for the treatment and prevention of bacterial infections. However, their consistent use has led to many bacteria becoming resistant, thus making antibiotics ineffective. Additionally, due to safety concerns, certain countries such as Japan and the European Union forbid the importation of food products that involve the use of antibiotics (Ina-Salwany *et al.*, 2019). As such, the use of antibiotics is becoming a forbidden and outdated treatment for bacterial infections, resulting in the need for alternative approaches.

Dusky kob (*A. japonicus*) is one of only four finfish species farmed in South Africa. Despite its economic importance, the production of farmed Dusky kob is limited to only four farms, which contribute approximately 2% of the total production of farmed seafood in South Africa. Due to the small scale at which Dusky kob are farmed in South Africa, very little is known about specific infections and diseases that afflict Dusky kob, with limited reports available discussing health related issues of this farmed fish (The Department of Agriculture Forestry and Fisheries (DAFF), 2017b). It has been reported that due to disease and other production problems, only 3 out of 10,000 juvenile Dusky kob reach market size (The Department of Agriculture Forestry and Fisheries (DAFF), 2017a). Therefore, the Food and Agriculture Organisation (FAO) and DAFF regards disease management as one of the single greatest threats to the finfish farming industry in South Africa (FAO, 2013, 2016).

Since the early 1990s, phage display has been shown to be a useful and vital technology for identification of diagnostic and therapeutically important antibodies to a variety of different human related pathogens. Despite this success, its application in other sectors such as aquaculture is limited. Therefore, phage display represents an ideal technology for the discovery of useful antibodies for disease management on Dusky kob farms.

The overall aim of this study was to identify antibodies against a pathogen to which Dusky kob would have had no prior exposure, which could be used for diagnostic and therapeutic applications, using an antibody phage display library. With this in mind, the aim of this chapter

was to develop a naïve phage display library displaying scFvs derived from combination pairings of Dusky kob V_H and V_L chains, sourced from the splenic B cells of healthy Dusky kob. The utilisation of a naïve phage display library will result in the display of antibody fragments that have not undergone affinity maturation, and thus, represent a repertoire for the identification of antibodies to almost any antigen.

To assess the potential of a Dusky kob scFv phage display antibody library as a means of identifying antibodies against emerging pathogens found in Dusky kob farms, the library was used to isolate antibodies against the plant virus, Brome mosaic virus (BMV), as a proxy for an emerging pathogen. Due to the nature of BMV, it is unlikely that the Dusky kob used to develop the library would have had any prior exposure, and would have no natural immunity to BMV. Following the construction of the Dusky kob phage display library, the library was used to answer the following questions:

- i. *Is it possible to identify antibodies from a Dusky kob scFv phage display antibody library to a pathogen of which the donor has had no prior exposure?*

By identifying antibodies to the plant virus BMV, to which the donor Dusky kob had had no prior exposure, the potential of the phage display antibody library as a source of antibodies to emerging pathogens that threaten Dusky kob farms in South Africa could be determined.

- ii. *What is the sensitivity and specificity of the anti-BMV antibodies identified from this library?*

The sensitivity and specificity of the antibodies identified from this phage display antibody library will allow us to determine whether the library has potential as a source of antibodies that may have diagnostic and therapeutic applications.

2.2. Materials and Methods

2.2.1 Construction of a Dusky kob phage display scFv library

2.2.1.1 Primer design

Since genome sequence or Ig gene data does not currently exist for Dusky kob that can be utilised for designing PCR primers for V_H and V_L gene amplification, a database consisting of published Ig genes from other fish species was compiled and used for primer design. Candidate

fish species were selected based on the availability of annotated published Ig gene information and on the phylogenetic relationship of those species to Dusky kob (Appendix B.1.4). To ensure the success of the primer design, a highly conserved sequence of the V_H and V_L chain gene fragments was generated using published Ig genes of various fish species that are both closely and distantly related to Dusky. A total of seven fish species were selected based on the above-mentioned criteria: Pacific bluefin tuna (*Thunnus orientalis*), orange-spotted grouper (*Epinephelus coioides*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Siberia sturgeon (*Acipenser ruthenus*), Channel catfish (*Ictalurus punctatus*), and zebrafish (*Danio rerio*).

A nucleotide multiple sequence alignment (MSA) was constructed based on the Ig mRNA gene sequences of the above-mentioned fish species using CLC Main Workbench (v7.7) (Qiagen, Germany). A consensus sequence was generated from the MSA based on the sequence similarity and sequence conservation of the aligned sequences. Both the V_H and V_L chain genes were annotated based on published fish V_H and V_L chain gene sequence data, in order to indicate the various framework and CDRs (Hordvik *et al.*, 1997; Solem *et al.*, 2001; Solem and Jørgensen, 2002; Brown, Kaattari and Kaattari, 2006; Wu *et al.*, 2013). In order to generate variability within the V_H and V_L chain gene products, as well as generating multiple V_H and V_L chain gene products that could mimic the variability naturally found in Dusky kob, alternative primers were designed based on the original V_H and V_L primer pairs. Alternative primers were designed based on various base changes observed in the commercial Mouse IgG library primer set (PROGEN, Germany), by altering between three and four bases of the V_H forward primer and two bases of the V_L forward primer. Thus, a total of six V_H and three V_L forward primers were constructed that could successfully amplify Dusky kob V_H and V_L chain gene fragments (Table 2.1).

2.2.1.2 RNA extraction and synthesis of cDNA

The spleens of healthy Dusky kob were kindly donated by Ocean Choice, Eastern Cape, South Africa. Spleens were stored in RNA_{later}TM stabilisation solution (Invitrogen, USA), according to the manufacturer's instructions, and stored at -80°C prior to RNA isolation. All Dusky kob were screened to ensure that they displayed no symptoms of disease and showed no presence of bacterial or parasitic infections prior to being sacrificed.

Total RNA was purified from the spleens of five healthy Dusky kob, using the RNeasy Mini Kit (Qiagen, Germany) as per a modified version of the manufacturer's instructions. Briefly,

~15 mg of fish spleen was suspended in 800 μ L of RNeasy Lysis (RLT) buffer and homogenised for 3 min at 5 m/s in a TissueLyser LT homogeniser (Qiagen, Germany). The lysate was centrifuged for 3 min at 11,500 $\times$ g, and the supernatant transferred to a fresh microcentrifuge tube. Subsequent centrifugation steps were performed at 6,000 $\times$ g. Total RNA was eluted from the spin column using 35 μ L of RNase-free dH₂O. The nucleic acid concentration was determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Inc). To determine the purity and integrity of the RNA, 500 ng of total RNA was resolved on a MOPS/formaldehyde agarose gel at 80 V for 2 h at 4°C. Complementary DNA (cDNA) was synthesised from 500 ng of total RNA using the QuantiTect Reverse Transcription kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA was stored at -20°C until use.

2.2.1.3 Amplification of V_H and V_L chain genes

PCR was performed to amplify the Dusky kob IgM gene using six heavy chain and three light chain forward primers and one reverse primer for each (Table 2.1). The PCR mixture consisted of 1 $\times$ Phusion High Fidelity Master Mix (Qiagen, Germany), ~250 ng template cDNA, 0.5 μ M PCR primers (MCB DNA synthesis facility), and nuclease-free water, to a final reaction volume of 25 μ L (Table 2.2). PCR was performed using a Bio-Rad MyCycler™ thermal cycler (Bio-Rad, USA) as follows: initial denaturation of 30 s at 98°C, followed by 35 cycles of 98°C for 1 min, 52.7°C for heavy chain and 53.7°C for light chain for 30 s, and 72°C for 1 min, respectively, and a final extension step of 72°C for 7 min. The amplified DNA product was analysed on a 0.8% (w/v) ethidium bromide (EtBr) stained agarose gel. PCR products were stored at 4°C prior to gel extraction.

Table 2.1 Primer sequences

Primer	Sequence (5'-3')	Product size (bp)
VHAF	AGGACTGCTGCTTTTAACTCTCTG	~350
VHBF	AGG T CTGCTG G TTATAACTCTCTG	
VHCF	AGG T CTG G TGCTTTTAACT T TCTG	
VHDF	CT GACTGCTGCTTT G TACTCTCTG	
VHEF	AGG T CTGCTGCTTTTAACTC A TGG	
VHFF	AGGAC A G T TGCTTTTAACT T TCTG	
VHRvs	CCTCGAGCACAATAATAAACAGC	
VLAF	TCACCATCTTCATCTGGACAC	~350
VLCF	TCACCATCT C CATCTGGAT A C	
VLEF	TCACCAT A T C CATCTGGACAC	
VLRvs	CACTCGCTCCTCTTGATGGACT	

The red letters indicate the location of base changes to generate alternative primers. VH: Heavy variable chain; VL: Light variable chain; F: Forward primer; Rvs: Reverse primer; A: Primer A; B: Primer B; C: Primer C; D: Primer D; E: Primer E; F: Primer F.

2.2.1.4 Gel purification of PCR amplicons

PCR products were resolved on a 1% (w/v) EtBr-stained agarose gel at 110 V for 1 h. The desired amplicons were excised, and the products purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's instructions. DNA was eluted in 35 µL of nuclease free water. The concentration and purity of the eluted DNA was determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Inc).

2.2.1.5 Addition of restriction sites to the amplified heavy and light chain fragments

The primers listed in Table 2.2 were used to introduce restriction sites onto the purified V_H and V_L chain gene sequence described in Section 2.2.1.4. *Nco*I and *Hind*III were added to all V_H gene products, while *Not*I and *Mlu*I were added to all V_L gene products, to allow for assembly

of the scFv gene fragments in pSEX81 (Appendix B 1.1). PCR was performed using a Bio-Rad MyCycler™ thermal cycler (Bio-Rad, USA). An initial denaturation step at 98°C for 30 s was performed, followed by 35 cycles of denaturation, annealing, and elongation (10 s at 98°C, 30 s at 53.7°C for amplification of the heavy chain and 55.5°C for amplification of the light chain, and 1 min at 72°C); a final elongation step was performed at 72°C for 7 min. The PCR mixture containing nuclease-free water instead of target DNA was used as a negative control. The PCR products were visualised with EtBr following agarose gel (0.8%) electrophoresis to verify the presence of the amplified product. PCR products were gel purified as described in Section 2.2.1.4.

Table 2.2 Primer sequences used for the addition of restriction sites to the V_H and V_L chain Ig genes

Primer	Sequence (5'-3')	Product size (bp)
VHREAF	TTATAT <u>CCATGG</u> AGGACTGCTGCTTTTAACTC	~380
VHREBF	TTATAT <u>CCATGG</u> AGGTCTGCTGGTTATAACTC	
VHRECF	TTATAT <u>CCATGG</u> AGGTCTGGTGCTTTTAACTT	
VHREDF	TTATAT <u>CCATGG</u> CTGACTGCTGCTTTGTACTC	
VHREEF	TTATAT <u>CCATGG</u> AGGTCTGCTGCTTTTAAATG	
VHREFF	TTATAT <u>CCATGG</u> AGGACAGTTGCTTTTAACTT	
VHRER	TAAATT <u>AAGCTT</u> CCTCGAGCACAATAATAACAGC	~380
VLREA	ATTATA <u>ACGCGT</u> CACCATCTTCATCTGGACAC	
VLREC	ATTATA <u>ACGCGT</u> CACCATCTCCATCTGGATAC	
VLREE	ATTATA <u>ACGCGT</u> CACCATATCCATCTGGACAC	
VLRER	AATATT <u>GCGGCCG</u> CATCTTCAGCCTGGACTCC	

The bold font indicates the recognition sites for the restriction enzymes (*Nco*I, *Hind*III, *Mlu*I, *Not*I); VH: Heavy variable chain; VL: Light variable chain; RE: Restriction enzyme; A: Primer A; B: Primer B; C: Primer C; D: Primer D; E: Primer E; F: Primer F. F: Forward primer; R: Reverse primer.

2.2.1.6 Assembly of scFv gene fragments in pSEX81

2.2.1.6.1 Assembly of V_H chain gene fragments in pSEX81

For assembly of the Dusky kob scFv gene fragments, 1 µg of purified V_H chain fragments and pSEX81 were digested with *Nco*I-HF[®] (New England Bio-Labs Inc, USA) and *Hind*III-HF[®] (New England Bio-Labs Inc, USA) overnight at 37°C. Following digestion, the pSEX81 phagemid vector was resolved on a 1% agarose gel and stained with EtBr. The linearised vector was excised and gel purified as described in Section 2.2.1.4. Ligation of the *Nco*I and *Hind*III digested V_H chain gene fragments to vector DNA was carried out using T4 DNA Ligase (New England BioLabs Inc, USA), using a 1:4 vector to insert ratio, overnight at room temperature. Half the ligation mixture was then transformed using the heat-shock technique described by Seidman *et al.* (2001) into *E. coli* JM109 cells that were made competent according to the protocol described by Dagert and Ehrlich (1979). Transformed cells were plated onto Luria agar (LA) supplemented with 100 µg/mL of ampicillin and incubated at 37°C overnight. The PCR products from each V_H primer set were digested and ligated into pSEX81 and transformed into *E. coli*. Therefore, a total of six transformations were conducted, one per V_H primer pair.

To determine whether the resulting colonies contained the desired plasmid, transformants were screened by colony PCR (cPCR) as follows: Individual colonies were randomly selected and resuspended in 10 µL of dH₂O. A 2 µL aliquot of the resuspended culture was then added to a PCR reaction mixture as described in Section 2.2.1.3, and PCR was conducted using the primers listed in Table 2.3. Colonies from each transformation were pooled and inoculated into 10 mL Luria broth (LB) overnight and plasmids were extracted using the QiAprep Spin Miniprep Kit (Qiagen, Germany) as per the manufacturer's instructions. Purified plasmids from each transformation set were pooled and stored at -20°C.

Table 2.3 pSEX81 sequencing primers.

Primer	Nucleotide sequence (5'-3')	Product size (bp)
ColEF	GGAAGAGCGCCCAATACG	~1200
pIIIR	CATTCCACAGACAGCCCTC	

2.2.1.6.2 Assembly of V_L gene fragments in pSEX81

Following the assembly of the Dusky kob V_H chain gene fragments in pSEX81, the purified pSEX81+V_H was prepared for the addition of the Dusky kob V_L chain gene fragments. One-microgram of purified V_L chain gene fragments (described in Section 2.2.1.4), and 1 µg of pSEX81+V_H were digested with *NotI*-HF[®] (New England Bio-Labs Inc, USA) and *MluI*-HF[®] (New England Bio-Labs Inc, USA) overnight at 37°C. Following digestion, the recombinant pSEX81+V_H phagemid vector was resolved on a 1% agarose gel stained with EtBr. The resolved vector was excised and purified as described in Section 2.2.1.4.

Ligation of the *NotI* and *MluI* digested V_L chain gene fragments to vector DNA was carried out overnight at room temperature using T4 DNA Ligase (New England BioLabs Inc, USA) and a 1:5 vector to insert ratio. The recombinant pSEX81_V_HV_L scFv phagemid vector was then transformed into competent *E. coli* JM109 cells using the heat-shock technique described by Seidman *et al.* (1997). Transformed cells were plated onto LA supplemented with 100 µg/mL of ampicillin and incubated at 37°C overnight.

In order to generate an antibody library with a large degree of diversity that would mimic that of a living organism, the V_H and V_L chain gene fragments (described above) were randomly assembled in pSEX81 by what is known as a chain shuffling method. A full length scFv fragment was assembled by matching one of the sets of six V_H chain gene fragments (A-F) with each of the three V_L chain gene fragments (A, C, E) (Fig. 2.1). Each pairing was prepared in triplicate, resulting in a total of 54 combinations.

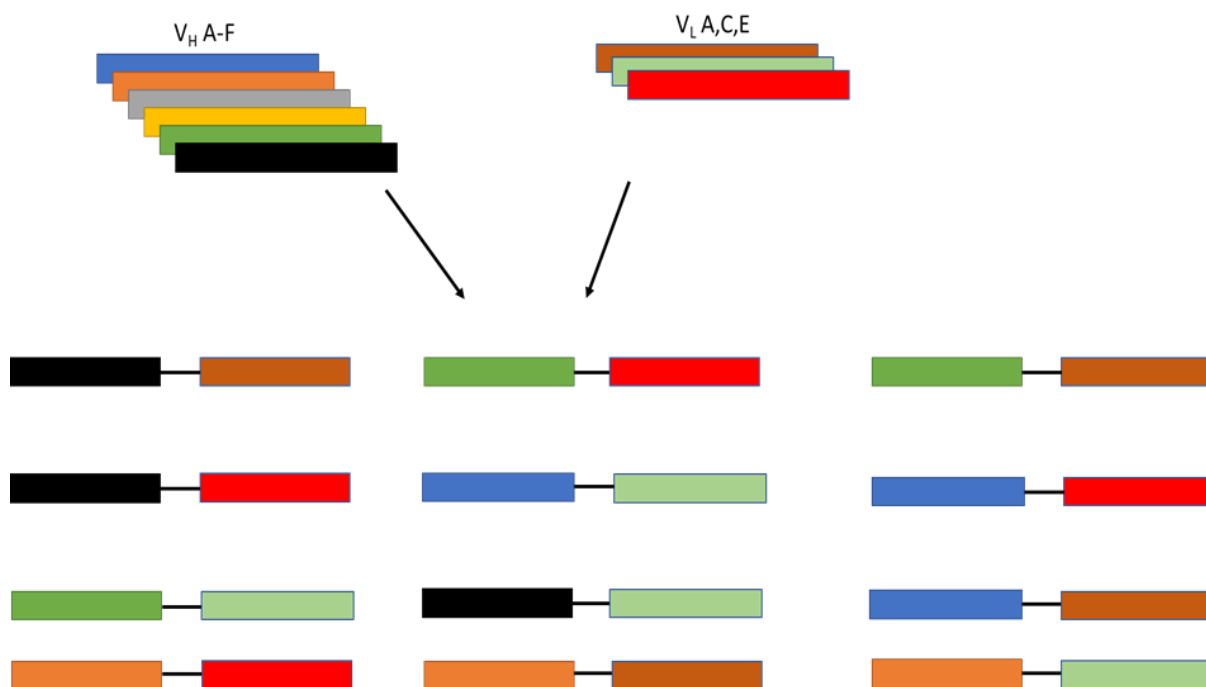


Figure 2.1 Schematic diagram depicting the assembly of Dusky kob scFvs by random shuffling of some of the V_H and V_L chain gene fragments. Not all possible combinations are shown.

Colonies were screened by cPCR as described in Section 2.2.1.6.1. Ten clones that displayed amplicons of the appropriate size (~1200 bp) were selected and their plasmids purified as described in Section 2.2.1.6.1. The isolated pSEX81_V_HV_L scFv phagemids were sent for sequencing by Macrogen Europe. The nucleotide sequences of the V_H and V_L chain gene fragments were analysed using CLC Main Workbench software and used to derive the amino acid sequences of their putative peptide products. A BLASTp search was conducted, using the NCBI non-redundant protein sequence database to confirm the identity of the V_H and V_L antibody fragments and determine their similarity to annotated V_H and V_L protein sequences.

2.2.2 Selection of anti-BMV scFvs

2.2.2.1 Antigen acquisition and storage

The BMV plant virus was generously supplied by Prof. E. Rybicki, Director of the Biopharming Research Unit located in the Molecular & Cell Biology Department, University of Cape Town. The concentration of BMV was determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Inc), whereby 5.2 OD₂₆₀ = 1 mg/mL. The concentration of BMV was found to be 0.3 mg/mL. BMV was stored in phosphate buffered saline (PBS) at 4°C until needed.

2.2.2.2 Transformation of recombinant pSEX81 plasmids into competent *E. coli* TG1

A concentration of 6.5 µg/µL of the pooled pSEX81_{V_HV_L} scFv phagemids were transformed into 1 mL of *E. coli* TG1 cells that had been made competent according to the protocol described by Dagert and Ehrlich (1979) and transformed using the heat-shock technique described by Seidman *et al.* (2001). Following the transformation, the transformants were inoculated into 9 mL of 2×TY broth and incubated in a water bath at 37°C for 1 h. Following incubation, the transformants were inoculated into 40 mL of 2×TY broth supplemented with 100 µg/mL of ampicillin (final conc.) and incubated overnight at 37°C with shaking.

2.2.2.3 Phage rescue

In order to rescue sufficient phages for antibody selection, the overnight *E. coli* TG1 culture, as described in Section 2.2.2.2, was inoculated into 950 mL of 2×TY broth supplemented with 100 µg/mL of ampicillin and 4% glucose (final conc.). The culture was subsequently incubated at 37°C with shaking until the desired OD₆₀₀ of 0.5. A 100 mL aliquot of the log phase *E. coli* TG1 culture was placed in a 1 L conical flask and inoculated with 1 mL of (1×10¹² p.f.u/mL) of reconstituted Hyperphage M13 K07ΔpIII (PROGEN, Germany), which was then incubated for 1 h at 37°C. The remaining 900 mL of culture was incubated for a further 3 h at 37°C with shaking. The cells were collected by centrifugation for 15 min at 3500 *x g*. The cells were resuspended in 9 mLs of 2×TY and an equal volume of glycerol, and stored at -80°C.

Following incubation with the Hyperphage M13 K07ΔpIII (PROGEN, Germany), the *E. coli* TG1 culture was centrifuged for 15 min at 3500 *x g* at 20°C. The cell pellet was resuspended in 1 L of 2×TY broth supplemented with 100 µg/mL of ampicillin, 50 µg/mL of kanamycin, and 0.1% glucose (final conc.). The culture was incubated at 30°C with shaking for 16-20 h. Following incubation, the cells were centrifuged for 10 min at 3500 *x g* at 20°C and the bacterial cell pellet was discarded. The supernatant was centrifuged for a further 10 min at 8,500 *x g* at 20°C to remove any residual bacteria. The supernatant was collected and added to a sterile 1 L container. The phages were precipitated from the supernatant by adjusting the pH to 4.2 using HCl and was subsequently collected by centrifugation at 9,000 *x g* for 10 min at 20°C. The phage pellet was resuspended in 200 mL of phosphate-buffered saline (PBS) pH 7.4, and stored overnight at 4°C.

2.2.2.4 Biopanning the scFv library for antibodies to BMV

A Maxisorp Immunosorbent Tube (Nunc, Denmark) was coated overnight at 4°C with 100 µg/mL of BMV in 4 mL PBS. Following overnight incubation, unbound antigen was discarded, the tube was washed three times with PBS, and blocked with 2% BSAPBS (PBS supplemented with 2% bovine serum albumin [BSA]) overnight at 4°C. The following day, the blocking solution was discarded and the immunotube washed twice with PBS-T (PBS supplemented with 0.1% Tween20), followed by a final two washes with PBS. The rescued phage library (described in Section 2.2.2.3) was centrifuged for 10 min at 9,000 $\times$ g at 20°C. In order to pre-absorb for binders to BSA, the phage pellet was resuspended in 5 mL of BSAPBS-T (PBS supplemented with 2% BSA/0.1% Tween 20) and pre-incubated for 30 min at room temperature. The pre-incubated phages were then added to the immunotube and incubated for 30 min at room temperature with continuous rotation. Then, the tube was left to stand for 90 min, without rotation, in a vertical position to allow for sufficient binding to the coated antigen. Unbound phages were discarded and non-specifically bound phages were removed by washing the tube ten times with 4 mL of PBS-T buffer, followed by ten washes with PBS.

After washing, the bound phages were eluted by adding 4 mL trypsin solution (1 µg/mL in 50 mM Tris pH 8, 1 mM CaCl₂) and incubated for 30 min at room temperature with gentle agitation. The eluted phages were used to infect 30 mL of an exponentially growing culture of *E. coli* TG1 cells (OD₆₀₀ = 0.5) by incubating with the cell culture for 1 h at 37°C without shaking. Cells were centrifuged at 3500 $\times$ g for 5 min and the cell pellet resuspended in 1 mL of 2 $\times$ TY broth. The resuspended cells were serially diluted, ranging from 10⁰ to 10⁻⁴. A 100 µL of each dilution was plated in duplicate onto LA supplemented with 100 µg/mL of ampicillin (final conc.) and incubated overnight at 37°C. To determine the number of positive binders to BMV, the number of colonies was determined for each dilution series and the average colony-forming units (CFU) calculated. After the titre for each plate was determined, the bacterial cells were collected from all the plates by adding 1 mL of 2 $\times$ TY broth to each plate and suspending the cells using a glass spreader. Harvested cells were resuspended in a final volume of 10 mL of 2 $\times$ TY broth. A further round of selection was performed using the colonies obtained from the first round of biopanning. The bacterial culture was inoculated into 100 mL of 2 $\times$ TY broth supplemented with 100 µg/mL of ampicillin and 4% glucose (final conc.) to an OD₆₀₀ of 0.1 and incubated at 37°C with shaking until an OD₆₀₀ of 0.5 was reached. Exponentially growing cells were infected with 1 mL (1 $\times$ 10¹² PFU/mL) of reconstituted Hyperphage M13 K07 Δ pIII (PROGEN, Germany). Phages were rescued as described in Section 2.2.2.3 and biopanning

was repeated as described above. A total of two rounds of biopanning were performed to enrich the library and identify stronger binders to BMV. After each round of biopanning, the resulting phages were acquired and used to infect *E. coli* TG1, from which glycerol stocks were prepared and stored at -80°C.

2.2.2.5 Polyclonal phage ELISA

To determine the degree of enrichment after each round of biopanning, the phage outputs generated after each round of biopanning were pooled and tested for antigen-specific binding by indirect ELISA. Prior to the ELISA, the pooled phage scFv recombinant library generated from each round of biopanning was prepared as described in Section 2.2.2.3. A Nunc polysorp 96-well plate was coated overnight at 4°C with 100 µL of 100 µg/mL BMV in PBS. Wells coated with PBS-only served as a negative control. The following day, the coating solution was removed, and the wells were washed three times, 300 µL/well, with PBS-T. Non-specific binding was blocked with 300 µL/well of 2% BSAPBS for 1 h at 37°C. The wells were washed three times with PBS-T. Each pooled scFv recombinant library derived from each round of biopanning was mixed with 4% BSAPBS-T and 100 µL of the phage dilution was added to each well. After incubation for 1 h at 37°C, the wells were washed three times with PBS-T. The bound phages were then probed with 100 µL/well of a 1:1000 dilution of rabbit anti-FD bacteriophage monoclonal antibody (3.3 mg/mL) (Sigma-Aldrich, USA) in BSAPBS-T, and incubated at 37°C for 1 h. The wells were washed three times with PBS-T. The bound anti-FD antibody was detected by adding 50 µL/well of a 1:5000 dilution of an alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma-Aldrich, USA) in BSAPBS-T, and incubated at 37°C for 1 h. After washing the wells three times with PBS-T and a final single wash with PBS, 200 µL/well of enzyme substrate p-nitrophenyl phosphate (pNPP; Sigma-Aldrich, USA) was added and the plates incubated at room temperature for 30 min in the dark. After 30 min, the absorbance was measured at 405 nm on the Multiskan Go Spectrophotometer (Thermo Scientific, USA).

2.2.3 Validating the sensitivity and specificity of anti-BMV scFvs

2.2.3.1 Validating the sensitivity of anti-BMV scFvs by monoclonal phage ELISA

To identify which phage displayed scFv clone had the strongest binding affinity to BMV, the binding affinity of individual phage displayed anti-BMV scFv clones was determined using a monoclonal phage ELISA. A total of 90 individual colonies were randomly selected from the output of the second round of biopanning, as described in Section 2.2.2.4, prior to pooling all the colonies, and inoculated into single wells of a sterile 96-well tissue culture plate containing

100 μ L of 2 $\times$ TY broth supplemented with 100 μ g/mL of ampicillin and 4% glucose (final conc.). The cultures were incubated overnight at 37°C with shaking. After overnight incubation, 2 μ L of each cell culture was transferred to a fresh 96-well plate containing 150 μ L of 2 $\times$ TY broth supplemented with 100 μ g/mL of ampicillin and 4% glucose (final conc.) in each well. The fresh plate was then incubated at 37°C for 2.5 h with shaking. Following incubation, 50 μ L of 2 $\times$ TY broth supplemented with 100 μ g/mL of ampicillin, 4% glucose (final conc.), and 2 μ L of 2×10^{12} pfu of Hyperphage M13 K07 Δ pIII (PROGEN, Germany) was added to each well and incubated at 37°C for 30 min with no shaking, to allow for infection. The infected bacterial cells were then centrifuged at 400 $\times$ g for 10 min. The supernatant was aspirated to remove any remaining phages, and the cell pellets resuspended in 150 μ L of 2 $\times$ TY broth supplemented with 100 μ g/mL of ampicillin, 50 μ g/mL of kanamycin, and 0.1% glucose (final conc.), and incubated overnight at 30°C with shaking. The plate was then centrifuged at 400 $\times$ g for 10 min. 25 μ L of the supernatant containing the phage particles was collected from each individual well and added to 25 μ L of 4% BSAPBS-T and used in an ELISA as follows:

A Nunc polysorp 96-well plate was coated overnight at 4°C with 100 μ L of 100 μ g/mL BMV in PBS. Wells coated with PBS only served as a negative control. The following day, the coating solution was removed, and the wells were washed three times with PBS-T. Non-specific binding was blocked with 300 μ L/well of 2% BSAPBS for 1 h at 37°C. Plates were washed three times with PBS-T. Phages displaying a monoclonal scFv, prepared as described above, were added at 50 μ L/well, to the appropriate wells and incubated for 1 h at 37°C. The wells were washed as previously described. The antigen bound phages were then probed with 100 μ L/well of a 1:1000 dilution of rabbit anti-FD bacteriophage monoclonal antibody (3.3 mg/mL) (Sigma-Aldrich, USA) in BSAPBS-T, and incubated at 37°C for 1 h. The wells were washed three times with PBS-T. The bound anti-FD antibody was detected by adding 50 μ L/well of a 1:5000 dilution of an alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma-Aldrich, USA) in BSAPBS-T, and incubated at 37°C for 1 h. After washing the wells three times with PBS-T and a final single wash with PBS, 200 μ L/well of enzyme substrate p-Nitrophenyl phosphate (pNPP; Sigma-Aldrich, USA) was added and the plates incubated at room temperature for 30 min in the dark. After 30 min, the absorbance was measured at 405 nm on the Multiskan Go Spectrophotometer (Thermo Scientific, USA).

2.2.3.2 Validating the specificity of anti-BMV scFvs by phage-western blot

2.2.3.2.1 Preparation of the C7 phage displayed anti-BMV scFv

To validate the specificity of the phage displayed anti-BMV scFvs to BMV, a phage-western blot was conducted. The clone that demonstrated the highest binding affinity to BMV, using the protocol described in Section 2.2.3.1, was selected for further analysis by phage-western blot. The clone C7 was prepared for phage-western blot analysis as follows: clone C7 was inoculated into 10 mL of 2×TY broth supplemented with 100 µg/mL of ampicillin and 4% glucose (final conc.) and incubated overnight at 37°C with shaking. The following day, 9 mL of 2×TY broth supplemented with 100 µg/mL of ampicillin and 4% glucose (final conc.) was inoculated with 1 mL of the overnight culture and incubated at 37°C for 2.5 h with shaking. The culture was infected with 100 µL of 2×10^{12} pfu Hyperphage M13 K07ΔpIII (PROGEN, Germany) to rescue the phage particles, incubated at 37°C for 30 min, and then centrifuged at 3500 x g for 15 min at 20°C. The pellet was resuspended in 5 mL of 2×TY broth supplemented with 100 µg/mL of ampicillin, 50 µg/mL of kanamycin, and 0.1% glucose (final conc.), and incubated overnight at 30°C with shaking. The overnight culture was centrifuged at 3500 x g for 10 min at 20°C. Five-millilitres of supernatant was retained and added to 5 mL of 4% BSAPBS-T. This phage solution was used for phage-western blot analysis.

2.2.3.2.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

BMV and tobacco mosaic virus (TMV) proteins were separated according to size by SDS-PAGE. The SDS-PAGE gels were prepared as follows. The stacking gel contained 0.375 M Tris-Cl/SDS pH 6.8, 4% (v/v) acrylamide 37:1, 0.05% (w/v) ammonium persulfate (AMPS), and 0.01% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED). The resolving gel was prepared as follows: 0.125 M Tris-Cl/SDS pH 8.8, 15% (v/v) acrylamide 37:1, 0.2% (w/v) AMPS, and 0.04% (v/v) TEMED. Protein samples were prepared by mixing each sample with 5× sample application buffer (SAB) (1 M Tris-HCl, pH 6.8, 50% v/v glycerol, 25% w/v SDS, 5 mg Bromophenol Blue, 21% w/v dithiothreitol) to yield a final 1× SAB concentration and a final BMV concentration of 100 µg/mL. The running buffer consisted of 25 mM (w/v) tris, 0.192 M (w/v) glycine, and 3.4 mM (w/v) SDS. The BMV and TMV protein samples were boiled at 95°C for 15 min prior to electrophoresis at 100 V for 2 h at 4°C or until the dye front present in the SAB reached the bottom of the gel.

To visualise the protein, the gels were stained with Coomassie stain (0.5 g Coomassie blue G-250, 225 mL methanol, 50 mL acetic acid, and 225 mL dH₂O) at 37°C for 1 h, and then destained with a destaining solution (300 mL methanol, 100 mL acetic acid, and 600 mL dH₂O)

overnight at room temperature. Gels were then visualised on a Bio-Rad ChemiDoc™ XRS+ (Bio-Rad, USA) system and analysed using Image Lab V5.2.1 (Bio-Rad, USA).

2.2.3.2.3 Phage-western blot of the C7 phage displayed anti-BMV antibody

BMV proteins separated by SDS-PAGE were prepared for phage-western blot analysis by rinsing the SDS-PAGE in cold Towbin buffer (25 mM (w/v) tris, 192 mM (w/v) glycine, 20% (v/v) methanol). The BMV and TMV proteins were then electrophoretically transferred onto a nitrocellulose membrane at 10 V for 1 h, using a Trans-Blot SD Semi Dry Transfer Cell (Bio-Rad). The membrane was blocked overnight at room temperature in blocking buffer (2% (w/v) BSA, PBS) with gentle agitation.

The membrane was washed with PBS-T for 15 min at room temperature three times, followed by incubation with the recombinant phage solution, prepared in Section 2.2.3.2.1, for 1 h at 37°C with gentle agitation. Unbound phages were removed by washing the membrane with PBS-T for 15 min at room temperature three times, followed by a final wash with PBS for 10 min. Bound phages were probed by incubation with rabbit anti-FD bacteriophage (Sigma-Aldrich, USA) diluted 1:2000 in blocking buffer (2% BSAPBS/0.1% Tween20) for 1 h at 37°C with gentle agitation. The membrane was washed with PBS-T for 15 min at room temperature three times, followed by a final wash with PBS for 10 min to remove excess background signal. The membrane was incubated with goat anti-rabbit peroxidase conjugated IgG (Rocklands) diluted 1:5000 in blocking buffer for 1 h at 37°C with gentle agitation. The membrane was washed three times with PBS-T for 15 min at room temperature, followed by a final wash with PBS for 10 min. Chemiluminescent signals were detected by adding 2 mL of TMB membrane peroxidase substrate and allowing to develop in the dark for up to 30 min. The membrane was visualised on a Bio-Rad ChemiDoc™ XRS+ system immediately following application of detection reagent and analysed using Image Lab V5.2.1 software (Bio-Rad, USA).

