UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Selection of chicken single-chain antibody fragments directed against recombinant VP7 of bluetongue virus

By

Molemaisago Rakabe

A dissertation submitted in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE (Veterinary Science)

in the

Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
South Africa

Date submitted: October 2008

CONTENTS

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following individuals, without whose contribution this work would not have been a success:

1. Dr. Jeanni Fehrsen, my promoter, for continuous guidance and mentorship throughout this project. Your guidance and help especially during the preparation of this dissertation is greatly appreciated. Thank you.

2. Dr. Dion du Plessis, Head of Immunology Section, for allowing me to pursue this research. I thank him for his guidance, valuable discussions, resources and expertise which made this research possible. I also appreciate his time for proofreading this dissertation.

3. Dr. Antony Musoke, Research and Technology Manager, I thank him for his constant motivation and encouragement and for arranging funds throughout the duration of this project. His involvement contributed immensely to the success of this research.

4. Dr. Christian Potgieter, for his kindness in providing VP7 of BTV 4, his advice and valuable discussions.

5. Dr. Jacques Theron of the University of Pretoria, Microbiology Department, for advice and guidance which provided me with an insight into microbiology.

6. A special word of thanks to Wouter Van Wyngaardt, for assistance, patience and guidance. Thank you for the training in phage display technology as well as for providing anti-BTV sera, and proteins.

7. Cordelia Mashau, for her patience and time in training me on phage display panning.

8. Shirley Smith, Virology Section, for providing anti-BTV-sheep serum and for her valuable discussions.

9. Joy Sixholo for training and assistance in microbiology techniques.

10. The Department of Science and Technology for funding and the Agricultural Research Council for resources and expertise.

11. I am grateful to the DST students at OVI and the rest of the Immunology staff for their support throughout the duration of this project.

12. Cassandra Olds and Dr. Victor Bagla for their kindness and time in proofreading part of this dissertation.

13. Last but not least, I am sincerely grateful to my parents and friends for their love, patience and continuous support. Thank you! It could not have been possible without you.

ABSTRACT

Selection of chicken single-chain antibody fragments directed against recombinant VP7 of bluetongue virus

By

Molemaisago Rakabe

Promoter : Dr. Jeanni Fehrsen
Faculty : Veterinary Science
Department : Veterinary Tropical Diseases
Onderstepoort
Degree : Master of Science

Viral protein seven (VP7) is a major core protein and a group-reactive antigen that can be used for the diagnosis of bluetongue virus. VP7 gene of bluetongue virus serotype 4 was expressed in *E. coli*. Using phage display technology, anti-VP7st4 scFvs were selected from a chicken scFv library (*Nkuku*®) following different panning strategies. Polyclonal phage ELISA showed that VP7st4-specific scFvs were enriched after three rounds of panning. Six different scFvs (A1, H2, TA8, TC9, TD12 and SA12) were identified by sequence analysis. Stability of these scFvs was determined by incubation at different temperatures and after several freeze/thaw cycles. The scFvs were also tested in an inhibition ELISA. Inhibition with an anti-bluetongue virus guinea pig serum resulted in a 30% decrease in ELISA signal of A1. No inhibition was obtained with the rest of the scFvs when guinea pig and sheep serum were used. An anti-bluetongue virus chicken IgY inhibited the scFvs by 50% to 86%. A fragmented-gene library displaying peptides of VP7st4 was constructed. The library was subjected to three rounds of affinity selection against the anti-VP7st4 scFvs. Enrichment of clones specific to each scFv was observed. The clones were identified by sequence analyses. The regions on VP7st4 to which the scFvs bind could not be identified since no duplicate clones were selected.

Presentations:

(1) Poster

Magdeline Rakabe and Jeanni Fehrsen

Chicken single-chain antibody fragments directed against recombinant VP7 of bluetongue virus. Presented at the 7th Molecular and Cellular Biology Group Symposium, October, 2007 at the University of Pretoria, Department of Medical Virology.

(2) Oral presentation

This work has been presented three times in parts after every six months at the Department of Science and Technology Progress Reviews and also at the Annual Project Review of the Agricultural Research Council-Onderstepoort Veterinary Institute (OVI) held at the OVI.

(3) Manuscript for publication representing part of this project was prepared for the fulfillment of the MSc degree.

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

LIST OF TABLES

ABBREVIATIONS

AHSV	African horse sickness virus
APS	Ammonium persulphate
A, 492nm	Absorbance at 492nm
BSA	Bovine serum albumin
BTV	Bluetongue virus
CDR	Complementarity determining region
CFU	Colony forming unit
C_H	Constant domain of the heavy chain
C_L	Constant domain of the light chain
cDNA	Complementarity DNA
D	Diversity
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
dsRNA	Double stranded ribonucleic acid
E. coli	*Escherichia coli*
EDTA	Tris-acetate
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment of antibody
FR	Framework region
Fv	Fragment variable
h	Hour
HRP	Horseradish peroxidase
IgG	Immunoglobulin class G
IgY	Immunoglobulin class Y
IPTG	Isopropyl ß-D-thiogalactopyranoside
J	Joining
kDa	Kilo dalton
KLH	Keyhole limpet
LB	Luria Bertani
mA	Milliamperes
MAb	Monoclonal antibody
mRNA	Messenger ribonucleic acid

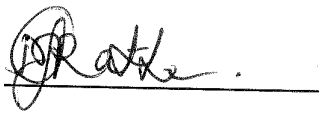
MP	Milk powder
Mw	Molecular weight
Ni-NTA	Nickel nitrilotriacetic acid
Ni-TED	Nickel tris-carboxymethyl ethylene diamine
NS	Nonstructural
OD	Optical density
ON	Overnight
OPD	*0*-phenylenediamine dihydrochloride
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pIII	Protein three of a filamentous phage
pIX	Protein nine of a filamentous phage
pfu	Plaque forming units
pVI	Protein six of a filamentous phage
pVII	Protein seven of a filamentous phage
pVIII	Protein eight of a filamentous phage
rATP	Recombinant adenosine 5´-triphosphate
RF	Replicative form
rpm	Revolution per minute
RT	Room temperature
ScFv	Single-chain antibody fragment
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SN	Supernatant
ssDNA	Single stranded deoxyribonucleic acid
T	Tween
TAE	Tris-acetate
TEA	Triethylamine
TEMED	N,N,N',N'-Tetramethyl-thylenediamine
Tris	Tris(hydroxymethyl) aminoethane
TY/AG	2×TY medium with 100 µg/ml ampicillin and 2% glucose
TY/AK	2×TY with 100 µg/ml ampicillin and 25 µg/ml kanamycin
TYE/AG	TYE agar plates with 100 µg/ml ampicillin and 2% glucose
V	Volts

V_L	Variable light
V_H	Variable heavy
VP	Viral protein

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DECLARATION

I, **Molemaisago Rakabe**, declare that this dissertation entitled "Selection of chicken-single-chain antibody fragments directed against recombinant VP7 of bluetongue virus" is my own work. It has been submitted to the University of Pretoria for the degree of Master of Science. It has not been submitted by me before for any degree or examination at any other University. I further declare that any sources of my information are indicated in the text and references list.



Signature

15/10/2008

Date

CHAPTER ONE

LITERATURE REVIEW

1.1. BLUETONGUE

Bluetongue is an insect-borne viral disease of domestic (e.g. cattle and sheep) and wild ruminants (e.g. deer). It is an economically important disease of sheep caused by bluetongue virus (BTV; Spreull, 1905; Bowne *et al*., 1966, 1967; Erasmus, 1975; Hourrigan & Klingsporn, 1975, 1975a). Bluetongue was observed in South Africa soon after Merino sheep from Europe were introduced to the Cape region in the 19th century. It was first described as "*Malarial Catarrhal Fever of sheep*" by Hutcheon (1881) and is characterised by fever and symptoms including lesions of the mouth and inflammation of the feet (Theiler, 1906). A bluetongue outbreak can be costly if not properly controlled. The disease can be severe in sheep resulting in wool breakage, weight loss and death. Bluetongue is of international concern because of its infectious nature and has been an Office International des Epizootic listed disease since the outbreak in Cyprus in 1943 (Gambles, 1949). Severe trade restrictions have been placed on countries known to have bluetongue. This affects the export of live animals, semen, embryos and other animal products. Testing is required to certify that animals and animal products are free from the disease.

Enzyme-linked-immunosorbent assays (ELISAs) have been developed as useful tools to detect BTV antibodies (Lunt *et al*., 1988; Afshar *et al*., 1995). Monoclonal antibodies (MAbs) play an important role in the diagnosis of BTV and in the development of vaccines. They have been produced using classical hybridoma technology (Köhler & Milstein, 1975) or alternatively, by phage display technology (Smith, 1985; McCafferty *et al*., 1990).

1.2. BLUETONGUE VIRUS

1.2.1. Classification and morphology

BTV is classified in the genus *Orbivirus* of the Family *Reoviridae*. Other orbiviruses which are closely related to this virus includes Epizootic Haemorrhagic Disease Virus of deer and African Horse Sickness Virus (Verwoerd, 1970; Borden *et al*., 1971; Murphy *et*

al., 1971). The morphology of BTV resembles that of *Reoviruses* (Studdert *et al*., 1966). The core particle is 54 nm and the whole virus is 70 nm in diameter (Els & Verwoerd, 1969; Grimes *et al*., 1995; Verwoerd, 1970). The BTV genome consists of 10 dsRNA segments each encoding for a different viral protein (Verwoerd, 1970a). The outer capsid plays a role in the attachment and penetration of the cell (Verwoerd, 1970; Verwoerd *et al*., 1972).

The capsid consists of seven structural viral proteins (VP), namely VP1, VP2, VP3, VP4, VP5, VP6 and VP7. They form a double-layered whole capsid which encloses the genome. The other three proteins, (NS1, NS2 and NS3) are nonstructural (Verwoerd *et al*., 1972). The viral proteins have varying molecular weights between 25 602 to 149 588 daltons (Roy, 1989). VP2 and VP5 form a diffuse layer while the remaining polypeptides VP1, VP3, VP4, VP6 and VP7 are found in the core particle of the virus, which obeys the rules of icosahedral symmetry. VP3 and VP7 are the major components in the core whereas VP1, VP4 and VP6 are minor components (Verwoerd *et al*., 1972; Mertens & Diprose, 2004; Figure 1.1).

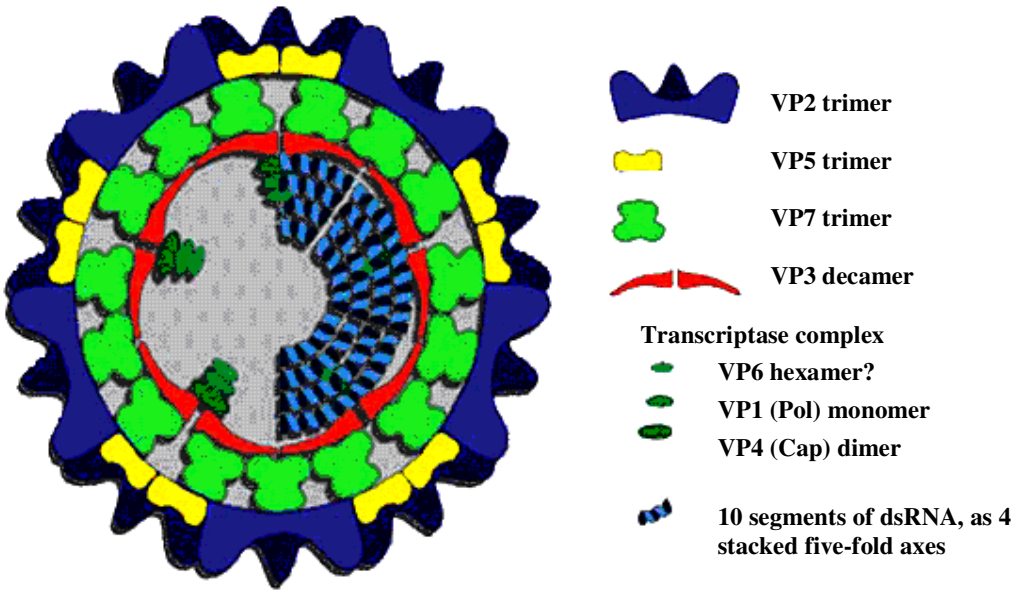


Figure 1.1: Schematic diagram showing the structure of the BTV particle (Mertens & Diprose, 2004).

The major structural protein, VP7, is a 349 amino acids protein encoded by genome segment seven (Yu *et al*., 1988). This protein is of importance to this study. Comparison of the deduced amino acid sequences of the VP7 coding gene of BTV-2, -10, -11, -13 and -17 shows that it is highly conserved (>94%; Eaton *et al*., 1991; Kowalik & Li, 1991).

The structure of VP7 has been elucidated by electron microscopy at a resolution of 23 Å. The viral core consists of 260 trimers of VP7. The X-ray crystallographic structure of the BTV core at a resolution of 3.5 Å further revealed the arrangement of VP7 as trimers (Grimes *et al*., 1995, 1997, 1998).

Twenty-four serotypes of BTV are recognised globally. Different serotypes occur in different geographic regions. In South Africa, there are 21 serotypes with types 17, 20 and 21 not yet having been isolated (Verwoerd & Erasmus, 1994). Antisera against all 24 BTV serotypes recognise the conserved VP7 (Oldfield *et al*., 1990). This makes it a group-reactive antigen, hence it is used in the detection of BTV. Several VP7-specific MAbs have been produced (Huismans & Erasmus, 1981). For example, MAb 20E9 recognises a discontinuous epitope used at present for BTV diagnosis. The epitope is conserved amongst all the serogroups (Lunt *et al*., 1988; Eaton *et al*., 1991; Nagesha *et al*., 2001).

1.2.2. Transmission and epidemiology

The vector of BTV was identified by Du Toit in 1944. He demonstrated that BTV can be transmitted to susceptible sheep by biting midges which have fed on a sheep infected with the virus. In Africa, Asia and Europe, the most efficient vector species is *Culicoides imicola* (Du Toit, 1944; Sellers, 1984; Breard *et al*., 2004). Bluetongue may be introduced to other areas by movement of ruminants or by infected midges blown by wind over great distances (Sellers, 1984). The occurrence of BTV outbreaks is influenced by factors such as climate, the presence of reservoir and amplifying hosts and vectors (Baylis, 2002; Sellers, 1984; Gibbs & Greiner, 1994). For example, in South Africa, bluetongue outbreaks are most prevalent during the summer months especially when it is wet. The level of humidity and temperature influence the breeding of BTV insect vectors and the increased population of these vectors favours the prevalence of the disease (Theiler, 1906; Erasmus, 1975; Hourrigan & Klingsporn, 1975).

1.2.3. Bluetongue distribution

For many years, bluetongue was thought to be confined to Africa. The first well-documented occurrence outside Africa was reported in Cyprus in 1943 (Gambles, 1949). Since then, bluetongue has been recognised amongst cattle and sheep in Palestine and

Israel (Boulanger & Frank, 1975) and in Texas in the United States, (Price & Hardy, 1954). It was later confirmed in Portugal, Spain, Pakistan, Japan, Peru and India (Boulanger & Frank, 1975). Subsequently, BTV has been found in several other regions including the Middle East, China, Asia and Australia (Verwoerd & Erasmus, 1994). Since 1999, at least 10 Mediterranean countries including France, Italy and Greece, have been affected by a new bluetongue epidemic. These countries were previously BTV-free and the outbreak has resulted in the loss of over 300 000 sheep (Mellor & Wittmann, 2002; Breard *et al*., 2004). More recently, there have been several other outbreaks. For example, BTV was re-introduced in Portugal in 2004 (Barros *et al*., 2007). The disease was also diagnosed in goats and in cattle in The Netherlands (Dercksen *et al*., 2007).

1.2.4. Clinical signs

In infected sheep, clinical signs differ. Fever, swelling of lips and tongue, weakness and weight loss are amongst symptoms observed. Animals may have difficulties in breathing and the feet become inflamed. Clinical signs in cattle depend on the strain of the virus while other domestic animals generally show few clinical signs (Spreull, 1905; Theiler, 1906; Verwoerd & Erasmus, 1994). It is important to differentiate bluetongue from related orbiviral infections as their clinical signs can often be confused (Verwoerd & Erasmus, 1994; Scott, 1998).

1.2.5. Diagnosis

Initial diagnosis of BTV is done through examination of the clinical signs. The presence of the virus can be confirmed by electron microscopy or immunological tests such as complement fixation tests or direct and indirect fluorescent antibody staining techniques (Foster & Luedke, 1968; Verwoerd & Erasmus, 1994). These techniques can differentiate different orbiviruses, but are unable to distinguish between different serotypes of BTV (Boulanger & Frank, 1975; Huismans & Erasmus, 1981). The Office International des Epizootic recommends detecting serogroup-specific antibodies using agar-gel-immunodiffusion or competitive enzyme-linked-immunosorbent assay (Afshar *et al*., 1991, 1992). If a test sample is positive in the assay, it can be typed subsequently by using a virus-neutralisation test employing a panel of serotype-specific antisera (Huismans & Erasmus, 1981; Afshar *et al*., 1991). Indirect or competitive ELISA is used to detect group-reactive antibodies to BTV due to its high specificity and sensitivity. It is

inexpensive, easy to operate and allows for rapid analysis. The success of this assay depends entirely on the availability of suitable reagents, particularly MAbs specific for the highly conserved VP7 (Afshar *et al*., 1991; Martyn *et al*., 1991).