2.2.4 Statistical analysis

In order to determine if there was a significant enrichment of BMV binders after each round of selection, a one-way analysis of variance (ANOVA), followed by a Bonferroni post-hoc test, was conducted using Prism 5 software (GraphPad, USA). The detected signal density for each selection round is presented as mean $\pm$ standard deviation ($n = 2$). A p value of less than 0.05 ($p \leq 0.05$) is considered significant to the preceding round of antibody selection.

2.3 Results

2.3.1 Dusky kob scFv primer design

To generate the Dusky kob V_H and V_L chain gene fragment library, a nucleotide sequence alignment was constructed based on the Ig gene sequences from seven fish species, in order to generate a highly conserved consensus sequence. A consensus sequence of ~420 bp based on the alignment was generated and used to design primers to amplify the V_H and V_L chain gene fragments from Dusky kob (Appendix B 1.2 and B 1.3). Based on the alignment for the V_H chain genes, a forward primer to amplify the V_H chain was designed and was observed to have an average sequence conservation of ~60% to the region found upstream of the first framework region (Appendix B 1.2). Additionally, a reverse primer was designed, which was found to have an average sequence conservation of ~99% to the desired region which overlapped the third framework and CDR of the V_H chain (Appendix B 1.2). The forward primer sequences were subsequently modified by changing between 3-4 bases of the V_H chain gene primer and 1-2 bases of the V_L chain gene primer in order to generate V_H and V_L chain gene products with a high degree of diversity. In total, six V_H and three V_L forward primers were constructed. Due to the highly conserved nature of the 3' end of the variable chains, only one V_H and V_L reverse primer was constructed that amplified the Dusky kob V_H and V_L chain gene fragments (Table 2.1).

2.3.2 Dusky kob scFv phage display library construction

A Dusky kob phage display scFv antibody library was constructed from healthy Dusky kob spleen B cells. cDNA was synthesised from RNA that was extracted from a total of five healthy Dusky kob and pooled. The V_H and V_L chain gene fragments were amplified from the cDNA (Fig. 2.2) using the set of primers listed in Table 2.1. The resulting 350 bp gene fragments were purified and used as a template in subsequent PCRs conducted to add *Nco*I and *Hind*III restriction sites to the V_H chain gene fragments, and *Not*I and *Mlu*I restriction sites to the V_L chain gene fragments, using the primers listed in Table 2.2

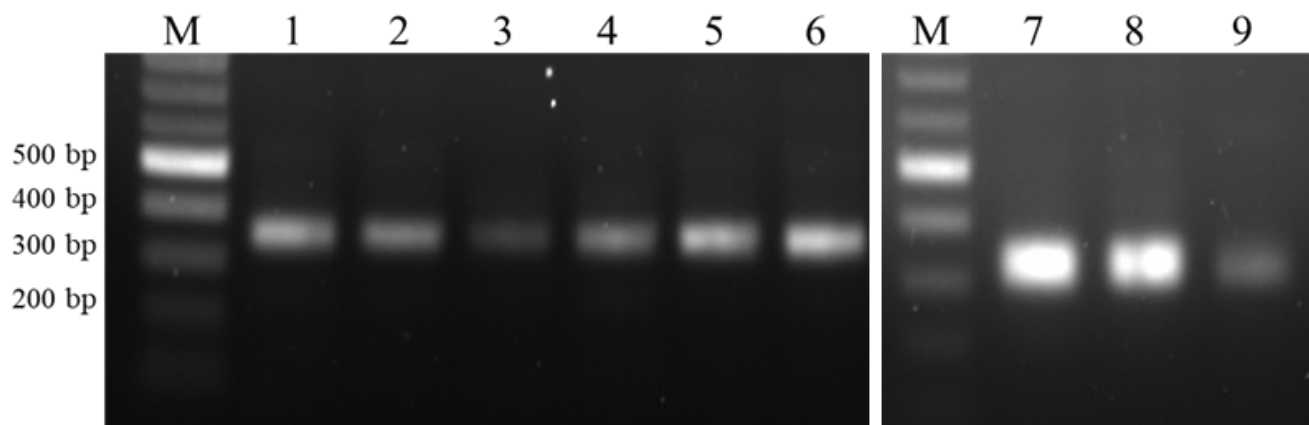


Figure 2.2 PCR amplification of Dusky kob heavy and light variable fragments used for the construction of the Dusky kob scFv phage display library. M = 100 bp DNA marker, New England BioLabs®, 1-6 = heavy chain (V_H) products (~350 bp), 7-9 = light chain (V_L) products (~350 bp).

2.3.2.1 Assembly of scFv in pSEX81

With the goal of developing a diverse Dusky kob antibody library that mimicked the natural diversity of a healthy Dusky kob repertoire, each of the six sets of V_H chain gene fragments, from the six V_H primer sets, were paired with each of the three sets of V_L chain gene fragments, from the three V_L primer sets. This resulted in eighteen unique combinations, with each pairing being performed in triplicate, for a total of 54 pairings. A strategy was employed in which each of the six V_H chain gene fragment sets were ligated into the phagemid vector pSEX81 and transformed into *E. coli* TG1. Agarose gel analysis of the PCR products obtained by cPCR of ten randomly selected transformants, two from each set of pSEX81_Heavy chain A, B, C, and D, and one for each set of pSEX81_Heavy chain E and F, yielded an expected amplicon product (~1200 kb), confirming the success of the ligation (Fig. 2.3A). Full scFv gene fragments were assembled following cloning of the V_L gene fragments into each of the pSEX81_ V_H recombinant phagemid vectors, which would link a V_H chain fragment to a V_L chain fragment via the Yol-tag. After transformation of the scFv gene library into *E. coli* TG1 cells, the resulting library size was calculated to be 1.33×10^7 colony forming units per mL (CFU/mL). Colony PCR of 10 randomly selected clones from the library showed that the plasmids possessed a DNA insert of the expected size (~1200 bp) (Fig. 2.3B).

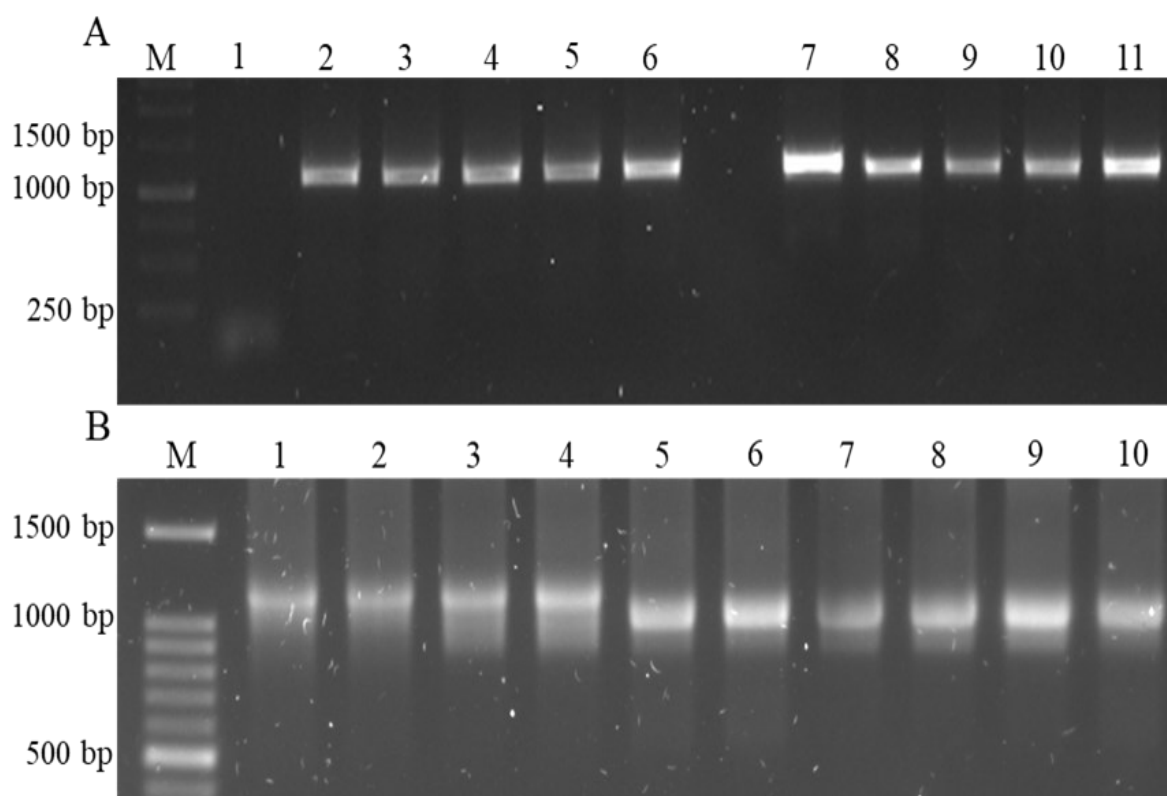


Figure 2.3 A) PCR amplification of recombinant pSEX81 containing a Dusky kob V_H chain gene fragment and a human V_L chain gene fragment. M = OneMark 100 (GeneDirex) 100 bp DNA marker, 1 = no-template control, 2 = pSEX81 Heavy A, 3 = pSEX81 Heavy B, 4 = pSEX81 Heavy C, 5 = pSEX81 Heavy D, 6 = pSEX81 Heavy E, 7 = pSEX81 Heavy F, 8 = pSEX81 Heavy A2, 9 = pSEX81 Heavy B2, 10 = pSEX81 Heavy C2, 11 = pSEX81 Heavy D2, (~1200 bp); B) Ten clones were randomly chosen from the fully constructed library, pSEX81 + V_H and V_L . M = OneMarkB (GeneDirex) 1 kb DNA marker, 1-10 = amplified Dusky kob scFvs from randomly selected clones (~1200 bp).

Ten fully assembled scFv clones were selected for sequence analysis. The amino acid sequences of the peptides expressed by the cloned Dusky kob cDNA fragments were derived from the ORF sequences. Of the ten clones, eight clones had been correctly sequenced and could be assembled, while the other two sequences failed to assemble. The amino acid sequence alignment of the eight sequences demonstrated that both the V_H and V_L chains possessed significant diversity (Fig. 2.4). Clones HALA 2 and HCLE were both observed to have an incorrect linker sequence, “KKVNFQKHAS”, instead of the expected “EEGEFSEAR” amino acid sequence. Clones HFLE and HELA were observed to have identical V_H chain genes, but different V_L chain genes, while clones HELA and HCLA were observed to have identical V_H and V_L chain gene fragments, despite being derived from different primer pairings. Additionally, HCLE had a truncated light chain, while HFLE included two stop codons in the

V_L chain sequence (Fig. 2.4). With clones HALA1 and HALA2, despite being derived from the same primer pairings, observed to have different V_H and V_L chain genes (Fig. 2.4). For all observed clones, with regards to the V_H chain gene sequence, the highest level of similarity was found in the FW2, FW3, and CDR3 regions, while the highest level of diversity was found in the FW1, CDR1, and CDR2 regions of the V_H chain gene (Fig. 2.4). With regards to the observed V_L chain gene sequences, the highest level of similarity was noted to be in the CDR1, FW2, and FW3 regions of these sequences, while the highest level of diversity was observed to be in the FW1 and CDR2 regions of the V_L chain gene sequences (Fig. 2.4). No CDR3 region was observed for any of the V_L chain gene sequences of the 8 clones.

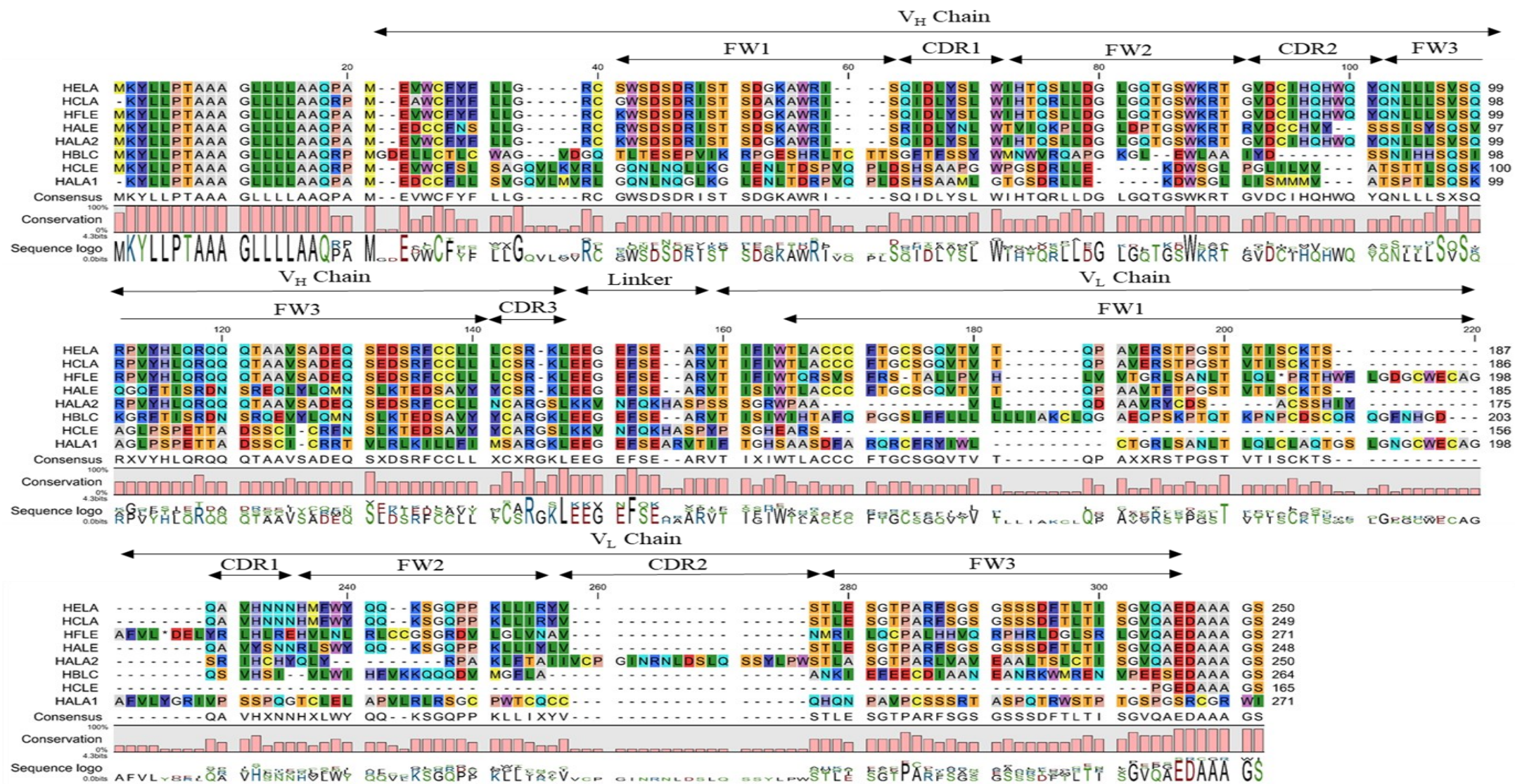


Figure 2.4 Amino acid sequence alignment of randomly chosen clones from the Dusky kob phage display scFv antibody library. The V_H and V_L chain gene fragments are joined together by a short amino acid linker (EEGEFSEAR) to form the scFv antibody gene fragment. The framework (FW) regions and complementarity determining regions (CDR) for each variable fragment are indicated.

2.3.3 Selection and characterisation of specific antibodies

2.3.3.1 Selection of anti-BMV scFvs by biopanning

To test the overall viability of the Dusky kob antibody phage display library as a source of antibody fragments, the naïve Dusky kob scFv phage display library was subjected to two rounds of selection. The panning process was performed by coating an immunotube with BMV followed by incubation with the naïve Dusky kob library, after which bound phages were eluted and recovered. After each round of selection, the rescued phages were re-infected back into *E. coli* TG1 cells. To determine the overall titre of BMV bound phages recovered after each round, the re-infected *E. coli* TG1 cells were plated across ten LB plates. The average output (CFU/mL) after each round of selection was recorded, as well as the difference in outputs observed between the two rounds of biopanning. The average number of bound phages eluted and reinfected into *E. coli* TG1, after the first and second round of biopanning, was 9.37×10^3 CFU/mL and 9.31×10^3 CFU/mL, respectively, with a 0.99% decrease in the average number of eluted phages observed in round two compared to round one of selection.

The rescued phages obtained after each round of biopanning were tested using a polyclonal phage ELISA to determine whether enrichment of phages displaying specifically anti-BMV scFvs had occurred with each successive round of selection. The binding of output phages to BMV increased with each successive round of biopanning (Fig. 2.5). Phages obtained after the first round of selection had a significantly higher binding to BMV than the pre-selected round, which is the Dusky kob library without selection to BMV ($p < 0.05$), while there was a significantly greater increase in scFv binding to BMV between biopanning rounds two and round one, as well as the pre-selected round ($p < 0.0001$) (Fig. 2.5).

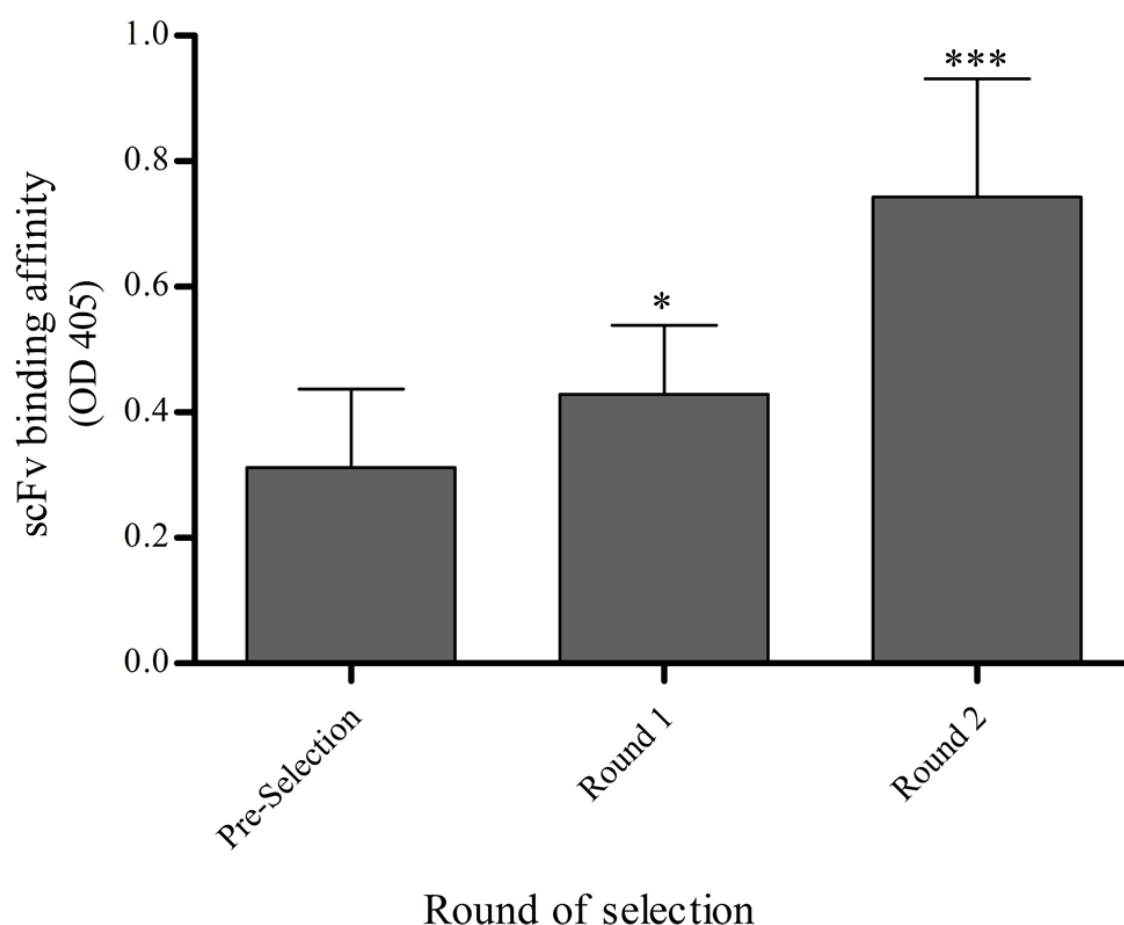


Figure 2.5 Polyclonal phage ELISA showing an overall increase in affinity to BMV with successive rounds of biopanning. Error bars represent the standard deviation of the means of two biological repeats. Statistical significance was determined for each round of selection compared to the preceding rounds of antibody selection. Statistical significance is indicated by an asterisk (*) above the bars as determined by one-way ANOVA. * = $p < 0.05$, *** = $p < 0.0001$; $n = 2$.

2.3.3.2 Binding affinity of anti-BMV scFvs

After two rounds of biopanning, 90 clones were randomly selected and their binding to BMV assessed using a monoclonal ELISA. After correcting for the no BMV and no phage controls, clones that were observed to be equal to or greater than the overall mean binding ($OD_{405} \geq 0.116$) were considered to have reacted positively with BMV.

Of the 90 clones, 21 (red bars) reacted positively with BMV (Fig. 2.6) and were selected for a second round of analysis to determine the binding affinity of each individual phage relative to a negative control (BSA) for that particular phage to more accurately determine the strongest

binder. After analysing the top 21 clones using a monoclonal ELISA conducted in duplicate, it was observed that 18 of the 21 clones demonstrated high binding affinity to BMV (Fig. 2.7). Of the top 18 clones, C7, D7, E9, E12, and F6 were selected for further analysis.

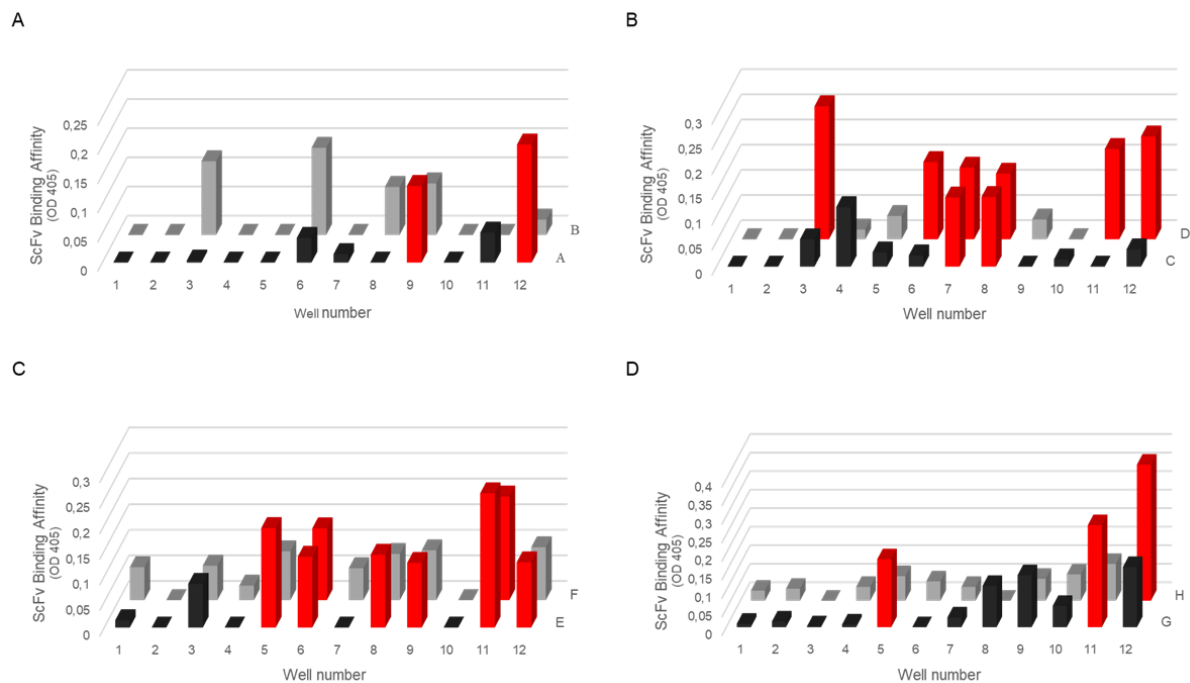


Figure 2.6 Monoclonal phage ELISA showing the binding affinity to BMV of 90 randomly selected clones after two rounds of biopanning. A) Individual clones tested in rows A and B of the 96-well plate. B) Individual clones tested in rows C and D of the 96-well plate. C) Individual clones tested in rows E and F of the 96-well plate. D) Individual clones tested in rows G and H of the 96-well plate. Red bars indicate the phages with a high BMV affinity, which were thus selected for further analysis.

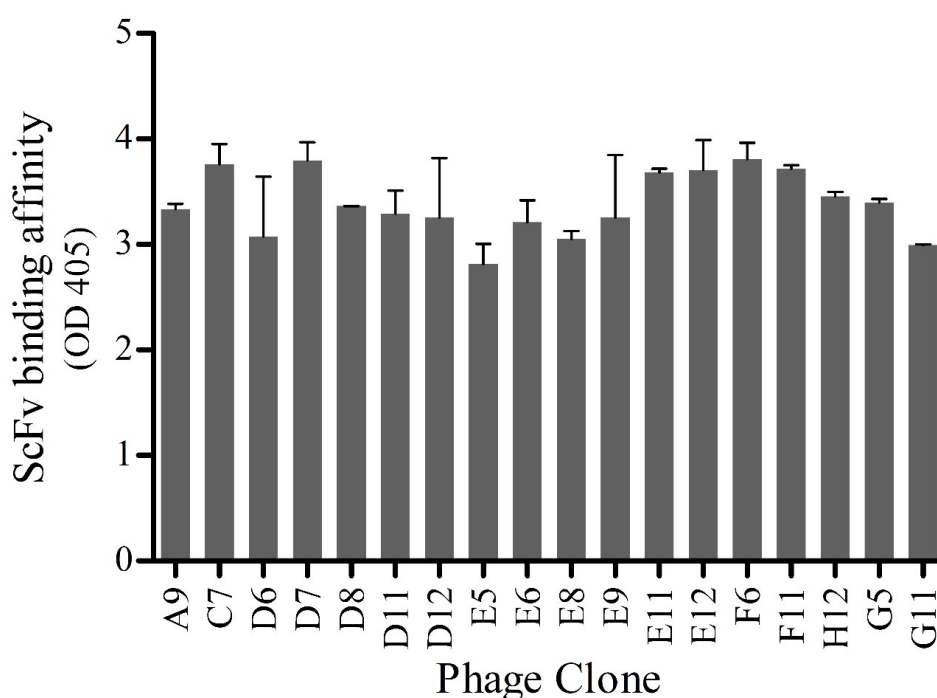


Figure 2.7 Monoclonal phage ELISA showing the binding affinity of the top 18 clones to BMV. Error bars represent the standard deviation of the means of two biological replicates.

2.3.3.3 Sequence analysis of isolated clones

The identity of the top five BMV binders, namely C7, D7, E9, E12, and F6, obtained after two rounds of selection against BMV, was determined by sequence analysis. The scFv genes of the five clones were sequenced, and subsequently, the deduced amino acid sequences were aligned using CLC Main Workbench 7 (Fig. 2.8). The amino acid sequences of the five clones revealed the presence of a complete V_H chain fragment and that all clones, apart from E12, possessed the correct linker sequence downstream of the V_H domain. Of the nine amino acids comprising the linker sequence, eight were found to be mutated in clone E12 (Fig. 2.8). The V_L chain fragment was truncated in all five clones. Although the expected size of the V_L domain is ~100 amino acids (Fig. 2.4), the size range of the V_L chain fragment in the five clones was between 14 and 55 amino acids (Fig. 2.8).

Three CDRs and three framework (FW) regions were identified in each of the V_H chain fragments, while no CDRs and FW regions were identified in any of the V_L chain fragments. Following sequence alignment, clones C7 and F6 were found to have similar V_H chain CDRs and FW regions. Clones E9 and E12 had slightly similar FW1 regions, while clone D7 was found to lack a FW1 region. Regarding the CDR1 region, clones E9 and E12 were noted to

have highly similar sequences within this region, with only a single amino acid difference between the two clones. Furthermore, it was observed that clone D7 was lacking the CDR1 region when compared to the other sequenced clones. Analysis of the FW2 region demonstrated that all clones, with the exception of E9, were found to have highly similar sequence identity. Only clones C7 and F6 were observed to have similar CDR2 sequence identity, while clones E9 and E12 had the greatest CDR2 sequence diversity. All five clones were observed to have similar sequence identity with regards to FW3, with clones C7 and F6 possessing identical FW3 regions. Clones C7 and F6 were observed to have an identical CDR3 region, as well as clones E12 and D7, while clone E9 was observed to have a unique CDR3 sequence compared to the other four clones (Fig. 2.8).

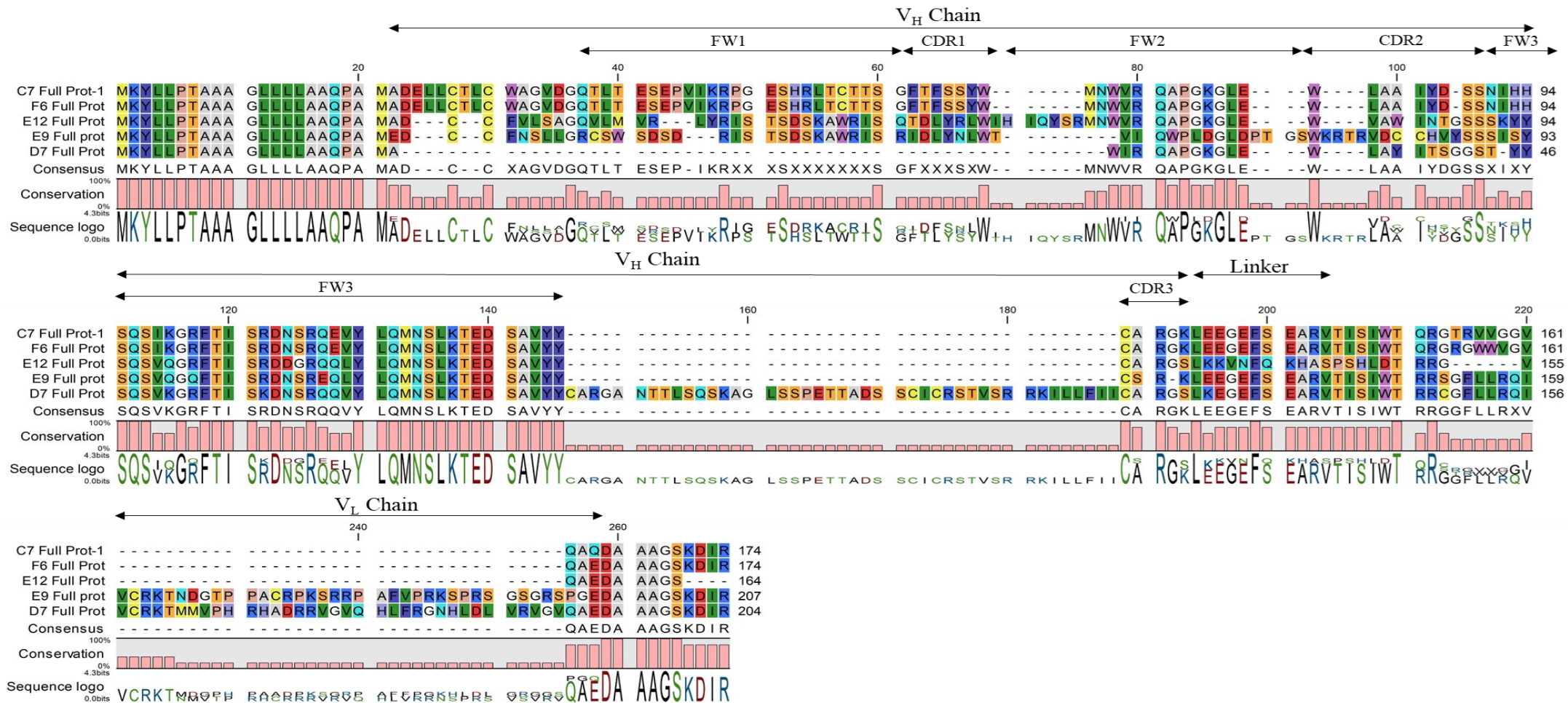


Figure 2.8 Amino acid sequence alignment of Dusky kob anti-BMV scFvs E9, E12, C7, F6, and D7, depicting the sequence similarity among the various framework (FW) and complementarity determining regions (CDRs) of each variable fragment.

To validate the identity of the gene fragments of the selected clones, a protein BLAST analysis was conducted using the NCBI Blast tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The analysis demonstrated that the gene fragments sequenced from all five clones were most homologous to the V_H chain of various fish species.

From the analysis, the protein sequences from the five selected clones had a sequence identity range of between 49.57% - 90.62%, with an average sequence identity of 82.6%, for 9 different fish species (Table 2.4). Of the 9 different fish species, *Epinephelus coioides*, *Echeneis naucrates*, *Oreochromis niloticus*, *Dicentrarchus labrax*, were observed to have the highest sequence identity (90.62%, 88.06%, 88.64%, and 88.89%, respectively) to the five query sequences.

As clones C7 and F6 have identical amino acid sequences (Fig. 2.6), the highest query coverage (95%), lowest average E values (2.07 e-64 and 1.40 e-62) (Table 2.4), and display high binding affinity to BMV (Fig. 2.7), clone C7 was selected for further analysis.

Table 2.4. Top three hits in a Blastp analysis of five cloned putative Dusky kob scFvs

Clone	BLAST Hit	Query Cover (%)	E Value	Percentage Identity (%)	Accession
C7	IgM heavy chain VH region [<i>Oreochromis niloticus</i>]	95	2,00E-64	86,11	AHI18433.1
	Immunoglobulin mu heavy chain [<i>Dicentrarchus labrax</i>]	95	2,00E-63	88,89	ARC77253.1
	IgM heavy chain VH region [<i>Oreochromis niloticus</i>]	95	6,00E-62	84,26	AHI18458.1
E9	IgM heavy chain VH region [<i>Oreochromis niloticus</i>]	95	2,00E-18	49,57	AHI18463.1
	IgM heavy chain VH region [<i>Oreochromis niloticus</i>]	39	3,00E-17	88,64	AHI18432.1
	Immunoglobulin mu heavy chain [<i>Thunnus orientalis</i>]	67	4,00E-17	62,67	AHC31461.1
F6	IgM heavy chain VH region [<i>Oreochromis niloticus</i>]	95	1,00E-64	86,11	AHI18433.1
	Immunoglobulin mu heavy chain [<i>Dicentrarchus labrax</i>]	95	2,00E-63	88,89	ARC77253.1
	IgM heavy chain VH region [<i>Oreochromis niloticus</i>]	95	4,00E-62	84,26	AHI18458.1
D7	immunoglobulin heavy chain variable region [<i>Epinephelus coioides</i>]	59	5,00E-35	90,62	ADZ99128.1
	Immunoglobulin heavy chain variable region [<i>Salmo salar</i>]	61	1,00E-33	85,07	AAG21239.1
	Immunoglobulin mu heavy chain [<i>Oncorhynchus mykiss</i>]	61	2,00E-33	86,76	ABH09016.1
E12	Immunoglobulin heavy variable [<i>Collichthys lucidus</i>]	57	7,00E-33	83,08	TKS91816.1
	Ig heavy chain [<i>Echeneis naucrates</i>]	58	8,00E-33	88,06	XP_029365147.1
	Immunoglobulin heavy chain [<i>Thunnus orientalis</i>]	57	3,00E-32	86,15	AHC31464.1

2.3.3.4 Specificity of Dusky kob scFv binding to BMV

To determine the specificity of the phage-displayed anti-BMV dAb, the phage clone with the highest binding affinity to BMV was chosen for phage-western blot analysis. As clones C7 and F6 have identical sequences (Fig. 2.8) and similar binding affinities to BMV, clone C7 was chosen for further analysis. To validate the specificity of the C7 phage display anti-BMV, the dAb was tested against BMV and a second plant virus, TMV.

SDS-PAGE showed that the BMV coat protein (~20 kDa) was present in the lanes loaded with BMV, while the TMV coat protein (~17 kDa) was detected in the TMV lanes (Fig. 2.9A). Additionally, a BMV coat protein dimer (40 kDa) was observed in the BMV lanes.

The phage-western blot analysis of the C7 phage-displayed anti-BMV dAb detected two clear bands in the BMV + phage lanes, which coincide with the position of the 20 and 40 kDa proteins detected by SDS-PAGE, while no bands were observed in the TMV + phage lanes of the phage-western blot (Fig. 2.9B). Thus, the C7 phage displayed anti-BMV dAb binds specifically to the BMV coat protein.

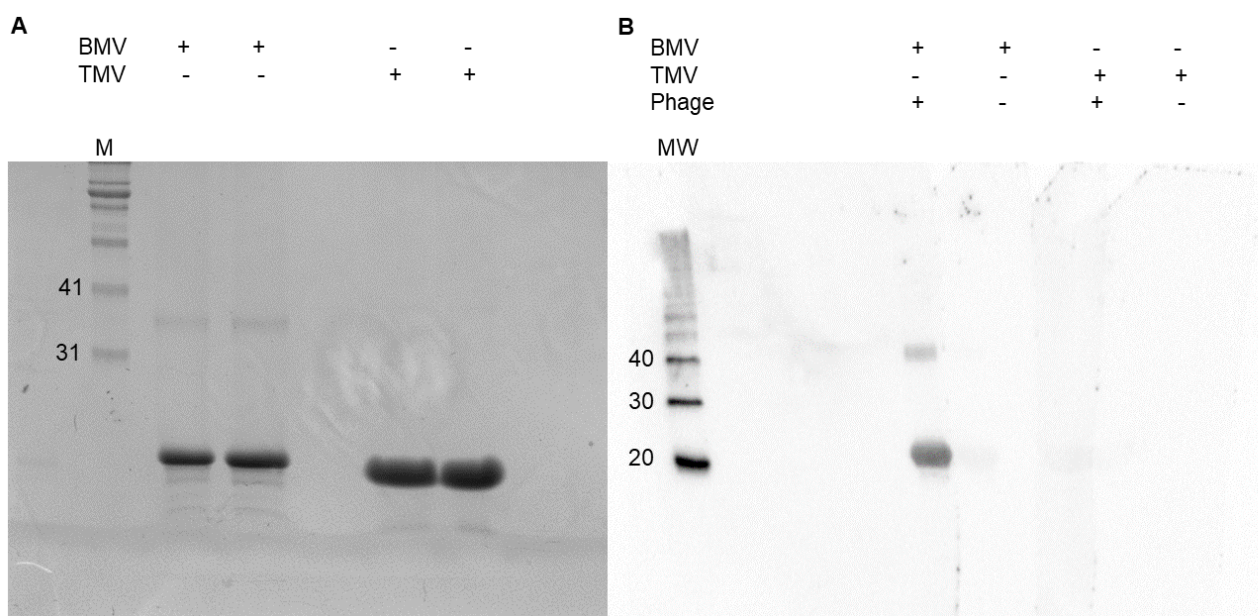


Figure 2.9 Phage-western blot and SDS-PAGE of BMV and TMV viral particles. A) Coomassie stain of an SDS-PAGE. M: Hi Mark™ Pre-stained Protein Standard (ThermoFisher Scientific); BMV (100 µg/mL) or TMV coat protein (100 µg/mL). B) Phage-western blot. MW: MagicMark™ XP Western Standard (ThermoFisher Scientific); Denatured BMV or TMV coat protein was probed with the C7 phage clone. The “+” symbols depict the presence of antigen or phage antibody, while the “-” symbol depicts their absence.

2.4 Discussion

Phage display antibody technology is becoming increasingly popular for the discovery and development of useful diagnostic and therapeutic antibodies. However, the majority of modern-day antibody libraries are based on human Ig genes (Van Wyngaardt *et al.*, 2004; Almagro *et al.*, 2019), with limited focus on the use of phage display technology in the aquaculture industry. The development of a Dusky kob phage display scFv antibody library may aid in controlling and mitigating disease outbreaks that affect finfish farms in South Africa. The phage display scFv antibody library developed in this study was used to successfully identify and isolate antibody fragments against the plant virus BMV, which served as a proxy for a novel emerging pathogen. This study highlights the potential application of this phage display antibody library for the development of diagnostic and therapeutic antibody fragments to various fish pathogens.

2.4.1 Antibody library construction

2.4.1.1 Primer design

At the time of library creation, no Ig sequence information for Dusky kob was available and there was a clear paucity of annotated fish Ig sequences available that could be utilised to generate any sort of consensus sequence, which in turn, could be used to develop a set of primers that would amplify the V_H and V_L chain fragments of Dusky kob. Therefore, in order to develop primers that could successfully amplify the V_H and V_L chain fragments of Dusky kob, and subsequently be used to construct a naïve Dusky kob phage display scFv antibody library, a highly conserved consensus sequence based on available V_H and V_L sequence data of various fish species, namely Pacific bluefin tuna (*T. orientalis*), orange-spotted grouper (*E. coioides*), Atlantic salmon (*S. salar*), rainbow trout (*O. mykiss*), Siberia sturgeon (*A. ruthenus*), Channel catfish (*I. punctatus*), and zebrafish (*D. rerio*), was generated. These fish species had a variable degree of taxonomic relatedness to Dusky kob (Appendix B.1.4), which allowed for the generation of a highly conserved consensus sequence for the creation of primers that would successfully amplify the V_H and V_L chain gene regions of Dusky kob.

A total of six V_H and three V_L forward primers, as well as one V_H and V_L reverse primer, were constructed that could successfully generate Dusky kob V_H and V_L chain gene fragments (Table 2.1). The naïve Dusky kob phage display scFv antibody library was generated by amplification of the V_H and V_L chain gene fragments from a pool of splenic mRNA isolated from five healthy Dusky kob. All five fish were screened to ensure that they displayed no symptoms of potential illness and that no parasites were present prior to the fish being sacrificed, to ensure that their V-gene repertoires were not skewed towards any infectious pathogen. This is important since an antibody repertoire sourced from a previously infected donor would be restricted to generating antibodies against that particular pathogen, thus significantly limiting the library as a source of antibodies to other antigens (Carmen, 2006; Rami *et al.*, 2017b). The use of disease free donors allows for the generation of naïve, single-pot libraries that can produce antibodies against any antigen, including toxins and self-antigens (Ponsel *et al.*, 2011; Chan *et al.*, 2014).

2.4.1.2 Assembly of the Dusky kob scFv antibody library

Library size and diversity are important factors that influence the overall success and power of an antibody library (Azzazy and Highsmith Jr., 2002; Norbury *et al.*, 2019). Naïve antibody libraries are unique in the sense that the antibodies in this library have not undergone affinity maturation, and are therefore a source of antibodies to any possible antigen (Ledsgaard *et al.*, 2018; Norbury *et al.*, 2019). However, a lack of affinity maturation results in antibodies having

a lower affinity than those isolated from other library types (Hoogenboom, 2002). It has been reported that the affinity of antibodies isolated from a naïve library is directly proportional to the size of the library, with antibodies isolated from smaller libraries having a lower affinity to those isolated from larger ones (Griffiths *et al.*, 1993). Therefore, the size of the library is essential to its success in producing antibodies with strong affinity (Bobkov, Woning and Haard, 2018).

The size and diversity of antibody libraries can be increased in multiple ways. For instance, it was discussed by Tohidkia *et al.* (2012) and Kumar *et al.* (Kumar *et al.*, 2019) that isolating V-genes from multiple donors, and isolating V-genes from multiple sources (spleen, bone marrow, lymphoid tissue, and blood), would aid in the creation of large, diverse naïve antibody libraries of greater than 10^8 transformants. It has been reported that combining different V_H and V_L chain gene fragments (Fig. 2.1), generates high diversity within the antibody library. This random mixing of V_H and V_L chain gene fragments is necessary to create as many gene combinations as possible, as well as creating unique antibody fragments which may not occur naturally (Bratkovič, 2010; Bradbury *et al.*, 2011; Lim *et al.*, 2016). The repertoire of a typical naïve antibody library is derived from healthy donors. However, it is unlikely to find an individual with a history of perfect health, and therefore it is highly likely that all naïve libraries are skewed in one way or the other to a particular antigen. Lim *et al.* (2016) proposed that random mixing of the V_H and V_L chains from a large number of donors would effectively normalise the skewing effect (Lim *et al.*, 2016). Alternatively, reports have shown that the use of multiple primer sets aids in the creation of V-gene repertoires that mimic the host's natural immune system (Azzazy and Highsmith Jr., 2002). However, Tohidkia *et al.* (2012) stated that the use of multiple primers may lead to the creation of potentially unwanted codons within the V_H and V_L chain sequences, which may lead to unwanted frame shifts and deletions within these variable fragments.

Various naïve antibody libraries have been generated, ranging in size from 10^7 to 10^{10} clones, that were successful in isolating antibodies with strong affinities to various antigens (Reiter *et al.*, 1999; D'Angelo *et al.*, 2018; Norbury *et al.*, 2019). In this study, we describe the construction of a naïve Dusky kob library from the V_H and V_L chain fragment genes of Dusky kob Ig sourced from five individuals. Initial estimates showed that the size of the Dusky kob phage display scFv antibody library was $\sim 1.33 \times 10^7$ clones. The relatively small size of this library may be due to the limited efficiency of chemical transformation. In a study by Russo *et al.* (2009), the authors highlighted their observation that higher transformation efficiencies can

be achieved by electroporation compared to chemical procedures (Russo *et al.*, 2009). Therefore, it is plausible that a larger library size could be generated through transformation by electroporation. Regardless, based on the library size criteria described by Omar and Lim (2018), it can be noted that the naïve library described in this study falls within the recommended working range (10^7 - 10^9 clones) and therefore will be efficient in identifying antibodies against an emerging pathogen of Dusky kob (Omar and Lim, 2018). Moreover, further validation that the library constructed in this study is of sufficient size comes from Norbury *et al.* (2019) who reported a naïve library of similar size (3.5×10^7 clones) that was successfully used to identify several scFv antibody fragments that could bind to the parasite *Fasciola hepatica* with strong affinity (Norbury *et al.*, 2019).

2.4.1.3 Diversity of the Dusky kob phage display scFv antibody library

To assess the diversity within the V_H and V_L chain fragments of the Dusky kob phage display scFv antibody library, ten random full length scFv clones (Fig. 2.3) were selected for sequence analysis (Fig. 2.4). The deduced amino acid sequences of the gene products demonstrated a high level of sequence diversity within the Dusky kob phage display scFv antibody library, with no two clones having identical scFv amino acid sequences. With regards to the V_H chain gene fragment, the highest degree of diversity within the three identified CDRs (CDR1, CDR2, and CDR3) was in CDR1, with an average codon conservation percentage of 56.6%. CDR2 and CDR3 were observed to have an average codon conservation percentage of 65.6% and 77%, respectively (Fig. 2.4). The CDR3 of humans is noted to have the highest variability among the CDRs, and on average is composed of fifteen amino acids. Furthermore, the human CDR3 is responsible for most of the antigen recognition and antigen binding (Van Wyngaardt *et al.*, 2004). However, the CDR3 region of the Dusky kob V_H chain described in this study shows a high level of conservation (77%) and is comprised of only six amino acids (Fig. 2.4). The length and variance of the CDR3 of higher order vertebrates, such as humans and mice, are greater than compared to the CDR3s of lower vertebrates, especially cold-water vertebrates such as fish (Chiraporn, Sasimanas and Prapansak, 2015). The CDR3 region of the V_H chain gene reported in this study is significantly truncated, likely due to the reverse primer, described in Table 2.1, annealing to the start of the CDR2 region, therefore significantly limiting the CDR3 generated. However, several reports have indicated that the CDR2 region is highly susceptible to mutations, which are known to significantly affect the binding ability of an antibody to its target antigen (Chen, Roberts and Rittenberg, 1992a, 1992b; Brown *et al.*, 1996; Wiens *et al.*, 1997; Bach *et al.*, 2015). Brown *et al.* (1996) demonstrated that a mutation at two sites in CDR2

was sufficient for generating a nonbinder phenotype, highlighting the significant role of CDR2 in antigen binding. Therefore, despite the absence of a complete CDR3 region in the V_H chain gene, there should be little to no loss of the antigen binding function of these domain antibodies due to the presence of a complete CDR2 region.

A higher degree of diversity was observed in the Dusky kob V_L chain fragments (Fig. 2.4). The FW regions of the V_L chain had between 39.4 and 53.6% conservation, while sequence conservation for CDR1 and CDR2 was between 43 and 50%, respectively. CDR3 was not present in any of the sequenced V_L chain fragments, which is a consequence of the primers being designed upstream of this region. Greater diversity in the CDR and FW regions was observed in the V_L chain domain compared to that of the V_H chain domain. Analysis of clone HCLE indicated that the clone was missing the V_L chain gene, and that clone HFLE, while having a full length V_H chain fragment, contained multiple stop codons within its V_L chain fragment, which would ultimately result in incomplete expression of the pIII and therefore failure to be displayed on the surface of the phage (Fig. 2.4). The presence of this variant in the sequencing analysis indicates the likelihood of other variants in the library that will not express on the surface of the phage, thus resulting in a lower predicated library diversity.

Only five of the ten sequenced Dusky kob clones possessed full scFv fragments. It can therefore be expected that approximately 50-60% of the library has the potential to express in-frame scFvs. This suggests that the Dusky kob phage display scFv antibody library has an effective size of between 50-60% of the observed library size, and therefore, the true effective size of the Dusky kob phage display scFv antibody library is approximately 1.3×10^6 clones. However, it is possible that the effective size of the scFv antibody library may actually be between 60-70%, as it is theoretically possible that clones such as HCLE, which lack a complete V_L chain, can produce a functional V_H domain. It was revealed by Almagro *et al.* (2019) that due to errors in library construction, the effective size of phage display scFv libraries is between 60-90%, and that the actual library size is one order of magnitude less than the absolute size of the library (Almagro *et al.*, 2019). This is in accordance with a study by van Wyngaardt *et al.* (2004), which reported that from a phage display library comprised of semi-synthetic chicken scFvs, approximately 65% of 70 randomly sequenced clones had the potential to express in-frame scFvs, giving their library an effective size of 65%. The effective library size described in the present study is in accordance with the aforementioned studies. Libraries with smaller effective sizes have been described, as in the study by Zhang *et al.* (2006). When evaluating the effective size of their library, Zhang *et al.* (2006) observed that out of fifteen randomly selected clones,

only five contained inserts that had the potential to express in-frame scFvs, resulting in a library size that was 33% of the absolute library size (Zhang, Wang, Wu, M. Li, *et al.*, 2006).

2.4.2 Antibody selection

To evaluate the potential application of the phage display library as a source of antibodies against emerging pathogens that threaten fish farming in South Africa, the plant virus BMV was used to represent an emerging pathogen of Dusky kob. BMV, and not a known Dusky kob pathogen, was used because Dusky kob are not the natural hosts or reservoirs of BMV, and therefore it is highly unlikely that the naïve gene library developed from healthy Dusky kob would be enriched or biased for antibodies to BMV. Thus, the ability of the naïve library to act as a source of antibodies to a new infection or an emergent pathogen could be tested without the confounding factor of acquired immunity due to a previous infection.

Following two rounds of selection, a significant increase in the enrichment of BMV-specific scFv antibodies was observed (Fig. 2.5). It is recommended that the selection process be repeated 2-5 times to ensure that highly specific binders are enriched from a phage display library (Azzazy and Highsmith Jr., 2002; Sioud, 2019). Multiple rounds of selection ensures that the number of clones that bind non-specifically or bind with low affinity, would be removed with each subsequent round, thereby improving the overall enrichment that can be achieved per round (Azzazy and Highsmith Jr., 2002; Carmen, 2006). However, due to the unavailability of Hyperphage M13 K07ΔpIII (PROGEN, Germany) due to import issues into South Africa, the number of rounds of selection was limited to two. Regardless, after only two rounds of selection, a significant increase in enrichment was observed between the first and second round of biopanning (Fig. 2.5). A similar result was reported by Lim *et al.* (2016), in which a significant increase in enrichment was observed after only two rounds of selection, indicating that further rounds were unnecessary. Based on a similar increase in enrichment following two rounds of biopanning and the unavailability of Hyperphage M13 K07ΔpIII (PROGEN, Germany), no further rounds of selection were performed.

After the second round of selection, a total of 90 colonies were randomly selected to evaluate their binding to BMV using phage-ELISA. Of the 90 clones evaluated, a total of 18 were observed to have strong binding to BMV (Fig. 2.6). While approximately 20% of the clones isolated from the Dusky kob phage display scFv antibody library showed affinity to BMV relative to the negative control, approximately 80% of the clones were found to be target-unrelated antibodies and thus, could be considered potential false positives. This finding of 20%

target-specific binders is in agreement with other studies that showed that the percentage of clones that recognised the target antigen after selection varied from 1-60% (Schwimmer *et al.*, 2013; Lim *et al.*, 2016; Almagro *et al.*, 2019; Valadon *et al.*, 2019; Rizzo *et al.*, 2020).

The discrepancy between the large percentage of negative clones compared to the small percentage of positive clones obtained following two rounds of selection is due to clones binding to off-target components during the selection process. Ideally, off-target clones are removed during the wash steps conducted as part of the selection protocol, and only those specific to the target antigen are selected for. Those that bind non-specifically are removed through multiple rounds of selection, thus only selecting and amplifying target-related clones. While two rounds of biopanning was sufficient for significant enrichment of binders, it had a corresponding consequence of producing a low percentage of antigen binders. This was in accordance with the study reported by Lim *et al.* (2016) in which the authors demonstrated that, while a significant degree of enrichment was observed after two rounds of biopanning, only a single positive clone out of a total of 91 tested was shown to bind to their target antigen.

While multiple rounds of biopanning could have decreased the percentage of non-target binders, a report by Menendez and Scott (2005) noted that a typical phage display library potentially contains a distinct population of clones that display “target-unrelated-antibodies”, which may react to other components of the screening system. The authors further stated that these off-target clones were responsible for the occurrence of false positive clones isolated after selection. These off-target clones mainly have an affinity to either the plastic of the plates or tubes, or to the blocking agents used in the screening process. The prevalence of these off-target binders may be controlled by blocking unoccupied surfaces with a suitable agent, such as BSA. Additionally, binding to the blocking agent could be prevented through pre-incubation of the phage libraries with the blocking agent prior to any selection process (Menendez and Scott, 2005; Vodnik *et al.*, 2011). Although these measures were employed in this study to mitigate the selection of off-target binders, it was still found that 80% of the clones isolated were false positives, which may represent a population of plastic and/or BSA binders, as well as insufficient rounds of biopanning or insufficient wash cycles. Regardless, it is easy to recognise and differentiate false positives from true positives based on a lack of reactivity by ELISA analysis.

2.4.2.1 Sequence analysis of positive clones

The sequence diversity of the variable domains of five clones with high affinity to BMV were analysed. The amino acid sequence of the five clones indicated that the FW regions were less variable than those of the CDRs, with the greatest variability observed in CDR1 (Fig. 2.8). Furthermore, the similar affinity of clones C7 and F6 to BMV (Fig. 2.7) and their close sequence identity, suggests that these two antibody fragments may recognise the same BMV epitope.

Interestingly, all five clones that bound to BMV lack a V_L chain, and thus, are essentially dAbs (Fig. 2.8). A similar observation was reported by Cai and Garen (1995 and 1996), who described the construction of a phage display scFv antibody library for the selection of anti-melanoma antibodies. Here, it was found that during construction of the library, restriction sites in the V_L cDNA resulted in cleavage and deletion of the V_L chain gene, resulting in an antibody library that consisted partially of V_H chain domains only. Similar observations were reported by van Wyngaardt *et al.* (2004) and Cai and Garen (1995 and 1996), in which the authors observed the isolation of antibody fragments that consisted of either a V_H or V_L domain only. Both studies noted that despite the lack of the opposite domain, the dAbs maintained high affinity to their target antigen, with their binding affinity found to be indistinguishable from other full length scFv antibodies isolated from the same library (Cai and Garen, 1995, 1996; van Wyngaardt *et al.*, 2004). Interestingly, Cai and Garen (1996) examined the effect of adding a V_L domain to one of their positive V_H domains that showed strong affinity to melanoma cells; the addition of the V_L domain from the original library either reduced or completely eliminated the affinity of that clone (Cai and Garen, 1996).

It is evident that the loss of the V_L chains in the five selected clones reported in this study may be a consequence of the strategy employed to construct the library. The absence of V_L domains might have been explained by the presence of restriction sites in the V_L cDNA, which may result in cleavage and deletion of the V_L chain gene (Cai and Garen 1995). However, *Mlu*I and *Not*I restriction sites, used in the present study, were not identified within the V_L chain gene sequence. Additionally, as Dusky kob is a non-model organism, no annotated V_H and V_L chain gene sequences were available from which to generate primers. Therefore, the primers in this study (Table 2.1) were designed from the consensus sequences generated with the V_H and V_L chain gene sequences of various fish species. Since the length of the Dusky kob V_H and V_L chain gene is unknown, the primers used in this study would therefore be amplifying products of unknown and various sizes. It is therefore plausible that a few of the resulting V_H and V_L chain gene products, when inserted into pSEX81, may have shifted the reading frame of the

scFv sequence resulting in the occurrence of a number of stop codons within the generated sequences. This is clear in Figure 2.4, where a number of stop codons can be observed in clone HFLE. Furthermore, such frameshifts can be observed that have altered the reading frame of the entire scFv gene product, such as in HALA2 and HCLE, where it can be observed that a frameshift has resulted in a change in the amino acid sequence of the linker from “EEGEFSEAR” to “SLKKVNFQKHAS”, therefore, completely altering the products produced (Fig. 2.4).

Due to selective pressure during biopanning, all clones which contain incorrect or altered reading frames, such as clones HCLE and HFLE (Fig. 2.4), would be lost. As such, variants which consist of only V_H chains are more likely to be selected for as these variants have fewer opportunities for frameshift errors compared to full scFvs. Therefore, it is likely that the majority of the library described in this study consists of V_H chain only variants. This is evident as all five of the clones analysed after biopanning consisted of only V_H chain genes (Fig. 2.8). Similar studies have reported that antibody libraries consisting of only a V_H chain domain can be used to isolate dAbs with high affinity and specificity to their target antigen, with similar properties to the full parental antibody (Cai and Garen, 1995; Reiter *et al.*, 1999; Holt *et al.*, 2003). Therefore, despite the loss of the V_L chains in the five clones, the V_H chain antibodies may still be effective as potential diagnostic and therapeutic antibodies, and they may have better tissue penetration and biophysical properties compared to larger antibody fragments.

2.4.2.2 Specificity of anti-BMV single domain antibody to BMV

Western blot analysis is a frequently used approach for the identification of specific proteins. This technique provides information regarding the putative size of the target protein, which can confirm the putative identity of the target (DING *et al.*, 2005). Along with ELISA and immunofluorescence antibody tests, western blot analysis is popular due to its sensitivity and specificity as a diagnostic technique (Desoubeaux *et al.*, 2017; Persichetti *et al.*, 2017). To assess the specificity of the dAbs that demonstrated strong binding to BMV, a phage-western blot experiment was conducted employing clone C7. Phages displaying the C7 dAb were used as immunological reagents in a western blot analysis against BMV and TMV, the latter serving as a negative control to test binding specificity. Both TMV and BMV are positive strand RNA plant viruses, and share significant sequence homology with one another within the methyltransferase, helicase, polymerase, and plant movement proteins, as well as to several other positive strand RNA plant viruses of the Sindbis superfamily (Strauss and Strauss, 2008). Although the structural proteins of these two viruses are different, with TMV possessing a rod

shaped structure and BMV an icosahedral capsid structure, it has been noted that TMV and BMV comprise proteins that have considerable homology to each other, namely the plant virus movement, methyltransferase, and helicase proteins (Cornelissen and Bol, 1984; Strauss and Strauss, 2008). The phage-western blot demonstrated that the C7 dAb could specifically identify and bind to the coat protein of BMV (~22 kDa) and showed no specificity to TMV (Fig. 2.7). This result indicates that the naïve Dusky kob phage display library can be utilised to identify antibody fragments that are highly specific to a target antigen to which the library has no bias. Overall, the library described in this study appears to be an ideal tool for the identification of diagnostic and therapeutic antibodies to an emerging pathogen.

2.4.3 Conclusions

Phage display is a powerful tool for the identification and isolation of antigen specific recombinant antibodies, which can serve as potential diagnostic and therapeutic reagents. Despite the lack of Ig sequence information, a Dusky kob phage display scFv antibody library was successfully created based on the V_H and V_L chain sequence information of related fish species. Colony and sequence analysis demonstrated that the library was of moderate size and diversity. The observed library size and diversity may be a result of the number of primers used to generate the Dusky kob scFv fragments. The use of multiple primers may have resulted in the inclusion of undesired codons, which were identified in a few sequences in this study. Regardless, the size and diversity of this library is in accordance with similar studies describing the construction of phage display libraries. Here, we demonstrate the potential of isolated antibody fragments to bind to BMV with strong affinity. Additionally, we demonstrate the potential of the C7 dAb to detect BMV with good specificity. The C7 dAb specifically bound to the coat protein of BMV, which is known to be multifunctional and is involved in many aspects of viral infection and virus-host interactions (Yi *et al.*, 2009). This demonstrates the potential of the C7 dAb to act as a therapeutic agent that would prevent BMV infection of its host as the dAb could bind the coat protein of the virus, preventing BMV binding to host cells and thus inhibiting virus-host interactions.

Although the antibody fragments isolated in this study have been shown to have a strong affinity and specificity to BMV, further analysis of these antibody fragments is needed to determine their potential as diagnostic and therapeutic reagents. This will demonstrate the potential of this library as a resource for the production of useful diagnostic and therapeutic reagents to emerging fish pathogens that threaten the fish farming industry in South Africa.

Chapter 3

Expression of the Dusky kob derived anti-BMV single domain antibody in bacterial expression and yeast display systems

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3.1 Introduction

A fundamental aspect of phage display is the linkage between the phenotype of the displayed protein and the genotype encoding that molecule (Azzazy and Highsmith Jr., 2002). Once a scFv or dAb has been identified and isolated from a phage display library, the binding domains can be characterised based on their affinity, epitope, and biological activity; additionally, their genes can be easily manipulated to further improve the binding affinity of the antibody fragments (Powers *et al.*, 2001; Ahmad *et al.*, 2012). An important application of antibodies isolated from phage display is their potential roles as diagnostic and therapeutic agents. For most applications, scFvs or dAbs can be used without modifications, as their small size allows for effective tissue penetration and rapid clearance from the blood (Teng *et al.*, 2020). Alternatively, scFvs or dAbs can be engineered into other formats, such as a full-length IgG, to take advantage of the effector function and the prolonged serum half-life of a traditional Ig (Holt *et al.*, 2003). Depending on the intended application, the displayed antibodies can either be utilised in the original phage format, or the genes encoding the antibody fragment can be subcloned into a suitable expression system that will allow mass production of the antibody fragment as a stand-alone protein.

For diagnostic purposes, phages displaying antibody fragments are ideal for antigen detection and recognition in ELISA and western blot analyses. Detection signals can be significantly enhanced through the use of an anti-M13 antibody, since it detects the pVIII coat protein of filamentous phages, of which 2700 copies are produced per phage, thereby increasing the sensitivity of the assays (Zhang, Wang, Wu, M. Li, *et al.*, 2006; Ahmad *et al.*, 2012). Moreover, phage displayed antibodies can be easily stored for extended periods of time without the need for expensive and/or specialised equipment.

Phage displayed antibody fragments can also be used directly as therapeutics, as phage particles are good immunogens. However, large quantities of recombinant antibody fragments are required for therapeutic purposes, making the cost and time required to manufacture these fragments on such a large scale impractical due to the large amounts of helper phage needed (Andersen and Krummen, 2002; Ahmad *et al.*, 2012). Although a large number of therapeutic proteins have been produced in mammalian cell culture systems, there has been an increase in the use of microbial systems, such as yeast and bacteria, for therapeutic protein production due to their comparative simplicity and low production costs (Frenzel, Hust and Schirrmann, 2013).

3.1.1 Bacterial expression systems

The first, functional, bacterially-expressed antibody fragment was reported in 1988 by Skerra and Plückthun, who successfully expressed the variable domains of the phosphorylcholine-binding antibody in *E. coli*, which retained an affinity identical to that of the native antibody (Skerra and Pluckthun, 1988; Skerra, 1993). Following this work, *E. coli* bacterial systems have been employed to produce antibody fragments, such as antigen-binding fragments (FABs) and scFvs. *E. coli* has a faster growth rate than mammalian cells, which results in a shorter turnaround time for the production, purification, and analysis of the expressed proteins (Verma, Boleti and George, 1998). Due to decades of research, *E. coli* is a well-established organism for the expression of antibody fragments, and numerous strategies are available to express recombinant antibody fragments in these cells (Ahmad *et al.*, 2012).

The cytoplasm and periplasm are the two cellular compartments favoured for protein production in *E. coli* (Skerra, 1993; Maynard and Georgiou, 2000). Due to its large size, the cytoplasm would be ideal for the mass production of recombinant antibodies in bacteria, were it not for the reducing conditions of the cytoplasm that inhibit disulphide bond formation necessary for functional antibodies (Maynard and Georgiou, 2000; Jeong, Jang and Velmurugan, 2011; Frenzel, Hust and Schirrmann, 2013). The majority of the recombinant antibodies produced in the cytoplasm are produced as insoluble inclusion bodies that require laborious methods for refolding, resulting in decreased production yields (Verma, Boleti and George, 1998; Chadd and Chamow, 2001). This limitation can be somewhat overcome by the incorporation of a signal peptide that directs the secretion of the recombinant antibodies into the periplasmic space. The periplasmic space is regarded as the favoured compartment for antibody production in bacteria as it provides a suitable oxidative environment for the formation of disulphide bonds, and contains important chaperone and disulphide-isomerase proteins that aid in folding the recombinant antibodies (Ahmad *et al.*, 2012).

Field *et al.* (1990) demonstrated that the secretion of their recombinant antibody fragments into the periplasm of *E. coli* still resulted in a large aggregation of insoluble proteins (Field, Yarranton and Rees, 1990). Furthermore, Tsumoto *et al.* (1998) noted that some antibody fragments could not be expressed in the periplasm of *E. coli*, not even as insoluble fractions (Tsumoto *et al.*, 1998, 2003). Numerous refolding strategies are available for the recovery of insoluble and active antibody fragments, with most involving the isolation of the inclusion bodies, which are subsequently solubilised with a denaturing agent such as urea and then refolded in a suitable environment that promotes sufficient disulphide bond formation (Tsumoto *et al.*,

1998; Verma, Boleti and George, 1998; Sánchez *et al.*, 1999). However, the concentrations of the recovered proteins can either be very low or the protein fragments cannot be effectively refolded (Golovanov *et al.*, 2004; Lee and Jeong, 2015). Thus, protein expression and purification strategies have to be optimised on a case-by-case basis. Regardless, antibody fragment production in bacterial systems is often favoured over other expression systems due to their fast cell growth, short expression times, and the ease of genetic manipulation, making them an extremely cost-effective system for antibody production (Chadd and Chamow, 2001; Jeong, Jang and Velmurugan, 2011).

3.1.2 Yeast expression systems

Similar to bacteria, yeast species are favoured hosts for recombinant protein production due to their short generation time, ease of genetic manipulation, and relatively low cultivation costs (Frenzel, Hust and Schirrmann, 2013). However, unlike bacteria, yeast are eukaryotic and thus are equipped with their own glycosylation machinery, allowing for proper folding and post-translational modifications (PTMs) of recombinant antibodies (Çelik and Çalik, 2012; Lee and Jeong, 2015). Yeast cells are equipped with sophisticated glycosylation machinery and PTM processes, such as protein folding in the oxidizing environment of the endoplasmic reticulum (ER) performed in the presence of molecular chaperones, which aids in the formation of disulphide bonds, enhancing the solubility of the expressed protein (Boder, Raeeszadeh-Sarmazdeh and Price, 2012). Furthermore, studies have reported that the addition of glycans to antibody molecules is necessary for maintaining the solubility and conformation of the molecule, as well as improving the antigenicity, immunogenicity, and clearance of the molecule (Goochee *et al.*, 1991; Arnold *et al.*, 2007). This was further highlighted in 2009, when Harmsen *et al.* reported that the N-glycosylation of a dAb improved its neutralizing capacity to foot and mouth disease virus, highlighting the overall importance of glycosylation in antibody production (Harmsen, Van Solt and Fijten, 2009). The production of highly soluble proteins that undergo effective protein folding and glycosylation makes yeast systems ideal for the production of recombinant antibodies (Jeong, Jang and Velmurugan, 2011; Çelik and Çalik, 2012).

In 1988, Horwitz *et al.* (1988) first described the production of fully functional, recombinant IgG and F(ab)₂ antibody fragments in *Saccharomyces cerevisiae* yeast cells. This study not only demonstrated the potential of yeast as a system for production of correctly folded and functional antibodies, but also demonstrated the ability of yeast systems to express full-length antibodies without any loss of function (Horwitz *et al.*, 1988). To date, several yeast expression systems

have been identified for the production of recombinant proteins, each one with its own unique advantages and disadvantages (Çelik and Çalik, 2012). Among these, a significant majority of the eukaryote-produced therapeutic antibodies that are approved by the FDA and the European Medicines Agency (EMA) are produced by *S. cerevisiae* (Gerngross, 2004; Liu *et al.*, 2012; Frenzel, Hust and Schirrmann, 2013). In addition to being an extremely well characterised organism, *S. cerevisiae* is “generally recognised as safe” (GRAS) and is widely used in food and beverage fermentation processes. It is also used as a probiotic for both humans and animals, and as an effective oral delivery system for therapeutic proteins (Blanquet *et al.*, 2001). Additionally, *S. cerevisiae* is noted to be tolerant to several abiotic factors, such as low pH, high ethanol concentrations, high osmotic pressure, and low oxygen levels, making it suitable for the production of therapeutic antibodies at an industrial level (Liu *et al.*, 2012).