Often, antigens for ELISA are produced by infecting cultured cells with BTV followed by extraction and purification of the virus or viral antigens. The procedure can be laborious and expensive while the amount and quality of the virus produced differ between preparations. Furthermore, the virus may still be infectious. However, the use of heterologous expression systems such as baculoviruses circumvents the above limitations. ELISAs based on BTV recombinant VP7 expressed in baculovirus have been developed to detect antibodies against BTV in hyperimmune sera (Oldfield *et al*., 1990; Mecham & Wilson, 2004). The test relies on the fact that BTV-specific antibodies present in test sera block the binding of a bluetongue serogroup-reactive MAb to immobilised antigen. Since, antigenic cross-reactivity between orbiviruses often leads to misdiagnosis, antigen capture competitive ELISAs tend to overcome this problem (Lunt *et al*., 1988; Eaton *et al*., 1991; Afshar *et al*., 1992; Mecham & Wilson, 2004; Fehrsen *et al*., 2005).

1.2.6. Control

Eradicating bluetongue is nearly impossible due to the existence of known and unknown reservoirs. Although target animals can be protected from contact with *Culicoides* vector species by using insecticides, immunisation is the most effective and practical control measure in endemic regions (Verwoerd & Erasmus, 1994). Sheep recover from natural infections and remain immune to the homologous serotype and are partially immune to similar, but not all, heterologous types. Protective immunity is associated with neutralising antibodies that are serotype-specific and may persist for many years. Group-specific antibodies disappear after a few months (Verwoerd & Erasmus, 1994).

Modified-live or attenuated virus vaccines are widely used in South Africa and the USA. These vaccines are produced in cell culture. They are known to induce effective and long lasting immunity when administered to susceptible animals especially when only one serotype of BTV is involved (Van Dijk, 1993). However, multiple serotypes occur and different levels of cross-protection between serotypes are achieved by vaccination. Therefore, the serotypes incorporated into the vaccine must be similar to those that are present in the field. In South Africa, the polyvalent vaccine used today contains 15

attenuated live viruses of the 21 serotypes that occur in the country. Most sheep develop immunity to all serotypes (Verwoerd & Erasmus, 1994). Strains of the virus can, however, sometimes interfere with each other resulting in immuno-suppression. Vaccinating ewes during their early pregnancy may cause foetal abnormalities. It is not possible to distinguish between animals that have recovered from the disease and those that have been vaccinated. This is an international problem because it limits the transportation of animals to other countries free from bluetongue. Such problems can potentially be overcome by using alternative effective inactivated viruses, subunit vaccines or recombinant viruses (Van Dijk, 1993; Scott, 1998; Verwoerd & Erasmus, 1994, 2004).

1.3. MONOCLONAL ANTIBODIES

Antibodies are traditionally produced by injecting a laboratory animal with an antigen. The resulting polyclonal antibodies are separated from the blood serum. Each host animal responds uniquely and any immunological assays that use this preparation for each bleeding need to be re-optimised. Köhler and Milstein (1975) were first to produce a cell line which was immortal and able to produce specific antibodies. A myeloma or tumor cell that is able to replicate continually but does not produce antibodies was fused with a single mouse spleen cell that produced antibodies. This fusion was called a "hybridoma" and could in theory produce antibodies endlessly (Köhler & Milstein, 1975). Blocking ELISAs have been developed to detect BTV antibodies in hyperimmune sera using BTV specific MAbs. This overcomes serological cross-reactions among related orbiviruses (Lunt *et al*., 1988; Reddington *et al*., 1991; Naresh & Prasad, 1995; Mecham & Wilson, 2004). Reports have also described hybridoma cell fusions from rabbits (Spieker-Polet *et al*., 1995) and chickens (Matsushita *et al*., 1998; Matsuda *et al*., 1999). The use of non-rodents in hybridoma technology is usually not as robust in efficiently generating MAbs as with rodents (Tsurushita *et al*., 2004). The production of hybridoma fusions, however, is laborious. The resulting fusions can be unstable, media and reagents are expensive. Each antigen requires that new fusions be prepared (Köhler & Milstein, 1975; Bhardwaj *et al*., 1994). Hence, an alternative has been developed and will be discussed in section 1.4.

1.3.1. The structure of the antibody molecule

Immunoglobulins have a basic structure which consists of an identical pair of heavy (H) chain and a pair of light (L) chain polypeptides joined together by disulphide bridges and non-covalent bonds (Frangione *et al*., 1968; Figure 1.2). Avian immunoglobulin G (IgG) is closely related to the mammalian IgG based on their functional and structural properties. Avian IgG, however, has one additional constant domain in its H chain but lacks the hinge region. Each H chain is encoded by variable (V_H), diversity (D), joining (J_H) and constant (C_H) genetic segments. The light chain on the other hand is encoded for by V_L, J_L and C_L segments (Parvari *et al*., 1988). When the H chain V-D-J regions and the L chain V-J regions are paired, an antigen-binding site is formed (Reynaud *et al*., 1985).

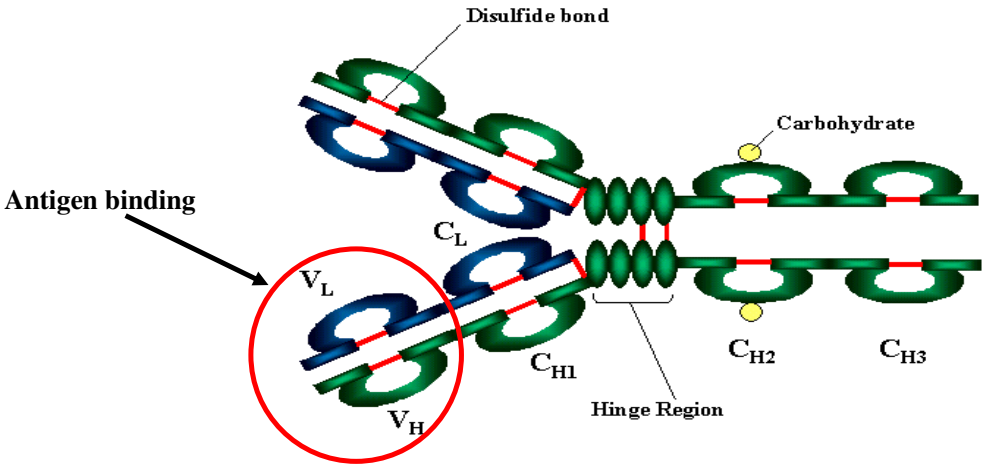


Figure 1.2: A schematic representation of a mammalian IgG (http://pathmicro.med.sc.edu /mayer/stru-4.jpg, accessed 15 July 2008).

1.3.2. Antibody fragments

The antibody molecule can be digested with proteases to yield antigen-binding fragments of antibody (Fab) and variable fragments (Fv). Porter (1959) used papain to cleave a rabbit IgG into a Fab, containing the antibody binding site, and an Fc. A Fab consists of V_H-C_{H1} and V_L-C_L segments which are linked by disulphide bonds while an Fv is a smaller molecule and it is composed of the V_L and V_H regions. A single-chain variable fragments (scFv) is the recombinant version of the Fv. The two variable regions of the scFv are linked by a flexible 15 amino acid peptide linker containing the sequence $(Gly_4Ser)_3$ (Huston *et al*., 1988; McCafferty *et al*., 1990; Figure 1.3). These fragments have been expressed as fusions displayed on the surface of a filamentous phage. They have also been used to construct phage display libraries for selection against different

antigens (McCafferty *et al*., 1990; Hoogenboom *et al*., 1991; Sheets *et al*., 1998; Van Wyngaardt *et al*., 1998).

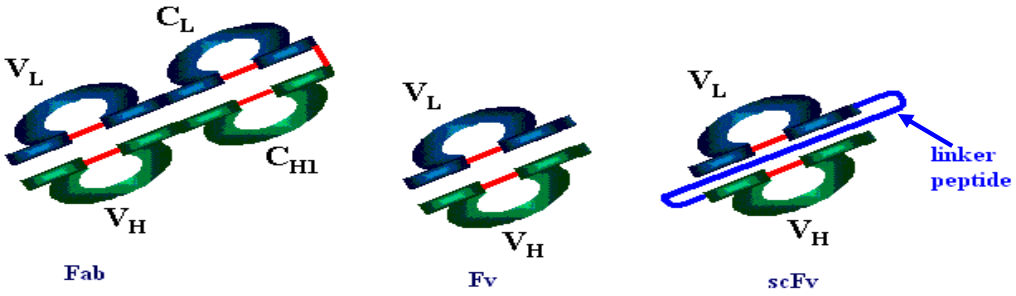


Figure 1.3: Structure of antibody fragments Fab, Fv and scFv (Modified from http://pathmicro.med.sc.edu /mayer/stru-4.jpg, accessed 15 July 2008).

1.4. PHAGE DISPLAY

Phage display technology was first introduced in 1985 by George Smith. It has been used extensively to identify novel antibodies and peptides for use in diagnostics and vaccine development (Parmley & Smith, 1988; Van Wyngaardt *et al*., 2004; Fehrsen *et al*., 2005, Tan *et al*., 2007), epitope mapping (Fack *et al*., 1997; Irving *et al*., 2001; Du Plessis *et al*., 1995; Wang *et al*., 1995) and engineering the binding affinity of proteins (Marks *et al*., 1992; Burton, 1995). This technology physically couples the phenotype of a phage-displayed peptide or protein to the genotype encoding that molecule packaged within the same virion (Smith, 1985; Hoogenboom *et al*., 1998). Antigen-specific binders can be selected and their binding characteristics can be manipulated which was not possible with the traditional hybridoma technology (Parmley & Smith, 1988).

1.4.1. Filamentous phage

Phages are viruses that infect bacterial cells such as *Escherichia coli* (*E. coli*). Infection begins when pIII of the phage attaches to the tip of the F-pilus of the host bacterium and injects its genome into the cytoplasm of the bacterium (Marvin & Hohn, 1969). Many have been characterised, but the best studied phages are the *E. coli* Ff class namely, the f1, fd and M13. These phages are collectively known as the Ff phages due to their similarity and their dependence on the F^+ plasmid for infection of Gram-negative bacteria (Smith, 1985; Hoogenboom *et al*., 1998). They have 98% nucleotide sequence homology. A wild-type Ff phage particle is 6.5 nm in diameter and 930 nm in length (Webster, 1996). The non-lytic M13 (Marvin & Hohn, 1969) has been extensively used to display peptides and

antibody fragments (McCafferty *et al*., 1990). Other phage display systems based on lambda (Cicchini, *et al*., 2002), T4 (Malys, *et al*., 2002) and T7 (Castillo, *et al*., 2001) have also been developed. M13 has a covalently closed circular, single-stranded (ss) DNA genome packaged in a tube containing many copies of the major coat protein pVIII (~ 2700 copies per phage). This protein is 50 amino acid residues long. The phage is closed at the ends by the minor coat proteins pIII, pIV, pVII and pIX, between three and five copies of each per phage (Smith, 1985; Parmley & Smith, 1988; reviewed by Russel, 1991; Figure 1.4). The antibody fragments are commonly fused to the amino terminus of pIII which encodes a 406 amino acid peptide (Hoogenboom *et al*., 1998). Foreign proteins are expressed on the surface of the phage particle as fusions and can therefore interact with other molecules. The phages displaying foreign proteins are identified by affinity selection and sequencing (Smith, 1985; McCafferty *et al*., 1990).

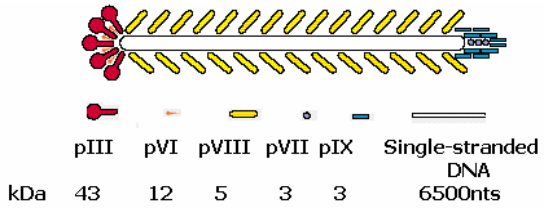


Figure 1.4: Schematic representation of a filamentous phage (Irving *et al*., 2001).

Antibody phage libraries allow for the isolation of MAbs. In 1990, McCafferty and co-workers constructed an antibody library displaying scFv antibody fragments as fusions to pIII on the surface of a phage particle (Figure 1.5). Subsequently, libraries displaying Fab fragments were constructed (Hoogenboom *et al*., 1991). Two types of display vectors are commonly used to construct phage libraries, namely, the phage and phagemid vectors.

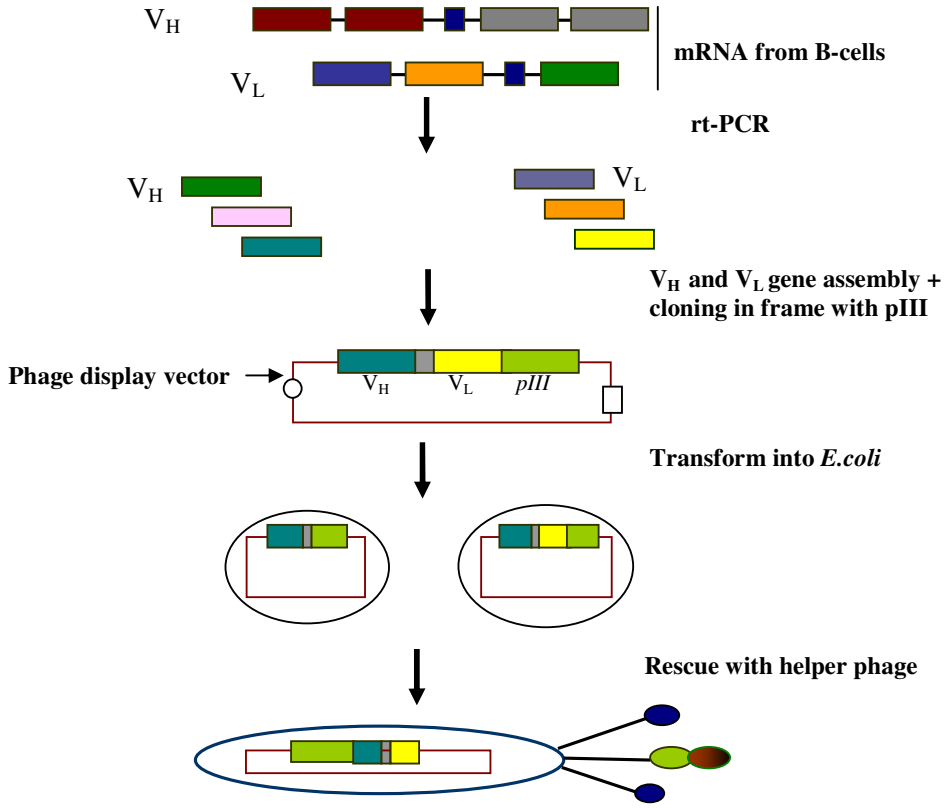


Figure 1.5: Diagram showing steps followed in constructing an antibody phage library as described by Hoogenboom *et al*., 1998. mRNA is isolated from B-lymphocytes from naïve or immune donors. cDNA is synthesised from the mRNA using reverse transcription PCR. The genes encoding for the V_H and V_L chains are amplified using primers, joined to form an scFv and then inserted into a phage display vector in frame with pIII. The resulting fusion is introduced into the cell and infection with a helper phage results in the display of antibody fragments as fusions to the phage particle (McCafferty *et al*., 1990).

1.4.2. The phage vectors

Vectors based on filamentous phages have been developed. The DNA encoding the desired peptide or protein is usually inserted at the 5' end of gene *pIII*. In some vectors, fusions are made at the 5' end of gene *pVIII* which results in a higher level of expression (Parmley & Smith, 1988; Markland *et al*., 1991; Wilson & Finlay, 1998; Irving *et al*., 2001). Phage vectors such as fdtet (Figure 1.6A) or phagemid vectors such as pHEN1 (Figure 1.6B) have been used to construct large display libraries (Hoogenboom *et al*., 1991). A higher display level of foreign peptides or antibody fragments per phage can be achieved with phage vectors rather than phagemids. This multivalent display allows for higher avidity to multivalent antigens. In these vectors, a natural phage promoter and a

transcriptional terminator allows for expression of the antibody-pIII fusion and no helper phage is required to provide packaging proteins (Hoogenboom *et al*., 1991). Phage vectors, however, have limitations in that the presence of large foreign peptide fragments at the N-terminus of pIII can limit the interaction between pIII which plays a major role in early infection and F pili on the bacterium (O'Connell *et al*., 2002).

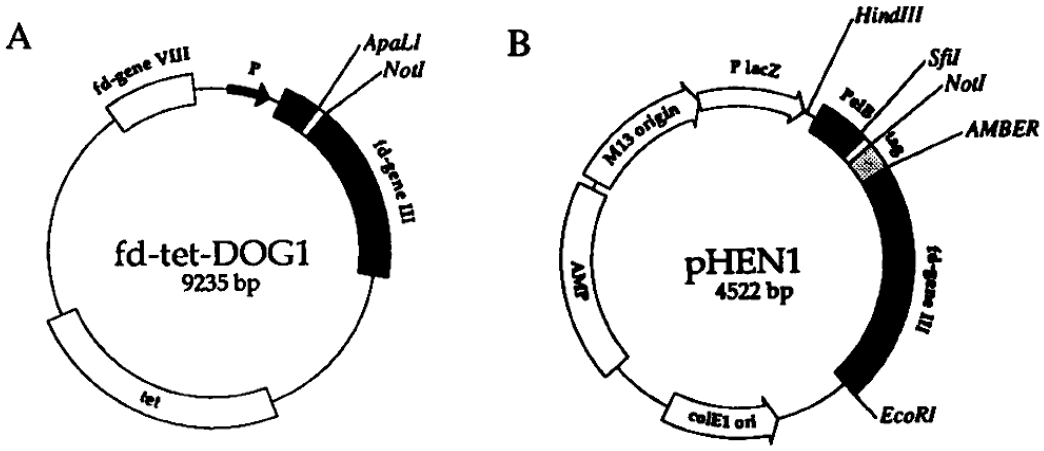


Figure 1.6: Phage and phagemid vectors used for display of antibody fragments on the surface of phage. Structure of vector fd-tet (A) and pHEN1 (B; Hoogenboom *et al*., 1991).