3.1.2.1 Yeast surface display

Although conventional yeast expression systems are successful for the production of recombinant antibodies, the recovery of these antibodies remains a challenge due to the rigid integrity of the yeast cell wall (Omara *et al.*, 2010). In addition to being primarily composed of mannoproteins and β -linked glucans, agglutinins, which function as adhesion proteins, are found on the cell walls of budding yeasts. During mating events agglutinins promote the aggregation of opposite protein types (Chen *et al.*, 1995; Sheehan and Marasco, 2015). In 1997, Boder and Wittrup demonstrated that by exploiting the properties of agglutinins, proteins of interest could be tethered to the outer surface of the yeast cell wall, allowing them to interact with other proteins and small molecules in solution, thereby removing the need to extract expressed proteins (Kieck *et al.*, 1997; Boder, Raeeszadeh-Sarmazdeh and Price, 2012). This expression method was termed “yeast surface display” and is an alternative to recombinant protein expression and purification. Yeast surface display is characterised by proteins of interest expressed as a fusion to the Aga2p mating agglutinin protein, which links directly to the Aga1p protein of the yeast cell wall by two disulphide bonds upon secretion (Colby *et al.*, 2004). The recombinant fusion vector is subsequently transformed into a yeast strain that has the Aga1p gene integrated into its chromosome (Xin and Shusta, 2005). The expression of both agglutinin proteins is controlled by a galactose inducible (GAL1) promoter, and in the presence of galactose the target protein is displayed on the yeast cell surface, with each cell displaying approximately 50,000 copies of the target protein (Gera, Hussain and Rao, 2013; Sheehan and Marasco, 2015) (Fig. 3.1).

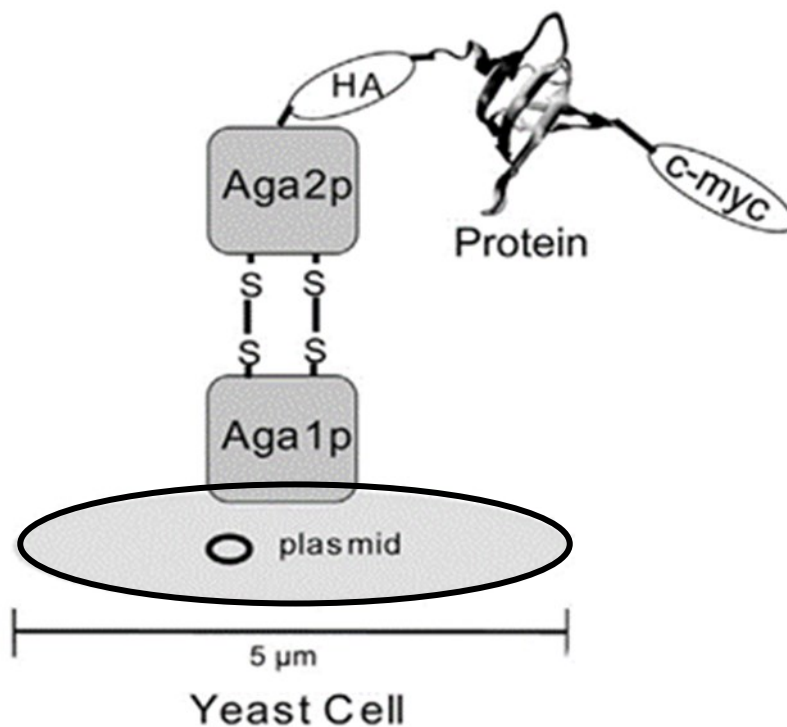


Figure 3.1 Yeast surface display of target protein. The target protein is displayed on the surface of yeast cells as a fusion protein with Aga2p. An N-terminal hemagglutinin (HA) and a C-terminal c-myc tag allow for immunofluorescence detection (Gera, Hussain and Rao, 2013).

Currently, yeast surface display is used as an alternative to phage display for the isolation of antibody fragments, as the yeast ER ensures that displayed proteins are correctly folded. Additionally, desired antibodies can be rapidly isolated and quantified by fluorescence-activated cell sorting (FACS) using epitope tags fused to the fusion product (Colby *et al.*, 2004) (Fig. 3.1). Furthermore, larger antibody fragments, such as F(ab)₂ and whole IgGs, have been displayed on the surface of yeast cells, which is not possible using phage display (Sheehan and Marasco, 2015). In 2010, Sun *et al.* proposed an alternative use of yeast surface display as a potential carrier of therapeutic proteins, which could be orally administered to patients (Sun *et al.*, 2010; Corchero *et al.*, 2013). The authors reported that oral administration of yeast surface-displayed salmon calcitonin induced a long term hypocalcaemic effect in Wistar rats, similar to that of a commercial salmon calcitonin, without any adverse effects (Sun *et al.*, 2010). Furthermore, yeast surface display has been employed as a means to develop whole-cell live vaccines by expressing recombinant antigens on yeast cell surfaces (Luo *et al.*, 2015). The yeast surface display of various recombinant antigens has been shown to be a successful means of developing vaccines against various marine pathogens such as RSIV, grass carp reovirus, and

Vibrio harveyi (Tamaru *et al.*, 2006; Zhu *et al.*, 2006; Luo *et al.*, 2015). These studies demonstrate the potential of yeast surface display as not only a low-cost method for the isolation and production of therapeutic proteins, but also as a safe and low-cost delivery system that can be directly administered without the addition of potentially undesirable adjuvants.

3.1.3 Aims of this chapter

Although phage display is an effective system for the isolation of useful proteins and antibody fragments, its direct use as a diagnostic or therapeutic agent is limited. Thus, alternative protein expression systems are required for the production of beneficial proteins isolated by phage display, for use in downstream applications. Therefore, the aim of this chapter was to identify an effective means of expressing and producing the anti-BMV dAb selected by phage display in Chapter 2. The Dusky kob gene encoding the anti-BMV dAb was subcloned and expressed in an *E. coli* expression system as well as on the surface of *S. cerevisiae* yeast cells. Furthermore, this chapter aimed to confirm that the expressed anti-BMV dAb retained its biological function by way of retaining affinity to the plant virus BMV, as it has been reported that the biological function of recombinant antibodies can be affected when expressed in other expression systems and not expressed on the surface of a phage, leading to decreased antibody affinity (Van Wyngaardt *et al.*, 2004).

The identification of an expression system that not only allows for the rapid, high-yield production of a recombinant antibody, but also retains its biological function, will aid in the development of a pipeline whereby antibodies can be rapidly identified and expressed for appropriate application.

3.2 Methods and Materials

3.2.1 Expression of an anti-BMV single domain antibody gene in *E. coli*

3.2.1.1 Cloning of an anti-BMV single domain antibody gene into the vector pPROEX-Htb

The gene encoding the anti-BMV dAb in clone C7 was amplified using pSEX81 sequence primers described in Table 2.3 (Chapter 2, Section 2.2.1). Following amplification, the PCR products and the vector pPROEX-Htb (Appendix B.2.1) were digested with *Nco*I-HF[®] (New England Bio-Labs Inc, USA) and *Hind*III-HF[®] (New England Bio-Labs Inc, USA) at 37°C overnight. Following digestion, the PCR products and vector were resolved through a 1% (w/v) EtBr stained agarose gel at 110 V for 1 h. The resolved products of the appropriate size were excised from the agarose gel and purified using the Wizard[®] SV Gel and PCR Clean-Up System

(Promega, USA) following the manufacturer's instructions. DNA was eluted in 35 µL of nuclease-free water. The concentration and purity of the eluted DNA was determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Inc).

Following purification, the ligation reaction of purified vector and the purified anti-BMV dAb gene, where the gene of interest was cloned between the polyhistidine tag and stop codon of the vector, was carried out overnight at room temperature using T4 DNA Ligase (New England Bio-Labs Inc, USA) and a 1:5 molar ratio of vector to insert. The recombinant pPROEX_HTb-dAb vector was then transformed into competent *E. coli* DH5α cells using the heat-shock method described by Seidman *et al.* (1997). The transformed cells were plated onto LA supplemented with 100 µg/mL of ampicillin (final conc.) and incubated at 37°C overnight.

To determine if the resulting colonies contained the desired plasmid, the transformants were screened by cPCR using the primers listed in Table 3.1 and were inoculated in LB and incubated overnight at 37°C with shaking. The overnight culture was collected following centrifugation and plasmids were extracted using the QiAprep Spin Miniprep Kit (Qiagen, Germany) as per the manufacturer's instructions. To determine if the anti-BMV dAb gene was correctly inserted into the pPROEX-Htb plasmid, between the polyhistidine tag and stop codon, extracted plasmids were sent to Macrogen Europe for sequencing.

Table 3.1 pPROEX_Htb sequence primers and anti-BMV dAb gene specific forward primer

Primer	Nucleotide sequence (5'-3')	Product Size (bp)
M13 Forward primer	CGCCAGGGTTTCCCAGTCACGAC	~500 bp
pTrcHis Reverse primer	GATTTAATCTGTATCAGG	
dAb Forward primer	GCCATGGCTGACGAGCTGCTTT	

3.2.1.2 Expression and extraction of anti-BMV dAb in *E. coli* BL21

Prior to the expression of the anti-BMV dAb, the sequence identity and reading frame of the recombinant plasmids was confirmed by sequence analysis using CLC Main Workbench (v7.7), to ensure the correct product was expressed. Plasmids shown to have the correct reading frame and sequence identity were selected and transformed into competent *E. coli* BL21 cells using the heat-shock method described by Seidman *et al.* (2001). Transformed cells were plated

onto LA supplemented with 100 µg/mL of ampicillin (final conc.) and incubated at 37°C overnight.

A single positive colony was inoculated into 10 mL LB supplemented with 100 µg/mL of ampicillin (final conc.) and incubated overnight at 30°C with shaking. Following incubation, the overnight culture was inoculated into 25 mL LB supplemented with 100 µg/mL of ampicillin, to a final OD_{600nm} of 0.1. The 25 mL culture was incubated at 37°C with shaking, until an OD_{600nm} of 0.6 was reached. Isopropyl-β-D-thiogalactoside (IPTG; final concentration of 2 mM) was added to stimulate the induction of the *trc* promoter in the pPROEX-Htb vector. Following induction, the culture was incubated at 37°C for 6 h with shaking, and 1 mL samples were collected every 2 h. The cells were harvested by centrifugation at 6000 *x g* for 20 min.

The cell pellets were resuspended in 2.5 mL of a native buffer solution (100 mM Tris-Cl, 10 mM sodium chloride). The resuspended cells were lysed by sonication using the following conditions: amplitude between 6 and 10 µm, sonication conducted over 30 s intervals followed by 15 s pauses for a total of 10 min. Following sonication, the cell suspension was centrifuged at 7000 *x g* for 15 min at 4°C and the supernatant collected. The cell pellet was resuspended in 10 mL of a urea lysis buffer (8 M urea, 10 mM Tris base, 100 mM NaH₂PO₄) to determine whether any target protein formed inclusion bodies and remained after cell lysis. The resuspended cells were incubated in the urea lysis buffer for 30 min, followed by centrifugation at 7000 *x g* for 30 min at room temperature. The supernatant was collected and stored at -20°C until needed. Protein samples were analysed by SDS-PAGE and western blotting.

3.2.1.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE gels were prepared as follows: the stacking gels comprised 0.375 M Tris-Cl/SDS pH 6.8, 4% (v/v) acrylamide 37:1, 0.05% (w/v) AMPS, and 0.01% (v/v) TEMED. The resolving gels comprised 0.125 M Tris-Cl/SDS pH 8.8, 15% (v/v) acrylamide 37:1, 0.2% (w/v) AMPS, and 0.04% (v/v) TEMED. The protein samples were diluted in 5× sample application buffer (1 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 25% w/v SDS, 5 mg Bromophenol Blue, and 21% w/v dithiothreitol) to a final concentration of 100 µg/mL, and boiled at 95°C for 15 min. The recombinant proteins were resolved on a 15% resolving gel. Electrophoresis was performed at 4°C at 100 V for 2 h or until the dye front reached the bottom of the gel. Once resolved, the gels were prepared for either visualisation by Coomassie staining, or for western blot analysis.

To visualise the presence of total proteins, gels were stained with Coomassie stain (0.5 g Coomassie blue G-250, 225 mL methanol, 50 mL acetic acid, and 225 mL dH₂O) at 37°C for 1 h, and then destained overnight at room temperature in destaining solution (300 mL methanol, 100 mL acetic acid, and 600 mL dH₂O). Gels were visualised on a Bio-Rad ChemiDoc™ XRS+ (Bio-Rad, USA) and analysed using Image Lab V5.2.1 (Bio-Rad, USA).

3.2.1.4 Western blot analysis

Western blot analysis was conducted to confirm the presence of the expressed anti-BMV dAb in the samples extracted under native and denaturing conditions. The proteins separated by SDS-PAGE were electrophoretically transferred onto a nitrocellulose membrane at 10 V for 1 h, using a Trans-Blot SD Semi Dry Transfer Cell (Bio-Rad, USA). The membranes were blocked overnight at room temperature in blocking buffer (2% w/v BSA, PBS) with gentle agitation.

The membranes were washed three times with PBS-T for 15 min at room temperature, followed by incubation with the primary antibody, rabbit anti-6-His antibody (Sigma-Aldrich, USA) diluted 1:2000 in blocking buffer (2% BSAPBS/0.1% Tween20) for 1 h at 37°C with gentle agitation. Following incubation, membranes were washed three times with PBS-T for 15 min at room temperature, followed by a final wash with PBS for 10 min. Bound primary antibodies were probed for 1 h at 37°C by incubation with a secondary antibody, goat anti-rabbit IgG (whole molecule) peroxidase antibody (Sigma-Aldrich, USA) diluted 1:5000 in blocking buffer, with gentle agitation. The membranes were washed three times with PBS-T for 15 min at room temperature, followed by a final wash with PBS for 10 min. Chemiluminescent signals were detected by adding 2 mL of TMB membrane peroxidase substrate and allowing it to develop in the dark for up to 30 min. Membranes were visualised on a Bio-Rad ChemiDoc™ XRS+ system immediately following application of the detection reagent, and analysed using Image Lab V5.2.1 software (Bio-Rad, USA).

3.2.1.5 Purification of His-tagged anti-BMV dAb from *E. coli*

In order to purify the His-tagged anti-BMV dAb expressed in *E. coli* BL21 cells, 10 mL of protein lysate was prepared following the resuspension of induced *E. coli* BL21 cell pellets in 10 mL of urea lysis buffer, as described in Section 3.2.1.2. Following the preparation of the protein lysate, batch purification of His-tagged anti-BMV dAb was performed according to the method described by Spriesterbach *et al.* (2015). Briefly, 1 mL of resuspended HisPur™ Ni-NTA Resin (Thermo Fischer Scientific) was added to 10 mL of protein lysate and incubated at

room temperature for 1 h with continuous rotation. Following incubation, the binding suspension was transferred to a Poly-Prep® Chromatography Column (Bio-Rad). The bottom cap of the column was removed and the flow-through was collected. The column was washed three times with 5 mL of urea wash buffer (8 M urea, 10 mM Tris base, 100 mM NaH₂PO₄, and 20 mM imidazole), with 1.5 mL fractions collected. Finally, bound His-tagged proteins were eluted with a urea elution buffer (8 M urea, 10 mM Tris base, 100 mM NaH₂PO₄, and 250 mM imidazole).

The absorbance at 280 nm of each fraction was determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Inc), and an elution profile was generated. The success of the purification was analysed by SDS-PAGE.

3.2.1.6 Renaturation of purified anti-BMV dAb

To renature and refold the purified anti-BMV dAb, the urea solution was removed by dialysis using a D-Tube™ Dialyzer Maxi (Merck) with a molecular weight cut-off of 6-8 kDa, following the manufacturer's instructions and the method described by ShengFeng *et al.* (2003). Briefly, purified proteins were dialysed against renaturing buffer A (5 M urea, 10 mM Tris base, and 100 mM NaH₂PO₄) at 4°C for 12 h, followed by a change to renaturing buffer B (3 M urea, 10 mM Tris base, and 100 mM NaH₂PO₄) for a further 12 h. The renatured anti-BMV dAb was finally dialysed against PBS (pH 8.0) for 24 h, changing the buffer every 12 h. The concentration of the purified protein was determined by SDS-PAGE analysis against BSA protein standards that ranged in concentration from 2.358 to 0.189 mg/mL. The resulting purified protein concentration was converted to “total yield of protein” using the following equation:

Total yield of protein (mg/L)

$$= \frac{(\text{Concentration of purified protein (mg/L)} \times \text{Final volume of purified protein (L)})}{\text{Initial culture volume (L)}}$$

The renatured anti-BMV dAb in PBS was tested for activity using ELISA.

3.2.1.7 ELISA to test the binding affinity of renatured anti-BMV dAb

A Nunc polysorp 96-well plate was coated overnight at 4°C with 100 µL/well of decreasing concentrations of BMV (50 µg/mL – 0.39 ng/mL). Wells coated with PBS-only served as negative controls. The following day, the coating solution was removed, and the wells were washed three times with PBS-T. Non-specific binding was blocked with 300 µL/well of 2% BSAPBS and incubated at 37°C for 1 h. The ELISA plates were washed three times with PBS-

T. Bound BMV was probed with 100 μ L/well of refolded anti-BMV dAbs diluted to either 100 μ g/mL, 10 μ g/mL, or 1 μ g/mL, and incubated at 37°C for 1 h followed by three washes with PBS-T. The bound anti-BMV dAbs were detected with 100 μ L/well of rabbit anti-6-His antibody (Sigma-Aldrich, USA) diluted 1:2000 in blocking buffer (2% BSAPBS/0.1% Tween20) for 1 h at 37°C. Wells were washed three times with PBS-T. The bound anti-His antibody was detected by adding 50 μ L/well goat anti-rabbit IgG probed with alkaline phosphatase (Sigma-Aldrich, USA), diluted 1:5000 in BSAPBS-T, and incubated at 37°C for 1 h. After washing three times with PBS-T, and a final single wash with PBS, 200 μ L/well of enzyme substrate pNPP (Sigma-Aldrich, USA) was added, before incubation at room temperature for 30 min in the dark. After 30 min, the absorbance was measured at 405 nm on the Multiskan Go Spectrophotometer (Thermo Scientific, USA).

3.2.2 Yeast surface display of a Dusky kob anti-BMV single domain antibody

3.2.2.1 Cloning and expression of an anti-BMV single domain antibody gene in pYD1

The anti-BMV dAb gene in pPROEX_Htb was subcloned into the yeast display expression vector pYD1 by PCR using the primers listed in Table 3.2. These primers were designed to include the restriction sites, *KpnI* and *ApaI*, required for subcloning into pYD1 (Appendix B.2.2). Following amplification, the PCR products and the yeast vector pYD1 were digested with *KpnI*-HF[®] (New England Bio-Labs Inc, USA) and *ApaI*-HF[®] (New England Bio-Labs Inc, USA) at 37°C overnight. Following digestion, the PCR products and vector were resolved on a 1% (w/v) EtBr-stained agarose gel at 110 V for 1 h. The desired products were excised from the agarose gel and purified using the Wizard[®] SV Gel and PCR Clean-Up System (Promega, USA) following the manufacturer's instructions. DNA was eluted in 35 μ L of nuclease-free water. The concentration and purity of the eluted DNA was determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Inc).

Following purification, the ligation reaction of purified vector and gene products was carried out using T4 DNA Ligase (New England Bio-Labs Inc, USA), using a 1:5 molar ratio of vector to insert, overnight at room temperature. The recombinant pYD1_dAb vector was then transformed using the heat-shock method, as described above in Section 3.2.1.2.

The transformed colonies were screened by cPCR (described in Chapter 2, Section 2.2.1.6.1) and sequenced, as previously described, using the primers listed in Table 3.2.

Table 3.2 Primers used to subclone the anti-BMV dAb gene product into pYD1 and pYD1 sequencing primers

Primer	Nucleotide sequence (5'-3')	Product size (bp)
pYD1 BMV Fwd	GATCG <u>GTACC</u> AGACGAGCTGCTTTGTA	~350
pYD1 BMV Rvs	GAG <u>GGCCC</u> CACCTCGAGCACAATAAT	
pYD1 Seq Fwd	AGTAACGTTTGTCTAGTAATTGC	~670 (with insert)
pYD1 Seq Rvs	GTCGATTTTGTACATCTACAC	~400 (no insert)

The bold, underlined font indicates the recognition site for the restriction enzyme *Kpn*I included in the forward primer and *Apa*I in the reverse primer.

3.2.2.2 Expression and display of anti-BMV dAb on the surface of *S. cerevisiae*

3.2.2.2.1 Preparation of competent *S. cerevisiae* EBY100

In order to produce competent *S. cerevisiae* EBY100, a single colony of *S. cerevisiae* EBY100 was inoculated into 10 mL of YPD broth (1% yeast extract powder, 2% peptone powder, and 2% dextrose (D-glucose) [final conc.]) and incubated overnight at 30°C with shaking at 250 rpm. Following incubation, the overnight culture of *S. cerevisiae* EBY100 was made competent using the S.C EasyComp™ Transformation Kit (Invitrogen). Briefly, the OD₆₀₀ of the overnight culture was diluted to an OD₆₀₀ of 0.2 in a fresh 10 mL volume of YPD media. The culture was incubated at 30°C with shaking at 250 rpm, until a final OD₆₀₀ of 1.0 was achieved. Following incubation, the culture was centrifuged at 300 $\times$ g for 5 min at room temperature. The cell pellet was resuspended in 1 mL of Solution 1 and the cells were made competent by the addition of 1 mL of Solution II according to the manufacture's protocol. The competent yeast cells were stored at -80°C until needed.

3.2.2.2.2 Transformation of competent *S. cerevisiae*

Competent *S. cerevisiae* cells were transformed with either pYD1 or recombinant pYD1_dAb using the S.C EasyComp™ Transformation Kit (Invitrogen) according to the manufacturer's instructions. Briefly, a total of 5 µg of plasmid was added to 50 µL of competent *S. cerevisiae* cells, followed by 500 µL of Solution III, according to the manufacturer's instructions. The yeast cells were vortexed briefly and incubated at 30°C for 1 h, with mixing every 15 min. A volume of 100 µL of the transformation reaction was plated on minimal dextrose plates (0.67% yeast nitrogen base (YNB) (supplemented with ammonium sulphate and lacking amino acids), 2% glucose, 0.01% leucine, and 1.5% agar [final conc.]) and incubated for up to 4 days at 30°C.

3.2.2.2.3 Induction of recombinant *S. cerevisiae* cells

A single transformed yeast colony was inoculated into 50 mL of YNB-Casamino acids (CAA) (0.67% YNB (with ammonium sulphate, without amino acids), 0.5% CAA, and 2% glucose) and incubated at 30°C with shaking at 250 rpm for approximately 24 h until a desired OD₆₀₀ of between 2 and 5 was reached. At the desired OD₆₀₀ of between 2 and 5, the cells were induced by the addition of 2% galactose (final conc.) and incubated at 20°C with shaking at 250 rpm for a 72 h period. One-millilitre samples were collected every 24 h to determine the optimum induction time by observing the binding affinity of the yeast displayed anti-BMV dAb to BMV using an ELISA. Each 1 mL sample was centrifuged at 3500 $\times$ g at 4°C for 10 min and the pellets resuspended in 1 mL PBS. The cells were stored in the dark at 4°C, until needed, for no longer than one week. The optimal induction time was found to be 72 h post-induction (P.I.).

3.2.2.3 Binding affinity of yeast displayed anti-BMV dAb to BMV

A sandwich ELISA was conducted to determine the binding of the yeast displayed anti-BMV dAb to BMV. Recombinant pYD1_dAb yeast cells were induced for a 72 h period, with samples collected at 48 and 72 h. Acting as the capture antibody, wells of a Nunc polysorp 96-well plate were coated with 100 μ L/well of serially diluted yeast cells displaying the anti-BMV dAb, and incubated overnight at 4°C. Thus, the wells were coated with either 6×10^7 , 3×10^7 , 2.4×10^7 , 1.5×10^6 , 6×10^5 or 3×10^5 yeast cells/mL. A negative control was included that consisted of 6×10^7 yeast cells transformed with pYD1. Following overnight incubation, the wells were washed three times with PBS-T. Non-specific binding was blocked by the addition of 300 μ L/well of 2% BSAPBS followed by a 1 h incubation at 37°C. The wells were washed as before, followed by the addition of 100 μ L/well of 175 ng/ μ L BMV, before incubation for 1 h at 37°C. The bound BMV was detected by probing with a rabbit anti-BMV monoclonal antibody (generously supplied by Prof. E. Rybicki, Biopharming Research Unit, Molecular & Cell Biology Department, University of Cape Town), diluted 1:1000 in blocking buffer (2% BSAPBS/0.1% Tween20) for 1 h at 37°C. The wells were washed three times with PBS-T. The bound anti-BMV antibody was detected by adding 50 μ L/well goat anti-rabbit IgG probed with alkaline phosphatase (Sigma-Aldrich, USA), diluted 1:5000 in BSAPBS-T, for 1 h at 37°C. After washing three times with PBS-T, and a final single wash with PBS, 200 μ L/well of enzyme substrate p-NPP (Sigma-Aldrich, USA) was added, before incubation at room temperature for 30 min in the dark. Then, the absorbance was measured at 405 nm on a Multiskan Go Spectrophotometer (Thermo Scientific, USA).

3.2.2.4 Competition ELISA

A Nunc polysorp 96-well plate was coated with 100 μL /well of 10 $\mu\text{g}/\text{mL}$ of BMV diluted in PBS, and incubated overnight at 4°C. Following overnight incubation, wells were washed three times with PBS-T. Non-specific binding was blocked with 300 μL /well of 2% BSAPBS and incubated at 37°C for 1 h. After blocking, 100 μL /well of either a 1:5000, 1:10,000, 1:15,000, or 1:20,000 dilution of anti-BMV IgG and 100 μL /well of yeast cells displaying the anti-BMV dAb at a titre of either 6×10^7 , 3×10^7 , 2.4×10^7 , 1.5×10^6 , 6×10^5 or 3×10^5 yeast cells/mL were added to each well and allowed to compete for binding to BMV for 1 h at 37°C. Yeast cells transformed with pYD1 (6×10^7 cells/mL) and wells incubated with anti-BMV IgG only (without any yeast cells added as competition) served as negative controls. After incubation, the wells were washed three times with PBS-T. Bound anti-BMV IgG mAb was detected by adding 50 μL /well goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, USA), diluted 1:5000 in BSAPBS-T, and incubated at 37°C for 1 h. After washing three times with PBS-T, and a final single wash with PBS, 200 μL /well of enzyme substrate pNPP (Sigma-Aldrich, USA) was added, and the microtitre plate incubated at room temperature for 30 min in the dark. Then, the absorbance was measured at 405 nm on a Multiskan Go Spectrophotometer (Thermo Scientific, USA).

3.2.2.5 Transmission electron microscope (TEM) imaging of yeast displayed anti-BMV single domain antibody bound to BMV

Recombinant pYD1_dAb yeast cells were induced as described in Section 3.2.2.2.3 for a 72 h period. Non-induced yeast cells, transformed with pYD1-dAb, grown for a 72 h period, served as a negative control. After the 72 h induction, a total of 4 $\times$ 1 mL aliquots, taken from each experimental sample, were collected. The cells were subjected to two rounds of centrifugation at 3600 $\times$ g for 5 min at 4°C and resuspended in a final volume of 1 mL ice-cold PBS. The yeast cells taken from the experimental and control samples were incubated with 350 ng/mL of BMV for 2 h at room temperature with gentle agitation. After incubation, the yeast cells were centrifuged at 500 $\times$ g for 5 min at 4°C to remove any unbound virus. The cell pellets were stored for TEM analysis.

To prepare for TEM, the cell pellets were fixed by resuspension in a primary fixation solution (2.5% glutaraldehyde, in 0.1 M phosphate buffer, pH 7.4) and incubated for 16 h at 4°C. Following incubation, the samples were washed twice (5 min each) in wash buffer (0.1 M phosphate buffer, pH 7.4). After the wash steps, the samples were fixed by resuspending the cell pellets in a secondary fixation solution (1% osmium tetroxide in 0.1M phosphate buffer,

pH 7.4) and incubating them for 1 h at room temperature. Following incubation, the samples were washed twice in wash buffer (5 min per wash) as described above, followed by an additional two washes in distilled water (5 min per wash). Following fixation, samples were dehydrated as follows: the samples were resuspended in 30% ethanol and incubated for 10 min. The samples were centrifuged at 6000 $\times$ g for 5 min and were resuspended and dehydrated using an ethanol gradient, beginning with 50% ethanol for 10 min, followed by 70% for 10 min, 90% for 10 min, and 95% for 10 min. The samples were centrifuged at 6000 $\times$ g for 5 min after each step and the cells were resuspended in the appropriate ethanol dilution. A final dehydration step consisted of resuspending and dehydrating the cells in 100% ethanol for 10 min, followed by a final dehydration step in 100% ethanol for 10 min. The samples were further dehydrated for 2 $\times$ 10 min in acetone. The samples were briefly centrifuged and resuspended in a 1:1 ratio of Spurr's resin (Sigma-Aldrich, USA) to acetone and incubated overnight at room temperature. Following overnight incubation, the samples were added to a 2:1 resin to acetone solution and incubated for 8 h at room temperature, followed by an overnight incubation in a 3:1 ratio of resin to acetone. The following day, the samples were incubated in a 10:1 ratio of resin to acetone for 8 h, followed by centrifugation. Samples were resuspended and incubated in 100% resin overnight at room temperature. After incubation, the samples were added to fresh resin (100%) and incubated for 2 h. The samples were then placed into BEEM[®] capsules (Agar scientific, UK) and polymerised overnight at 60°C.

Following polymerisation, 100 nm sections of each of the induced and non-induced yeast samples were prepared using an ultramicrotome (Reichert FCS Cryo Ultramicrotome, Leica Microsystems). The sections were placed onto copper grids and stained with 3% (w/v) uranyl acetate and lead citrate for 30 min prior to being examined by TEM (Tecnai F20 model instrument, FEI Co).

3.2.3 Statistical analysis

In order to determine if there was a significant level of binding inhibition of the anti-BMV IgG to BMV, as a result of the binding of the yeast displayed anti-BMV dAb, a one-way ANOVA, followed by a Bonferroni post-hoc test, was conducted using Prism 5 software (GraphPad). The percentage binding inhibition for each antibody dilution is presented as mean $\pm$ standard deviation ($n = 3$). A p value of less than 0.05 ($p \leq 0.05$) is considered significant of the binding inhibition of the yeast displayed anti-BMV dAb to a set concentration of anti-BMV IgG.

3.3 Results

3.3.1 Expression of a Dusky kob anti-BMV single domain antibody in a bacterial expression system

3.3.1.1 Expression of a Dusky kob anti-BMV dAb in *E. coli* BL21

The Dusky kob gene encoding the anti-BMV dAb was sub-cloned into the pPROEX-Htb vector and subsequently transformed into *E. coli* BL21 cells for expression of the anti-BMV dAb. Two randomly selected transformants were chosen to confirm the open reading frame and identity of the inserted gene by cPCR analysis and sequencing using the dAb forward and pTrcHis reverse primers (Table 3.1).

Agarose gel analysis of the cPCR products showed an amplicon size of ~350 bp (Fig. 3.2) which was the size expected when using the primers described in Table 3.1. No products were observed for the pPROEX-Htb-only sample (Fig. 3.2), indicating that the selected colonies contained the desired recombinant pPROEX-Htb_dAb. This was confirmed by sequencing the recombinant vectors. A BLASTn analysis identified the gene to have a high level of homology to an IgM heavy chain of the European seabass (*Dicentrarchus labrax*) and sequence analysis using CLC Main Workbench 7 confirmed the polyhistidine epitope used for protein detection and purification was in the correct reading frame to allow for translation of the dAb gene (Fig. 3.3).

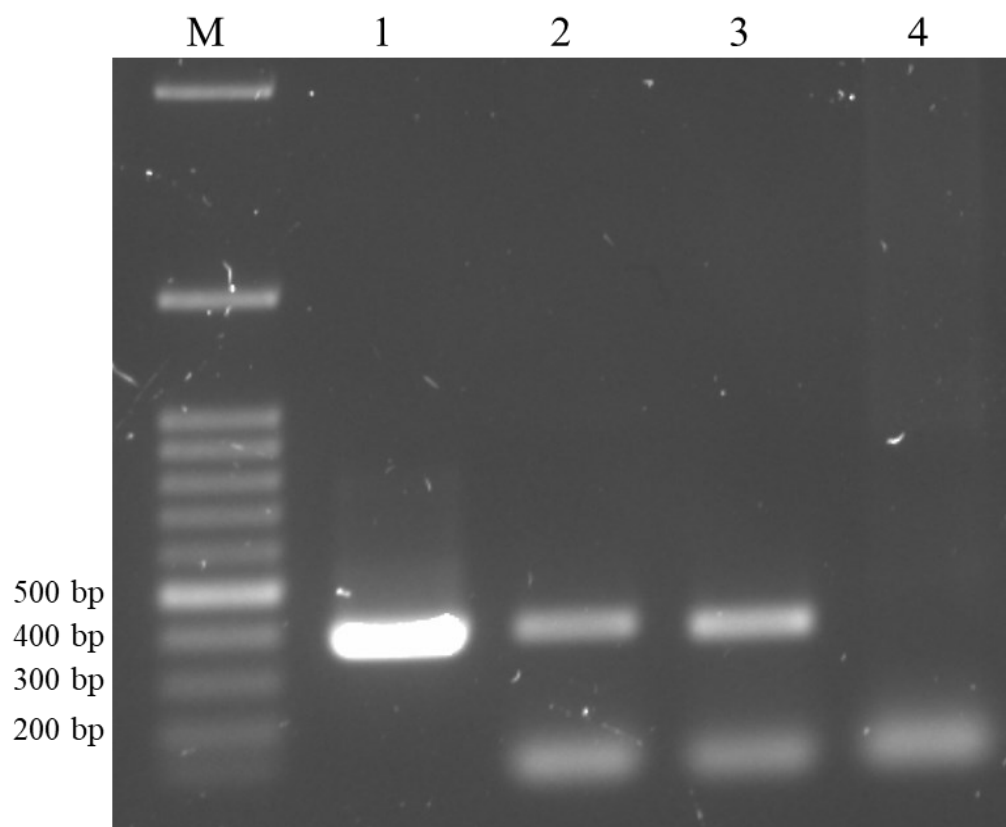


Figure 3.2 Colony PCR of recombinant *E. coli* BL21 pPROEX-Htb_dAb cells using a gene specific dAb forward primer and the vector specific pTrc His Reverse primer. M = One Mark B (GeneDirex) 100 bp DNA marker. 1: pPROEX-Htb_dAb (~350 bp) positive control; 2-3: randomly selected recombinant *E. coli* BL21 colonies (~350 bp); 4: pPROEX-only negative control.

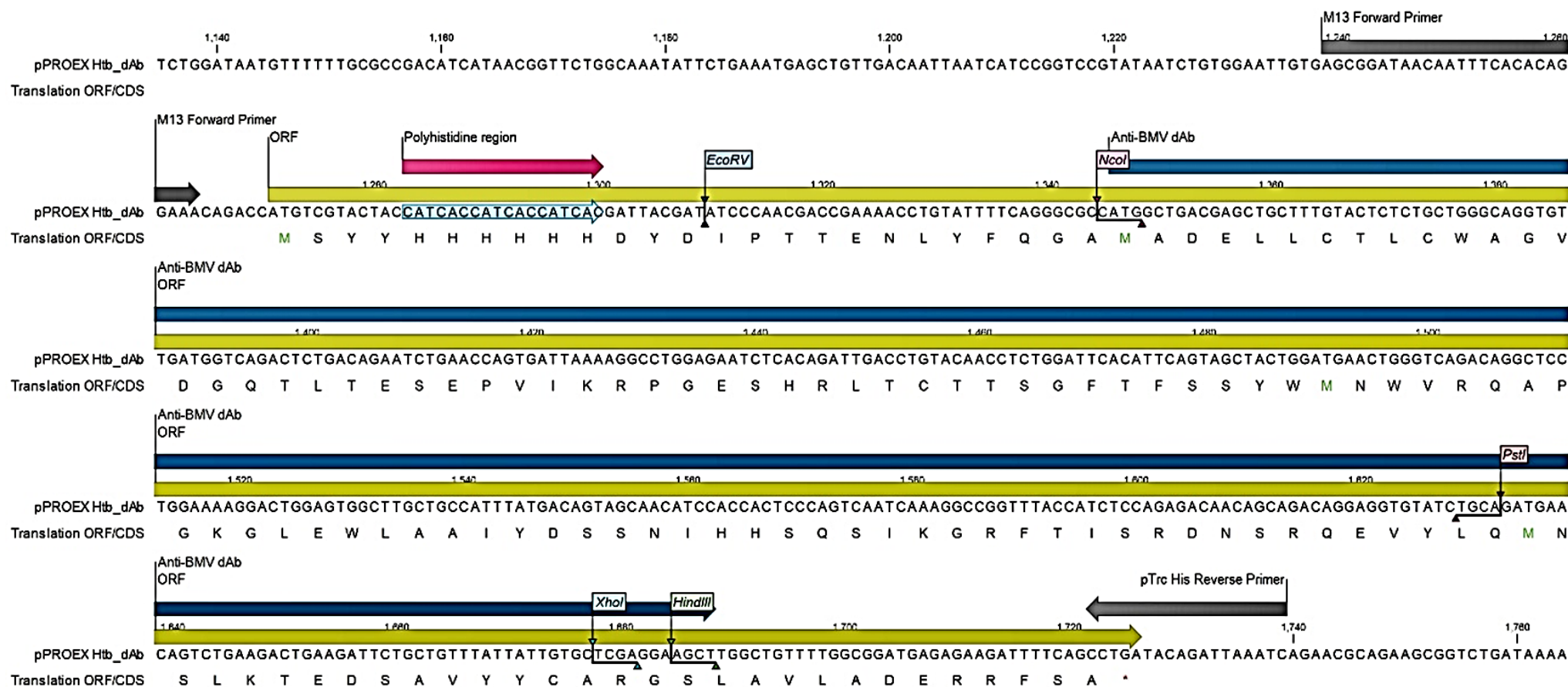


Figure 3.3 Fully annotated gene and protein sequence of recombinant pPROEX-Htb_dAb. The polyhistidine epitope region used for detection and purification of expressed proteins is indicated by the red arrow.

In order to determine the timepoint at which the anti-BMV dAb was optimally expressed in *E. coli* BL21 cells, the transformants were induced with IPTG for a period of 6 h at 37°C, with samples collected every 2 h. The samples collected at each time point were lysed with either a native or denaturing buffer to determine whether or not the protein was expressed as inclusion bodies. Expression of the putative anti-BMV dAb at 2, 4, and 6 h P.I. was indicated by the presence of a distinct band of approximately 17 kDa in the samples that were lysed with the denaturing buffer (Fig 3.4A 1-4). Optimal expression of the antibody fragment was observed at 6 h P.I. (Fig. 3.4A). No expression of anti-BMV dAb was observed in the samples lysed with the native buffer at any of the sampled time points (Fig. 3.4A 6-8).

The identity of anti-BMV dAb was further validated by western blot analysis, probing with an anti-6-his tag antibody. The putative anti-BMV dAb was detected in the 2, 4, and 6 h P.I. samples lysed with the denaturing buffer (Fig. 3.4B 1-4). In the samples lysed with the native buffer, the anti-BMV dAb band was only observed faintly in the sample extracted 4 h P.I. (Fig. 3.4B 7). No bands of approximately 17 kDa were observed in the *E. coli* BL21 negative control cells.

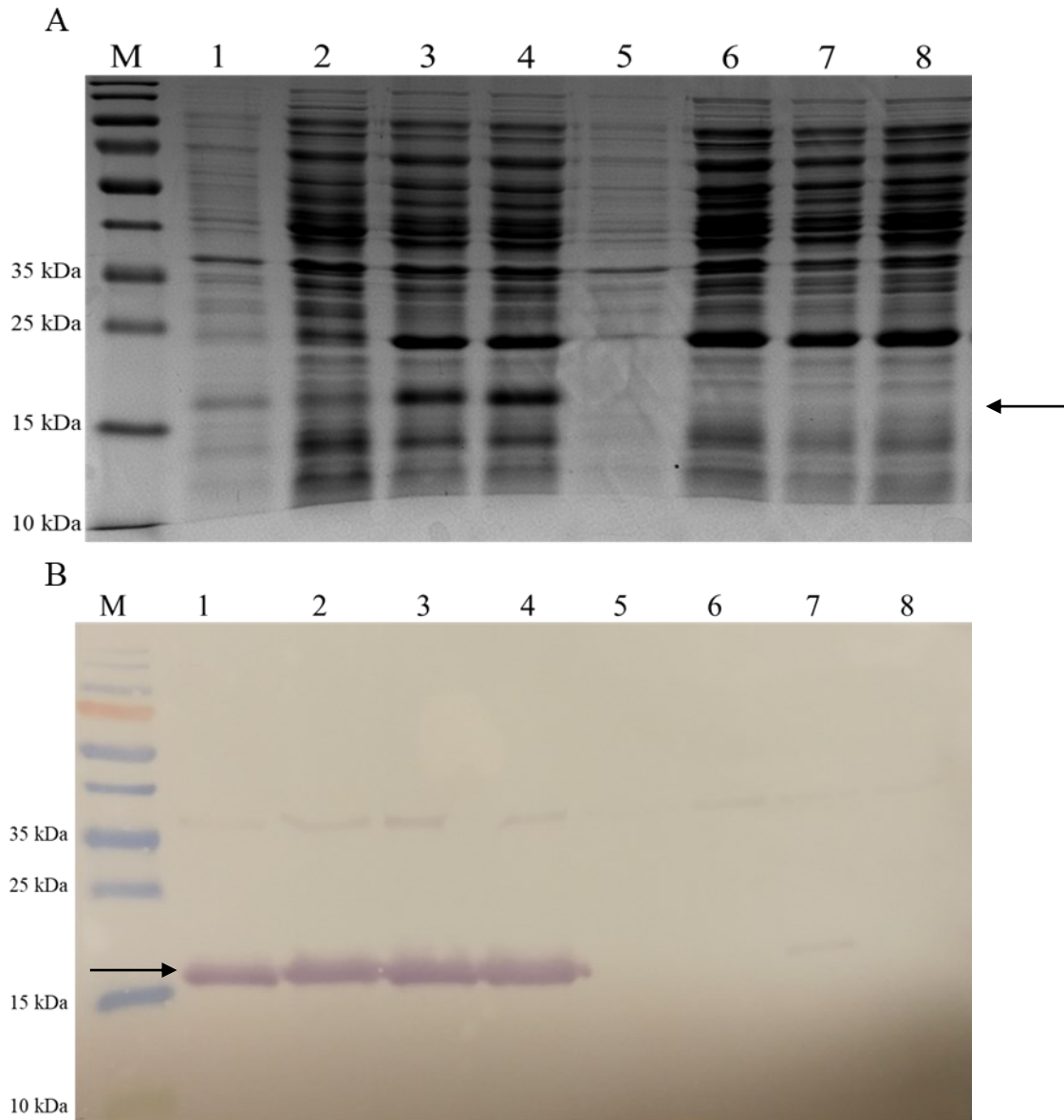


Figure 3.4 A) SDS-PAGE and B) western blot analysis of extracted proteins from recombinant *E. coli* BL21 induced with 2 mM IPTG over a 6 h period, sampling every 2 h. Proteins were extracted with either a denaturing buffer (lanes 1-4) or a native buffer (lanes 6-8). Lanes 1 and 6 were collected 2 h P.I., lanes 2 and 7 were collected 4 h P.I., and lanes 3, 4, and 8 were collected 6 h P.I. M: Hi Mark™ Pre-stained Protein Standard (ThermoFisher Scientific); 5: non-recombinant *E. coli* BL21 cells lysed with a native buffer. B) Extracted proteins were probed with an anti-6-His antibody (Sigma-Aldrich, USA) to detect expression of the putative anti-BMV dAb (~17 kDa). Black arrows indicate the protein of interest.

3.3.1.2 Batch purification of the putative Dusky kob anti-BMV single domain antibody by immobilised metal affinity chromatography (IMAC)

The putative anti-BMV dAb was expressed in *E. coli* BL21 cells following induction with 2 mM of IPTG for 6 h at 37°C. IMAC was used to purify the His-tagged anti-BMV dAb, using a HisPur™ Ni-NTA resin. The His-tagged putative anti-BMV dAb was successfully purified and eluted in the presence of 250 mM imidazole (Fig. 3.5B and C). Elution fractions E1-8 are presented in Figure 3.5B, and elution fractions E10, 11, 12, 13, and 9 are presented in Figure 3.5C. Due to loading issues, elution fraction E9 was loaded in the final lane of Figure 3.5C. Non-target proteins of approximately 25 kDa were eluted together with the target protein (putative anti-BMV dAb) in fractions E4, E5, and E6, but were absent in fractions E7-13 (Fig. 3.5C). However, other non-target proteins were observed to be present in fractions E7-13, which has the same banding pattern as the wash fractions (W3, 6, 10, and 13), indicating weak, non-specific binding of non-target proteins to the HisPur™ Ni-NTA resin (Fig. 3.5A+B). The presence of the target protein was observed in the flow-through, wash, and elution lanes when compared to the control lane (Con) which consisted of induced untransformed *E. coli* BL21 cells (Fig. 3.5A, B, and C). Although a band corresponding to 17 kDa was observed in the control lane (induced untransformed *E. coli* BL21 cells), the band is much fainter than that of the induced transformed *E. coli* cells.

Following purification of the putative anti-BMV dAb, refolding of the protein was attempted following dialysis of the pooled elution fractions E7-13. The refolded protein was analysed by SDS-PAGE and the concentration of the refolded proteins determined by densitometric analysis using a series of BSA protein standards. The concentration of the refolded protein was determined to be 0.334 mg/mL (Fig. 3.6).

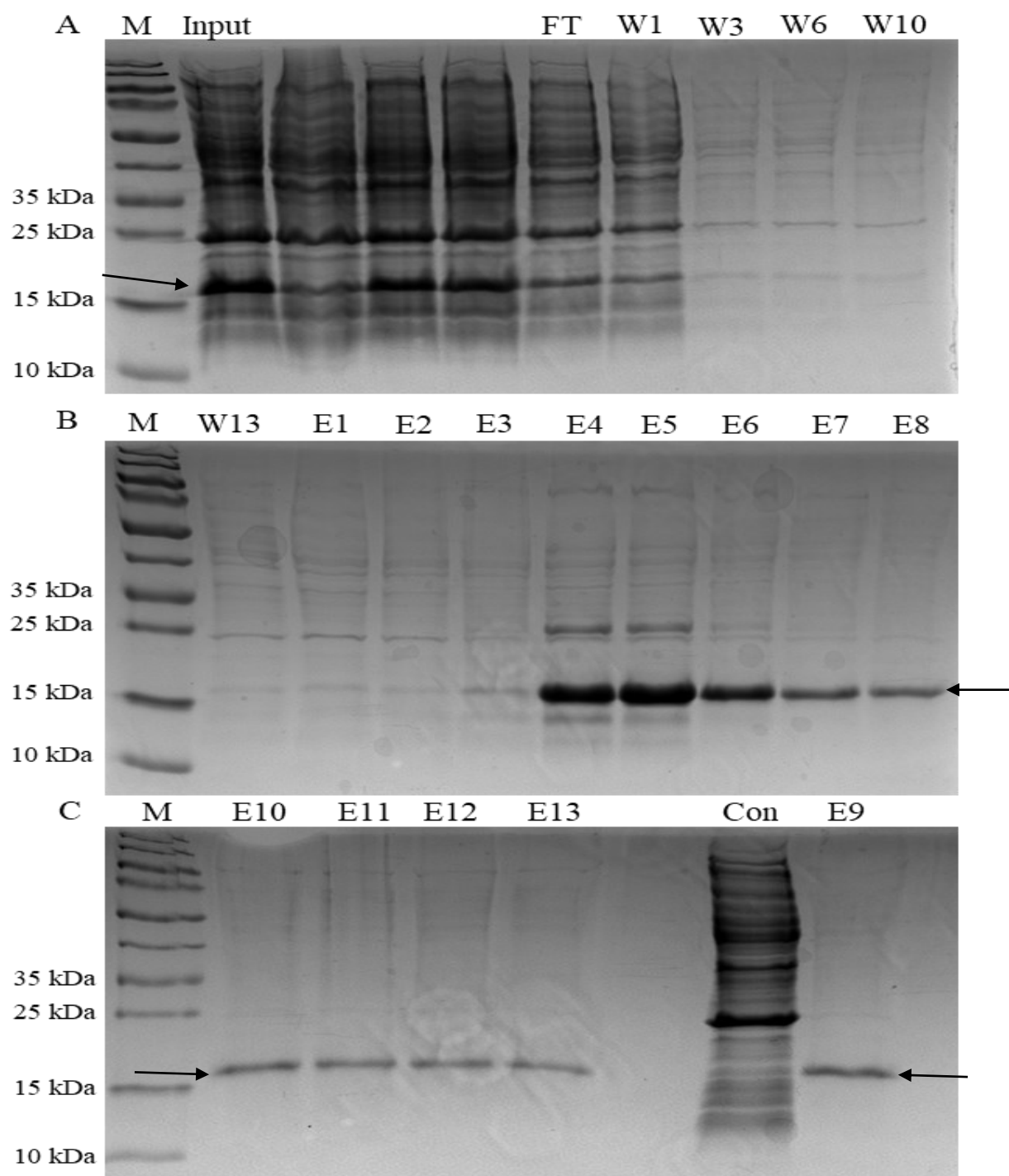


Figure 3.5 SDS-PAGE of eluted proteins obtained after batch purification using a HisPur Ni-NTA resin. Fractions were collected at each step of the purification and analysed by SDS-PAGE. Input: input sample; FT: flow-through; W: wash; E: elution; Con: Induced untransformed *E. coli* BL21 control; M: Hi Mark™ Pre-stained Protein Standard (ThermoFisher Scientific).

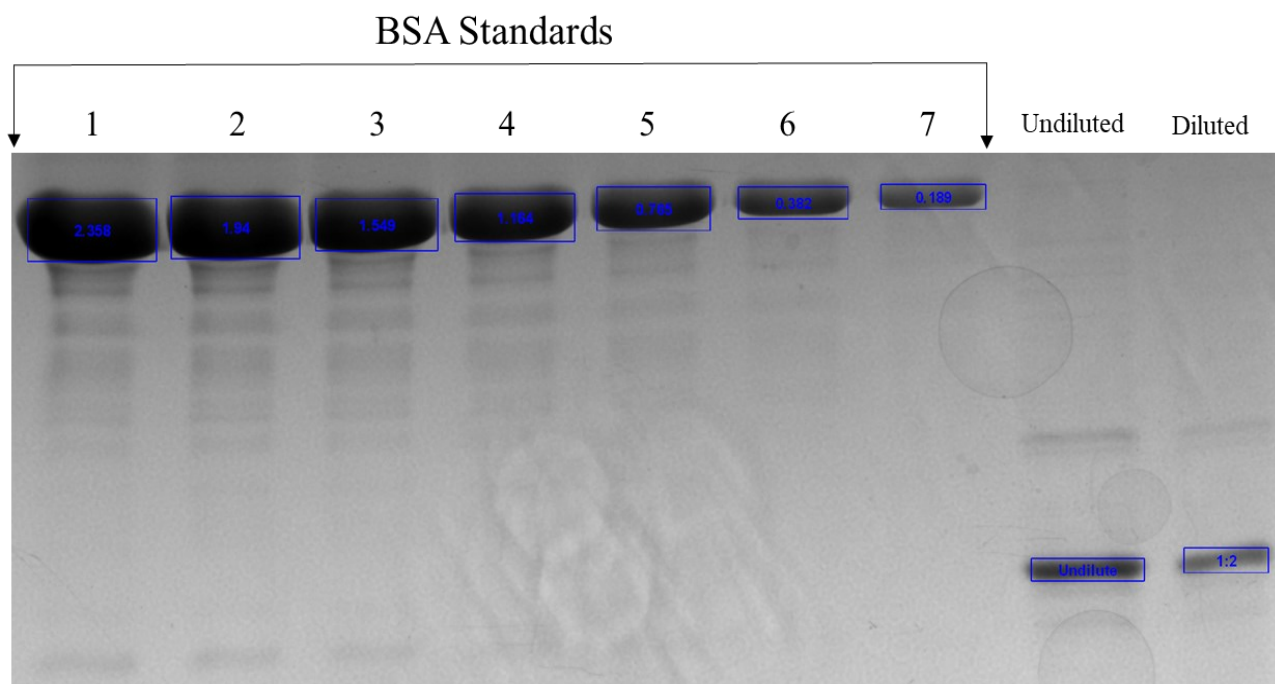


Figure 3.6 SDS-PAGE analysis of the purified and refolded anti-BMV dAb, compared to various known concentrations of BSA. BSA standards (mg/mL): lane 1: 2.358; lane 2: 1.940; lane 3: 1.549; lane 4: 1.164; lane 5: 0.765; lane 6: 0.382 and lane 7: 0.189. Undiluted and diluted (1:2) purified anti-BMV dAb were analysed.

3.3.1.3 Binding affinity of purified Dusky kob anti-BMV dAb to BMV

To measure the success of renaturation and refolding of the putative anti-BMV dAb by testing its functionality, an ELISA was conducted to determine whether the obtained putative anti-BMV dAb retained binding affinity to BMV. Compared to the no BMV (PBS only) control, it was observed that for the three different antibody concentrations tested, none were observed to bind to BMV (Fig. 3.7), even though it has previously been shown that the OD₄₀₅ of the phage displayed C7 anti-BMV antibody was above 3 (Appendix B 1.5).

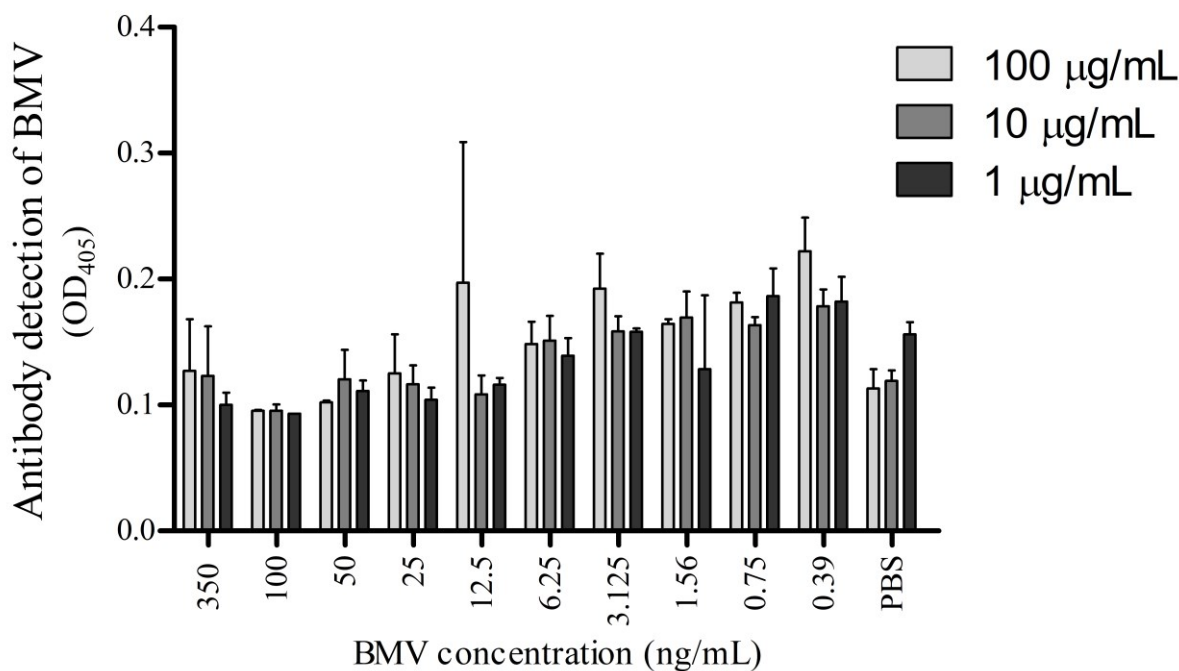


Figure 3.7 ELISA showing the detection of BMV with refolded putative anti-BMV dAb at three different concentrations (100 µg/mL, 10 µg/mL, and 1 µg/mL). Wells coated with PBS served as a negative control to which statistical significance was determined. Error bars represent the standard deviation of the means of two biological repeats.

3.3.2 Yeast surface display of a Dusky kob anti-BMV single domain antibody

3.3.2.1 Expression of anti-BMV single domain antibody in *S. cerevisiae* EBY100

The gene encoding the Dusky kob anti-BMV dAb was sub-cloned from pPROEx-Htb into pYD1 following PCR amplification. The recombinant pYD1_dAb vector was transformed into *E. coli* DH5α cells. A total of four colonies were randomly selected from the transformants for cPCR and sequence analysis. Agarose gel analysis of the PCR products obtained by cPCR of the four transformants confirmed the sizes of the expected amplicon (~672 bp) generated by the set of primers described in Table 3.2, thereby confirming that the recombinant plasmids contained the desired insert DNA (Fig. 3.8). The pYD1 vector served as a negative control.

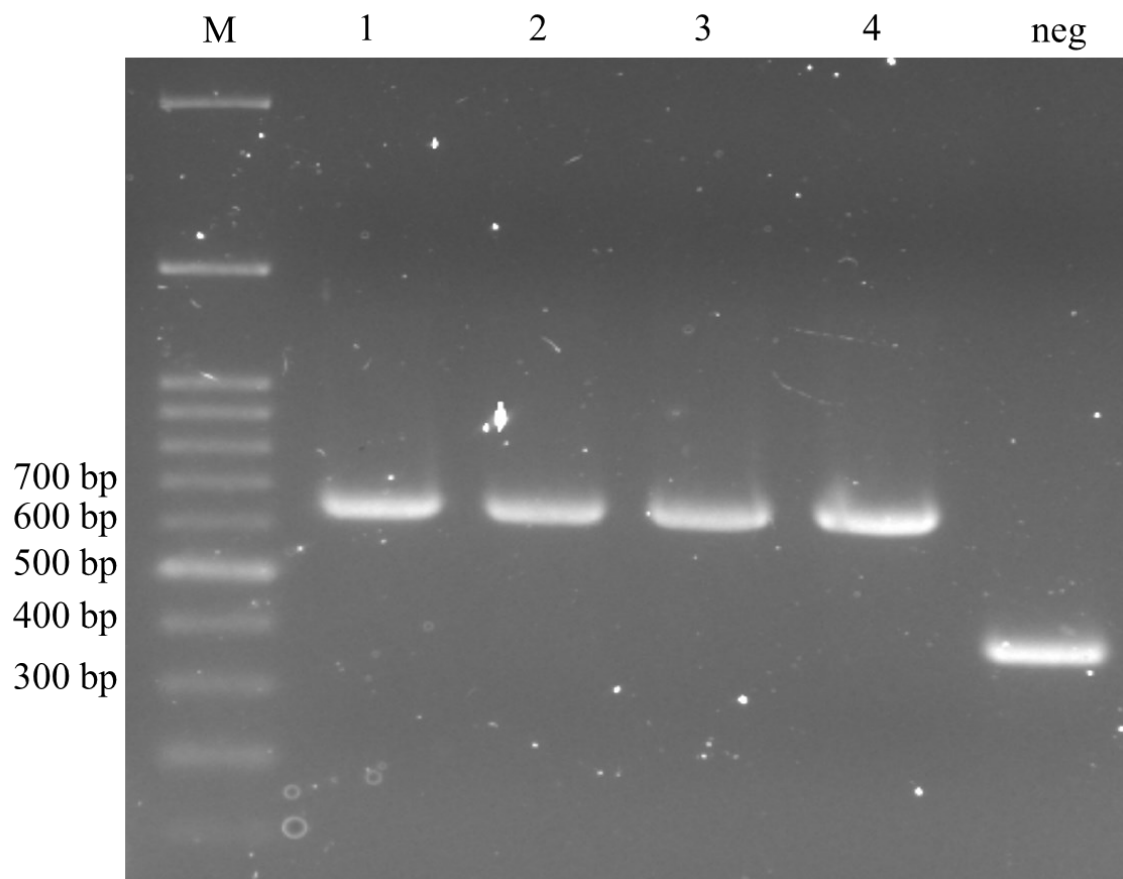


Figure 3.8 Colony PCR amplification of randomly selected colonies transformed with recombinant pYD1 containing a Dusky kob gene encoding the anti-BMV single domain antibody. M: One Mark B (GeneDirex) 100 bp DNA marker; lanes 1–4: pYD1_dAb (~672 bp); neg: pYD1 only (~399 bp).

The identity of the four clones was determined by sequence analysis. All four recombinant plasmids were observed to contain the correct putative anti-BMV dAb gene following a BLASTn analysis. Furthermore, it was observed that the anti-BMV dAb gene was in-frame with the Aga2 peptide gene, the glycine-serine linker gene, and the polyhistidine gene region (Fig. 3.9).

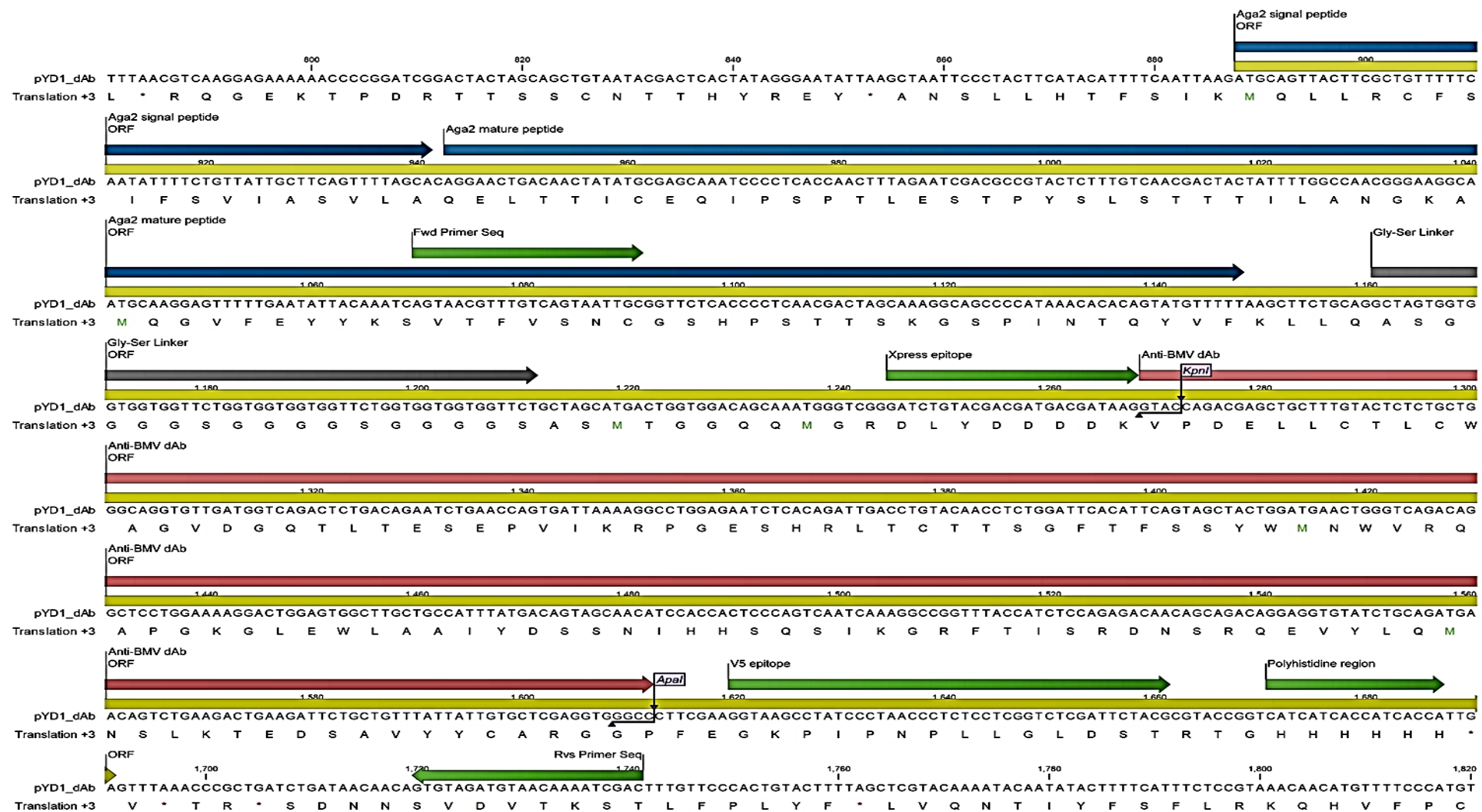


Figure 3.9 Fully annotated gene and protein sequence of recombinant pYD1_dAb. The Aga2 peptide is linked to the 5' end of the anti-BMV dAb region by a short glycine-serine linker. The Aga2 peptide is linked to the Aga1 peptide, found on the surface of *S. cerevisiae* EBY100 cells. The forward sequence primer can be found at position 1070-1091, and the reverse primer can be found at position 1722-1745. Epitopes (V5, Xpress, and polyhistidine) for the detection of displayed proteins are indicated by the green arrows.

3.3.2.2 Competition ELISA

To determine whether the binding affinity of the putative anti-BMV dAb is comparable to that of a commercial antibody, a competition ELISA was conducted by exposing BMV to a mixture of yeast displayed anti-BMV dAbs and a commercial anti-BMV IgG, at varying concentrations of each. The negative controls were as follows: induced yeast cells transformed with pYD1 were mixed with the commercial anti-BMV IgG to detect non-specific interactions. Furthermore, anti-BMV IgG alone (anti-BMV IgG not mixed with any yeast cells) served as a positive control.

Anti-BMV IgG binding was inhibited by the yeast displayed anti-BMV dAb to varying degrees depending on the dilution of the anti-BMV IgG and the concentration yeast cells displaying the anti-BMV dAb. The yeast displayed anti-BMV dAb samples did not inhibit anti-BMV IgG binding at a concentration of 2 $\mu\text{g/mL}$ (Fig. 3.10). Yeast concentrations of 6×10^7 cells/mL, 3×10^7 cells/mL, and 2.4×10^7 cells/mL significantly inhibited the binding of anti-BMV IgG at a concentration of 1 $\mu\text{g/mL}$ (Fig. 3.10). These yeast titres were noted to have a percentage inhibition of 40%, 16%, and 11%, respectively (Fig. 3.10).

At a concentration of 0.5 $\mu\text{g/mL}$ anti-BMV IgG, yeast concentrations of 6×10^7 cells/mL and 3×10^7 cells/mL were observed to significantly inhibit the binding of anti-BMV IgG (Fig. 3.10). The percentage binding inhibition of anti-BMV IgG when exposed to yeast cell concentrations of 6×10^7 cells/mL and 3×10^7 cells/mL was observed to be 40% and 34%, respectively. Finally, samples tested against 0.25 $\mu\text{g/mL}$ anti-BMV showed a significant degree of binding inhibition at yeast concentrations of 6×10^7 cells/mL, 3×10^7 cells/mL, and 2.4×10^7 cells/mL, with a percentage inhibition of 49%, 40%, and 31%, respectively (Fig. 3.10).

Yeast displayed anti-BMV dAb at a cell concentration of 6×10^7 cells/mL was observed to have the highest binding inhibition to anti-BMV IgG concentrations of 1 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, and 0.25 $\mu\text{g/mL}$. Yeast displayed anti-BMV dAb at a titre of less than 1.5×10^6 cells/mL showed no significant binding inhibition at any of the anti-BMV IgG concentrations (Fig. 3.10).

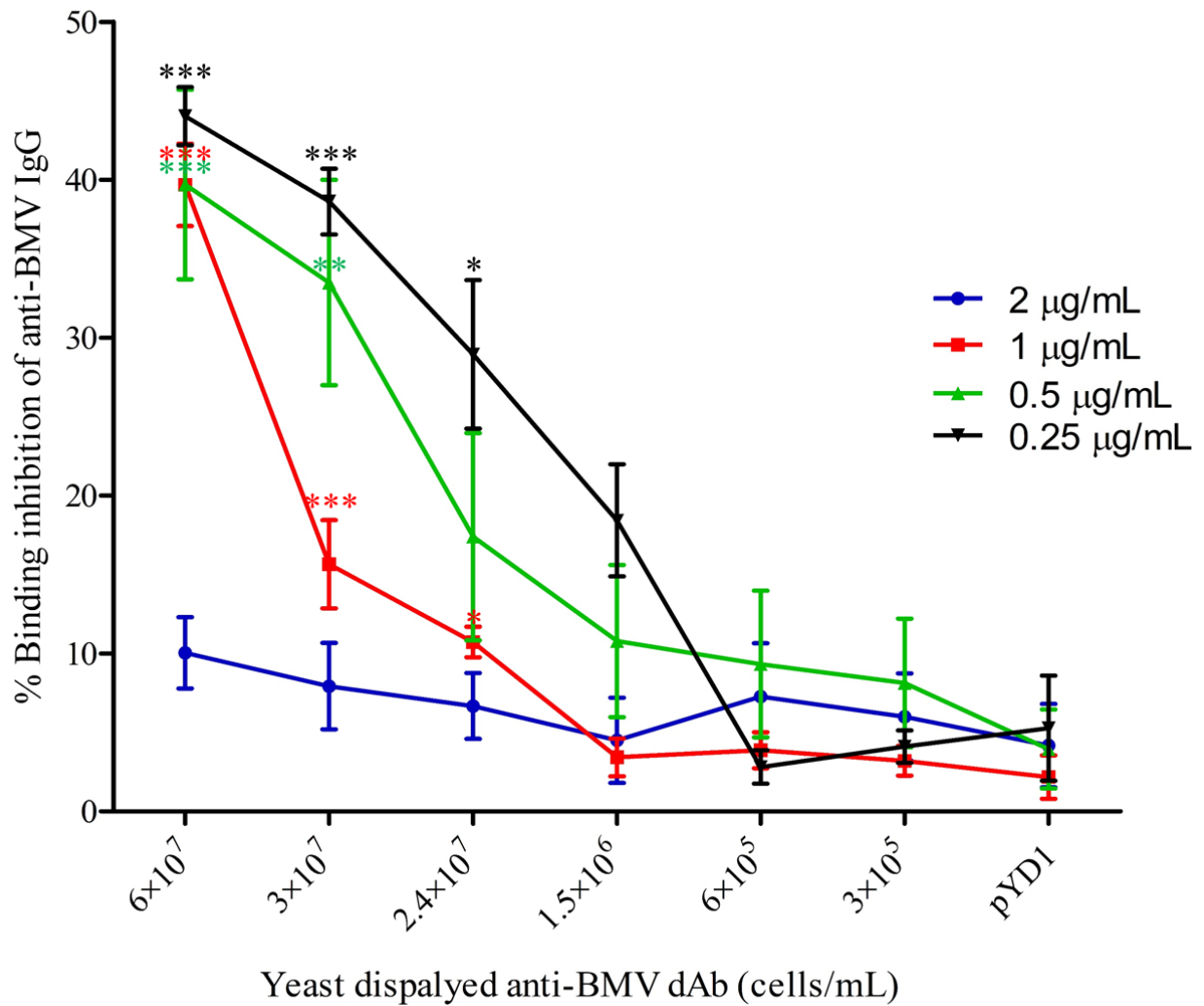


Figure 3.10 Percentage binding inhibition of a commercial anti-BMV IgG to BMV by a yeast-displayed putative anti-BMV dAb. Various concentrations of yeast cells displaying the anti-BMV dAb were tested for competitive binding to 10 µg/mL of BMV against 2 µg/mL anti-BMV IgG, 1 µg/mL anti-BMV IgG, 0.5 µg/mL anti-BMV IgG, and 0.25 µg/mL anti-BMV IgG. Anti-BMV IgG only (no competition) and induced yeast cells transformed with pYD1 served as negative controls, both of which were used to calculate the percentage binding inhibition. Statistical significance was determined by the ability of yeast displayed anti-BMV dAb to inhibit the binding of anti-BMV IgG, at a specific concentration, to BMV, compared to yeast transformed with pYD1. Significance is indicated by an asterisk (*) as determined by one-way ANOVA. * = $P < 0.05$, ** = $p < 0.005$, *** = $p < 0.0001$. Error bars represent the standard error of the mean (SEM); $n = 3$.