On the other hand, phagemids are hybrids of phage and plasmid vectors. They contain the origin of replication from the M13 phage, plasmid origin of replication from *E. coli,* gene *pIII* or *pVIII* for formation of fusion proteins, appropriate multiple cloning sites and an antibiotic-resistance gene. Phagemids use the *lac*Z promoter to direct expression of antibody-pIII fusions to the periplasm using a leader sequence (Hoogenboom *et al*., 1991). They are grown and stably maintained as plasmids in bacteria (Parmley & Smith, 1988; Hoogenboom *et al*., 1998; O'Connell *et al*., 2002). These vectors lack genes that encode proteins required to produce a complete phage particle. Their origin of replication is activated during infection with helper phages such as M13KO7 by a process termed "rescuing of phages". M13KO7 supplies essential proteins to facilitate the packaging of recombinant Ff phage DNA and the display of fusion proteins (Webster, 1996). The wild-type pIII competes with antibody-pIII fusion protein for incorporation into the phage particle. As a result, the majority (>90%) of rescued phage display no antibody and those displaying fusion products contain a single copy. This monovalent display allows for selection of high affinity antibodies. Phagemids have high transformation efficiency and are therefore ideal for use in constructing large libraries (Hoogenboom *et al*., 1998). In addition, they are easy to clone, amplify, manipulate and isolate from bacteria when

compared to phage vectors. For example, display libraries such as the *Nkuku*® library which represent a large repertoire of antibodies of different specificities have been successfully constructed using these vectors (McCafferty *et al*., 1990; Hoogenboom *et al*., 1998; Van Wyngaardt *et al*., 2004).

To select and identify scFv binders from a phage displayed library, a process called "panning" is used (Figure 1.7). The library displays millions of antibodies on the surface of a filamentous phage particle. The genotype and phenotype are physically linked, therefore antigen-specific antibodies can be enriched by selection on immobilised antigens. The phage DNA encoding the antibody fragment is sequenced and their amino acid sequence is deduced. An increase in the number of target-binding phages indicates whether enrichment occurred after each round of screening (Parmley & Smith, 1988; Van Wyngaardt *et al*., 1998).

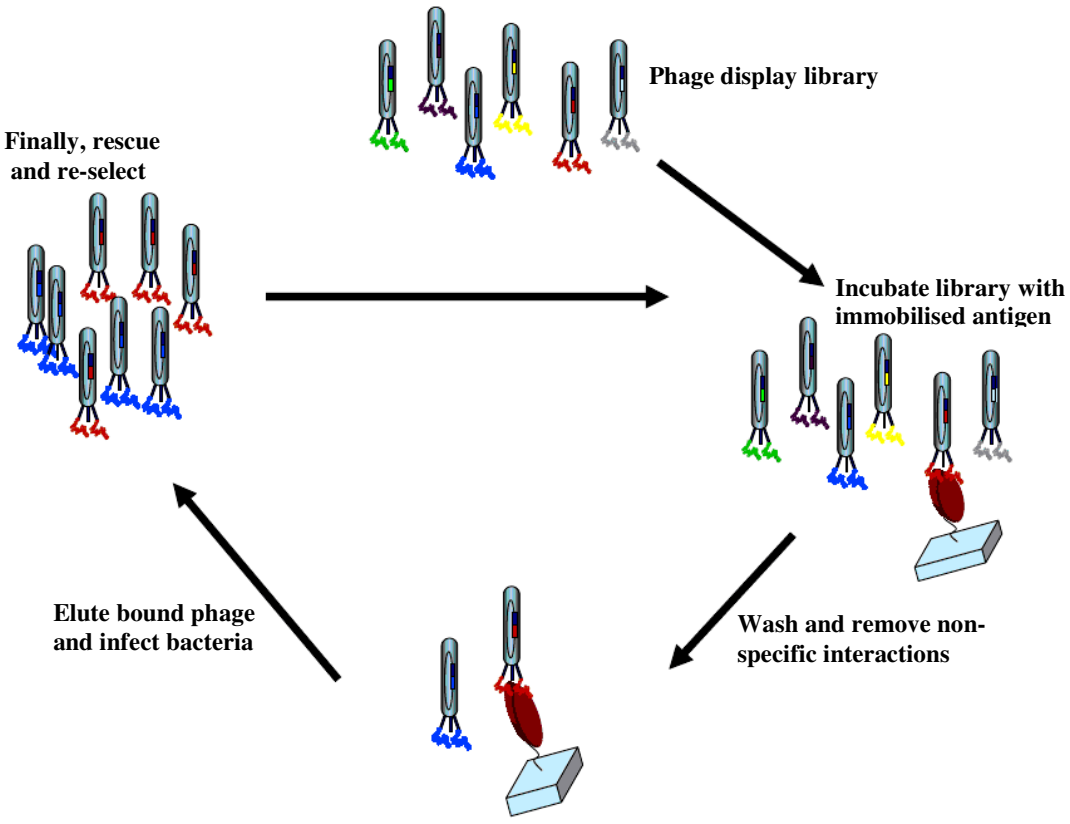


Figure 1.7: Diagram showing steps followed in panning to enrich for antigen-specific phage–displayed scFvs (http://www.imcb.a-star.edu.sg/antibodiestalk/DLtalks.pdf, accessed 18/01/2008). The library of phage particles is exposed to an immobilised antigen to which antigen-specific antibodies will bind. Non-specific binders are removed by washing. Bound phages are eluted, neutralised and then amplified by transfecting into *E. coli* TG1 cells. The phage particles are rescued using M13KO7 helper phage and used for the next round of panning. The antigen-specific clones are enriched when this process is repeated several times (Parmley & Smith, 1988).

1.4.3. Phage display libraries

1.4.3.1. Antibody libraries

Antibody libraries reduce the time and cost required to produce antibodies and to select the corresponding gene fragments. Several reports have described the construction of different types of libraries, namely, immune, synthetic and naïve.

Immune libraries are based on the variable genes of the mRNA from B-cells of an immunised donor. The antibodies represented here are those produced against epitopes recognised by the immune system of the donor. These antibodies have undergone affinity maturation by the immune system. Therefore, clones of high affinity can be selected efficiently using small libraries. Animals have to be immunised with the antigen and the resulting library will depend on the animal's immune response. The limitation of immune libraries includes the inability to obtain antibodies to toxic and self-antigens and to non-immunogenic structures. In addition, a new library has to be created for every new antigen (Clackson *et al*., 1991, Yamanaka *et al*., 1996).

Naïve antibody libraries on the other hand are constructed using variable genes derived from the mRNA obtained from B-cells of an unimmunised donor (Davies *et al*., 1995). They have more advantages compared to immune libraries as they are not biased to an antigen-specific immune response of the donor and no immunisation is required. Such libraries serve as a single source that can be used for selection of binders against many antigens (Nissim *et al*., 1994; Hoogenboom *et al*., 1998; Sheets *et al*., 1998; Van Wyngaardt *et al*., 2004). In addition, antibodies to self-antigens, toxic antigens and non-immunogenic antigens can be isolated during panning. However, the library must be exceedingly large ($>10^9$) in order to obtain antibodies of high affinity (Vaughan *et al*., 1996).

Synthetic libraries are artificially constructed *in vitro* by combining the V and D or J genes and randomising the CDR. It is often CDR3 of the V_H chain which is thought to play a major role in the antigen-binding and it is usually the most diverse. Such libraries allow antibodies against almost any antigen to be selected (Marks *et al*., 1992; Nissim *et al*., 1994; Sheets *et al*., 1998; Van Wyngaardt *et al*., 2004). An example is the *Nkuku*®

library which is a large semi-synthetic scFv phage display repertoire derived from a naïve chicken mRNA and include a synthetically randomised heavy chain CDR3. Antibodies specific for many antigens have been selected from this library (Van Wyngaardt *et al*., 2004). For example, BTV-specific scFv F10 has been selected by panning this library on purified BTV serotype 10 virus particles and has shown the potential for use as a diagnostic reagent in immunoassays. Low affinity antibodies can also be selected, however, their affinity can be improved (Fehrsen *et al*., 2005).

1.4.3.2. Random peptide libraries

Random peptide libraries are constructed using synthesised degenerate oligonucleotides cloned into filamentous phage DNA such that the encoded peptide is expressed on the surface of a phage. Scott and Smith (1990) constructed a library displaying hexapeptides (6-mer) fused to pIII of a filamentous phage. Subsequently, random peptide libraries displaying peptides of different lengths on pIII have been constructed. Examples include a 6-mer (Cwirla *et al*., 1990), 15-mer (Devlin *et al*., 1990; Böttger *et al*., 1995), 20-mer (Jellis *et al*., 1993; Böttger *et al*., 1995) and 38-mer (Kay *et al*., 1993). Others have constructed libraries displaying peptides on pVIII of the phage particle (Felici *et al*., 1991; Petrenko, 2008). The DNA which codes for the peptide can be identified by sequencing. Consensus sequences of the expressed peptides can be matched to the amino acid sequence of the original protein. These libraries have been used to identify the epitopes of MAbs recognising short continuous linear epitopes. Cwirla and co-workers (1990) identified an epitope of MAb 3-E7 specific for the N-terminus of β-endorphin. MAbs specific for VP7 of BTV (Du Plessis *et al*., 1994) have also been identified. D'Mello and co-workers (1999) used a 15-mer random peptide library to identify a discontinuous epitope recognised by a MAb specific to the envelope protein gp120 of feline immunodeficiency virus. The selected peptides, however, were found to be mimotopes (Burton, 1995; D'Mello *et al*., 1999).

1.4.3.3. Fragmented-gene libraries

A fragmented-gene library can also be used to map epitopes as an alternative to a random peptide library. Wang and co-workers (1995) were first to report on fragmented-gene libraries displaying peptides fused to the N-terminus of pIII of the fUSE2 phage vector. An epitope recognised by a MAb specific for BTV VP5 was identified (Wang *et al*.,

1995). In a fragmented-gene library, the gene coding for the protein of interest is randomly fragmented using an endonuclease such as DNase I in the presence of manganese ions (Stanley & Herz, 1987). Several authors have constructed this type of library to map epitopes for MAbs specific for BTV VP7 (Du Plessis *et al*., 1995); MAb215 specific for *Drosophila* RNA polymerase II, mouse MAb specific for human p53 protein and human cytokeratin 19 protein (Petersen *et al*., 1995); MAb specific for glutathione-S-transferase (Gupta & Chaudhary, 1999); MAb specific for VP2 of African Horse Sickness Virus serotype 3 (Bentley *et al*., 2000); and scFv F10 specific for VP7 of BTV serotype 1 (Fehrsen *et al*., 2005). In 1998, Blüthner and co-workers used polyclonal serum to identify epitopes on the sp100 nuclear primary biliary cirrhosis antigen. Discontinuous epitopes, such as those on VP2 of infectious bursal disease virus, have also been identified (Cui *et al*., 2003). In addition to fragmented-gene libraries, genome-targeted libraries have also been constructed (Fehrsen *et al*., 1999).

In 1997, the study by Fack and co-workers compared the efficiencies of mapping epitopes recognised by four MAb, each raised against different antigens using fragmented-gene and random peptide phage display libraries (6-mer and 15-mer). The fragmented-gene library proved to be efficient since the epitopes recognised by all four MAbs were identified. Two MAbs could be mapped using the 6-mer and 15-mer random peptide libraries, while the rest failed to be mapped. Although fragmented-gene libraries are less diverse than random epitope libraries, they are more effective at mapping linear epitopes (Fack *et al*., 1997).

1.4.3.4. Advantages of phage display libraries

The production of MAbs through phage display antibody libraries has several advantages. In addition to obtaining novel antibodies of high affinity against different targets, MAbs to non-immunogenic or toxic antigens can be obtained for potential use in diagnostics, vaccine development and therapeutics (Andris-Widhopf *et al*., 2000). In naïve and synthetic display libraries, immunisation is eliminated. Once the cDNA library is obtained it can be frozen and screened or re-amplified again for future experiments. Screening recombinant antibodies is relatively inexpensive and the recombinants can be expressed in different hosts such as yeast, bacteria and insect cells (Verma *et al*., 1998). Even if yields, binding affinity and avidity of scFvs are low, their ability to bind to different targets can be improved *in vitro* (Marks *et al*., 1992; Yamanaka *et al*., 1996, Hoogenboom *et al*.,

1998; Jung *et al*., 1999; Park *et al*., 2000; O' Connell *et al*., 2002; Wang *et al*., 2004) and their specificity can also be altered (Karu *et al*., 1995).

1.4.4. Chicken monoclonal antibodies

Phage display technology allows for the isolation of MAbs from various species. Reports on antibodies derived from rabbits (Ridder *et al*., 1995), cattle (O'Brien *et al*., 1999), llamas (Tanha *et al*., 2002; reviewed by Harmsen & De Haard, 2007), sheep (Li *et al*., 2000) and chickens (Yamanaka *et* al., 1996; Andris-Widhopf *et al*., 2000) have been well documented. Chickens remain an attractive source for generating MAbs (Sapats *et al*., 2003) because their immunoglobulin genes consist of one variable segment at each of the H and L chain loci. The loci can rearrange into functional immunoglobulin V-D-J or V-J genes respectively (Reynaud *et al*., 1985). In order to create somatic diversity in the V regions, the rearranged genes undergo gene conversions which involve V-region pseudogenes (Ψ) as sequence donors (Reynaud *et al*., 1987; 1989; Thompson *et al*., 1987; McCormack *et al*., 1993). These pseudogenes are found upstream of the V-regions genes of both the H and L chains and their sequences are similar to those of the V_H and V_L chains. They lack functional recombination signals or are truncated in the V-regions and hence they donate their sequences to the rearranged genes (Reynaud *et al*., 1987; 1989). Therefore, one set of primers for each of the H and L chain coding genes of the antibody is sufficient to amplify the V gene segments in chickens (Reynaud *et al*., 1985; Sapats *et al*., 2003; Van Wyngaardt *et al*., 2004; Shimamoto *et al*., 2005).

Many reports have focused on generating recombinant chicken antibodies from phage display libraries derived from immunised chickens. In a study by Yamanaka and co-workers (1996), a library was constructed from chickens immunised with murine serum albumin. Three libraries encoding scFvs and Fab fragments were constructed from chickens immunised with flourescein-BSA to demonstrate that different antigen-specific antibodies can be obtained from a single source (Andris-Widhopf *et al*., 2000). Recombinant chicken antibody fragments against antigens such as mammalian prion protein (Nakamura et al., 2004); Foot and Mouth Disease Virus (FMDV) non-structural protein 3ABC (Foord *et al*., 2007); *Eimeria acervulina* (Wieland *et al*., 2006); severe acute respiratory syndrome associated coronavirus spike protein (Lee *et al*., 2007); domoic acid (Finlay *et al*., 2006) and *Eimeria tenella* sporozoites (Abi-Ghanem *et al*., 2008) have been isolated by phage display. Chicken antibodies have been used to develop

sensitive competitive ELISAs. For example, an ELISA was developed using a recombinant chicken antibody specific to very virulent infectious bursal disease virus. This scFv recognised a broad spectrum of the virus but showed no reactivity to classical, variant and vaccine strains. Therefore, it can be used as a powerful diagnostic reagent for the differentiation of very virulent infectious bursal disease virus (Sapats *et al*., 2006). Such findings demonstrated that phage display technology allows for the generation of chicken antibodies of high specificity and that minor antigenic differences can be detected between closely related epitopes. Foord *et al*., (2007) developed an inhibition ELISA for FMDV aimed at differentiating between vaccinated and infected animals. Recombinant chicken scFv CRAb-FM27 detected antibodies in naïve, infected and vaccinated sera independent of the species used to derive the sera and the serotype used (Foord *et al*., 2007).

Recombinant chicken antibodies of high affinity and specificity have also been selected from naïve libraries (Davies *et al*., 1995). In this way, immunisation is eliminated but the library needs to be exceedingly large. ELISAs based on these kind of antibodies have also been developed (Van Wyngaardt & Du Plessis, 1998; Van Wyngaardt *et al*., 2004; Fehrsen *et al*., 2005). Advantages of chicken antibodies include the fact that cross-reactions, false positive and false negative results in ELISAs are eliminated (Larsson *et al*., 1992; Shimamoto *et al*., 2005). In human diagnostics, chicken antibodies are advantageous as they do not cross-react with complement proteins or rheumatoid factor (Larsson *et al*., 1991).

1.5. MOTIVATION, AIM AND OBJECTIVES

Immunodiagnostic tests that are well characterised, easily reproducible and safe without infectious viruses are needed to detect BTV. This problem can be addressed using recombinant reagents since they are cost effective, specific and easily manipulated (Hust *et al*., 2002; Sapats *et al*; 2003; Van Wyngaardt *et al*., 2004; Foord *et al*., 2007). For example, previously, an scFv called F10 was selected from the *Nkuku*® library against purified BTV particles. It has been validated in an inhibition ELISA to detect BTV antibodies by the Virology Section at the OVI. ScFv F10 recognises soluble baculovirus-expressed VP7 of BTV serotype 1 when trapped with rabbit IgG which meant it could be used without being purified. The supply of IgG is finite and F10 does not recognise the protein when it is adsorbed directly to a plastic surface in immunoassays, possibly as a

result of epitope distortion (Parbosky *et al*., 1996; Qian *et al*., 2000; Fehrsen *et al*., 2005). The baculovirus construct encoding this protein was lost. Immunoassays based on recombinant VP7 without the need of a trapping antibody are needed for use as alternatives. This is the basis for this study. A new preparation of VP7 of BTV 1 was cloned in a different baculovirus system and histidine tags were introduced to facilitate purification (done by Dr Potgieter, Virology Section, OVI in collaboration with Biovac, personal communication). However, low yields were obtained with these new constructs. Therefore, it was decided to express BTV 4 VP7 (VP7st4) in *E. coli* for use as an alternative. The VP7 sequence is conserved among different BTV serotypes. The amino acid sequence of BTV 1 VP7 is 100% similar to that of BTV 4 (personal communication, Dr. Potgieter, Virology Section, OVI). The aim of this study was to select additional anti-VP7 scFvs from the *Nkuku*® library by phage display following various panning strategies; test whether the binding of the scFvs to recombinant VP7st4 can be inhibited by immune serum; and to map the epitopes to which they bind on the protein. The specific objectives were to express the VP7 of BTV serotype 4 gene in *E. coli*; purify the recombinant VP7st4 using Ni-NTA or Ni-TED columns; use the recombinant VP7st4 to select scFvs from the *Nkuku*® library following different panning strategies and characterise VP7st4-specific scFvs in ELISAs. To map the epitopes to which the VP7st4-specific scFvs bind, a fragmented-gene library displaying peptides derived from authentic VP7st4-sequence was constructed; the new sub-library was panned against anti-VP7st4 scFvs. The selected clones were sequenced to identify the region on the recombinant VP7st4 to which they bind.

1.6. POTENTIAL BENEFITS

Additional VP7-specific scFvs will be generated by phage display using recombinant VP7st4 adsorbed directly on plastic surfaces. This can provide an alternative way of obtaining well-characterised scFvs specific to VP7st4. The steps involved in immunoassays can be reduced since no trapping antibodies will be needed if the recombinant protein can react when adsorbed directly on plastic surfaces. Information on VP7st4 epitopes will also be generated using a fragmented-gene library. The knowledge of where the antibodies bind to their antigens offers the opportunity to focus on the specific regions in developing immunoassays to detect BTV antibodies.

CHAPTER TWO

EXPRESSION AND PURIFICATION OF VP7ST4

2.1. INTRODUCTION

Escherichia coli (*E. coli*) is widely used to express heterologous proteins since it is well characterised and easy to use. All proteins have unique amino acid sequences and therefore pose unique problems during their expression. The yield, solubility and stability are thus likely to differ with each recombinant protein expressed (Itakura *et al*., 1977; Goeddel *et al*., 1979; Georgiou & Valax, 1996; reviewed by Betiku 2006). An expression system which is suitable for one protein may therefore not be suitable for another. It is important to optimise the expression conditions for every new protein (De Boer *et al*., 1983; Verma *et al*., 1998).

In this study, the VP7 of BTV serotype 4 (VP7st4) encoding gene cloned into the pET-32a(+) vector (done previously by Dr. Potgieter) was expressed in *E. coli* Origami™ (DE3) pLysS strain (Novagen, USA). This *E. coli* strain has mutations in the thioredoxin reductase and glutathione reductase genes which promote proper folding of the protein during expression. The host is a lysogen of lambda DE3 and contains a gene for the T7 RNA polymerase gene under the control of the lacUV5 promoter. The pLysS indicates that the T7 lysozyme protein controls the expression of the T7 promoter and protein expression is induced by isopropyl ß-D-thiogalactopyranoside (IPTG; Novagen). Previous studies have shown that expression in this strain leads to a high yield of soluble proteins (Xiong *et al*., 2003; 2005).

The amino acid sequence of VP7 is highly conserved amongst all 24 BTV serotypes (Oldfield *et al*., 1990). There are three conserved cysteine residues at the amino terminal end at position 15, 65 and 154. The cysteines at amino acid position 15 and 65 may be linked via a disulphide bond while the other cysteine exists freely (Kowalik *et al*., 1990; Eaton *et al*., 1991; Le Blois & Roy, 1993; Wang *et al*., 1994). Other studies, however, have shown that VP7 molecules exist as trimers (Grimes *et al*., 1995, 1997) and they are not linked via disulphide bonds (Eaton *et al*., 1991). These cysteines may be reduced by thioredoxin reductase and glutathione reductase in the cytoplasm of *E. coli*. The correct folding of proteins depends on the formation of stable disulphide bonds (Bessette *et al*., 1999) and this is improved in the *E. coli* Origami™ (DE3; Xiong *et al*., 2005). Therefore,

this type of strain will potentially help with folding of VP7 into the correct conformation. Sometimes heterologous proteins containing several cysteine residues form aggregates which accumulate into dense insoluble inclusion bodies (Rinas *et al*., 1992; Lilie *et al*., 1998) which are not biologically active (Georgiou *et al*., 1986). This can, however, be advantageous for several reasons; a high yield of the expressed protein can be obtained, and the protein can be protected from proteolysis. The separation of insoluble inclusion bodies can facilitate purification. In addition, the cell is protected from the toxicity of the protein being expressed (Lechtken *et al*., 2006; Forrer & Jaussi, 1998; Lilie *et al*., 1998).

The aim of this part of the study was to express VP7st4 in *E. coli*, to isolate and purify the recombinant protein for subsequent use in phage display panning experiments and immunoassays.

2.2. MATERIALS AND METHODS

2.2.1. Materials

The VP7st4, cloned into a pET-32a(+) vector and *E. coli* Origami™ (DE3) pLysS cells were obtained from Dr. C. Potgieter, Virology Section, OVI. The same vector without an insert was used as a control.

2.2.2. Expression of VP7st4 in *E. coli*

For expression of the gene, the plasmid DNA (~1ng) was mixed with competent *E. coli* Origami™ (DE3) pLysS cells (Novagen, USA) and incubated on ice for 30 min. The transformation mixture was heat-shocked at 42°C for 30 s and immediately transferred to ice. Luria-Bertani (LB) medium was added and incubated with shaking at 800 rpm at 28°C for 1 h (Thermomixer Comfort, Merck Chemicals). The transformants were then selected on LB-agar plates supplemented with antibiotics (50 µg/ml carbenicillin, 12.5 µg/ml tetracycline and 25 µg/ml kanamycin) and incubated overnight (ON) at 30°C. A single colony from the ON transformants was inoculated into LB medium and grown with shaking at 30°C ON. LB medium supplemented with 50 µg/ml carbenicillin was inoculated with the ON culture (1:50 dilution) and grown at 37°C until the optical density at 600 nm (OD_{600nm}) reached 0.6. The cultures were divided into two portions. Protein expression was induced in one culture by adding IPTG (Calbiochem, USA) to a final

concentration of 1 mM and the other was used as an uninduced control. The cultures were then grown for a further 4 h at 30°C. Induction was also performed at an OD_{600nm} of 0.9 with the same concentration of IPTG. The vector alone was also expressed and purified as described for VP7st4. This was to obtain the thioredoxin fusion tag alone to use as a control.

2.2.3. Purification of VP7st4

The induced and uninduced cells were harvested by centrifugation at 6000 rpm (Sorvall® GSA rotor) for 30 min. The cells were used immediately for SDS-PAGE or they were frozen at -20°C. The cell pellets were resuspended at room temperature (RT) in Bugbuster Protein Extraction Reagent (Novagen, USA) containing rLysonase Bio-processing Reagent (10 µl rLysozyme/5 ml Bugbuster/g cell pellet; Novagen, USA). The cell lysates were incubated on a rotating mixer (Stuart Scientific rotator drive STR4) at RT for 10 min. To determine the solubility, the insoluble cell debris was removed by centrifugation at 6000 rpm at 4°C for 30 min. The supernatants containing the soluble fraction were separated from the pellets containing insoluble proteins which were again resuspended in Bugbuster Reagent and then separated using a discontinuous 10% SDS-PAGE. In addition, the insoluble inclusion bodies containing VP7st4 were subjected to a series of wash steps (partial-purification) following Bugbuster manufacturer's instructions prior to column purification. A final pellet of the inclusion bodies was then purified via histidine-tagged protein purification procedures. The Protino® nickel tris-carboxymethyl ethylene diamine (Ni-TED) and nickel nitrilotriacetic acid (Ni-NTA) kits under denaturing conditions were used according to the manufacturer's instructions (Ni-TED, Macherey-Nagel; Ni-NTA, Qiagen). The inclusion bodies were solubilised or denatured with 8 M urea at pH 8 for 1 h at 4°C on a rotating mixer. The solubilised protein was applied to either one of the columns. The fractions collected were analysed on SDS-PAGE. The protein eluted from the histidine column was dialysed in phosphate buffered saline (PBS). The sample was loaded into a Slide-A-Lyser Dialysis Cassette (3.500 MW cutoff, Pierce, USA) that was pre-immersed in the dialysis buffer. The buffer was changed three times every 2 hrs followed by another ON dialysis at 4°C. The concentration of the protein was determined spectrophotometrically (NanoDrop® ND-1000 spectrophotometer).

2.2.4. Analysis by SDS-PAGE

The protein samples were mixed with an equal volume of 2×protein solvent buffer (see appendix). The samples were boiled at 100°C for 5 min in a heating block to denature the proteins before analysis using a discontinuous 10% SDS-PAGE. Following electrophoresis at 150 V, 400 mA for 1 h, the gel was stained with Coomassie Brilliant Blue stain on a rocker (Bio-rocker model IIOA, Denville Scientific Inc.) for 1 h at RT. It was then destained in 4% (v/v) acetic acid and the gel was photographed (UVP Bioimaging system, The Scientific Group).

2.2.5. Western blot analysis

Following separation using a discontinuous 10% SDS-PAGE, the proteins were transferred onto a PVDF membrane (Invitrogen, USA). The membrane was blocked in 2% (w/v) fat-free milk powder (MP) in PBS for 1 h at RT on a rocker. The membrane was washed three times for 5 min each with PBS/0.05% (v/v)Tween-20 (PBS/0.05%T). For detection of a histidine tag, the blot was incubated with an anti-histidine tag monoclonal antibody (Novagen, USA) diluted 1:500 in PBS/0.05%T containing MP for 1 hr at RT. The wash step was repeated followed by incubation with a polyclonal rabbit anti-mouse monoclonal antibody conjugated to horseradish peroxidase (HRP; Dakocytomation, Denmark) diluted 1:1000 in MP/PBS/0.05%T for another 1 h at RT. After a final wash, a substrate solution (0.06 g of 4-chloro-1-naphthol dissolved in 10 ml ice-cold methanol and mixed with 60 µl hydrogen peroxide in 100 ml PBS) was used to develop the reaction. A photograph of the blot was taken using the AutoChemi^{TM} System (UVP Bioimaging systems, USA).

2.2.6. ELISA with recombinant VP7st4 immobilised on Polysorp microtitre plates

A 96-well Polysorp microtiter plate (Nunc^{TM}, Denmark) was coated with 50 µl/well of 5 µg/ml or 50 µg/ml of the dialysed recombinant VP7st4 in PBS ON at 4°C. MP and undialysed VP7st4 (50 µg/ml) were included as controls. The coated wells were washed three times with PBS/0.1% (v/v) Tween-20 (PBS/0.1%T) followed by three washes with PBS and then blocked with MP in PBS for 1 h at 37°C. The washing step was repeated followed by 1 h incubation with an anti-histidine antibody (Dianova, Germany) diluted

1:500 in MP/PBS/0.1%T. The washing step was repeated followed by 1 h incubation with a polyclonal rabbit anti-mouse IgG conjugated to HRP (Dakocytomation, Denmark) diluted 1:1000 in MP/PBS/0.1%T. *o*-phenylenediamine dihydrochloride substrate (50 µl; 5 mg OPD tablet in 5 ml 0.1 M citrate buffer at pH 4.5 containing 2.5 µl of hydrogen peroxide) was added to each well. The signal was measured spectrophotometrically using BDSL Immunoskan MS (Labsystems) at 492 nm after the reaction was stopped with 2 M H_2SO_4 after 40 min.

The recombinant VP7st4 was also tested to determine whether it can react with polyclonal rabbit anti-BTV and negative rabbit sera diluted 1:100 as well as guinea pig anti-BTV and negative guinea pig sera diluted 1:500. A polyclonal swine anti-rabbit antibody (Dakocytomation, Denmark) conjugated to HRP diluted 1:1000 in MP/PBS/0.05%T used to detect rabbit antibodies while recombinant protein A (Zymed, USA) also conjugated to HRP, was used at 1:4000 dilution for guinea pig antibodies. The wells were developed as above.

2.3. RESULTS

2.3.1. Expression of VP7st4

The recombinant VP7st4 was expressed at detectable levels in *E. coli* Origami™ cells. The predicted size of VP7 without histidine tag is 37 kDa. Following induction, electrophoresis revealed that the cells produced a protein of 50 kDa within 4 h at 30°C (Figure 2.1A and B, lane 4). The protein was larger than the native protein due to the two histidine tags at the N- and C-termini of VP7st4 and the presence of thioredoxin fusion partner. The cells were lysed and the insoluble proteins separated by centrifugation. To check if the recombinant VP7st4 was soluble, the resulting supernatant and the pellet were analysed on SDS-PAGE. VP7st4 was found to be present in the form of insoluble inclusion bodies (lane 4). More VP7st4 was expressed when induction was performed at an OD of 0.9 (Figure 2.1B). Other expression conditions aimed at improving the yields of the protein in a soluble form were investigated. These included using IPTG at various concentrations (0.1 mM to 0.5 mM), incubating at a lower temperature (18°C) but VP7st4 remained insoluble (done by Dr. Potgieter, Virology Section, OVI).

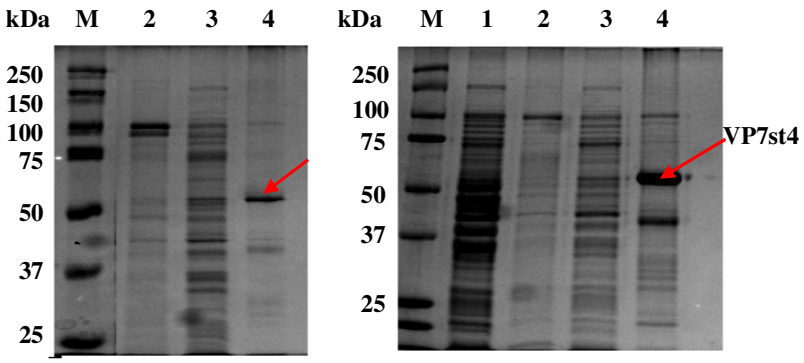


Figure 2.1: Coomassie stained discontinuous 10% SDS-PAGE of VP7st4 expressed in *E. coli*. The cells were induced at OD_{600nm} 0.6 (A) and at OD_{600nm} 0.9 (B). Lane M: Kaleidoscope Precision Marker; lane 1 (only B): supernatant of lysed bacteria before induction by IPTG (soluble); lane 2: bacterial pellet before protein expression was induced by IPTG (insoluble); lane 3: supernatant after induction by IPTG (soluble); and lane 4: bacterial pellet after induction by IPTG (insoluble). Equal volumes of samples were loaded per lane.

2.3.2. VP7st4 purification with Ni-TED

The recombinant protein was purified by Ni-TED affinity columns but the protein did not bind to the column since the 50 kDa band was observed in the column flow-through and wash fractions (Figure 2.2, lanes 3-4) and no protein in the eluted fractions (lanes 6-8).

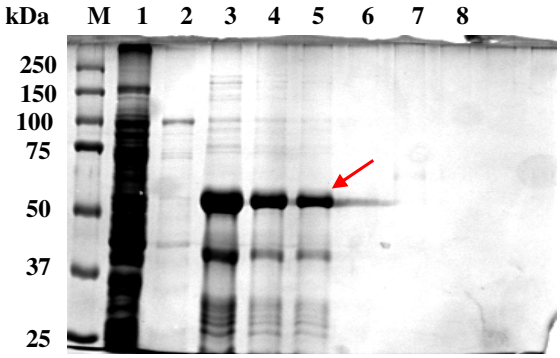


Figure 2.2: Coomassie stained SDS-PAGE of the recombinant VP7st4 purified by immobilised nickel affinity chromatography. Lane M: Kaleidoscope precision marker; lane 1: soluble fraction after Bugbuster extraction; lane 2: supernatant of insoluble inclusion bodies after washing with LEW buffer; lane 3: solubilised protein after insoluble proteins were denatured in solubilisation buffer (8 M urea, 50 mM NaH_2PO_4 and 300 mM NaCl at pH 8.0); lane 4: flow-through from the Ni-TED column; lanes 5-6: column wash fractions; lanes 7-8: fractions obtained by elution with 8 M urea containing 250 mM imidazole, 50 mM NaH_2PO_4 and 300 mM NaCl at pH 8.0. Equal volumes of samples were loaded per lane.

The presence of histidine-tagged recombinant VP7st4 of the expected size was confirmed in western blot using a commercial anti-histidine tag MAb (Figure 2.3). An anti-histidine antibody reacted with the recombinant VP7st4 confirming that the histidine tags were indeed present.

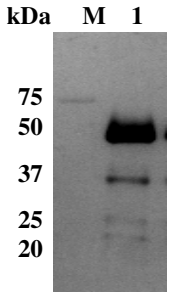


Figure 2.3: Immunoblot of VP7st4 separated by SDS-PAGE and detected by an anti-histidine tag antibody. Lane M: Kaleidoscope precision marker, Lane 1: flow-through collected from the Ni-TED column in denaturing buffer (i.e. same sample as in Figure 2.2, lane 3).

2.3.3. VP7st4 purification with Ni-NTA

Since recombinant VP7st4 did not bind to the Protino® Ni-TED column (above), a different Ni-NTA column was investigated instead. The flow-through, the wash fractions as well as the eluted fractions were analysed by SDS-PAGE (Figure 2.4) which showed that in this case the protein did indeed bind to the column. However, there are multiple bands in the eluted samples (lanes 5-7), which are present in the solubilised protein (lane 1). These protein bands were also recognised by a commercial anti-his tag antibody (lanes 5 to 7; green arrow; Figure 2.3).