3.3.2.3 Transmission electron microscope imaging of yeast displayed anti-BMV single domain antibody bound to BMV

To visualise BMV particles binding to anti-BMV dAb displayed on yeast cell surfaces, TEM analysis was conducted on induced and non-induced recombinant yeast cells that had been exposed to BMV. TEM analysis of BMV exposed to non-induced recombinant yeast cells did not reveal any BMV particles on the surface of the yeast cells (Fig. 3.11A). TEM analysis of BMV exposed to induced pYD1_anti-BMV_dAb yeast cells showed BMV particles attached to the yeast cell surfaces (Fig 3.11B). This demonstrates that the anti-BMV dAb expressed on the surface of yeast cells binds to BMV.

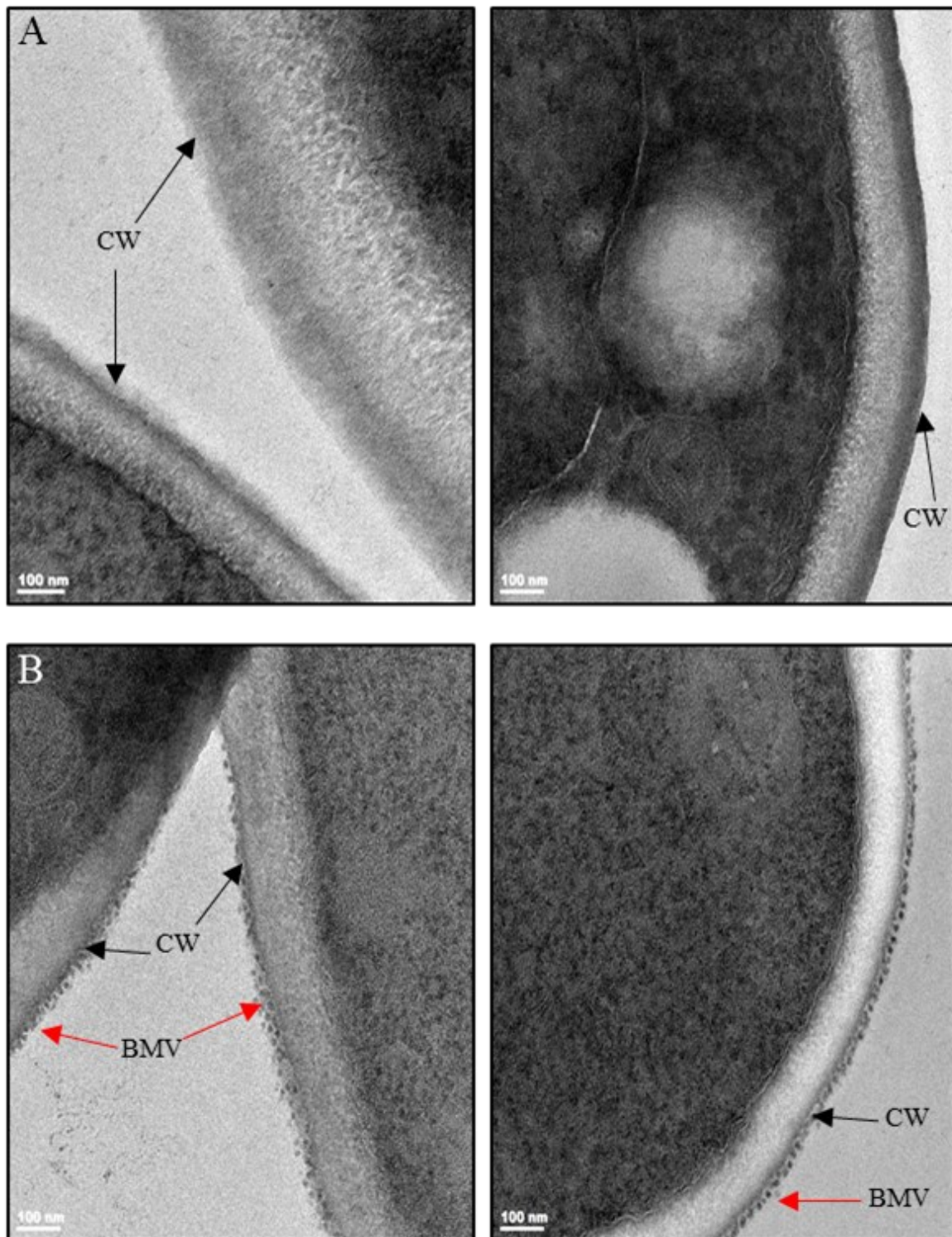


Figure 3.11 Transmission electron microscope imaging of (A) non-induced yeast cells and (B) induced yeast cells displaying anti-BMV dAb exposed to BMV. Black arrows indicate the cell wall (CW) of the yeast cell. Red arrows indicate the presence of BMV particles adhering to the surface of the yeast cells. Scale bars = 100 nm.

3.4 Discussion

Infectious diseases are a significant constraint on the development and sustainability of the aquaculture industry, not only in South Africa, but worldwide (Bondad-Reantaso *et al.*, 2005; Ina-Salwany *et al.*, 2019). In South Africa, Dusky kob farming is still regarded as a developing industry, with limited knowledge available regarding the epidemiology of emerging pathogens. The development of a pipeline that will allow for the rapid identification, isolation, and production of therapeutic and diagnostic antibodies is fundamental to controlling and managing disease outbreaks on Dusky kob farms. In this study, two expression systems, bacterial and yeast, were employed and compared to determine the ideal system for expression of the putative anti-BMV dAb identified in Chapter 2. This expression system will allow for the development of an effective, low-cost antibody production process for use in the Dusky kob farming industry.

3.4.1 Expression of a recombinant anti-BMV single domain antibody in *E. coli*

pProEX-HTb is an efficient plasmid vector that allows the expression of recombinant proteins in the cytoplasmic space of *E. coli* BL21 cells. However, it has been reported that the cytoplasm is not ideal for the production of functional recombinant antibodies that require disulphide bond formation for correct folding (Jeong, Jang and Velmurugan, 2011). To overcome this problem, the inclusion of a signal peptide, such as the leader sequence of outer membrane protein A (OmpA) or pectate lyase B (PelB) can be used for direct secretion of the target protein into the periplasmic space of *E. coli*. The periplasmic space of *E. coli* is reportedly better suited to the production of recombinant antibodies due to the presence of chaperone proteins and disulphide isomerases, which assist in the correct folding of recombinant proteins (Ahmad *et al.*, 2012; Rosano and Ceccarelli, 2014). However, Sánchez *et al.* (1999) reported that periplasmic expressed recombinant antibodies resulted in yields six times lower than those obtained in the cytoplasm, and only 60% of the proteins expressed in the periplasm were sufficiently folded, while those produced in the cytoplasm had a 56% refolding efficiency. However, despite the tendency of protein expressed using the pProEX-HTb expression system to form inclusion bodies, numerous studies have reported successful strategies, such as extracting proteins under denaturing conditions, that allow for the recovery of large amounts of proteins which can be subsequently refolded in order to maintain biological function (Maynard and Georgiou, 2000; ShengFeng *et al.*, 2003; Liu and Huang, 2018). Therefore, with this in mind, the Dusky kob gene encoding the anti-BMV dAb was subcloned into the *E. coli* expression vector pProEX-HTb to allow for sufficient expression of the antibody fragment.

It was observed that after induction with IPTG, the anti-BMV dAb was successfully expressed in *E. coli* cells from as early as 2 h P.I., with optimum expression at 6 h P.I. (Fig. 3.4). Most of the expressed recombinant protein was found in inclusion bodies, due to the high amount of protein observed when extracted under denaturing conditions compared to those extracted using native buffers (Fig. 3.4A). Although attempts were made to reduce the formation of aggregates and increase the solubility of the dAb by reducing the concentration of IPTG and the induction temperature, the formation of inclusion bodies persisted. As previously mentioned, the formation of aggregates is often due to expression of the recombinant antibody in the reducing environment of the cytoplasm due to the lack of a signal sequence (Verma, Boleti and George, 1998). Furthermore, it has been reported that dAbs have a tendency to form aggregates due to the presence of a large exposed hydrophobic region resulting from the absence of opposite chain pairs (Omidfar and Daneshpour, 2015).

In order to refold and restore functionality to the purified anti-BMV dAb, attempts were made to remove the urea denaturing solution from the recovered proteins and replace it with a non-denaturing buffer such as PBS. The removal of urea from the anti-BMV dAb aids in reforming disulphide bonds, which are crucial for the correct refolding of antibody proteins such as the anti-BMV dAb, and are responsible for the overall stability and structure of the recombinant protein (Verma, Boleti and George, 1998; Andersen and Krummen, 2002). Following the method outlined by ShengFeng *et al.* (2003), anti-BMV dAbs were refolded and recovered using dialysis. A total of 12.83 mg/mL of anti-BMV dAb protein was estimated to have been recovered after renaturing. Frenzel *et al.* (2013) reported that the production of recombinant antibodies in *E. coli* typically yields between 83-127 mg/L, while Lee and Jeong (2015) reported a production yield of between 1-400 mg/L for typical flask cultivation production and up to 3 g/L from fermentation or high cell density cultivation. Here, we demonstrated a recovery yield of 10.69 mg/L of Dusky kob anti-BMV dAb protein, which is at the lower end of protein production yields observed in *E. coli*.

To determine whether the recovered putative anti-BMV dAb retained biological activity after refolding, an ELISA was conducted to test the binding ability of the recovered antibody to BMV. To access the detection range of the recovered putative anti-BMV dAb, three concentrations (100 µg/mL, 10 µg/mL, and 1 µg/mL) of the recovered and refolded antibody were used to test BMV binding. It was found that for the three concentrations of refolded anti-BMV dAb tested, none bound significantly to BMV relative to the control (Fig. 3.7). Even at concentrations greater than 0.35 µg/mL, no significant binding was observed (data not shown). In a similar study by Sánchez *et al.* (1999), their refolded antibody was found to retain

functionality by significantly binding to hepatitis B at concentrations ranging between 1.5 to 15 µg/mL of the refolded protein. Additionally, a similar result was reported by ShengFeng *et al.* (2003), in that their refolded antibody was found to bind efficiently to the foot and mouth virus at concentrations as low as 3 µg/mL. The recovered and refolded antibody reported in this study was inefficient in binding to BMV, even at concentrations of 10 and 100 µg/mL, indicating that the refolded antibody could no longer bind to BMV. The inability of the protein to bind to BMV is likely a strong indication that the attempts to refold the antibody fragment were ineffective, which can be explained by the high amounts of amounts of protein aggregates observed when attempting to refold the protein. It has been reported by Goswami *et al.* (2009) that phage displayed proteins demonstrate altered characteristics when compared to their soluble counterparts. Furthermore, the authors highlight the importance of the pIII protein of the phage in providing a degree of structural support to the displayed protein, assisting it to acquire a unique conformation and binding feature. Upon expression in soluble form, the protein is devoid of any structural support from the pIII, leading to altered structural and binding characteristics (Goswami, Saini and Sinha, 2009). These characteristics were observed by McCafferty *et al.* (1991) and Corey *et al.* (1993), who displayed enzymatic proteins such as alkaline phosphatase and trypsin on phage surfaces as fusion proteins, respectively, observed altered affinities for their respective substrates when expressed as soluble proteins. This observation supports the observed loss in binding of the anti-BMV dAb when expressed in a soluble form. Therefore, it is clear that an anchor protein, such as the pIII of the phage, is beneficial to the structural and binding characteristics of the expressed protein. Consequently, it was decided to express the putative anti-BMV dAb using a yeast surface display system, as proteins such as AGA2 may provide structural support similar to that of the phage pIII protein. Additionally, a yeast display system would allow for expression of the desired protein without the need for complicated extractions methods.

3.4.2 Yeast surface display of a Dusky kob anti-BMV single domain antibody

Yeast surface display is an effective eukaryotic protein expression system that involves the expression and display of recombinant proteins on the surface of a yeast cell from where it can directly interact with the surrounding environment. Yeast surface display has been successfully used to discover and express novel antibody fragments, similar to that of phage display (Im *et al.*, 2019). Compared to bacteria, yeast expression systems are more appropriate for the expression of recombinant antibodies due to effective post-translational modification and glycosylation of expressed proteins (Boder, Raeeszadeh-Sarmazdeh and Price, 2012). In an attempt to display the anti-BMV dAb on the surface of yeast cells, the Dusky kob gene

encoding the anti-BMV dAb was subcloned into the yeast display vector pYD1 (Fig. 3.8). Sequence analysis confirmed that the anti-BMV dAb gene was successfully cloned in-frame with the Aga2 peptide (Fig. 3.9), and thus, anti-BMV dAbs could be displayed on the surface of yeast cells after induction with 2% galactose.

In order to validate the binding of the yeast displayed anti-BMV dAb to BMV, a TEM analysis was conducted by observing the binding of either an induced or non-induced pYD1_anti-BMV dAb transformed *S. cerevisiae* EBY100 cells to BMV. BMV was shown to bind to yeast displaying the anti-BMV dAb (Fig. 3.12B), which further indicates that the anti-BMV dAb was expressed and displayed correctly on the surface of yeast cells following induction. Furthermore, BMV binding was not observed on the surface of non-induced yeast cells (Fig. 3.12A), which confirms that BMV is binding specifically to the displayed antibody and not to the yeast cell wall.

The affinity between an antibody and its target antigen can be determined by inhibiting binding by the addition of an alternative antibody that targets the same antigen (van Wyngaardt *et al.*, 2004). Such assays demonstrate whether antibodies isolated by alternative methods can be used as a replacement for more traditional, hybridoma-derived antibodies. In this study, a competition ELISA was employed to determine whether a yeast displayed Dusky kob anti-BMV dAb could effectively compete for binding to BMV against an anti-BMV IgG. The displayed anti-BMV dAb (at yeast concentrations of 6×10^7 cells/mL, 3×10^7 cells/mL, and 2.4×10^7 cells/mL) was found to effectively inhibit anti-BMV IgG binding (at concentrations of 1 μ g/mL and 0.25 μ g/mL) to BMV (Fig. 3.11). A similar result was reported by van Wyngaardt *et al.* (2004) in which an anti-blue tongue virus (BTV) antibody, derived from a phage display library, could successfully inhibit the binding of BTV-specific antibodies derived from either a sheep or rabbit.

Overall, the results presented in this study demonstrate that a yeast displayed Dusky kob anti-BMV dAb could inhibit the binding of commercial anti-BMV IgG to BMV by between 10 and 45%, in a concentration-dependent manner. This result shows that a yeast display system can be used to successfully and rapidly express useful and specific antibody fragments without resorting to hybridoma, which is often regarded as expensive, labour intensive, and time consuming (Basu *et al.*, 2019).

3.4.3 Concluding remarks

The aim of this chapter was to identify an effective means for the expression and production of the Dusky kob anti-BMV single domain antibody identified in Chapter 2. The gene encoding

the anti-BMV dAb was sub-cloned into an *E. coli* expression system and a yeast surface display system. Despite various attempts to express the anti-BMV dAb in *E. coli*, the recovered protein lacked affinity to BMV. In contrast, the Dusky kob anti-BMV dAb was successfully expressed and displayed in a yeast surface display system and demonstrated strong binding to BMV. In conclusion, a yeast surface display system was shown to be an effective system for the expression of the Dusky kob anti-BMV dAb. Although both yeast and bacterial expression systems are suitable for the production of recombinant antibodies and similar biodrugs, yeast is favoured due to its eukaryotic status. With regard to yeast surface display, the recombinant antibody is displayed directly on the surface of the yeast cell wall, allowing interaction with its target antigen directly in solution, as demonstrated by the TEM analysis, without the need for purification steps characteristic of bacterial production systems. Furthermore, the composition of the yeast cell wall makes it resistant to gastric and pancreatic juices, and is also largely immunogenic. This makes yeast displayed recombinant antibodies a suitable system for the production and delivery of therapeutic antibodies.

Chapter 4

Diagnostic and therapeutic analysis of the anti-BMV single domain antibody derived from a Dusky kob phage display library

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4.1 Introduction

In a typical vertebrate system, antibodies are naturally occurring proteins that protect against infections from disease-causing pathogens, such as viruses and bacteria (Wootla, Denic and Rodriguez, 2014). Antibodies are responsible for neutralising and preventing infection by either inhibiting binding of the pathogen to the cellular receptors of the host cells, or through stimulating specific immune cells and triggering an immune response (Corti and Lanzavecchia, 2013). This natural antibody response to common antigens is highly heterogeneous and is produced by multiple B cells, resulting in the production of numerous antibodies that recognise different epitopes of the target antigen (Milstein, 1980; Wootla, Denic and Rodriguez, 2014). The process of antibody development was harnessed in 1975 by Köhler and Milstein, who developed the hybridoma method, which produces mAbs derived from a single B cell, that are highly specific to a single antigenic determinant (Köhler and Milstein, 1975).

Ever since their initial description, mAbs have been a pivotal technology for a variety of applications, including vaccine development (Aghebati-Maleki *et al.*, 2016), epitope mapping of antigens (Bottger and Bottger, 2009; Wu *et al.*, 2016), and the study of structural changes in plant cell walls during pre-treatment for biofuel production (Demartini *et al.*, 2011). One of the most important applications of mAbs is their use as diagnostic and therapeutic agents for the rapid detection and treatment of various disease-causing pathogens (Hoogenboom *et al.*, 1998; Azzazy and Highsmith Jr., 2002; Bazan, Całkosiński and Gamian, 2012). Typically, the diagnosis of a particular disease is based on the presentation of distinct clinical symptoms. However, in cases whereby a multitude of symptoms allude to a variety of different disease-causing pathogens, or patients are asymptomatic, the accurate diagnosis of disease is reliant on the quantitative identification or the localisation of particular pathogenic determinants in the cells, tissues, and body fluids of an infected host (Zhang *et al.*, 2014). Due to their highly specific nature, mAbs are frequently used in clinical diagnosis, more so than other methods due to their high accuracy, sensitivity, and reproducibility. Both hybridoma and phage display systems have been instrumental in the development of more rapid and accurate diagnostic tests for the detection of various disease-causing pathogens, including avian influenza (Yang *et al.*, 2008), Seneca valley virus (Yang, van Bruggen and Xu, 2012), and *Vibrio cholerae* (Qadri *et al.*, 1994).

In the late 1980s, the first therapeutic mAb, a mouse antibody, was approved for the treatment of acute transplant rejection in humans (Leavy, 2010). As a result, the development of therapeutic antibodies for the treatment of various cancers, autoimmune disorders, and

inflammatory diseases has increased (Leavy, 2010; Suzuki, Kato and Kato, 2015). To date, over 570 different therapeutic mAbs have been studied at various stages of clinical trials, with mAbs derived from phage display accounting for over 30% of the total number (Omidfar and Daneshpour, 2015; Lu *et al.*, 2020). As of 2020, approximately 79 therapeutic mAbs for the treatment of various human-related diseases have been clinically approved, nine of which are derived from phage display (Kaplon *et al.*, 2020; Lu *et al.*, 2020). Of these, the therapeutic mAb Adalimumab is credited with being the most successful therapeutic mAb to date. Adalimumab is a recombinant human IgG mAb used as a tumour necrosis factor (TNF)-inhibiting, anti-inflammatory drug for the treatment of rheumatoid and psoriatic arthritis and Crohn's disease (Omidfar and Daneshpour, 2015). Adalimumab was the first fully human mAb produced by phage display to be market approved, and in 2018 it was reported to have generated sales exceeding US\$19 billion (Weinblatt *et al.*, 2003; Omidfar and Daneshpour, 2015; Lu *et al.*, 2020), demonstrating the reliability and capability of phage display as a source of novel therapeutic antibodies.

The advent of mAbs has been fundamental in improving our understanding of the immune system of teleost fish. Their use in aquaculture dates back to 1983, when Secombes *et al.* demonstrated the existence of separate fish lymphocyte populations based on cell surface markers using mAbs raised against carp thymocytes and serum Igs (Secombes, van Groningen and Egberts, 1983; Coll and Dominguez-Juncal, 1995). The development of mAbs against fish serum Igs has created a better understanding of the fish immune system and has been found to be a powerful tool in determining the level of total and specific serum Ig levels in healthy, infected, and vaccinated fish (Li *et al.*, 2007). This has allowed farms to assess the general immunocompetence of vaccinated and diseased fish, leading to the development of more efficient vaccination programs, biosecurity measures, and overall disease management in aquaculture (Coll and Dominguez-Juncal, 1995; Scapigliati, Romano and Abelli, 1999). To date, mAbs to serum Igs of numerous economically important fish species, including rainbow trout (*O. mykiss*), red drum (*Sciaenops ocellatus*), Atlantic salmon (*S. salar*), sea bream (*S. aurata*), and tilapia (*O. niloticus*), have been successfully developed (Wu *et al.*, 2020).

The use of mAbs has also aided in the development of vaccines in aquaculture. Numerous studies have utilised mAbs to select bacterial clones expressing various recombinant antibody fragments specific to fish pathogens such as viral haemorrhagic septicaemia virus (VHSV) (Estepa, Thiry and Coll, 1994; Coll and Dominguez-Juncal, 1995) and infectious pancreatic necrosis virus (IPNV) (Lawrence *et al.*, 1989). From these studies, the authors were able to identify particular viral proteins that displayed higher immunogenicity than other proteins of

the same pathogen. This allowed for the development of better vaccine candidates that were able to induce a higher percentage of survival *in vivo* (Coll and Dominguez-Juncal, 1995). As phage display developed, it became possible to display entire viral and bacterial genomes on the surfaces of filamentous phages, which could be utilised to identify immunogenic pathogen proteins for the development of more efficient vaccines. For example, phage display has been used to develop a phage display library that displayed the entire RSIV genome (Takano *et al.*, 2019). From this library, an anti-RSIV mAb was identified by Takano *et al.* (2019) that could successfully identify the target antigen to which the anti-RSIV binds, which can be used to develop recombinant protein vaccines that elicit a greater immune response and convey better protection. However, despite their importance, the number of licensed vaccines available for disease prevention in fish aquaculture is limited. The World Organisation of Animal Health (OIE) lists a total of twelve pathogens that pose a significant threat to the fish aquaculture industry. However, of those twelve, there are vaccines available for only five of the pathogens that are commercially licensed, which include vaccines for RSIV, ISAA, salmonid alphavirus, *Oncorhynchus masou* virus, and IHNV (which is only licensed in North America) (OIE, 2019). While these vaccines have been useful in disease prevention to some degree, complete protection has not been successfully conveyed for the majority of them (Hoare *et al.*, 2016). Furthermore, current methods for the treatment of infection of these twelve commercially important fish pathogens is limited, with the majority having little to no chemotherapeutic or immunostimulant approach available for disease control. Combined with the lack of vaccines, this has resulted in many fish farms being reliant on resistant breeding, good general husbandry practices, and the means to rapidly and accurately detect the presence of disease-causing pathogens for sufficient disease prevention and control (OIE, 2019).

Rapid detection and identification of pathogenic outbreaks is regarded as the single most important step in disease management on all aquaculture farms. On fish farms, viruses are reported to cause the majority of disease-related mortalities, followed by bacteria and parasites (Coll and Dominguez-Juncal, 1995). Traditional methods for the diagnosis and identification of fish pathogens initially requires isolation and culturing of pathogens from infected tissues, from which the pathogen can be identified using various techniques such as microscopy and PCR-based assays (Coll and Dominguez-Juncal, 1995; Adams and Thompson, 2011; Hoare *et al.*, 2016). However, for the majority of fish pathogens, these methods are time consuming and expensive, and often require a combination of methods for a definitive diagnosis (Adams and Thompson, 2006). However, the use of mAbs in immunological approaches such as fluorescence antibodies, dot blot, western blot, and ELISA, has allowed for the rapid and highly

specific diagnosis of various fish pathogens without the need to first isolate the pathogen (Adams and Thompson, 2006; OIE, 2019). Due to the high specificity of mAbs, their use in aquaculture diagnostics has led to the identification of specific pathogens. mAbs are more sensitive than traditional methods in the identification of pathogens and can distinguish between pathogens that possess similar morphological, biochemical, and physiological characteristics. For example, Chen *et al.* (1992) developed mAbs against different *Vibrio* species that infect humans, fish, and shellfish. The authors were successful in identifying species and genus specific mAbs that were applied in a blind test to differentiate between *Vibrio* species (Chen *et al.*, 1992). A similar result was demonstrated by Phianphak *et al.* (2005), in which mAbs were successfully raised against *V. harveyi* and did not show any cross-reactivity to other *Vibrio* species or gram-negative bacteria. These studies demonstrate the versatility of mAbs as highly specific immunological tools for the rapid and specific detection of various fish pathogens. Antibody-based assays are an attractive alternative method for a low-cost, highly specific, on-site disease monitoring system for various aquaculture systems, that would result in improved disease management and the reduced need for antibiotics and hazardous chemical treatments (Adams and Thompson, 2006; Wangman *et al.*, 2017).

Neutralising antibodies are antibodies that have been raised against important proteins of infectious pathogens, such as the viral envelope and spike proteins of virions, and bind to them, preventing attachment and subsequent infection of the host cell (Kim *et al.*, 2004). Since the advent of mAbs, numerous studies have reported the use of antibodies in neutralisation assays to identify immunogenic proteins that are involved in infection, such as those of the pathogens WSSV and VHSV (Lorenzen, Olesen and Jorgensen, 1990; Huang *et al.*, 2005; Li *et al.*, 2005; Robalino *et al.*, 2006; Jiang, Zhan and Sheng, 2007). These studies successfully identified antibodies against individual viral proteins involved in infection. The findings of such studies has resulted in the exploitation of similar anti-viral neutralising antibodies in passive immunisation strategies for the control of viral infections by WSSV and VHSV (Li *et al.*, 2005; Ohtani *et al.*, 2013). In 2001, Van Hulten *et al.* demonstrated that an antibody raised against VP28, the major coat protein of WSSV, was able to convey 100% protection against shrimp infection (Van Hulten *et al.*, 2001). A similar result was reported by Kim *et al.* (2004), in which shrimp treated with an egg yolk antibody raised against VP28 exhibited a survival rate of up to 85%. These studies highlight the potential immunotherapeutic application of antibodies for the prevention of pathogenic infection in aquaculture. However, despite the clear neutralising effect of antibodies and their success in certain aquaculture systems, their use as therapeutic reagents in aquaculture have been limited. To date, no commercial antibodies are available for

protection against any fish pathogen (OIE, 2019). A potential explanation for the limited use of therapeutic antibodies in aquaculture in general may be the nature in which the majority of antibodies for aquaculture have been made. To date, the majority of antibodies used in aquaculture are derived from hybridomas, which are known to be a highly complicated, time-consuming, and expensive technology that requires highly trained technologists (Zhang, Wang, Wu, M Li, *et al.*, 2006). Other reports have demonstrated that changes in culture conditions and genetic mutations that occur over time may result in variations of the antibody structure (Kulabhusan *et al.*, 2017). Furthermore, limited knowledge regarding emerging pathogens has hindered the production of antibodies for disease management in aquaculture, particularly with regards to hybridomas, where detailed knowledge of the target pathogen is required.

Recombinant antibodies, with high specificity and affinity to a target antigen, can be effectively developed using a phage display library in an efficient, cost-effective manner and without the requirement of having any prior knowledge of the target pathogen. To date, studies have reported the use of phage display libraries to identify effective diagnostic antibodies to pathogens such as WSSV, IHNV, spring viraemia of carp virus, and the fish parasite *Myxobolus rotundus* (Dai *et al.*, 2003; Zhang, Wang, Wu, M Li, *et al.*, 2006; Liu *et al.*, 2013, 2014). However, the use of phage display as a source of therapeutics for aquaculture is limited to only a few studies (Yuan *et al.*, 2006; Ohtani *et al.*, 2013). Despite the success of phage display as a source of beneficial therapeutic and diagnostic antibodies for human diseases, its application in aquaculture is currently minor, and it presents a potential novel resource for the development of antibodies for the improved disease management of aquaculture farms.

4.1.1 Chapter aims

Monoclonal antibodies are increasingly becoming important diagnostic and therapeutic reagents due to their high specificity, affinity, and ability to bind to and neutralise crucial infectious proteins, thus preventing attachment of pathogens to host cells. Furthermore, phage display is gradually becoming the gold standard method for the identification of diagnostic and therapeutic antibodies, but its application in aquaculture is limited. A phage display library displaying scFvs derived from combination pairings of Dusky kob V_H and V_L chains was successfully constructed and utilised to identify antibodies against the plant virus BMV, which served as a proxy for an emerging pathogen of Dusky kob (Chapter 2). As antibodies to the proxy emerging pathogen were successfully identified, the aim of this chapter was to analyse and determine the diagnostic and therapeutic potential of a phage display derived anti-BMV recombinant antibody, thereby proving the potential of phage display as a useful technology for the identification of diagnostic and therapeutically important mAbs for fish aquaculture.

The first objective of this chapter was to investigate the diagnostic potential of a phage display derived anti-BMV antibody displayed on the surface of yeast cells, by detecting the presence of BMV viral particles in the blood serum of infected Dusky kob using immunological assays, including ELISA and western blot. As previously stated, mAbs are ideal for the detection of pathogens due to their sensitivity, and thus, it is hypothesised that the yeast displayed anti-BMV dAb will be successful in detecting BMV in the blood serum of infected Dusky kob.

The second objective of this study was to investigate the therapeutic potential of the anti-BMV antibody displayed on the surface of yeast cells (Chapter 3). Previous studies have demonstrated the role of antibodies in preventing viral attachment and host cell infection. It is therefore hypothesised that the yeast displayed anti-BMV antibody will mitigate BMV infection of barley plants (*Hordeum vulgare*), which is a natural host of the virus.

4.2. Materials and Methods

4.2.1 Diagnostic testing of a yeast displayed anti-BMV single domain antibody

4.2.1.1 Animal maintenance

A. japonicus (average weight ~300 g, average length 350 mm), were obtained from a commercial fish farm in South Africa and maintained at the Department of Environment, Forestry, and Fisheries (DEFF) research aquarium in Sea Point, Cape Town, South Africa. Animals were housed in 350 L plastic tanks with circulating filtered natural sea water at 24°C, pH 7.9. Animals were fed daily with a commercial, 4 mm Trout Grower dried-pellet, fish feed (Specialised Aquatic Feeds, South Africa).

4.2.1.2 BMV challenge of Dusky kob

Dusky kob (average weight ~400 g, average length 450 mm) were injected intravenously in the lateral cutaneous vein, using a 21 G 1½" hypodermic needle, coated with the anticoagulant heparin sodium (Fresenius Kabi, Canada), with a 1 mL syringe, with 100 µg/mL of purified BMV suspended in sterile PBS. A total of 12 Dusky kob were infected, three per time point. A total of three Dusky kob were injected with PBS only, which served as a negative control. Blood samples of infected Dusky kob were collected by venepuncture using a 21 G 1½" hypodermic needle, coated with heparin sodium (Fresenius Kabi, Canada), with a 1 mL syringe, at 0 h, 1 h, 3 h, and 5 h post-infection, and stored at 4°C overnight for the blood to coagulate and the sera to separate. The blood was then centrifuged at 400 x g for 10 min and the sera collected and stored at -20°C until needed. All needles and syringes were coated with heparin prior to infection or sampling, to prevent blood coagulation. All fish were anaesthetised with 0.75 mL of 2-phenoxyethanol (Sigma-Aldrich, USA), diluted in 5 L of sea water (1:6000),

prior to infection and sampling. The sampled fish were sacrificed by submerging in 4.5 mL of 2-phenoxyethanol (Sigma-Aldrich, USA), diluted in 5 L of seawater (1:1000).

4.2.1.3 Detection of BMV in blood serum of Dusky kob

The presence of BMV was detected in the sera of infected Dusky kob by ELISA. Ninety-six well plates were coated overnight at 4°C with 100 µL/well of Dusky kob serum. The wells were washed three times with PBS-T. Non-specific binding was blocked by adding 300 µL of 2% BSAPBS/well and incubating the plate at 37°C for 1 h. Following three washes with PBS-T, 100 µL/well of either yeast-displayed anti-BMV dAb (prepared as described in Section 3.3.2.1, Chapter 3) or anti-BMV IgG, was added to each well and incubated at 37°C for 1 h. Yeast cells transformed with pYD1 served as a negative control. Following three washes with PBS-T and a single wash with PBS, 100 µL of the secondary antibody, goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, USA), diluted 1:5000 in BSAPBS-T, was added to each well containing probed anti-BMV IgG and incubated at 37°C for 1 h. For the detection of bound yeast cells, yeast cells were probed with 100 µL HRP anti-V5 tag antibody (200 µg) (Abcam, UK), diluted 1:2000 in BSAPBS-T, added to the appropriate well and incubated at 37°C for 1 h. After washing three times with PBS-T and a final single wash with PBS, goat anti-rabbit IgG conjugated with alkaline phosphatase was detected by the addition of 200 µL/well of enzyme substrate pNPP (Sigma-Aldrich, USA) to the appropriate well. HRP anti-V5 tag bound antibodies were detected by the addition of 100 µL/well of KPL SureBlue™ TMB microwell peroxidase substrate (SeraCare Life Sciences, USA) to the appropriate well. Following the addition of both substrates, the 96-well plate was incubated at room temperature for 30 min in the dark. Following development with KPL SureBlue™ TMB microwell peroxidase substrate (SeraCare Life Sciences, USA), the reaction was stopped with the addition of 100 µL/well of 1N HCl. Absorbance was measured at 405 nm, for detection of bound goat anti-rabbit IgG and at 450 nm for the detection of bound HRP anti-V5 antibodies, on a Multiskan Go Spectrophotometer (Thermo Scientific, USA). The absorbance readings of the experimental samples (Dusky kob injected with BMV) were corrected for by subtracting the absorbance readings of the negative control (Dusky kob injected with PBS).

4.2.2 Investigation of yeast displayed anti-BMV dAb as a therapeutic antibody

4.2.2.1 Plant growth and maintenance

Barley (*H. vulgare*) seeds (Seeds for Africa) were germinated by soaking in water for 5 h at room temperature. The seeds were then drained and left to germinate overnight at room temperature. Germinated barley seeds were planted in growing medium (1 part vermiculite and 13 parts peat) in 9 cm pots and grown with a 16 h light: 8 h dark cycle under fluorescent lights in a grow room maintained at an average temperature of 22°C, with a relative humidity of 70%, and watered every second day. Plants were grown for approximately 2 weeks, or until they reached their second leaf stage, at a height of 15 cm.