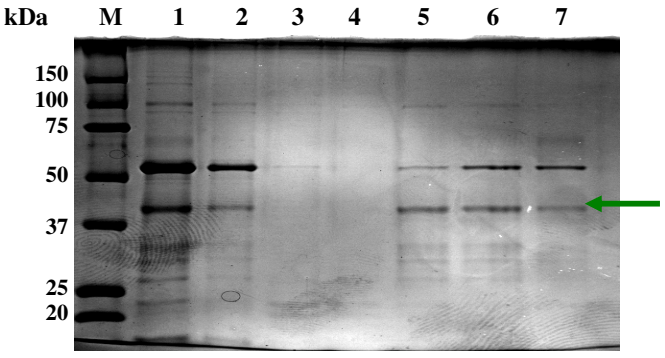


Figure 2.4: Coomassie stained SDS-PAGE of the recombinant VP7st4 purification by nickel affinity chromatography. Lane M: Kaleidoscope marker, lane 1: solubilised protein (in 8 M urea, 500 mM NaH_2PO_4 and 50 mM Tris-Cl at pH 8.0), lane 2: flow-through from the Ni-NTA column; lanes 3-4: column wash fractions; and lanes 5-7: fractions obtained by elution with 8 M urea containing 500 mM NaH_2PO_4 and 50 mM Tris-Cl at pH 4.5. Equal volumes of each sample were loaded per lane.

A log Mw versus Rf values was plotted to determine the size of VP7st4 as follows:

Table 2.1: List the Mw and calculated Log values of the standard, the distance of each protein migrated from the top of its lane as measured on an SDS-PAGE gel (lane 1 on Figure 2.4) and Rf values.

Mw (kDa) of known standard	Log Mw of known standard	Protein migration distance (mm)	Calculated Rf value
20	1.3	38	0.9
25	1.4	34	0.8
37	1.6	25	0.6
50	1.7	19	0.4
75	1.9	11	0.3
100	2.0	8.5	0.2

Table 2.2: List the distances of protein band above 37 kDa and 50 kDa (lanes 6 and 7 on Figure 2.4) measured from the top of the gel, the Rf values, Log Mw values from the graph as well as the calculated Mw in kDa.

Protein band	Protein migration distance (mm)	Rf value	Log Mw read from graph	Mw (kDa)
~50kDa	17	0.43	1.72	52
> 37kDa	23	0.55	1.64	44

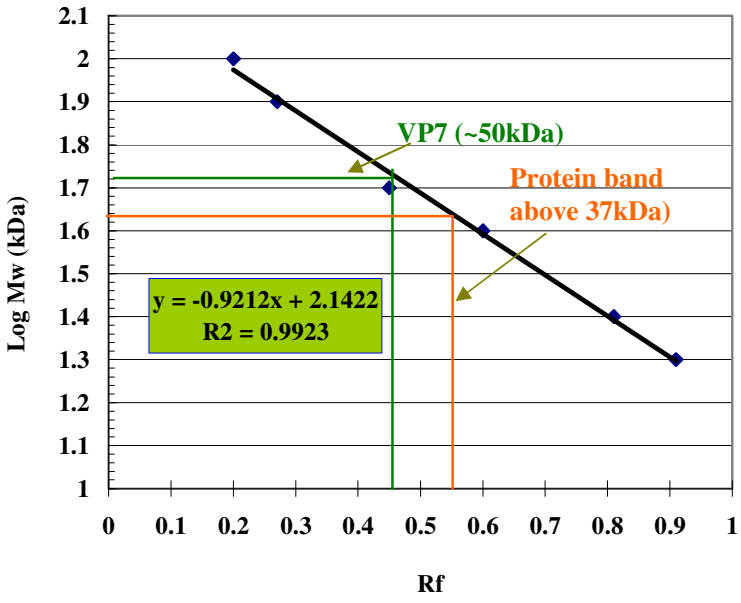


Figure 2.4a: The total distance migrated by a tracking dye was 42 mm. Each Rf value was calculated by dividing the distance of each protein by 42 mm. A graph of Log Mw versus Rf value was plotted and the known Rf value of each band was read on the x-axis of the graph and the corresponding y-axis Log Mw value was read off. The Mw of VP7st4 was found to be 52 kDa.

It was decided to use VP7st4 immediately after solubilisation in 8 M urea containing 500 mM NaH_2PO_4 and 50 mM Tris-Cl at pH 8.0 since affinity column purification does not increase the purity of the sample and there is sample loss during the purification. VP7st4 was dialysed against PBS to remove the excess urea which could potentially affect its function. Dialysis was for 36 h at 4°C with several buffer changes. The protein was then quantitated spectrophotometrically and ready for use in immunoassays.

2.3.4. Reactivity of recombinant VP7st4 in an ELISA

Since recombinant VP7st4 was to be used in panning experiments where it would be immobilised on plastic, it was tested in an ELISA to see whether the protein could be efficiently coated to plastic surfaces and be recognised by an anti-histidine tag antibody. It was added at concentrations of 50 µg/ml and 5 µg/ml to a Polysorp ELISA plate (Figure 2.5). The commercial anti-histidine tag antibody reacted strongly with the coated protein and resulted in a high ELISA signal of three even when VP7st4 was coated at 5 µg/ml. Dialysis of the recombinant protein was found to be essential as the pre-dialysed VP7st4 yielded a much lower signal in ELISA, less than 0.5.

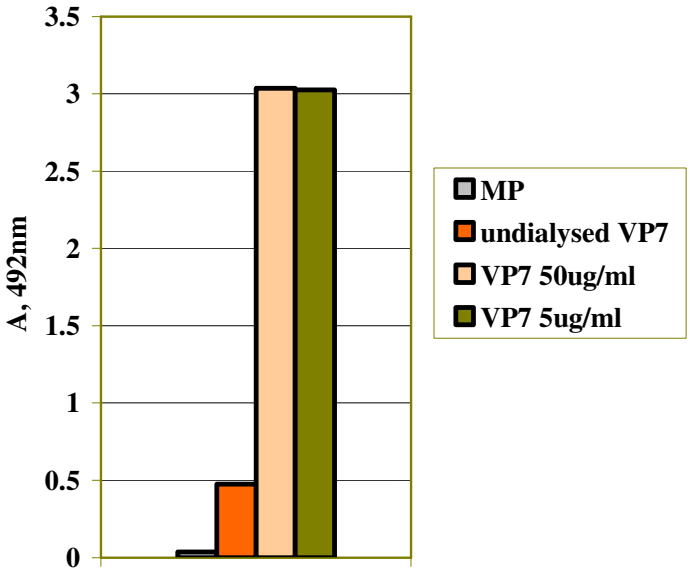


Figure 2.5: ELISA of an anti-histidine tag antibody reacting with histidine tagged recombinant VP7st4 before and after dialysis. MP was included as a control in the absence of VP7st4. VP7st4 was adsorbed directly on ELISA wells after dialysis at 50 µg/ml and 5 µg/ml. An undialysed sample was used at 50 µg/ml. An anti-histidine tag antibody detected the histidine tag on VP7st4.

The recombinant VP7st4 was also tested to ascertain whether there were any antibodies against VP7 in rabbit and guinea pig antisera raised against whole BTV (Figure 2.6).

ELISA showed that there were indeed antibodies in the antisera that could recognise denatured protein. This confirmed that VP7st4 prepared in this way does react with anti-BTV sera and can be used in panning.

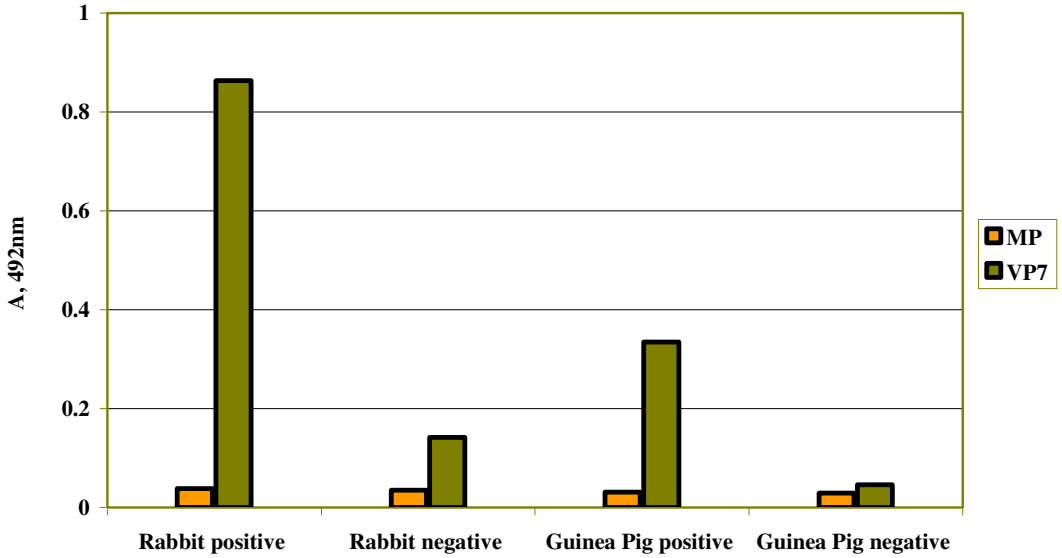


Figure 2.6: ELISA of rabbit and guinea pig anti-BTV serum reacting with recombinant VP7st4. MP was included as a control in the absence of VP7st4. Rabbit anti-sera was used at 1:100 and guinea pig antisera was used at 1:500 in MP/PBS/0.05%T. Bound antibodies were detected using commercial polyclonal swine anti-rabbit-HRP diluted 1:1000 for rabbit antisera. Recombinant protein A-HRP diluted 1:4000 was used for guinea pig antisera. Dilutions were in MP/PBS/0.05% T.

2.4. DISCUSSION

Proteins expressed in *E. coli* can potentially be obtained rapidly and at low cost. Those secreted in the periplasm of *E. coli* are often soluble, stable and folded in a native form (Courtney *et al*., 1984; reviewed by Marston, 1986; Cabilly, 1989). Some accumulate in the reducing environment of the cytoplasm, usually aggregating into an insoluble mass due to inefficient folding into the correct conformation (Goeddel *et al*., 1979; De Boer *et al*., 1983; Georgiou *et al*., 1986; reviewed by Marston, 1986; Rinas *et al*., 1992; Makrides, 1996; Van den Berg *et al*., 1999). VP7st4 was expressed at high levels in *E. coli* but it was found in the cytoplasm in the form of insoluble aggregates. This may have been as a result of inappropriate folding due to rapid over-expression and as a result of unbalanced equilibrium between aggregation and solubilisation (Kiefhaber *et al*., 1991; reviewed by Marston, 1986 and Rudolph & Lillie, 1996; Verma *et al*., 1998, Jonasson *et al*., 2002). Aggregation is caused by non-specific interactions between hydrophobic groups on unfolded proteins and by incorrect association of partially folded intermediates

(reviewed by Rudolph & Lillie, 1996). This is not uncommon as these findings correlate with several previous reports. For example, expression of human insulin (Goeddel *et al*., 1979), preS2-S'-β-galactosidase (Vasina & Baneyx, 1997) and rice lipoxygenase (Shirano & Shibata, 1990) in *E. coli* at 37°C, did not result in native, soluble and biologically active recombinant proteins. Instead the proteins accumulated in the cytoplasm of the cell as aggregates. The VP7st4 inclusion body preparation was highly enriched with the recombinant protein (Figure 2.1). This is in line with previous reports stating that when expression leads to inclusion body formation, the preparation will be dominated in quantity by the protein under study (reviewed by Marston, 1986; Lilie *et al*., 1998; Xiong *et al*., 2005). In addition, inclusion bodies are obtained at a relatively pure form because they are dense and thus easily collected by centrifugation at low speed. Another advantage is that the proteins enriched in inclusion bodies are protected against proteolysis by intracellular proteases (reviewed by Murby *et al*., 1996 and Rudolph & Lillie, 1996). Protein expression at lower temperatures has proved effective in facilitating the solubility of many proteins (Cabilly, 1989; Shirano & Shibata, 1990; Vasina & Baneyx, 1997 and references therein).

Production of recombinant proteins that are biologically active depends on the proper folding of the protein during expression. This is especially important if correct disulphide bonds and intramolecular interactions must be formed (Perry & Wetzel, 1986; Pigiet & Schuster, 1986; reviewed by Marston, 1986 and Messens & Collet, 2006). In the *E.coli* cytoplasm, proper folding is limited because of the reducing environment (reviewed by Marston, 1986 and Mergulhão *et al*., 2005). The correct pairing of cysteine residues contributes to the stability of proteins. Therefore stable disulphide bond formation during expression of proteins facilitates soluble expression (Stewart *et al*., 1998; Bessette *et al*., 1999). In the cytoplasm, there are thioredoxins and glutaredoxins which can be oxidised to initiate the formation of disulphide bonds. In the presence of thioredoxin reductase and glutathione these proteins are kept reduced (Stewart *et al*., 1998; Bessette *et al*., 1999; reviewed by Sørensen & Mortensen, 2005). Glutathione initiates the formation of random disulphide bonds in a reduced and denatured state. Thioredoxin oxidise and reduce disulphide rearrangement to the native form, and also allows interchange of incorrect disulphide bonds from the unfolded, reduced form or from randomly disordered protein conformations (Pigiet & Schuster, 1986; Stewart *et al*., 1998). The pH is also a critical factor for the formation of correct disulphide bonds because thiol/disulphide exchange occurs faster at alkaline pH (reviewed by Rudolph & Lilie, 1996).

The VP7st4 sequence has three cysteine residues at position 15, 65 and 154 two of which might be involved in disulphide bond formation while one exists freely and, therefore could result in improper folding of the protein (Kowalik *et al*., 1990; Bessette *et al*., 1999). According to Perry and Wetzel (1986), T4 lysozyme consists of two cysteine residues at position 54 and 97 which are not involved in disulphide bond formation. Insertion of a third cysteine at position three by side-directed mutagenesis allowed for three possible intramolecular interactions. A disulphide bond was formed between cysteines at positions 3 and 97 resulting in a properly folded molecule while cysteine at position 54 was involved in intermolecular thiol/disulphide exchange to stabilise the molecule (Perry & Wetzel, 1986).

Several studies have focused on improving the solubility of heterologous proteins expressed in the cytoplasm of *E. coli* (e.g. Origami™ DE3). For example, strains which are deficient in reductase enzymes allowed the expression of proteins with a single disulphide bond to be over 90% soluble. The expression of proteins containing free cysteines formed inclusion bodies even in Origami™ cells which they are not supposed to do (Xiong *et al*., 2003; 2005). This could also explain why VP7st4 was insoluble even when Origami™ cells were used. Therefore, in addition to the induction temperature, the choice of *E.coli* strain (Xiong *et al*., 2003), the concentration of IPTG, or the use of chaperones may improve the soluble production of other proteins (William *et al*., 1982; Thomas & Baneyx, 1996; Bessette *et al*., 1999; Carrió & Villaverde, 2003; Sørensen & Mortensen, 2005). Some BTV proteins are found as insoluble inclusion bodies regardless of the conditions used (personal communication, Dr Potgieter). The VP7st4 cloned in a pET32a(+) vector (by Dr. Potgieter) which contained a thioredoxin tag aimed at enhancing the formation of disulphide bonds in the cytoplasm and hence the solubility of the protein (pET manual; personal communication, Dr Potgieter) remained insoluble.

Although the recombinant protein was insoluble, it could be solubilised in a denaturing buffer (8 M urea) prior to purification with Ni-affinity columns. The recombinant protein did not bind to a Ni-TED column. There are several factors that could have caused the protein not to bind the Ni-TED column. These include the pH of the purification buffer and the absence of the histidine tag. The pH of the buffer was checked prior to use. The presence of the histidine tags on VP7st4 was confirmed on western blot. The lack of a functional histidine tag was not the reason why the protein was not binding to the nickel

affinity column. This result suggested that there could be other reasons as to why the protein was collected in the flow-through and wash steps. For example, the binding conditions such as the temperature also play an important role during purification. It was decided to use an alternative purification method, i.e Ni-NTA. Some of the protein was still found in the flow-through fraction (Figure 2.4., lane 2). This could be because the column was overloaded with sample. There was no protein in the wash fractions collected during purification which confirmed that the protein indeed bound to the Ni-NTA column. The protein was finally eluted, but there were additional bands which reacted with the anti-histidine tag antibody, indicating the presence of other histidine-rich proteins in the sample other than the 50kDa VP7st4. These bands could be degraded VP7st4 with one tag or even VP7st4 which was still in the process of being translated. Since VP7st4 was found in inclusion bodies, the preparation was already considered relatively pure. Consequently it was decided to use VP7st4 in immunoassays without Ni-NTA purification which resulted in unacceptible losses, but virtually no improvement in purity. Other workers reported improved expression of proteins directly in *E. coli* by fusion with bacterial genes such as β-galactosidase gene as demonstrated for somatostatin (Itakura *et al*., 1977) and insulin (Goeddel *et al*., 1979).

A protein which folds correctly such that the important epitopes are retained is needed in panning and immunoassays. ELISA showed that an anti-histidine tag antibody recognised recombinant VP7st4 absorbed directly on plastic surface. In addition, antibodies in the sera of rabbits and guinea pigs immunised with BTV also recognised the recombinant VP7st4. Although the ELISA signals obtained were low, there was an indication that the recombinant protein was reactive. This suggests that some VP7st4 epitopes were in the right conformation and it was decided to use this VP7st4 to pan for additional *Nkuku*® recombinant antibodies.