4.2.2.2 Preparation of yeast displayed Dusky kob anti-BMV single domain antibody

A single recombinant pYD1-anti-BMV dAb yeast colony was inoculated into 50 mL of YNB-CAA (0.67% YNB (with ammonium sulphate, without amino acids), 0.5% CAA, and 2% glucose), and incubated at 30°C with shaking at 250 rpm for approximately 24 h. Following incubation, the OD₆₀₀ of the overnight culture was determined. Once an OD₆₀₀ of 2-5 was reached, the cells were induced by the addition of 2% galactose (final conc.). The cultures were induced at 20°C with shaking at 250 rpm for a 72 h period. After induction, the cells were collected by centrifugation at 3500 x g at 4°C for 10 min. The cell pellets were resuspended in 50 mL of PBS and centrifuged as before in order to remove residual media and wash the cells. The cells were washed a total of three times with ice cold PBS, before being resuspended in 10 mL of ice-cold PBS and stored in the dark at 4°C until needed, for no longer than one week. Success of induction was determined by ELISA, as described in Section 3.2.2.3.

4.2.2.3 BMV challenge trial of barley plants

To determine the therapeutic effect of the yeast-displayed Dusky kob anti-BMV single domain antibody, three independent challenge experiments were conducted whereby barley plants were infected with various concentrations of BMV, incubated with either yeast-displayed anti-BMV dAb, yeast displaying pYD1 only, or with anti-BMV IgG. Control groups consisted of barley plants infected with BMV-only or were mock-infected with PBS.

Prior to plant infection, inocula were prepared as follows: a pre-determined concentration of BMV (350-0.68 ng/μL) was preincubated with either 6×10⁷ cells/mL of yeast displayed anti-BMV dAb (experimental), 6×10⁷ cells/mL of yeast cells transformed with pYD1 (negative control), or with 200 μg/mL of anti-BMV IgG (positive control), in a final volume of 2 mL. The concentration of yeast cells used was determined by the competition ELISA described in Chapter 3.3.2.2. The inocula were preincubated at room temperature for 1 h, with occasional

shaking. Following preparation of the inocula, 10-day post-germination barley plants, were divided randomly into experimental and control groups. The plants were infected by rubbing an approximately 2.5 cm area of the 2nd leaf with approximately 1 mL of the inoculum supplemented with celite, which acted as an abrasive regent, using a cotton swab. A total of two plants were infected for each experimental sample. The experiment was repeated a total of three times. Infected leaves were harvested 7 days post-infection, by removing 2.5 cm of the infected area of each plant leaf. Freshly harvested leaf matter for each sample were pooled and ground to a fine powder in liquid nitrogen with a mortar and pestle, and suspended in 1:1 (w/v) PBS/ 0.05% Tween 20/ 0.2% BSA.

4.2.2.4 Detection of BMV infection by double antibody sandwich ELISA (DAS-ELISA)

A 96-well plate (Nunc-Immuno™ MicroWell™ MaxiSorp™, Sigma-Aldrich, USA) was coated with 200 µL/well of 400 µg/mL anti-BMV IgG in PBS and incubated at 4°C overnight. The wells were washed three times with PBS-T. Non-specific binding was blocked with 300 µL/well of 2% BSAPBS and incubated at 37°C for 1 h. Plant extracts (Section 4.2.2.3) were incubated (200 µL/well) in antibody-coated wells for 1 h at 37°C. The plates were washed three times with PBS-T. A total of 200 µL/well of anti-BMV IgG (prepared by diluting 1:500 in PBS/0.1% Tween 20/2% BSA) was added to each well and incubated for 1 h at 37°C. The wells were washed three times with PBS-T. The secondary antibody, goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, USA), was diluted 1:5000 in BSAPBS-T and was added to each well at a volume of 50 µL/well and incubated at 37°C for 1 h. After washing three times with PBS-T and a final single wash with PBS, 200 µL/well of enzyme substrate pNPP (Sigma-Aldrich, USA) was added, before incubation at room temperature for 30 min in the dark. Wells coated with the capture antibody only, and not exposed to BMV, served as the negative control. The absorbance was measured at 405 nm immediately after development on a Multiskan Go Spectrophotometer (Thermo Scientific, USA). The capture antibody only wells, exposed to the secondary antibody, served as a negative control.

4.2.3 Statistical analysis

All statistical analyses were performed using GraphPad PRISM software. For the diagnostic comparison between the two different antibodies used to detect the presence of BMV, a standard curve (Supplementary Fig. B 2.1 and B 2.2) was used to convert the acquired OD values into the amount of BMV detected (ng/µL) in the blood serum of infected fish sampled at 1, 3, and 5 h post-infection. A one-way ANOVA was performed with a Bonferroni post-hoc test, to determine the statistical significance between each antibody at the different time points.

A two-way ANOVA was performed with a Bonferroni post-hoc test to evaluate the therapeutic effect of anti-BMV antibodies (anti-BMV IgG and Anti-BMV dAb) in barley plants infected with various concentrations of BMV compared to each other (anti-BMV IgG vs anti-BMV dAb). Final experimental readings were generated by subtracting the negative control (capture antibody only), prior to analysis. Additionally, statistical significance of antibody protection in infected plants was determined by comparing plants treated with anti-BMV antibodies to those infected with BMV not exposed to any antibody (Fig. 4.2). Significance was taken at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). All graphs were made using GraphPad PRISM and display mean $\pm$ SD.

4.3. Results

4.3.1 Detection of BMV in Dusky kob serum using a yeast displayed anti-BMV single domain antibody from Dusky kob

Healthy Dusky kob injected with BMV were used to validate the yeast displayed anti-BMV antibody from Dusky kob as a potential diagnostic reagent. Following injection with BMV, the blood of injected fish was extracted at 0, 1, 3, and 5 h post-injection. Analysis of the blood serum of the injected fish demonstrated that both the yeast displayed anti-BMV dAb and the anti-BMV IgG could successfully detect the presence of BMV in the sera of injected fish at all time points (Fig. 4.1). The anti-BMV IgG antibody detection of BMV in the sera of injected Dusky kob was significantly higher ($p < 0.0001$) compared to the yeast displayed anti-BMV dAb detection of BMV at all time points, where it was observed that the concentrations of BMV detected by the anti-BMV IgG were 2.03 ± 0.45 , 1.70 ± 0.12 , 1.58 ± 0.23 , and 1.47 ± 0.20 ng/ μ L at 0, 1, 3, and 5 h post-infection, respectively, while the concentrations of BMV detected by the yeast displayed anti-BMV dAb were 1.37 ± 0.17 , 1.16 ± 0.12 , 0.63 ± 0.075 , and 0.54 ± 0.040 ng/ μ L for the same timepoints (Fig. 4.1).

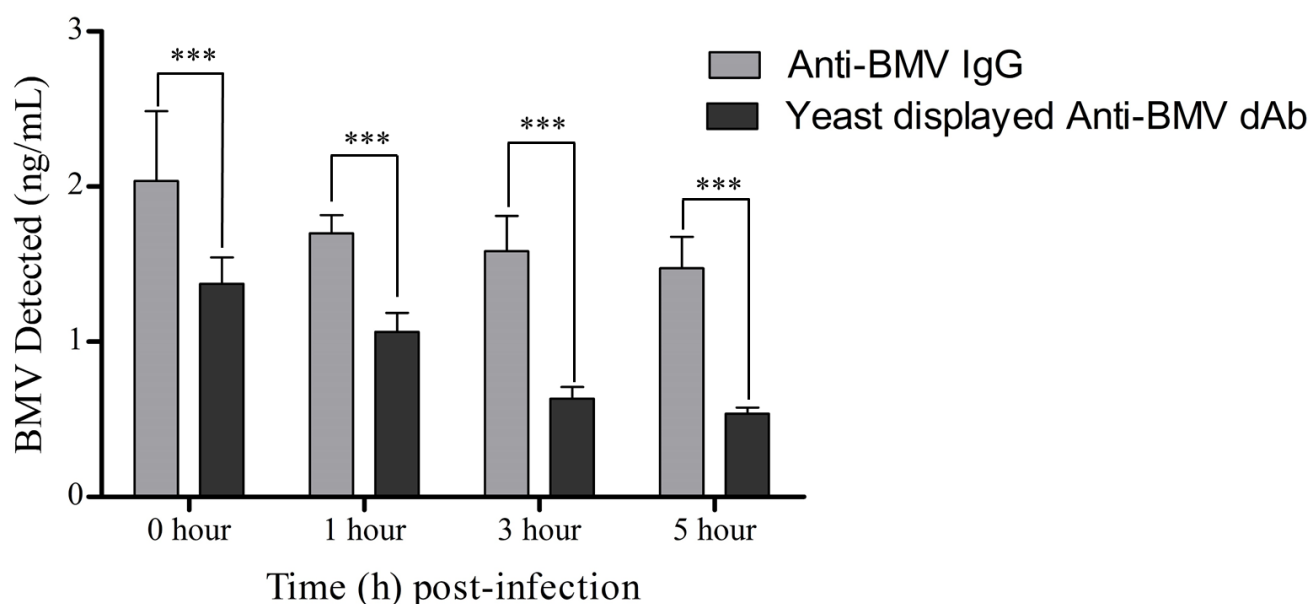


Figure 4.1 Detection of BMV in the sera of infected Dusky kob using either a commercially available antibody (anti-BMV IgG) or the yeast displayed Dusky kob anti-BMV dAb (yeast anti-BMV dAb). The antibodies were used to detect BMV in the blood of kob at 0, 1, 3, and 5 h post-infection. Data represents detection levels minus the negative control (Dusky kob injected with PBS). Error bars indicate the standard deviation of the means of three biological repeats. Statistical significance is indicated by an asterisk (*) above the bars, as determined by a one-way ANOVA. *** = $p \leq 0.0001$; $n = 3$.

4.3.2 Validation of yeast displayed Dusky kob anti-BMV single domain antibody as a therapeutic reagent

To determine the effectiveness of a yeast displayed anti-BMV dAb derived from a Dusky kob phage display scFv library as a therapeutic reagent, the ability of the yeast-displayed anti-BMV dAb to block and mitigate the BMV infection of barley plants was evaluated by DAS-ELISA. Barley plants were challenged with a range of BMV concentrations (0.68-87 ng/ μ L), each of which had been pre-incubated with either 6×10^7 cells/mL of yeast displayed anti-BMV dAb or 200 μ g/mL of anti-BMV IgG. Barley plants infected with either BMV pre-incubated with yeast cells transformed with pYD1 or BMV-alone served as negative controls. No significant difference ($p > 0.05$) was observed in antibody protection of barley plants infected with BMV at a concentration of 87 ng/ μ L when pre-incubated with the yeast displayed anti-BMV dAb, while a significant difference ($p \leq 0.05$) in antibody protection can be observed at the same BMV concentration when pre-incubated with the anti-BMV IgG commercial antibody (Fig. 4.2). A significant difference ($p \leq 0.001$) was observed in the antibody protection of barley plants that were infected with BMV, at all concentrations, when pre-incubated with the

anti-BMV IgG (Fig. 4.2). A significant difference in antibody protection of barley plants infected with BMV at concentrations between 43 ng/ μ L ($p \leq 0.05$) and 0.68 ng/ μ L ($p \leq 0.001$) was observed when pre-incubated with the yeast displayed anti-BMV dAb (Fig. 4.2). No significant difference ($p \geq 0.05$) in antibody protection of barley plants infected with BMV at concentrations of 1 and 0.68 ng/ μ L was observed when pre-incubated with either the yeast displayed anti-BMV dAb or anti-BMV IgG. No significant difference ($p > 0.05$) was observed in the amount of BMV detected in barley infected with either BMV-only and those that were infected with BMV pre-incubated with yeast transformed with pYD1, at all BMV dilutions.

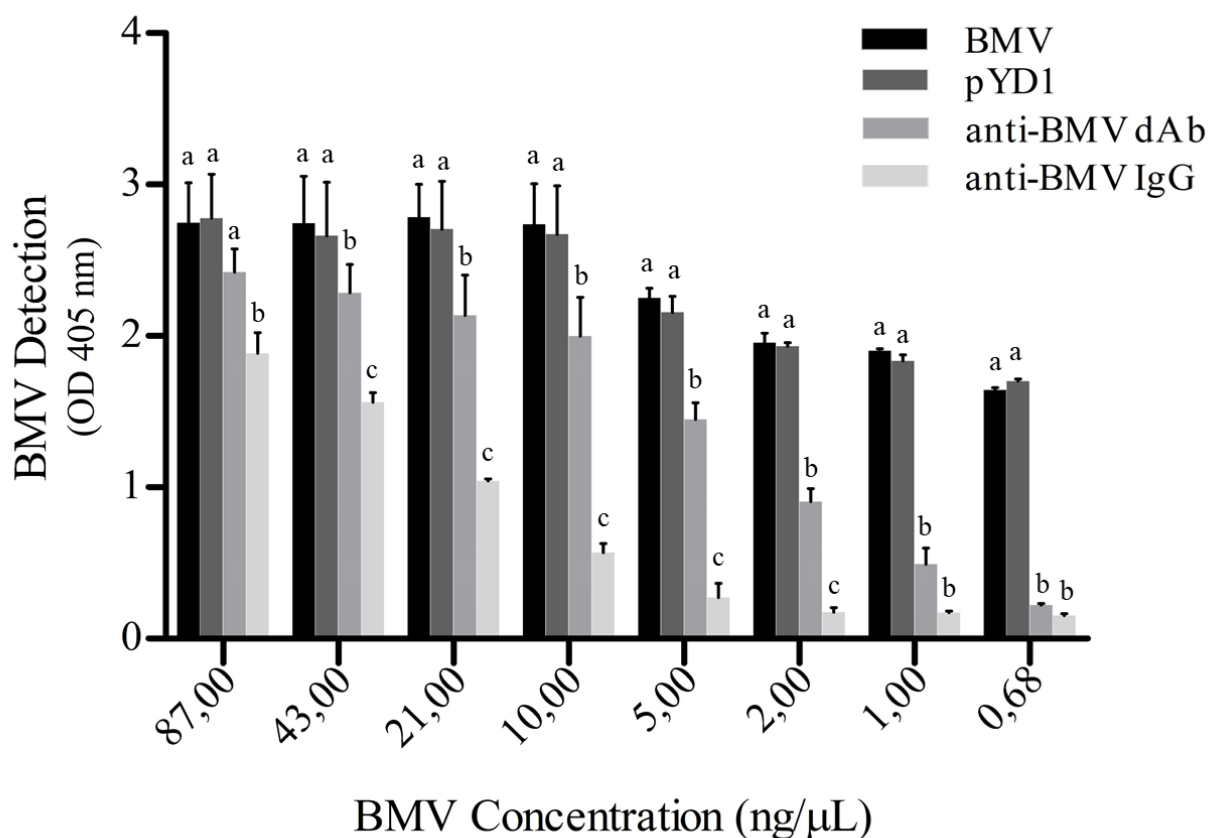


Figure 4.2 Detection of BMV in infected barley plants treated with either a commercial anti-BMV IgG or with a yeast-displayed Dusky kob anti-BMV dAb. BMV: barley infected with BMV alone; pYD1: barley infected with BMV pre-incubated with yeast-displayed pYD1; Anti-BMV dAb: barley plants infected with BMV pre-incubated with yeast-displayed anti-BMV dAb; Anti-BMV IgG: barley plants infected with BMV pre-incubated with anti-BMV IgG mAb. Error bars represent the standard deviation of the means. Bars with similar letters represent no significant difference ($p \geq 0.05$). Bars with different letters represent samples that are significantly different ($p \leq 0.05$) from each other; $n = 6$.

4.4 Discussion

Monoclonal antibodies have proven to be ideal tools for the diagnosis of various fish pathogenic infections (Bakopoulos, Adams and Richards, 1997; Kim *et al.*, 2004), as potential therapeutics (Yi *et al.*, 2003; Huang *et al.*, 2005), and in furthering the understanding of the fish immune system (Coll and Dominguez-Juncal, 1995; Chiraporn, Sasimanas and Prapansak, 2015). However, traditional methods of mAb production, such as hybridoma, are expensive, time-consuming, and require an animal host for production (Hairul Bahara *et al.*, 2013; Omidfar and Daneshpour, 2015; Unkauf *et al.*, 2016). Phage display technology is an ideal alternative for the production of antibody fragments for various applications, including, but not limited to, the production of diagnostic and therapeutic reagents for improved disease management in aquaculture. A number of studies have demonstrated the potential of phage display as a source of diagnostic (Dai *et al.*, 2003; Zhang, Wang, Wu, M Li, *et al.*, 2006) and therapeutic (Yuan *et al.*, 2006; Liu *et al.*, 2014) antibodies against various aquatic pathogens. However, the aforementioned studies utilised a scFv phage display library derived from murine V_H and V_L chains. There are currently no reports on the identification and evaluation of antibodies derived from a fish scFv phage display library, and the potential diagnostic and therapeutic capabilities of antibodies derived from a fish scFv phage display library remains to be elucidated. In this study, Dusky kob and barley plants were challenged with BMV, as a proxy of an uncharacterised emerging pathogen of the former and a viral pathogen of the latter, to evaluate the effect of a phage display-derived antibody (Chapter 2) in detecting BMV and/or mitigating infection.

4.4.1 Detection of BMV in the blood serum of infected Dusky kob

Immunological assays are gradually becoming the gold standard for the diagnosis of fish diseases, replacing more traditional methods that are considered costly, slow, and labour intensive (Adams and Thompson, 2011). Previously, pathogen detection typically took several days to complete, as pathogens would have to be meticulously extracted and cultured, all requiring complicated techniques and laboratory conditions (Liu *et al.*, 2014). The use of mAbs has allowed for the implementation of immunological-based assays that can be performed in a very short time without the need to isolate and culture target pathogens, thus significantly decreasing the need for specialised equipment and laboratory environments (Hoare *et al.*, 2016; Kulabhusan *et al.*, 2017). An ELISA is a popular diagnostic techniques due to its high sensitivity and specificity (Desoubaux *et al.*, 2017; Persichetti *et al.*, 2017). To evaluate the

diagnostic role of the yeast displayed anti-BMV dAb, an ELISA was conducted on the blood sera of Dusky kob injected with BMV.

Yeast cells displaying the anti-BMV dAb and an anti-BMV IgG mAb were used as detection reagents in an ELISA on BMV-infected blood serum from Dusky kob. Several reports have demonstrated the effectiveness of using entire display systems as biosensors rather than expressed soluble antibody molecules. This is due to greatly enhanced detection signals of display systems. This is evident in the yeast surface display system, whereby each cell displays ~50,000 copies of the recombinant protein, allowing for greater detection signals with an appropriate detection antibody (Siegel, 2002; Zhang, Wang, Wu, M Li, *et al.*, 2006; Nanduri *et al.*, 2007; Archer and Liu, 2009; Gera, Hussain and Rao, 2013; Liu *et al.*, 2013). In the present study, an ELISA was employed utilising a yeast displayed anti-BMV dAb and a commercial anti-BMV IgG antibody to detect the presence of BMV in the blood serum of Dusky kob injected with BMV. It was observed that both the commercial anti-BMV IgG antibody and yeast displayed anti-BMV dAb could successfully detect the presence of BMV in the blood serum of injected Dusky kob at 0 h, 1 h, 3 h, and 5 h post-infection (Fig. 4.1). While both antibodies were successful in detecting BMV in infected serum, it was observed that the commercial anti-BMV IgG antibody was more sensitive than the yeast displayed anti-BMV dAb and could effectively detect a higher concentration of BMV at all observed time points. It was found that at 0 h, 1 h, 3 h, and 5 h post-infection, the commercial anti-BMV IgG antibody could detect 2.03, 1.70, 1.58, and 1.47 ng/ μ L of BMV respectively (Fig. 4.1), while the yeast displayed anti-BMV dAb was effective at detecting 1.37, 1.16, 0.63, and 0.54 ng/ μ L of BMV at the same time points (Fig. 4.1). This suggests that the commercial anti-BMV IgG antibody is a slightly more sensitive diagnostic reagent than the yeast displayed anti-BMV dAb.

However, a significant advantage of yeast surface display over other expression and display systems is that it allows for rapid and easy affinity maturation of various antibody fragments, increasing their affinity and sensitivity to target antigens (Li, 2000; Gai and Wittrup, 2007; Ferrara *et al.*, 2012). This is often achieved through random or target specific mutagenesis of various regions, such as the CDR3, of the genes encoding the desired antibody fragment (Sun *et al.*, 2019). This has been demonstrated in various studies, including that by Van Den Beucken *et al.* (2003), where the authors successfully improved the affinity and sensitivity of an anti-streptavidin by up to 10.7-fold using yeast display. Therefore, although the yeast displayed anti-BMV dAb presented in this study was found to be slightly less sensitive than the commercial anti-BMV IgG antibody, the sensitivity of the anti-BMV dAb can be improved through affinity maturation. This will allow for the development of antibodies that not only

display a higher affinity and sensitivity when compared to a commercial antibody, but can be produced in a more cost-effective manner and over a shorter timeframe, without resorting to animals or hybridomas.

Similar studies have reported the success of various display systems, such as phage display and yeast display, in the detection of various pathogens using immunological assays, including studies demonstrating their use in the detection of aquatic pathogens (Zhang, Wang, Wu, M. Li, *et al.*, 2006; Liu *et al.*, 2013; Yuan *et al.*, 2014). For example, in a study by Ferrara *et al.* (2012), the authors describe the development of a yeast-displayed antibody to a tuberculosis biomarker, antigen 85 (Ag85), which could successfully detect Ag85 down to 6.1 nM in the absence of serum and 22.7 nM in the presence of serum. Additionally, in a study by Dai *et al.* (2003), a display system was successfully employed to display an anti-WSSV antibody to detect the presence of WSSV directly from the haemolymph of shrimp 24 h after infection. In the present study, it was demonstrated through an ELISA that yeast cells displaying an anti-BMV dAb could effectively detect the presence of BMV in the blood sera of Dusky kob injected with BMV at 0 h, 1 h, 3 h, and 5 h post-infection (Fig. 4.1). Despite only detecting the presence of BMV 5 h post-infection, as compared to the 24 h reported by Dai *et al.* (2003), it is important to note that BMV is not a natural pathogen to Dusky kob and therefore will not replicate within the fish, but rather would be rapidly cleared from the fish circulatory system. Regardless, the success of BMV detection at 5 h post-infection highlights the ability of these yeast-displayed antibodies to detect the presence of a foreign virus in the blood serum of an infected host. This demonstrates the effectiveness of a yeast-displayed antibody to act as a diagnostic reagent with an ELISA based assay using sera directly from infected fish, without prior culturing or preparation, allowing for a more rapid diagnosis.

4.4.2 Therapeutic capacity of the yeast-displayed Dusky kob anti-BMV dAb

The use of antibodies as a means to control pathogens has been widely utilised in a number of different systems, including the protection of mice and humans against western equine encephalitis (Burke *et al.*, 2018), the protection of figs against fig mosaic virus (Shahmirzaie *et al.*, 2020), and controlling infection by WSSV in cultured shrimp (Yuan *et al.*, 2006). Despite the lack of an adaptive immune system, antibodies (purified or in sera) reportedly have the ability to neutralise a number of plant pathogens (Kassanis, 1943; Gold and Duffus, 1967; Dietzgen and Sander, 1984; Erdag, Atalay and Çirakoglu, 2000; Boonrod *et al.*, 2004). In 1926, a study by Mulvania was the first to demonstrate that the addition of rabbit sera, extracted from rabbits immunised with TMV, decreased TMV infectivity in host plants (Kassanis, 1943). Similar studies have further demonstrated the use of antiserum in mitigating infection by

various plant viruses, such as the beet western yellow virus (Gold and Duffus, 1967), tomato bushy virus, and tobacco necrosis virus (Kassanis, 1943).

It is well understood that for an antibody to have a neutralising effect, the antibody needs to bind to key proteins of the pathogen, such as the envelope spikes of a virion, in order to prevent attachment and entry into host cells (Burton, Williamson and Parren, 2000; Van Hulten *et al.*, 2001). It is well documented that the key protein for viral infection of BMV is the coat protein (CP) (Dasgupta and Kaesberg, 1982). The CP of BMV plays an essential role, not only in infection, but is actively involved in viral replication and suppression of the innate immune system of host plants (De Jong and Ahlquist, 1995; Kao *et al.*, 2011). Thus, an antibody targeting the CP of BMV is expected to assist in preventing attachment and infection of the virus to plant host cells. As previously demonstrated, the anti-BMV dAb selected in this study was observed to bind specifically to the CP of BMV (Chapter 2, Section 2.3.3.4), therefore yeast displaying the anti-BMV dAb could potentially be used to prevent BMV infection of barley.

In the present study, it was observed that BMV could successfully infect barley at concentrations of 0.68-84 ng/ μ L (Fig. 4.2). However, BMV at a concentration of 21, 10, 5, and 2 ng/ μ L, pre-incubated with the yeast displayed anti-BMV dAb, was found to effectively mitigate BMV infection in barley (Fig. 4.2). Additionally, it was observed that infection of barley with BMV at concentrations of 1 ng/ μ L and 0.68 ng/ μ L could successfully be prevented when pre-incubated with the yeast displayed anti-BMV dAb (Fig. 4.2). However, the anti-BMV IgG antibody was found to be more effective in mitigating the infection of BMV in barley, when pre-incubated with BMV concentrations of 87-2 ng/ μ L (Fig. 4.2). This is possibly due to the anti-BMV IgG having a stronger affinity and sensitivity compared to the yeast displayed anti-BMV dAb. Regardless, a yeast displayed anti-BMV dAb was successfully found to mitigate BMV infection in barley plants. As it was previously shown that the anti-BMV dAb used in this study binds specifically to the CP of BMV (Chapter 2, Section 2.3.3.4), it is therefore plausible that the observed decrease in BMV infection of barley incubated with the yeast displayed anti-BMV dAb is due to the recombinant displayed antibody successfully binding to the CP of BMV, thereby preventing the virus from binding to and infecting the barley plants. A similar result was reported by Kassanis (1943), in which the author observed that when exposed to a TMV antiserum, the number of lesions in TMV-infected plants decreased from 53 to 23 when infected with a 10^{-3} dilution of TMV, and from 21 to 0 when infected with a 10^{-6} dilution of the virus, although the actual titre of TMV was not disclosed (Kassanis, 1943). Similarly, Gold and Duffus (1967) reported that an antiserum to western

yellow virus, mixed with the virus, could completely mitigate western yellow viral infection of beet plants. Overall, the results of the present study show that a yeast-displayed Dusky kob anti-BMV dAb can effectively mitigate and control the infection of barley plants by the plant virus BMV, thereby proving the therapeutic potential of the dAb.

4.4.3 Conclusions

In conclusion, it has been demonstrated that an anti-BMV dAb, derived from a Dusky kob phage display library, could successfully be used to detect the presence of BMV in the blood serum of BMV-infected Dusky kob by ELISA with similar sensitivity to a commercially available counterpart. This study also demonstrated the neutralising effect of the phage display derived anti-BMV dAb in reducing BMV infection in barley. To our knowledge, this is the first study to report the application of a Dusky kob phage display library as a source of diagnostic and therapeutic reagents aimed at BMV, which served as an emerging fish pathogen proxy. Overall, this study highlights the potential of a Dusky kob phage display library to be used as a source of diagnostically and therapeutically important antibodies against emerging pathogens that threaten the fish farming industry of South Africa.

Chapter 5

Conclusions and future prospects

Dusky kob is an economically important finfish species and is currently one of the only marine fish species farmed in South Africa. Despite its economic importance, the annual production of Dusky kob contributes less than 2% of the total production of all farmed seafood in South Africa (Department of Agriculture Forestry and Fisheries, 2017). It is believed, as with most aquaculture farms, that the occurrence and reoccurrence of disease outbreaks is a significant hinderance to the successful establishment of Dusky kob farms in South Africa. The establishment of Dusky kob farms is also impacted by a lack of information regarding disease-causing pathogens that infect Dusky kob, which limits pathogen detection and treatment. Technologies such as phage and yeast display allow for the rapid isolation of a range of beneficial antibodies without prior knowledge of the target antigen, and have contributed greatly to the development of diagnostic and therapeutic antibodies for a number of human-related diseases (Chester *et al.*, 1994; Jansen *et al.*, 2017; Jin *et al.*, 2017). However, application of the technology to other sectors, such as aquaculture, is lacking. To date, much of the work conducted on the use of phage display in aquaculture has focused on mapping epitopes or identifying antibody fragments specific to shrimp pathogens using a murine phage display library (Dai *et al.*, 2003; Yuan *et al.*, 2006; Liu *et al.*, 2014). Consequently, the application of antibodies derived from a phage display library in improving disease management in aquaculture farms remains limited. Thus, this study set out to investigate the potential of a Dusky kob scFv phage display library as a source of diagnostic and therapeutic antibodies to an emerging pathogen. With the development of a Dusky kob phage display library in mind, this study addressed the following aims:

- i. Construct a naïve phage display library displaying scFvs derived from combination pairings of *A. japonicas* V_H and V_L chains, to identify and isolate recombinant scFv antibodies against the plant virus BMV, which will serve as an emerging fish pathogen.
- ii. Identify an effective means of expressing and producing the antibody fragments isolated from the phage display library to produce sufficient quantities of the recombinant protein for downstream applications.
- iii. Validate the diagnostic and therapeutic potential of the identified antibody which in turn will demonstrate the antibody phage display library constructed in this study

as a source of diagnostic and therapeutic antibodies against emerging pathogens of farmed *A. japonicas*.

The main empirical findings of this study have been reported and summarised within the respective experimental chapters (Chapters 2-4). Overall, the findings of this study demonstrate the potential use of a Dusky kob phage display library to effectively identify candidate diagnostic and therapeutic antibodies to an emerging pathogen of farmed Dusky kob. This final chapter serves as a summary of the key findings of each chapter and their potential implications for Dusky kob farm disease management. Additionally, this concluding chapter highlights and addresses potential limitations of the study and offers possible solutions to improve future work in this field.

5.1 The development of an *A. japonicus* naïve single chain variable fragment phage display antibody library

Phage display is a powerful peptide display technology that has been effectively employed since its discovery in 1985, most notably for the display and identification of novel antibody fragments (Smith, 1985; Azzazy and Highsmith Jr., 2002). In comparison to other antibody development methods, such as hybridoma, phage display is cheaper, less labour intensive, and does not require the immunisation of animals, allowing for the identification of novel antibodies in a more cost-effective and humane manner. To date, therapeutic antibodies identified by phage display, which are currently at various stages of clinical trials, account for over 30% of the total number of antibodies being studied (Almagro *et al.*, 2019). However, the application of phage display in aquaculture is limited. With the success of phage display as a tool for the discovery of diagnostically and therapeutically important antibodies, it is believed that its application in aquaculture will allow for the development of novel reagents for better disease management.

The first aim of this study was to develop a Dusky kob scFv phage display library that could be used to identify novel antibodies to an emerging pathogen. Chapter 2 describes the successful construction of a Dusky kob scFv phage display library based on the V_H and V_L chain genes isolated from splenic B cells of healthy Dusky kob. Upon successful construction of the Dusky kob scFv phage display library, the library was screened for antibodies to the plant virus BMV to determine the potential of the library as a resource from which antibodies to emerging pathogens of Dusky kob could be obtained. As BMV is a plant virus, the probability of Dusky kob having any prior exposure to BMV is unlikely, and thus it is highly improbable that healthy Dusky kob would have antibodies to the virus. Therefore, BMV served as an ideal proxy for an emerging pathogen. The library described in this study was successfully

used to identify candidate antibodies with strong affinity and specificity to BMV. The findings of this study highlight not only the potential of this library as a source of antibodies to any emerging pathogen of Dusky kob, but also the overall power of phage display as a means of identifying antibodies to an antigen without resorting to hybridomas.

Alongside the positive outcome of Chapter 2, there were, however, certain limitations. The first major limitation of this study was the lack of Ig sequence information for Dusky kob. As a result, the generation of primers to amplify the V_H and V_L chain genes of Dusky kob was reliant on V_H and V_L sequence data from other fish species that were available at the time. As such, a limited number of sequences belonging to a limited number of fish species were utilised for the design of multiple primers. This hindered the design of ideal primers that could adequately amplify the V_H and V_L chain genes of Dusky kob, which may explain why limited coverage of the CDR3 regions of both V_H and V_L chains was observed. Furthermore, a number of sequences were identified to have missing or truncated V_L chain genes, which may be a consequence of using multiple primers, leading to the addition of unwanted nucleotides resulting from the addition of restriction enzyme sites, such as *Mlu*I and *Not*I, which would have led to partially or fully truncated sequences during library construction. The availability of specific Dusky kob immunological genetic information would allow for the construction of primers better suited to the amplification of Dusky kob V_H and V_L chain gene fragments, which would ensure adequate coverage of all necessary CDR and FW regions of the Dusky kob Ig, resulting in the development of potentially more effective antibody fragments.

Additionally, due to financial constraints and the limited availability of the Hyperphage reagent due to rigorous import restrictions, the number of rounds of selection was limited to two. Further rounds of selection would allow for further enrichment of positive binders to BMV and contribute to the identification of a greater number of specific binders. A total of five positive clones were selected after two rounds of biopanning and were screened by sequence analysis to assess the sequence diversity and identity, of which all five were found to consist of only V_H chain genes. By implementing more rounds of selection and screening more positive clones, it may be possible to identify antibody fragments that consist of full length V_H and V_L chain fragments. Regardless, V_H-only antibody fragments have been reported to have high specificity, high physico-chemical stability, and due to their small size, enhanced tissue penetration capabilities (Krah *et al.*, 2016). Despite the above-mentioned limitations, we have successfully constructed a Dusky kob scFv phage display library, which was used to identify a number of candidate antibodies to a virus to which Dusky kob would have had no prior exposure.

5.2 Expression of an anti-BMV single domain antibody using a bacterial expression plasmid and a yeast surface display system

Previous studies have shown the potential of phage-displayed antibodies as effective diagnostic reagents through ELISA and western blot analyses (van Wyngaardt *et al.*, 2004; Zhang, Wang, Wu, M. Li, *et al.*, 2006). Their ability to act as effective diagnostic reagents is in part due to the numerous copies of the PVIII coat protein of filamentous phages, which acts as the recognition antigen of the anti-M13 antibody. Due to the numerous copies of this recognition antigen, the detection signal is greatly enhanced, allowing for increased sensitivity. However, despite the benefit of using the antibody in a phage display format for diagnostic purposes, their use as therapeutics is less conventional due to the cost and time required to manufacture the phage-displayed antibodies on an appropriate scale for commercial application. Furthermore, the use of phages as a drug delivery system remains relatively unknown.

Previous studies have demonstrated the successful use of bacterial and yeast expression systems as a mean to produce large quantities of antibody fragments, identified by phage display, for various applications (Swartz, 2001; ShengFeng *et al.*, 2003; Shi *et al.*, 2003; Tsumoto *et al.*, 2003; Andersen and Reilly, 2004). Additionally, studies have demonstrated the effectiveness of yeast as a drug delivery system due to their strong cell walls as well as their ability to act as adjuvants, thereby stimulating the immune system upon delivery (Sun *et al.*, 2010; Zhao *et al.*, 2017). Therefore, the second aim of this study was to identify an effective means to express the anti-BMV dAb, outside of the phage display format, which could be effectively used as a therapeutic reagent. With this in mind, bacterial expression and yeast display systems were employed to effectively express the anti-BMV single domain antibody identified in Chapter 2.