CHAPTER THREE

SELECTION AND CHARACTERISATION OF SINGLE-CHAIN VARIABLE FRAGMENTS AGAINST VP7ST4

3.1. INTRODUCTION

Bluetongue remains an important viral disease of domestic animals worldwide. The recent outbreaks in Portugal (Barros *et al*., 2007) and the Netherlands (Dercksen *et al*., 2007) demonstrated the need to be able to control and diagnose this disease. Disease outbreaks can be devastating since they have economic implications. Therefore, novel reagents are continuously needed for applications in veterinary, agricultural and human diagnostics (Van Wyngaardt *et al*., 2004). It is necessary to generate antibodies specific to different disease causing agents rapidly and at low costs. This is especially important for applications in developing countries which have financial constraints and where proper facilities are limited or unavailable (Van Wyngaardt *et al*., 2004). The main purpose of this part of the study was to select recombinant antibodies against recombinant VP7st4 for potential use as immunodiagnostic reagents.

The selection of clones from a phage library is achieved by panning against a specific antigen as described in Section 1.4.2 (Smith, 1985; Van Wyngaardt *et al*., 2004). VP7st4 specific binders are to be selected from the *Nkuku*® phage display library. This semi-synthetic library represents a large repertoire of antibodies against potentially any antigen. Antibodies have been selected from this library against viruses, proteins and haptens. The *Nkuku*® library allows both phage-displayed scFvs and soluble fragments to be produced (Van Wyngaardt *et al*., 2004). An amber stop codon (TAG; Hoogenboom *et al*., 1991; Webster, 1996) is incorporated between the coding regions of the scFvs and the start of gene III. This stop codon allows for the expression of scFv-pIII fusions when *E. coli* suppressor stains (e.g. TG1) are used. Therefore, an infection with M13KO7 helper phage results in phage displaying the wild type gene III and scFv-pIII fusions. To ensure that phage displayed scFvs are selected, the soluble fragments are eliminated by precipitation of the phages with PEG. On the other hand, when non-suppressor strains are used, or when over-expressing with IPTG without rescue, only soluble scFvs are generated (McCafferty *et al*., 1990; Clackson *et al*., 1991; Winter *et al*., 1994; Sheets *et al*., 1998; Van Wyngaardt *et al*., 2004; Barderas *et al*., 2006).

An scFv that will be able to function in an inhibition ELISA is needed. For incorporation into an assay, a soluble scFv is the preferred format rather than a fusion protein displayed on the phage. In an inhibition ELISA, an immobilised antigen is allowed to react with an antiserum. The presence of antibodies against the antigen will block the binding of a specific scFv. The scFv is detected and the level of inhibition can be determined (Kweon *et al*., 2003; Kashiwazaki *et al*., 2004; Fehrsen *et al*., 2005). In this chapter, chicken scFvs specific to VP7st4 will be selected from the *Nkuku*® library following different panning strategies. The selected scFvs will be investigated in an inhibition ELISA to determine whether their binding to VP7st4 can be blocked by antibodies in the sera of guinea pigs, rabbits, chickens and sheep immunised or infected with BTV.

3.2. MATERIALS AND METHODS

3.2.1. Materials

The *Nkuku*® phage display library, *E. coli* TG1 cells, M13KO7 (2×10^{12} CFU/ml) helper phage, baculovirus-VP7 of BTV serotype 1, anti-BTV chicken IgY, rabbit and guinea pig serum were obtained from Wouter Van Wyngaardt (Immunology Section, OVI). Anti-BTV sheep serum was obtained from Shirley Smith of the World Reference Centre for BTV at the OVI.

3.2.2. Preparation of *E. coli* TG1 stock

E. coli TG1 cells were streaked on a M9 minimal medium plate and incubated at 30°C for 96 h. A single colony was grown in 2×TY medium ON at 37°C with shaking at 220 rpm. A glycerol stock of the cells containing 15% (v/v) glycerol was prepared and stored at -70 °C until use.

3.2.3. Preparation of TG1 mid-logs

Medium (2×TY, 5 ml) was inoculated with TG1 from a glycerol stock prepared (Section 3.2.2) and grown ON at 37°C at 220 rpm. The ON culture was diluted 1:100 into fresh 2×TY medium, grown at 37°C until the OD at 600 nm reached mid-log phase (OD_{600nm} of 0.5) and used immediately.

3.2.4. Large scale production of M13KO7 helper phage

TG1 cells at OD_{600nm} of 0.2 were infected with serial dilutions of M13KO7 stock (2×10^{12}) prepared in a 2×TY medium for 10 min at 37°C in a water bath. Following infection, the phage-infected cells were added to molten H-top agar pre-warmed at 42°C and mixed. The mixture was poured onto TYE plates which were pre-warmed at 37°C. The plates were incubated ON at 37°C. A single M13 plaque was inoculated in TG1 cells at OD_{600nm} 0.2 for 2 h at 37°C. The culture was inoculated into 500 ml of 2×TY medium and incubated for an additional 1 h. It was then incubated ON after 50 µg/ml kanamycin was added. The cells were collected by centrifugation at 6000 rpm for 30 min. The phages were precipitated for 1 h on ice by adding ¼ volume of 20% (w/v) PEG/2.5 M NaCl solution to the supernatant. The phages were collected by centrifugation (6000 rpm, 30 min), and the supernatant was discarded followed by a brief re-centrifugation to remove the remaining supernatant. The pellet was resuspended in 2 ml PBS and filtered through a 0.45 µm filter (Sartorius Minisart®, Germany). Glycerol was added to a final concentration of 50% (v/v) and the stocks were stored in aliquots at -70°C.

3.2.5. Titration of phages obtained after each selection round

For titration of the M13KO7 phage stock, 500 µl of two-fold serial dilutions were prepared in 2×TY medium from 10^{-8} to 10^{-12} and used to infect 500 µl TG1 cells at mid-log phase (ratio of 1:1) for 10 min at 37°C in a water bath. After infection, 100 µl of each M13KO7 dilution, 100 µl ON TG1 culture and 4 ml of molten H-top agar pre-warmed at 42°C were mixed and then poured onto TY agar plates which were incubated ON at 37°C. The titre of the phages was calculated by counting plaques on the plates.

3.2.6. Selection of scFvs against VP7st4 from the *Nkuku*® library by panning

Panning was performed as described previously (Van Wyngaardt *et al*., 1998). A Polysorp immunotube (Nunc™, Denmark) was coated with 20 µg/ml recombinant VP7st4 ON at 4°C. The immunotube was washed three times with PBS/0.1%T and thrice with PBS. The non-specific sites in the tube were blocked with MP at RT for 1 h. Simultaneously, the *Nkuku*® phage stock (10^{12} transducing units) was pre-incubated in 2% MP/PBS/0.2%Tween-20 (MP/PBS/0.2%T). The pre-incubated phage library was applied into the immunotube after the wash step and incubated for 30 min on a rotating turntable

(Stuart Scientific rotator drive STR4) at RT and left to stand for another 90 min. The immunotube was washed 20 times with PBS/0.1%T and then 20 times with PBS. Bound phages were eluted by adding 1 ml 100 mM triethyl amine (TEA) to the tube and rotating for 10 min. Eluted phages were neutralised with 500 µl 1 M Tris-HCl buffer (pH 7.4) and then added to 5 ml TG1 mid-log cells. The mixture was incubated at 37°C for 30 min in a water bath. An aliquot was used to infect the cells for titration of phages while the remainder was centrifuged for 10 min at 3100 rpm (Kubota 8100). The supernatant was removed and the pellet was resuspended in 600 µl 2×TY medium. The cells were plated out on TYE agar plates containing 100 µg/ml ampicillin and 2% glucose (TYE/AG) medium and incubated ON at 30°C. Glycerol stocks were prepared by scraping bacteria off the plates with 2×TY containing 100 µg/ml ampicillin and 2% glucose (TY/AG) by mixing bacterial cells and 60% glycerol (at a ratio of 3:1). The same panning method was also performed in His-Grab^{TM} nickel coated wells (Pierce).

For amplification of phages, the glycerol stock was used to inoculate fresh TY/AG medium to an initial OD between 0.03-0.05. The rest of the glycerol stock was stored at -70°C. The inoculated medium was grown until the OD_{600nm} reached 0.5. From this exponentially growing culture, a portion was infected at a ratio of 20:1 of M13 helper phage (2×10^{12} CFU/ml) to bacterial cells and incubated at 37°C for 30 min. Bacterial cells were collected by centrifugation at 3100 rpm for 10 min. The pellet was resuspended in 2×TY supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin (TY/AK). The culture was grown ON at 30°C with shaking at 220 rpm. Cells were collected at 3100 rpm for 20 min and the phages in the supernatant were precipitated by adding 1/5 volume of 20% (v/v) PEG/2.5 M NaCl and incubated for 1 h on ice. Precipitated phages were collected by centrifugation at 3100 rpm for 15 min. The pellet was resuspended in PBS after a brief re-centrifugation. These phages were then used for the second round of panning. Four successive rounds of panning were performed. Serial dilutions of phages obtained after each selection round were prepared in 2×TY medium from 10^{-8} to 10^{-12}. Titration of phages was as described in Section 3.2.5 except that the cells were infected for 30 min in a water bath and 100 µl of each dilution was plated on TYE/AG agar plates. The output in colony forming units (CFU) is calculated as the number of colonies on titre plates after each selection round × (total volume of cells infected with phages ÷ volume plated on selective agar plates). The output increase was calculated using the formula (R_n/ R_{n-1}) where n represent a selection round.

3.2.7. Screening of phages pools in a polyclonal phage ELISA to monitor the increase in VP7st4-specific phages

ELISAs were performed following methods described previously (Van Wyngaardt *et al.*, 1998). A 96-well Polysorp ELISA plate (Nunc™, Denmark) coated ON at 4°C with 40 µg/ml recombinant VP7st4 was blocked with MP for 1 h at 37°C. The plate was washed thrice with PBS/0.1%T and again with PBS. Phages from each selection round were diluted 100-fold and then mixed 1:1 in MP/PBS/0.2%T, added to the wells and incubated for 1 h at 37°C. The wells were washed followed by 1 h incubation with a mouse MAb B62-FE2 (Progen Biotechnik, Germany), specific to an epitope on phage coat protein VIII, diluted 1:1000 in MP/0.1%T. The wash step was repeated. A polyclonal rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP; Dakocytomation, Denmark) diluted 1:1000 was added to the wells and left for 1 h at 37°C. An OPD enzyme substrate solution (50 µl; 5 mg OPD tablet in 5 ml 0.1 M citrate buffer at pH 4.5 containing 2.5 µl of hydrogen peroxide) was added to each well. The signal was measured spectrophotometrically using BDSL Immunoskan MS (Labsystems) at 492 nm after the reaction was stopped with 2 M H_2SO_4 after 40 min.

3.2.8. Preparation of *E. coli* Origami™ cell proteins

E. coli Origami™ cells were inoculated into 10 ml 2×TY medium and grown ON at 30°C. The cells were collected by centrifugation at 3100 rpm for 20 min. The pelleted-cells were purified using Bugbuster Protein Extraction kit (see 2.2.3). The Origami™ cell proteins were lysed in 8 M urea containing 500 mM NaH_2PO_4 and 50 mM Tris-Cl at pH 8.0 and then dialysed ON in PBS as described for VP7st4 (Section 2.2.3). The proteins were then used as positive controls in monoclonal phage and scFv ELISAs to verify that the individual clones are specific to VP7st4.

3.2.9. Screening of individual phage-positive clones in a monoclonal phage ELISA using a MAb B62-FE2

After the fourth round of panning against recombinant VP7st4, individual clones were randomly picked from the titre plates of selection round three and four clones and inoculated into a 96-well sterile Polysorp ELISA plate (Nunc™, Denmark) containing TY/AG. Wells H11 and H12 were inoculated with an anti-keyhole limpet (KLH) as a

positive control. The plate was shaken at 220 rpm ON at 30°C. Fresh medium (100 µl) in another 96-well sterile plate was inoculated with the ON culture using a 96-well transferring device and shaken at 37°C for 150 min to amplify the individual clones. Glycerol was added to the master plate (ON plate) to a final concentration of 15% and stored at -70°C. Phages were produced in the newly inoculated plate by adding 50 µl TY/AG containing 2.8×10^{11} CFU/ml M13 helper phage per well and incubating for 30 min at 37°C without shaking. The plate was centrifuged at 1700 rpm (Kubota 8100) for 10 min and the supernatant was discarded followed by a brief re-centrifugation to remove the rest of the supernatant. The pellet was resuspended in TY/AK medium and grown ON at 30°C shaking at 220 rpm. The plate was again centrifuged at 1700 rpm for 10 min. The supernatants containing antibody-displayed phages were mixed with MP/PBS/0.2%T at a ratio of 1:1, added to Polysorp ELISA plates that were separately coated ON at 4°C with 10 µg/ml *E. coli* Origami™ cell proteins, 40 µg/ml VP7st4 or MP. The plates were blocked with MP for 1 h at 37°C followed by 1 h incubation at 37°C with a mouse MAb B62-FE2 specific for phage coat protein VIII (Progen Biotechnik, Germany) diluted 1:1000. Bound antibody was detected by an anti-mouse conjugated IgG to HRP diluted 1:1000. An enzyme substrate solution was added as in Section 3.2.7.

3.2.10. Screening of individual soluble fragments in a monoclonal scFv ELISA using an anti-c-*myc* tag MAb 9E10

Monoclonal scFv ELISA was done as described for the monoclonal phage ELISA except that soluble scFvs were induced by adding 2×TY containing 100 µg/ml ampicillin and 3 mM IPTG instead of rescuing phages with M13. Monoclonal scFv culture supernatant was added to ELISA wells that had been pre-coated at 4°C separately with 10 µg/ml *E. coli* Origami™ cell proteins, 40 µg/ml VP7st4 or MP. The wells were blocked with MP for 1 hr at 37°C. The c-*myc*-tag of the scFv was detected with an anti-c-*myc* mouse MAb (clone 9E10; Roche, USA) diluted 1:500 in MP/PBS/0.05%T to the wells followed by a polyclonal rabbit anti-mouse IgG conjugated to HRP. Substrate solution was added as above.

3.2.11. Sequencing of scFv genes

The DNA inserts of several clones tested in monoclonal scFv ELISA from selection round three and four were sequenced (ABI 3100 Genetic Analyzer at Molecular Biology, OVI).

Positive clones having the highest ELISA signal were inoculated from the master plates into 5 ml TY/AG and grown ON at 30°C. The culture was centrifuged and the pelleted DNA was extracted using a Qiaprep® Spin Miniprep Kit following the manufacturer's instructions (Qiagen). The primers OP52F (5´-CCCTCATAGTTAGCGTAACG-3´) and M13R (5´-CAGGAAACAGCTATGAC-3´) were used (Van Wyngaardt *et al*., 2004). The sequences obtained were aligned using BioEdit version 7.0.4 (Hall, 1999).

3.2.12. ELISA to verify whether the scFvs are specific to VP7st4

To test whether the anti-VP7st4 scFvs recognise recombinant VP7st4 or its fusion partner expressed as described for VP7st4 (Sections 2.2.2 and 2.2.3), 40 µg/ml of each protein was coated in Polysorp ELISA wells and incubated ON at 4ºC. The coated wells were washed three times with PBS/0.05%T followed by three times washing with PBS and then blocked with MP/PBS for 1 h at 37°C. The washing step was repeated followed by 1 h incubation with affinity purified anti-VP7st4 scFvs at 20 µg/ml (see Section 4.3.1). The washing step was repeated followed by another 1 h incubation with an anti-c-*myc* mouse MAb (clone 9E10) conjugated to HRP (Roche) diluted 1:1000 in MP/PBS/0.05%T. An OPD substrate (50 µl) was added to each well. The signal was measured spectrophotometrically at 492 nm after the reaction was stopped with 2 M H_2SO_4 after 40 min.

3.2.13. Large scale growth of individual soluble scFvs

Following DNA sequencing, representative positive clones were grown in large volumes for further characterisation. The ON cultures inoculated with the glycerol stock of the scFvs were diluted 1:100 in a fresh TY/AG and shaken at 37°C for 2 h until the OD_{600nm} reached 0.9. The bacterial cells were collected at 3100 rpm and the supernatant was removed followed by a brief re-centrifugation to remove the remaining liquid. The bacterial pellets of the scFvs A1, H2, TC9 and SA12 were resuspended in 10 ml 2×TY containing 100 µg/ml ampicillin and 1 mM IPTG. ScFv TD12 was induced in a 2 ml IPTG containing medium since the yield was low in 10 ml. The cells were grown ON at 30°C. Following induction, the cells were removed by centrifugation and 2% sucrose was added to the supernatants and stored at -20°C until use.

3.2.14. Stability of scFvs

The stability of the scFvs stored under different conditions was investigated. Following large scale growth of the selected scFvs, 2% sucrose was added to the supernatants containing each scFv and stored at -20°C, 4°C and RT respectively. Samples at -20°C were thawed, aliquots were taken and then frozen again. Aliquots were also taken daily from samples stored at 4°C and RT. The aliquots were then frozen at -20°C. This process was repeated daily for each scFv. The samples were then removed from -20°C, thawed and tested in an ELISA as described for monoclonal scFv ELISA in Section 3.2.14.

3.2.15. Inhibition ELISA with immune serum

The recombinant VP7st4 was coated at 40 µg/ml in a 96-well Polysorp ELISA plate (Nunc™, Denmark) ON at 4°C and blocked with MP. The plate was incubated with guinea pig anti-BTV or a negative guinea pig serum diluted 1:10 in MP/PBS/0.05%T for 1 h at 37°C. The scFv in 4% MP was added to the wells and incubated for 1 h and then detected with an anti-c-*myc* mouse MAb (clone 9E10) as in Section 3.2.10. An anti-BTV chicken IgY (20 µg/ml) from chickens immunised with whole BTV virus purified by sucrose-gradient (personal communication, Wouter Van Wyngaardt, Immunology Section, OVI) as well as sheep serum were also tested in an inhibition ELISA.