Initial attempts to express the anti-BMV dAb in a bacterial expression system were unsuccessful, as the antibody was expressed as inclusion bodies in the bacterial cells. Attempts to extract and refold the antibody were deemed unsuccessful, as the antibody obtained did not show any affinity to BMV. However, the antibody was successfully expressed and displayed on the surface of yeast cells, and was shown to have a strong affinity to BMV using ELISA, western blot analysis, and TEM. Thus, Chapter 3 demonstrated the efficacy of a yeast display system for the expression of an antibody identified by phage display. The benefit of utilising a yeast surface display system is that it simultaneously allows for cost-effective and routine production of a desired antibody while serving as an effective delivery system.

The limitations of this experimental chapter relate to the formation of inclusion bodies when attempting to express the anti-BMV dAb in bacteria. Consequently, the protein could not be correctly refolded, resulting in the expressed anti-BMV dAb being incapable of binding to BMV. Therefore, this bacterial system could not be utilised in subsequent downstream applications. It is possible that expressing the anti-BMV dAb together with an upstream signal peptide could allow the dAb to be exported and expressed in the periplasmic space of *E. coli*, which has been reported to be better suited for the production of antibodies due to the presence of essential proteins that assist in folding (Swartz, 2001; Liu and Huang, 2018).

5.3 Diagnostic and therapeutic analysis of the anti-BMV single domain antibody isolated from a Dusky kob phage display library

Thus far, we have successfully demonstrated that a Dusky kob scFv phage display library can be used to isolate antibodies to the plant virus BMV. Furthermore, we have shown that a yeast display system can be successfully used to express the anti-BMV antibody. To determine whether an antibody isolated from the phage display library can be used to improve disease management on Dusky kob farms, the diagnostic and therapeutic proficiency of the yeast-displayed antibody described in Chapter 2 and 3 needed to be verified. With this in mind, the diagnostic and therapeutic proficiency of the anti-BMV dAb was assessed in two challenge trials, with each trial aiming to investigate either the diagnostic or therapeutic aspect of the antibody. The diagnostic proficiency of the anti-BMV dAb was assessed by challenging Dusky kob with BMV. Following exposure, the blood serum of infected fish was analysed by ELISA using a yeast displayed anti-BMV dAb. Here, we successfully demonstrated that the displayed antibody could adequately detect the presence of the plant virus in the fish serum up to 5 hours post-infection. The therapeutic proficiency of the anti-BMV dAb was assessed by investigating BMV infection of barley plants using BMV that had been exposed to the yeast displayed anti-BMV prior to infection. It was found that BMV infection of barley plants was mitigated when treated with the Dusky kob anti-BMV dAb, highlighting the therapeutic potential of a yeast-displayed antibody originally identified and isolated from a phage display library.

Despite the success of this chapter, it was not without limitations. In determining the diagnostic proficiency of the anti-BMV dAb, Dusky kob were injected with BMV, which is unable to replicate in the fish as Dusky kob is not a host of this virus, which specifically infects cereal plants. As a result, the time at which the Dusky kob injected with BMV could be sampled was limited to 5 h post-injection, due to the fish rapidly clearing BMV from its system. Thus, future studies would focus on utilising a pathogen more specific to Dusky kob to further evaluate the sensitivity, and therefore, the overall detection limit of an antibody isolated by phage display.

Furthermore, in determining the therapeutic proficiency of the yeast displayed anti-BMV dAb, the antibody was mixed with BMV prior to exposure to the barley plant. Although this method of determining the therapeutic role of antibodies has been reported in previous plant infection studies (Kassanis, 1943; Gold and Duffus, 1967), the effectiveness of a yeast displayed dAb in mitigating a pathogenic infection in farmed Dusky kob remains to be tested.

5.4 Future studies

Although we have reported the effective use of a Dusky kob scFv phage display library as a source of therapeutic and diagnostic antibodies to improve overall disease management in Dusky kob farms, the continued use of a phage display library is not feasible due to the high cost of reagents, such as Hyperphage, and due to the time required in isolating antigen-specific antibodies. However, the use of a yeast display system can be easily implemented as an ideal replacement for phage display, without the requirement of expensive reagents. Thus, future studies will involve the generation of a Dusky kob dAb yeast display library, based off available sequence data of Dusky kob immunoglobulin genes, that will be used to isolate dAbs to pathogens that threaten Dusky kob, such as *Diplectanum oliveri*. Furthermore, yeast displayed dAbs can be used directly in diagnostic and therapeutic trials, without the need for an expression system, allowing for a rapid turn-around time between isolating an antigen-specific antibody and its immediate deployment as a diagnostic and therapeutic reagent.

5.5 Conclusions

This study describes the construction of a naïve phage display library of scFvs derived from the V_H and V_L chains of Dusky kob, which was successfully used to identify antibodies to BMV. Further, we showed that these antibodies could be used to detect the presence of BMV in Dusky kob serum and could be used to effectively mitigate BMV infection in barley plants. Although previous findings have reported the application of phage display in aquaculture (Dai *et al.*, 2003; Yuan *et al.*, 2006; Liu *et al.*, 2014), these studies were based on the use of a mouse phage display scFv antibody library. To the best of my knowledge, this is the first study to report on the construction of a fish scFv phage display library. Furthermore, this is the first study that has successfully reported on the identification of fish antibody fragments using a phage display system, in particular a single domain V_H chain antibody fragment, which can specifically bind to a plant virus that is completely foreign to the fish immune system. The findings of this study show that phage display is an effective tool for use in the identification of candidate diagnostic and therapeutic antibodies to any emerging pathogen of Dusky kob, and thus provides a rapid, targeted approach for mitigating significant fish stock loss due to pathogenic infection.

Appendix A

Media and Solutions

A.1 Media

A.1.1 2 × Tryptone yeast extract broth (2 × TY)

- 1.6 % (w/v) Tryptone
- 1% (w/v) Yeast extract
- 0.5% (w/v) NaCl

Make in dH₂O and autoclave.

A.1.2 2 × Tryptone yeast extract agar (2 × TY)

- 1.6 % (w/v) Tryptone
- 1% (w/v) Yeast extract
- 0.5% (w/v) NaCl
- 1.5% (w/v) Agar Bacteriological

Make in dH₂O and autoclave.

A.1.3 Luria-Bertani (LB) broth

- 1% (w/v) Tryptone
- 0.5% (w/v) Yeast extract
- 0.5% (w/v) NaCl

Make in dH₂O and autoclave.

A.1.4 Luria agar (LA)

- 1% (w/v) Tryptone
- 0.5% (w/v) Yeast extract
- 0.5% (w/v) NaCl
- 1.5% (w/v) Agar Bacteriological

Make in dH₂O and autoclave.

A.1.5 Minimal media plates

- 0.67% (w/v) Yeast Nitrogen Base (YNB) with ammonium sulphate, without amino acids
- 2% (w/v) D-glucose C₆H₁₂O₆
- 0.01% (w/v) Leucine
- 0.01% (w/v) Tryptophan (optional)

1.5% (w/v) Agar Bacteriological

Make in dH₂O and autoclave.

A.1.6 Minimal media broth

0.67% (w/v) Yeast Nitrogen Base (YNB) with ammonium sulphate, without amino acids

0.5% (w/v) Casamino acids

2% (w/v) D-Glucose C₆H₁₂O₆

Make in dH₂O and autoclave.

A.1.7 Minimal media broth (induction media)

0.67% (w/v) Yeast Nitrogen Base (YNB) with ammonium sulphate, without amino acids

0.5% (w/v) Casamino acids

2% (w/v) D-Galactose C₆H₁₂O₆

Make in dH₂O and autoclave.

A.1.8 Yeast extract, peptone, dextrose media (YPD)

1% (w/v) Yeast extract

2% (w/v) Peptone

2% (w/v) Dextrose (D-glucose) C₆H₁₂O₆

Make in dH₂O and autoclave.

A.2 Solutions

A.2.1 General Solutions

A.2.1 PBS stock solution (10×)

1.37 M (w/v) NaCl

27 mM (w/v) KCl

100 mM (w/v) Disodium phosphate (Na₂HPO₄)

18 mM (w/v) Monopotassium phosphate (KH₂PO₄)

Make in dH₂O, adjust pH to 7.4, and autoclave. Dilute 10× stock solution to 1× working concentration in dH₂O.

A.2.2 20% Tween 20

100% Tween 20 (Sigma-Aldrich)

Make up to volume in dH₂O. Do not autoclave.

A.2.3 50× TAE

2 M (w/v) Tris

100 mM (w/v) Ethylenediaminetetraacetic acid (EDTA)

5.7% (v/v) Glacial acetic acid

Make up to volume with dH₂O, autoclave, and store at room temperature. Dilute 50× stock solution to 1× working concentration (1× TAE) in dH₂O.

A.2.2 Solutions for SDS-PAGE, western blot, and ELISA analysis

A.2.2.1 4× Tris HCl/SDS pH 8.8

1.5 M (w/v) Tris

69.3 mM (w/v) SDS

Dissolve in 0.5 of the final volume of sterile dH₂O, adjust pH to 8.8, then make up to final volume. Filter sterilise and store at 4°C.

A.2.2.2 4× Tris HCl/SDS pH 6.8

0.5 M (w/v) Tris

69.3 mM (w/v) SDS.

Dissolve in 0.5 of the final volume of sterile dH₂O, adjust pH to 6.8, then make up to final volume. Filter sterilise and store at 4°C.

A.2.2.3 10% Ammonium persulfate

10% (w/v) Ammonium persulfate (Promega)

Prepare in dH₂O, do not autoclave. Filter sterilise and store at 4°C.

A.2.2.4 5× SDS running buffer

125 mM (w/v) Tris

0.96 M (w/v) Glycine

17.3 mM (w/v) SDS

Make up in dH₂O and store at 4°C.

A.2.2.5 40% (v/v) Acrylamide (Sigma-Aldrich)

Reconstitute lyophilised powder in dH₂O and filter sterilise. Store at 4°C.

A.2.2.6 1 M Tris (hydroxymethyl) aminomethane-hydrochloric acid (Tris-HCl) pH 6.8

1 M (w/v) Tris

Dissolve Tris in 0.6 volume of dH₂O. Adjust pH to 6.8, make up to final volume. Filter sterilise and store at 4°C.

A.2.2.7 5× Protein sample application buffer (SAB)

250 mM Tris-Cl pH 6.8 (Prepared from 1 M stock solution, Appendix 2.2.6)

500 mM (w/v) Dithiothreitol (DTT)

10% (w/v) SDS

0.5% (w/v) Bromophenol blue

67% (v/v) Glycerol

Prepare in dH₂O and store at -20°C.

A.2.2.8 Towbin buffer

25 mM (w/v) Tris

192 mM (w/v) Glycine

20% (v/v) Methanol

Prepare in dH₂O, do not autoclave. Store at 4°C.

A.2.2.9 Blocking buffer

4% (w/v) Bovine serum albumin

Make up fresh before every use in 1× PBS (Appendix A.2.1).

A.2.2.10 PBS-T

1× PBS (Prepared from 10× stock solution, Appendix A.2.1)

0.2% Tween 20 (Prepared from 20% stock solution, Appendix A.2.2)

A.2.2.11 Coomassie protein gel stain

45% (v/v) Methanol

10% (v/v) Glacial acetic acid

3 g/L Coomassie Brilliant Blue G-250 (ThermoFisher Scientific)

Make up to volume with dH₂O, filter sterilise, and store at room temperature.

A.2.2.12 Destain solution

50% (v/v) Methanol

10% (v/v) Glacial acetic acid

Make up to volume with dH₂O.

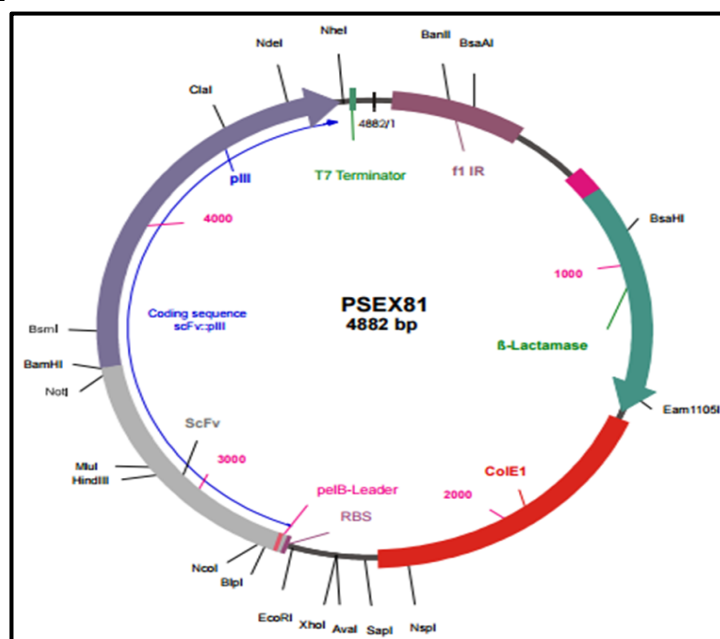
Appendix B

Supplementary data

B.1

Chapter 2 Supplementary information

A



B

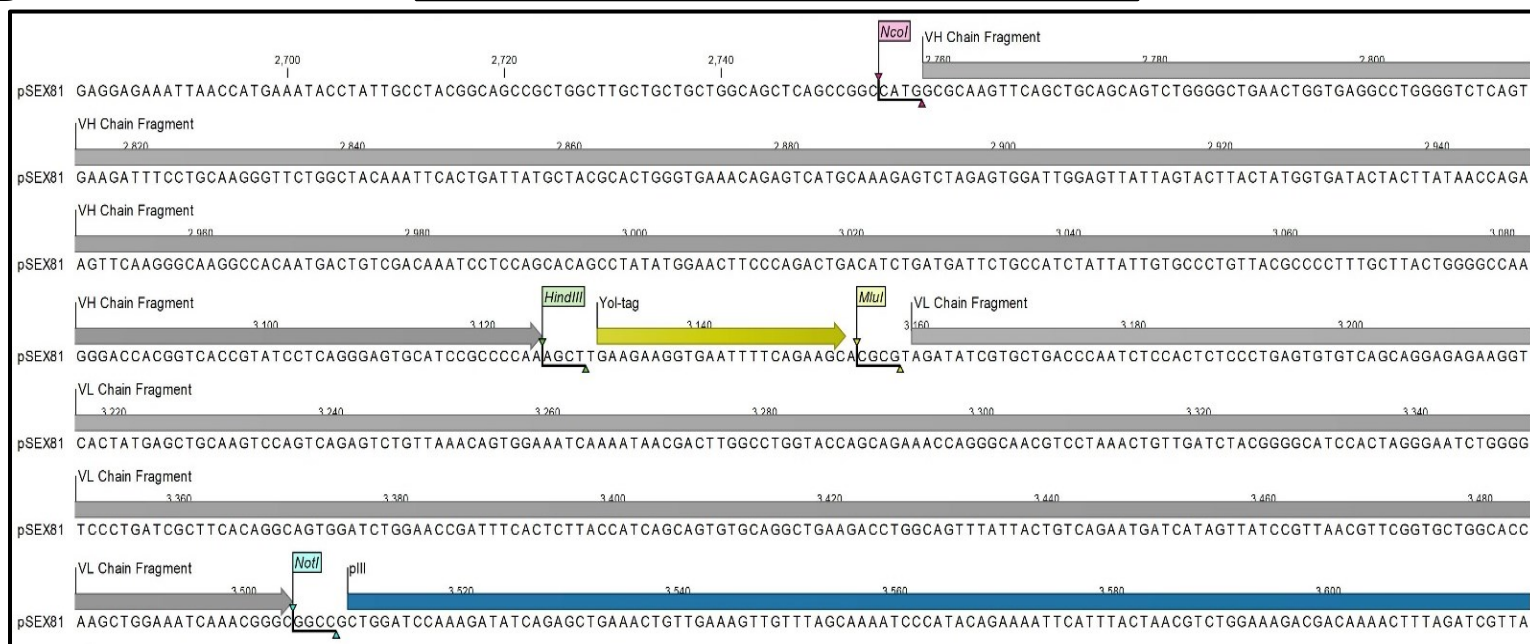


Figure B.1.1 A) Restriction map of the phagemid vector pSEX81 (PROGEN, Germany). Regions of interest include the human single chain variable fragment (grey), the phage minor coat protein gene pIII (purple). Blue arrow indicates the coding sequence of the scFv:pIII region. B) Expanded region of the cloning site for the V_H and V_L chain gene fragments (grey) which are linked by the Yol-tag (yellow). Restriction enzyme sites *NcoI*, *HindIII*, *MluI*, and *NotI* were used for inserting the V_H and V_L chain gene fragments.

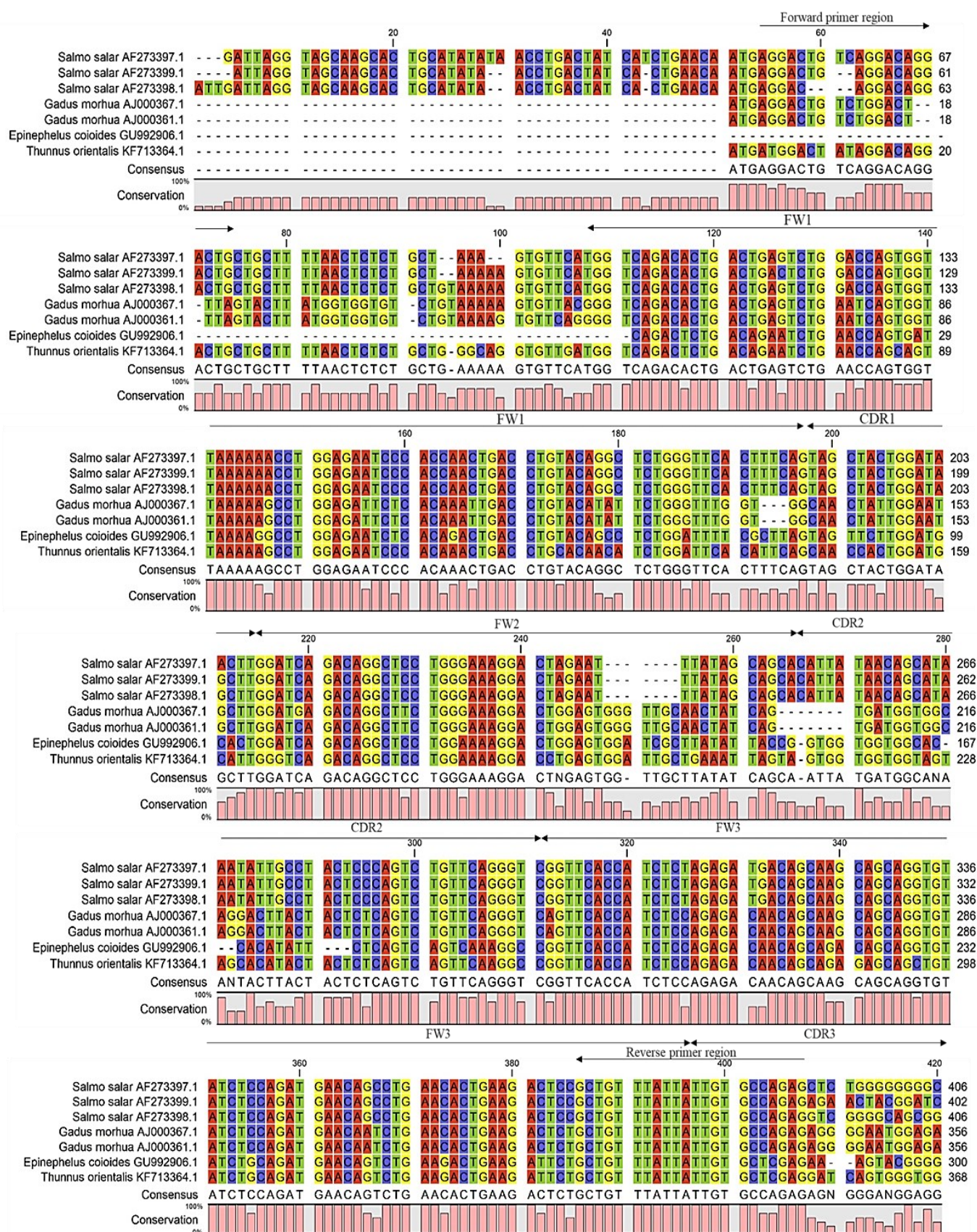


Figure B.1.2 Multiple sequence alignment of seven heavy chain variable sequences of four different fish species covering two V-gene families (IgH and IgM). The various framework and complementarity-determining regions are indicated above as well as the binding sites of the forward and reverse primers used to generate the variable heavy chain of Dusky kob.



Figure B.1.3 Multiple sequence alignment of eight light chain variable sequences of six different fish species covering two V-gene families (IgL and IgD). The various framework and complementarity-determining regions are indicated above as well as the binding sites of the forward and reverse primers used to generate the variable light chain of Dusky kob.

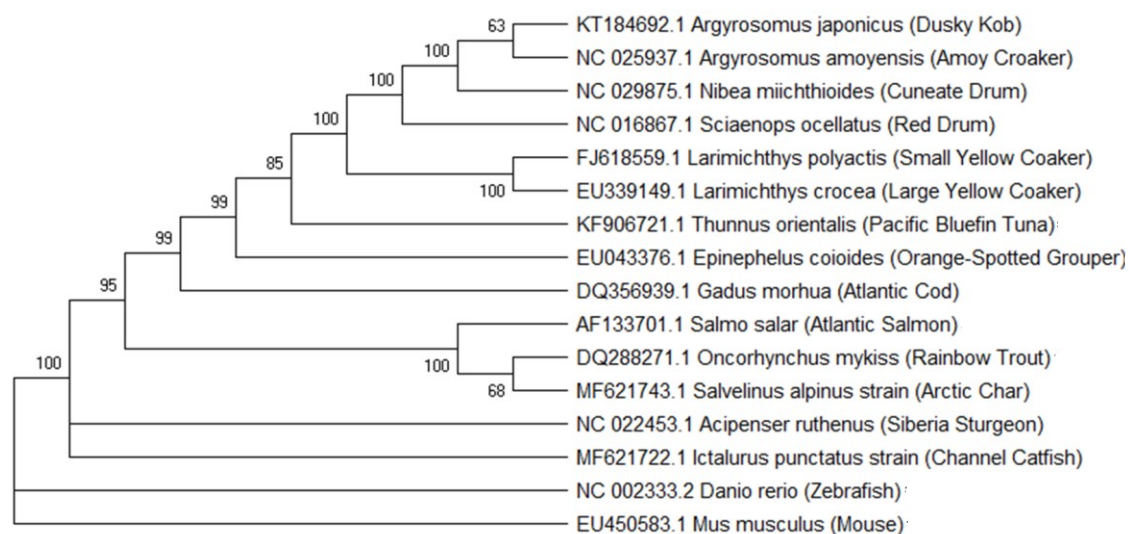


Figure B.1.4 Phylogenetic analysis of different fish species to ascertain the fish species closely and distantly related to Dusky kob for primer design. *Mus musculus* was used to root the tree.

B.2

Chapter 3 Supplementary information

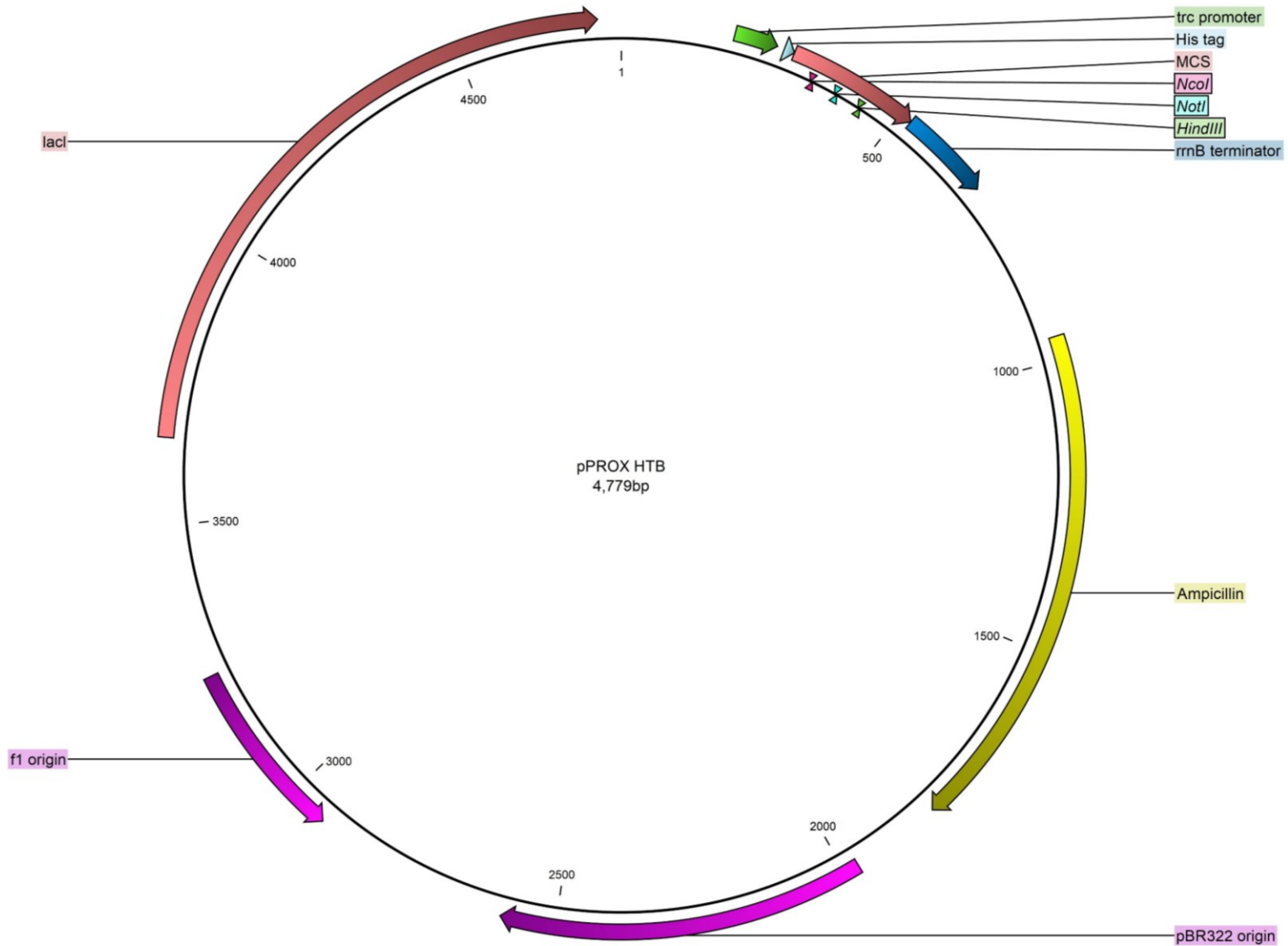


Figure B.2.1 Restriction map of the phagemid vector pPROEX-HTb (Invitrogen). Regions of interest include the (His)₆, N terminus tag (light blue), trc promoter (light green), Multiple-cloning site (pink). The stop codon can be located downstream of the MCS.

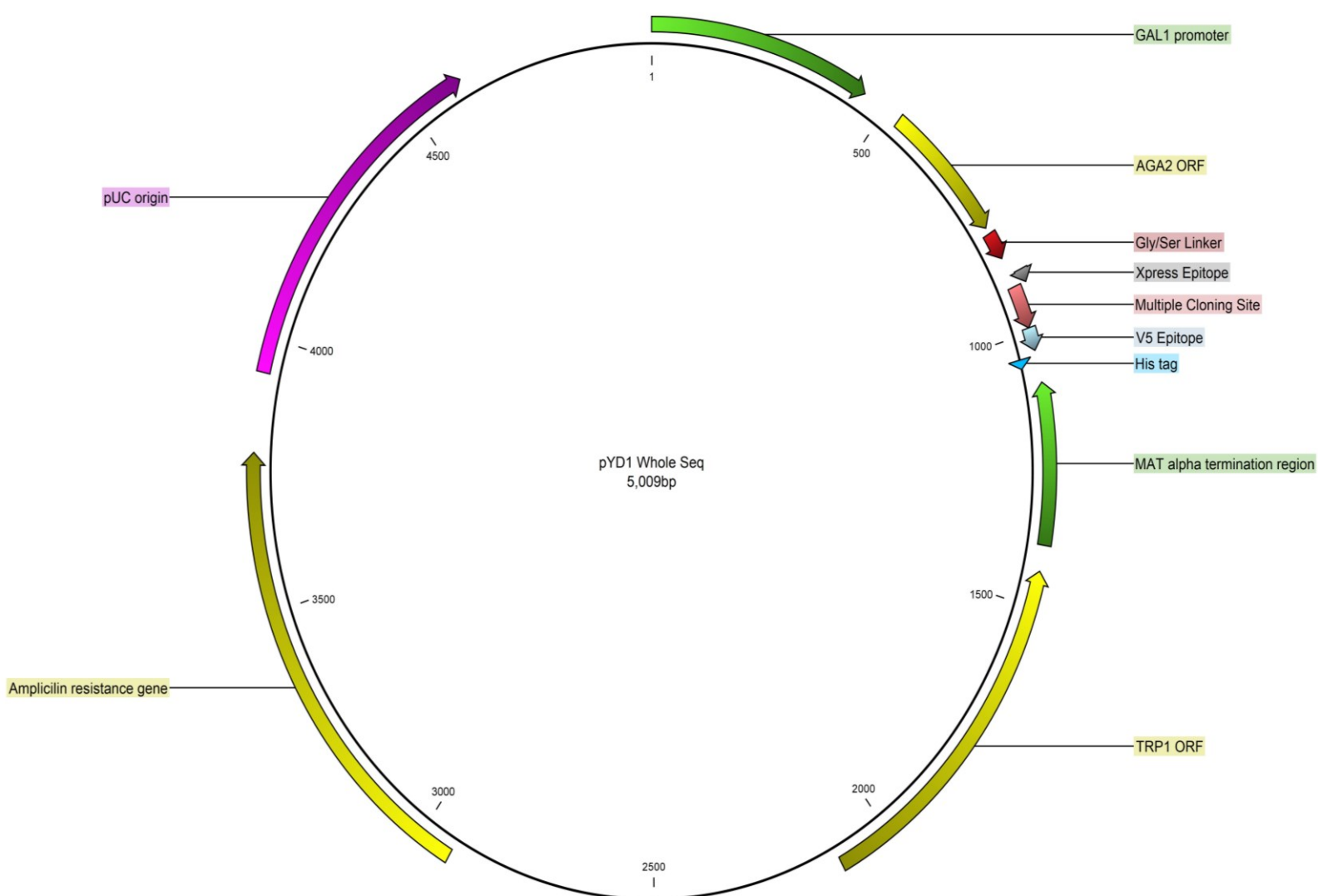


Figure B.2.2 Restriction map of the phagemid vector pYD1 (Addgene, plasmid #73447). Regions of interest include the GAL1 promoter, AGA2 ORF, Gly/Ser linker (red), MSC (pink), V5 Epitope (Grey), (His)₆ N terminus tag (light blue), MAT α transcription termination region (Green), and TRP1 ORF.

Chapter 4 Supplementary information

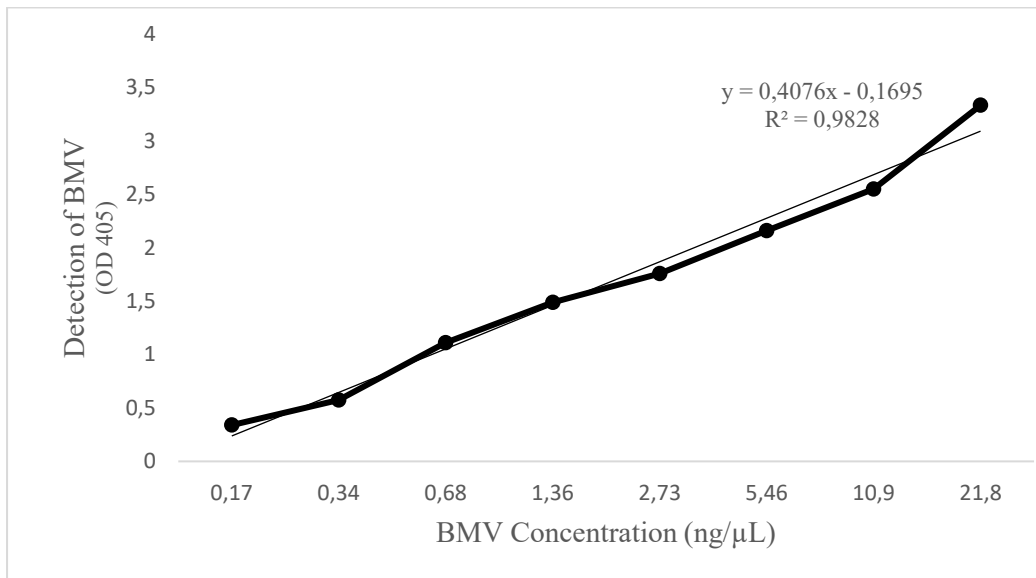


Figure B.3.1 Standard curve demonstrating the binding ability of the anti-BMV IgG to various concentrations of BMV.

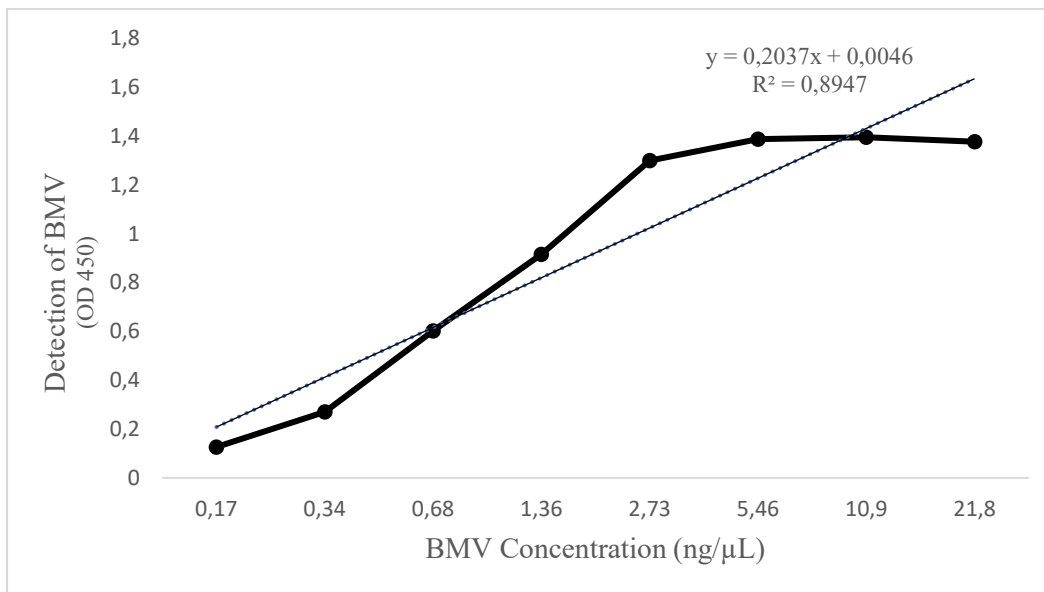


Figure B.3.2 Standard curve demonstrating the binding ability of the yeast displayed anti-BMV dAb to various concentrations of BMV.

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