3.3. RESULTS

3.3.1. Selection of scFvs against VP7st4 from the *Nkuku*® library

The *Nkuku*® phage display library was subjected to four rounds of panning against VP7st4. The titres of phages released after each round increased, implying that there was enrichment during panning. The phage pools obtained after each round were then tested in a polyclonal ELISA which resulted in a high signal of 2.5 confirming enrichment of VP7st4-specific binders (Figure 3.1). This indicated that the relative number of phages specific to VP7st4 increased with each consecutive round corresponding to an increase in the output phages.

Table 3.1: Number of phages binding to the recombinant VP7st4 released after each round of selection.

Selection round	Output (CFU)	Output increase (R_n/ R_{n-1})
Round one	2.20×10^3	
Round two	2.51×10^4	11.4 ×
Round three	3.44×10^6	137×
Round four	4.68×10^7	13.6×

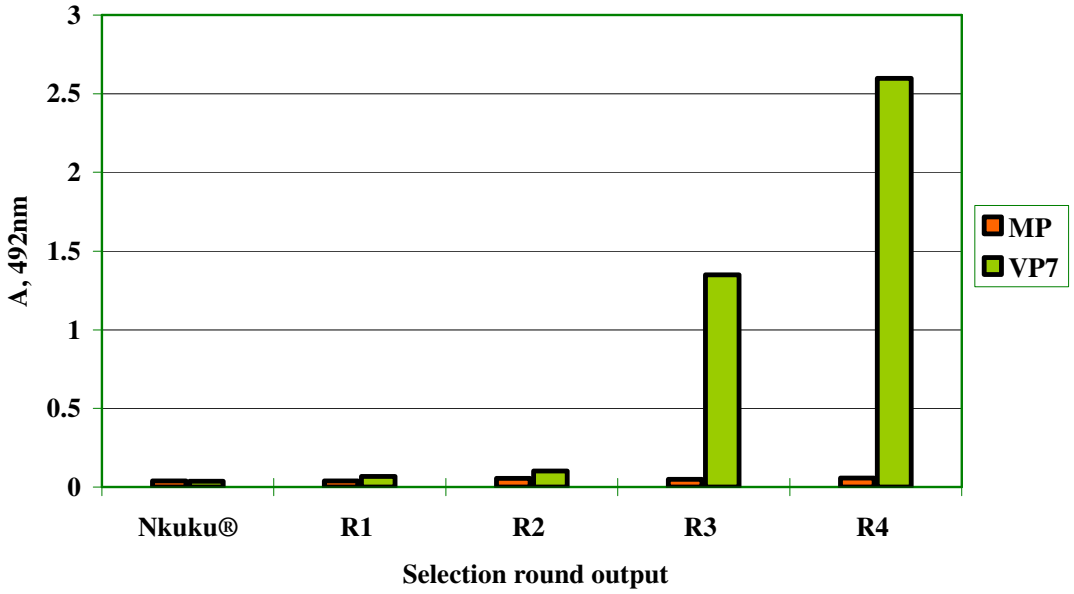


Figure 3.1: Polyclonal ELISA of phage pools after each round of selection reacting with VP7st4. A, 492 nm represent the absorbance at 492 nm. MP was used as a control. VP7st4 (40 µg/ml) and MP were adsorbed directly in Polysorp ELISA wells and incubated ON at 4°C. Non-specific sites were blocked with MP. Phages produced after each selection round were tested for binding to VP7st4 and MP including the phages from an unpanned library. B62-FE2, a mAb specific to the phage coat protein VIII was used to detect bound phages.

Single clones that were randomly picked from the titre plates of selection rounds three and four were tested individually in an ELISA to identify specific binders from the pool of binders. Screening of 96 individual clones was done in both the phage-displayed (Figure 3.2A and 3.5A) and soluble scFv formats (Figure 3.2B and 3.5B). Most of the individual clones which were positive in the phage format were also positive in the soluble scFv format (Figure 3.2; represented by green bars). There were some exceptions which were

positive as phages but not as soluble scFvs (Figure 3.2, sky blue bars) and vice versa (yellow bars). *E. coli* Origami™ cell proteins were included as controls (Figure 3.3 and 3.6) to verify that the binders were specific to VP7st4 since the recombinant VP7st4 used was expressed in these cells. The clones having an ELISA signal of at least twice that of the background were regarded as positive. The background refers to MP which was included as a negative control since it was used as a blocking solution in the panning (Figure 3.4 and 3.7). If the clones reacted with *E. coli* Origami™ cell proteins and MP controls, they were excluded from further investigation. An anti-KLH binder was tested in duplicate as a positive control for ELISA reagents (Figure 3.2 and 3.5, grey bars). This binder reacted strongly with KLH giving an ELISA signal of above three in the phage displayed scFv format but not in the soluble format (personal communication, Wouter Van Wyngaardt). None of the clones from round four reacted with the MP control (Figure 3.7). Most of the individual clones obtained after round four reacted strongly with recombinant VP7st4 both as phages and as soluble scFvs (Figure 3.5). The soluble scFvs yielded an ELISA signal double that obtained as phage binders.

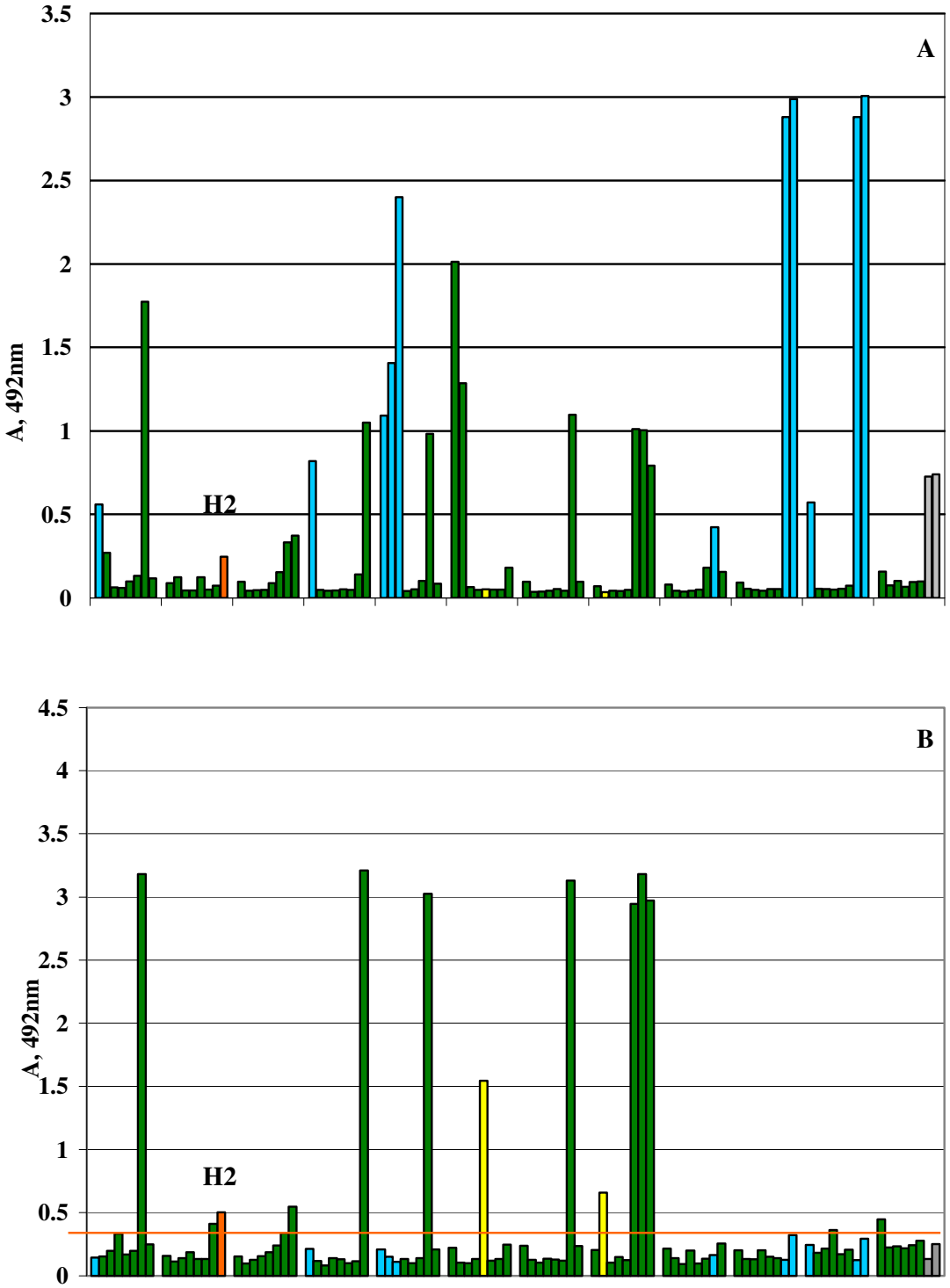


Figure 3.2: ELISA of individual phage displayed clones (A) and of soluble antibody fragments (B) against VP7st4 from selection round three. Each bar represents an individual clone that was randomly chosen from titre plates. Grey bars represent an ELISA signal resulting from an anti-KLH binder, a positive control. Sky blue bars represent clones which were positive as phages but not as soluble fragments and vice versa (yellow bars). ScFv H2 is shown in orange. Clones having an ELISA absorbance of above 0.4 were regarded as VP7st4-specific and were sequenced.

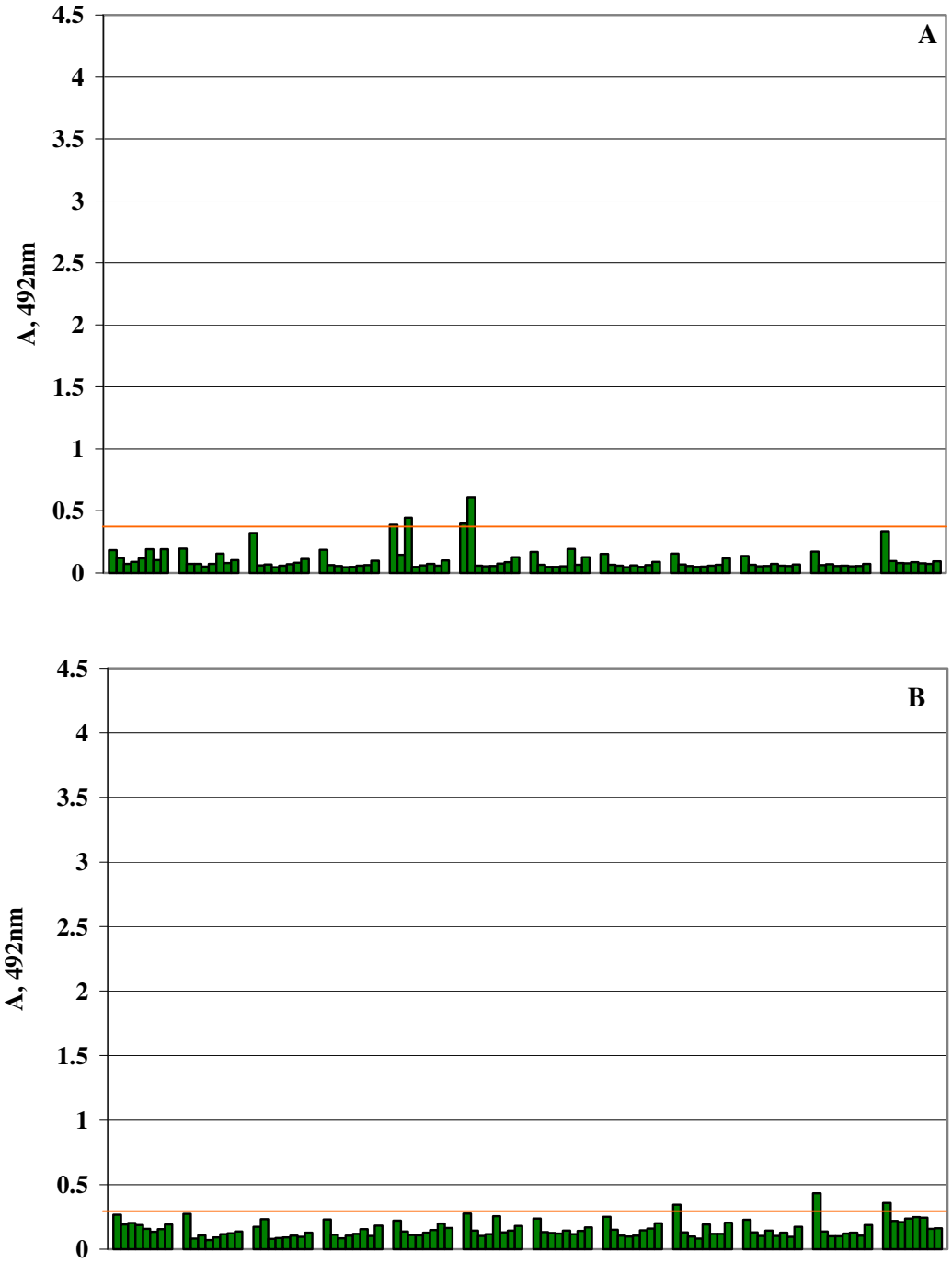


Figure 3.3: ELISA of individual phage displayed clones (A) and of soluble antibody fragments (B) from selection round three corresponding to those shown in Figure 3.2 tested against *E. coli* Origami™ proteins as a control instead of recombinant VP7st4. The orange line represent a cut-off, clones having ELISA absorbance above this line were regarded as having high background and were not sequenced and characterised further.

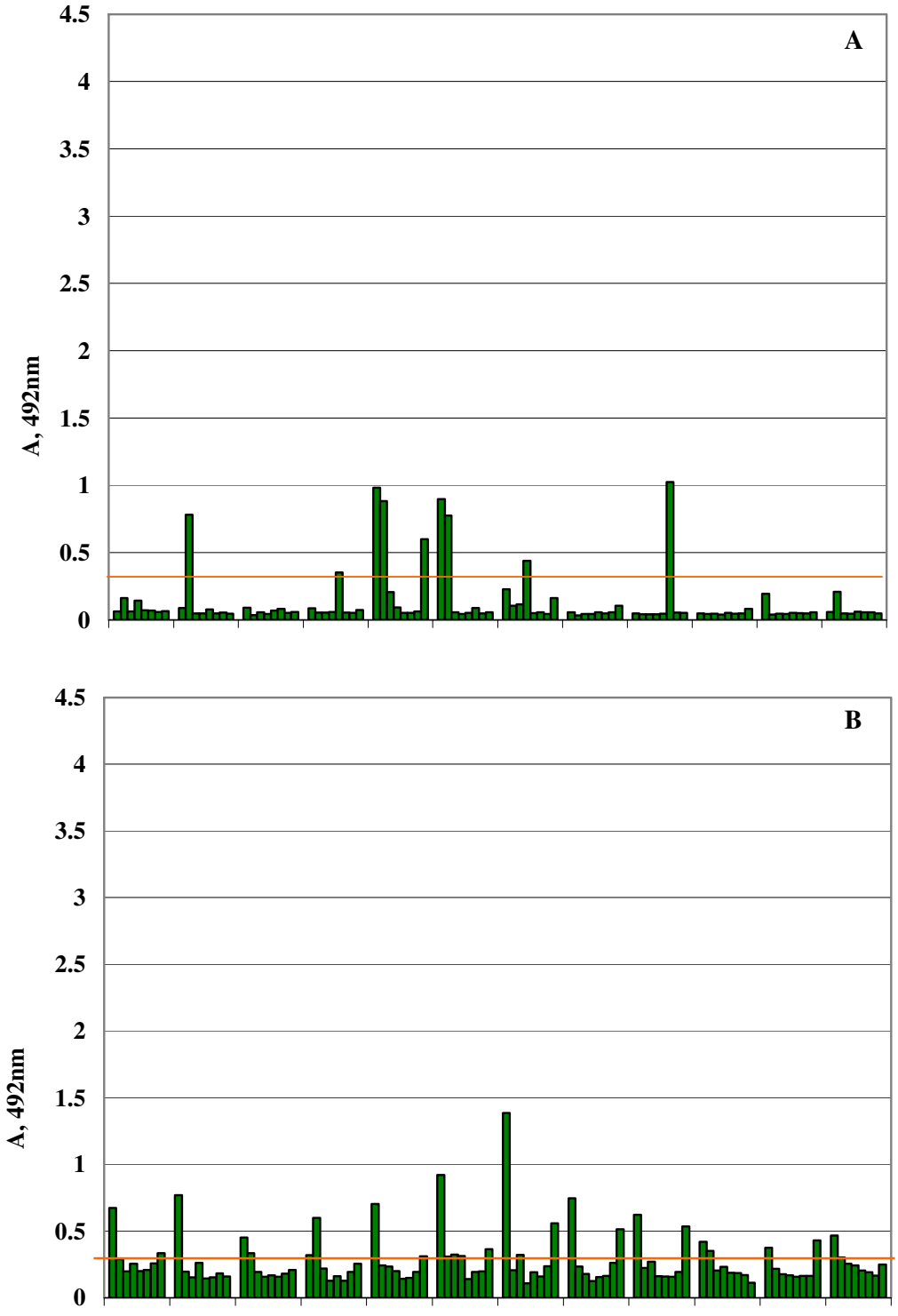


Figure 3.4: ELISA of individual phage displayed clones (A) and of soluble antibody fragments (B) corresponding to those shown in Figure 3.2 tested against MP as a control instead of VP7st4. The orange line represent a cut-off, clones having ELISA absorbance above this line were regarded as having high background and were not sequenced and characterised further.

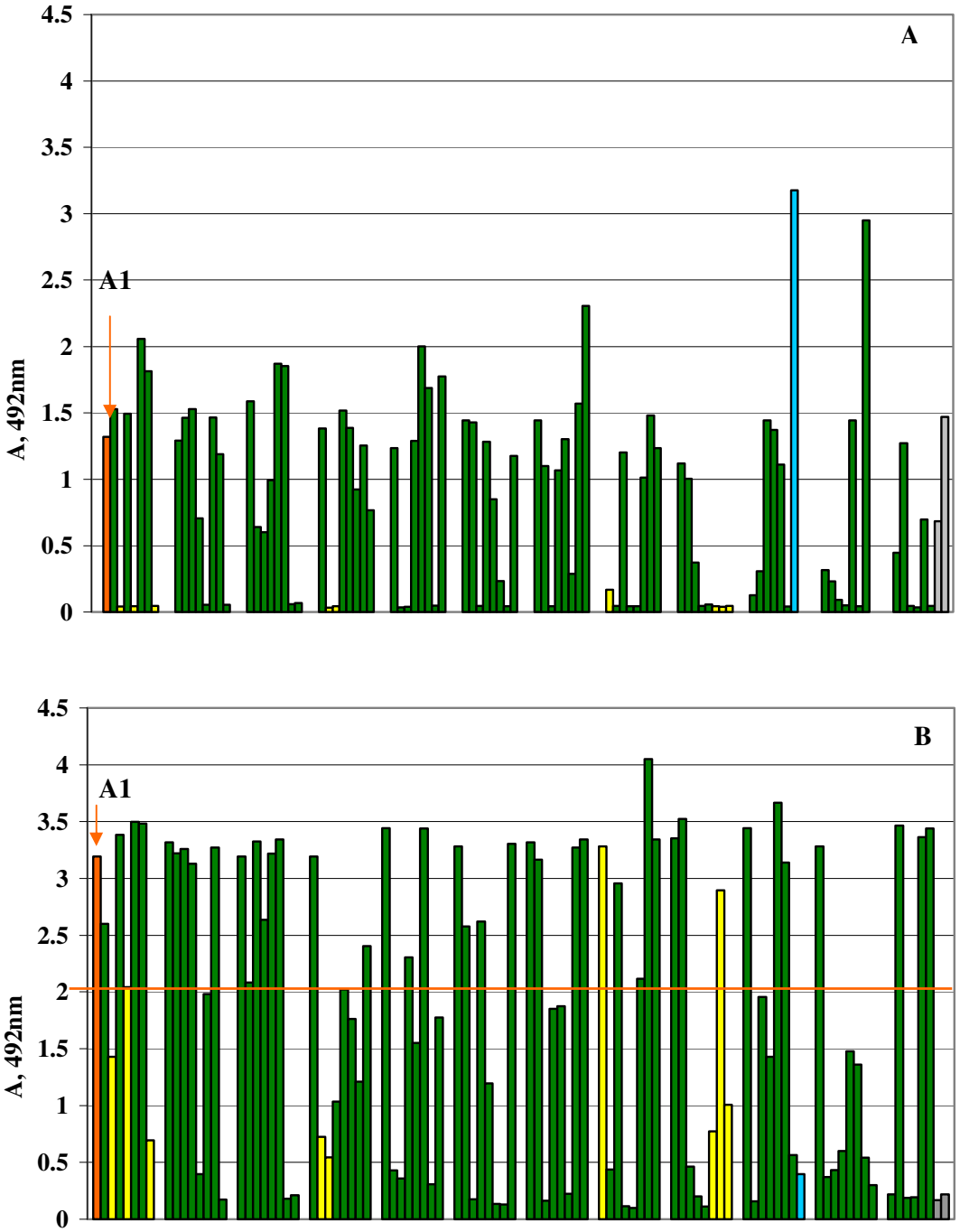


Figure 3.5: ELISA of individual phage displayed clones (A) and of soluble antibody fragments (B) against VP7st4 from selection round four. Each bar represents individually clones randomly chosen from titre plates. Grey bars represent an ELISA signal resulting from an anti-KLH binder, a positive control. Sky blue bars represent clones which were positive in a phage format but not as soluble fragments and vice versa (yellow bars). Clones having an ELISA absorbance of above 2 (orange line represent a cut-off) were regarded as VP7st4-specific and were sequenced or characterised further.

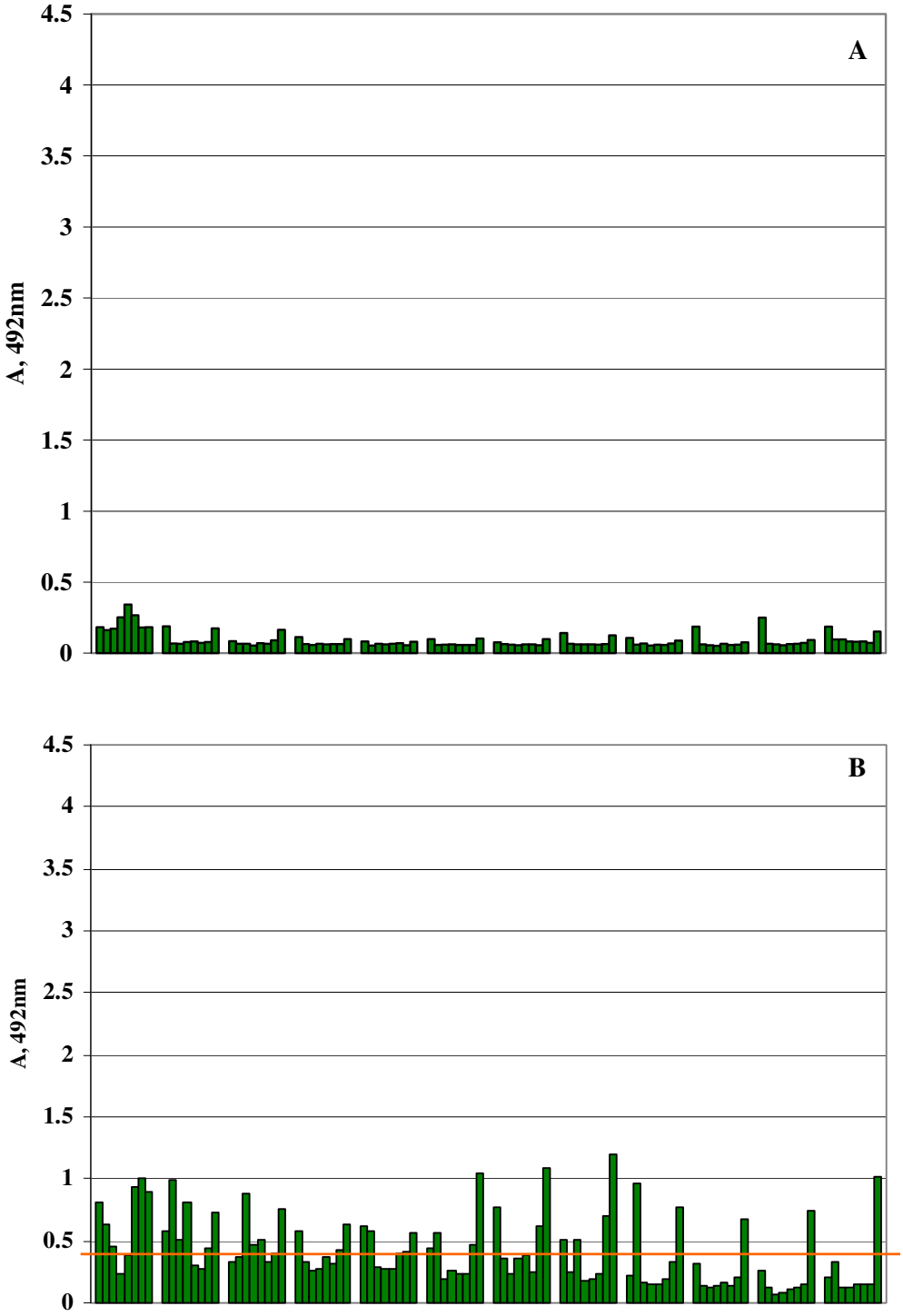


Figure 3.6: ELISA of individual phage displayed clones (A) and of soluble antibody fragments (B) from selection round four corresponding to those shown in Figure 3.5 tested against *E. coli* Origami™ proteins as a control instead of recombinant VP7st4. The orange line represent a cut-off, clones having ELISA absorbance above this line were regarded as *E. coli* binders and were not sequenced or characterised further.

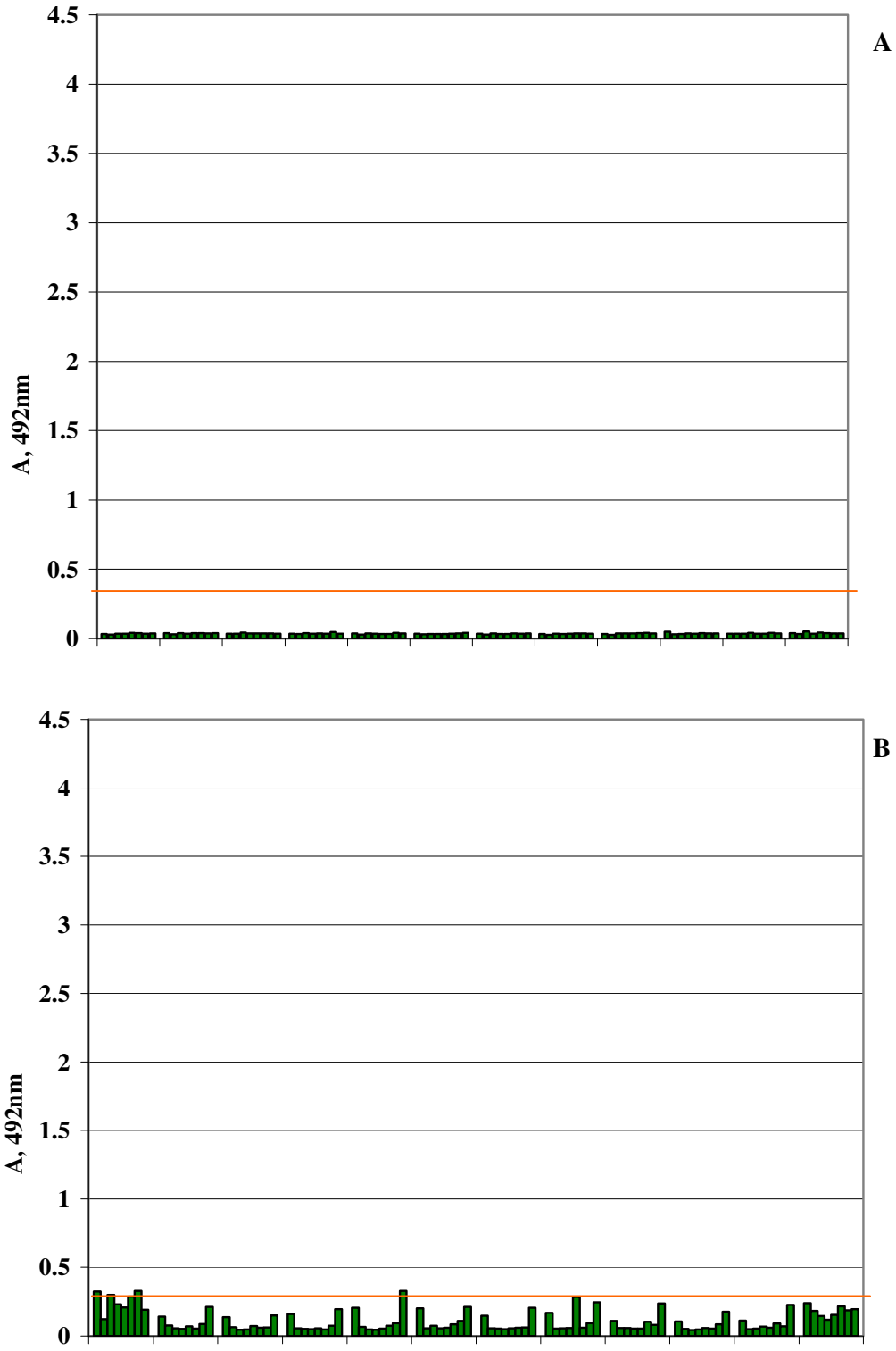


Figure 3.7: ELISA of individual phage displayed clones (A) and of soluble antibody fragments (B) from selection round three corresponding to those shown in Figure 3.5 tested against MP as a control instead of recombinant VP7st4. The orange line represent a cut-off, clones having ELISA absorbance above this line were regarded as having high background and were not sequenced and characterised further.

3.3.2. Sequences of different single clones

The DNA inserts of the 20 positive clones from selection rounds three and four that reacted as soluble fragments in ELISA (Figure 3.2B and 3.5B) were sequenced. Clone H2 from selection round three was found to be unique. The rest of the clones had the same sequence and clone A1 from selection round four was chosen as a representative clone (Figure 3.8). The sequence of the scFv F10 which was obtained by panning the *Nkuku*® library against BTV virus particles was included for comparison (Van Wyngaardt *et al*., 2004). Sequence alignment of the scFvs F10, H2 and A1 revealed that they differed with the major differences being found in their CDRs.

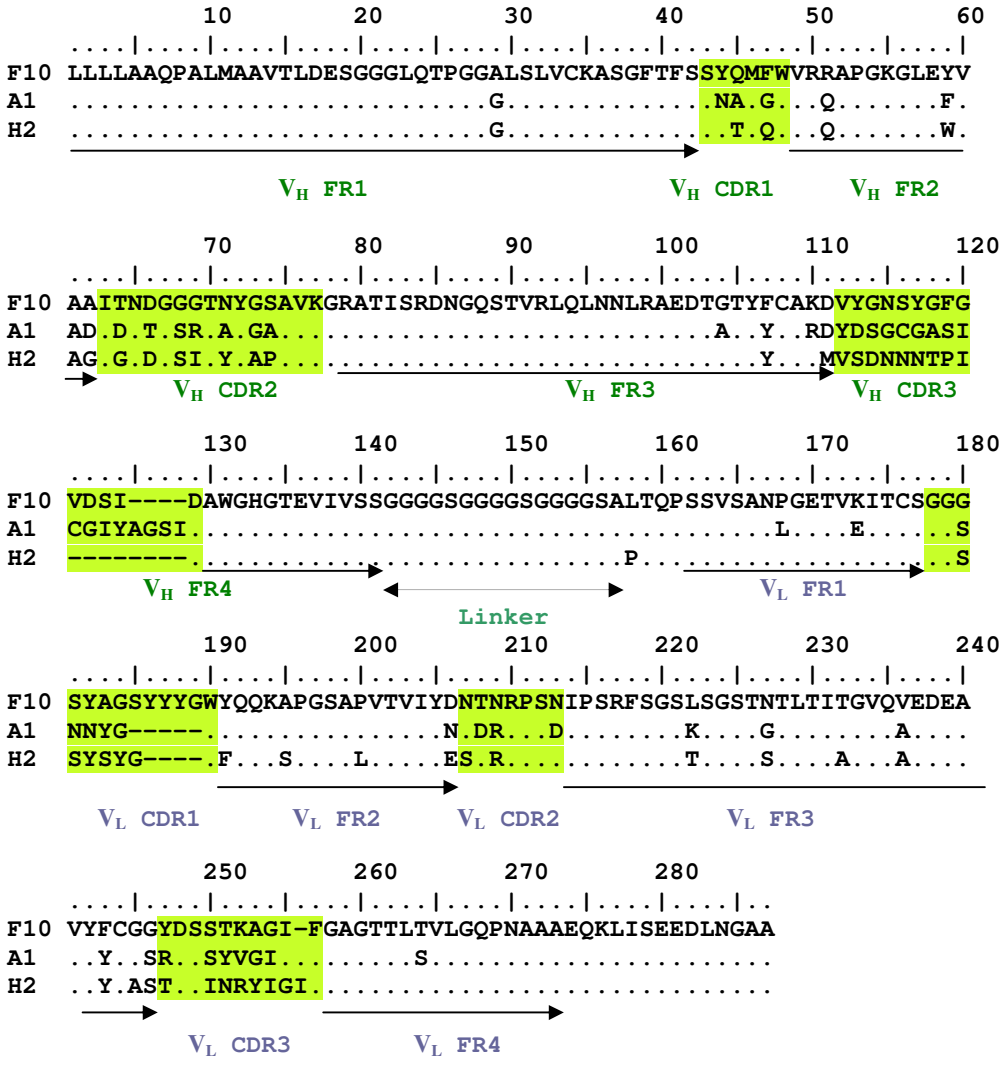


Figure 3.8: Deduced protein sequence of scFvs F10, H2 and A1. "." represents identical amino acids and "-" represents gaps in the alignment. The scFvs consists of the variable heavy (V_H) and light (V_L) chains each containing framework regions 1 to 4 (FR1 to FR4) and complimentary determining regions 1 to 3 (CDR1 to CDR3) as indicated. CDRs of the heavy and the light chains are shaded in green. The V_H and V_L are joined by a 15 amino acid linker $(Gly_4Ser)_3$.

3.3.3. Inhibition ELISA with immune serum

VP7st4-specific scFvs were obtained by selection against recombinant VP7st4 directly adsorbed on Polysorp ELISA plate. One aim of the study was to determine if the binding of these scFvs can be inhibited by anti-BTV immune sera. A homologous BTV serotype 4 guinea pig serum was used. The binding of scFv H2 to recombinant VP7st4 was inhibited by the anti-BTV serum resulting in a 67% decrease in the ELISA signal compared to the uninhibited signal (Figure 3.9). However, the negative serum also showed a 33% inhibition. Thus the inhibition might not be very specific and the ELISA signal of H2 was weak.

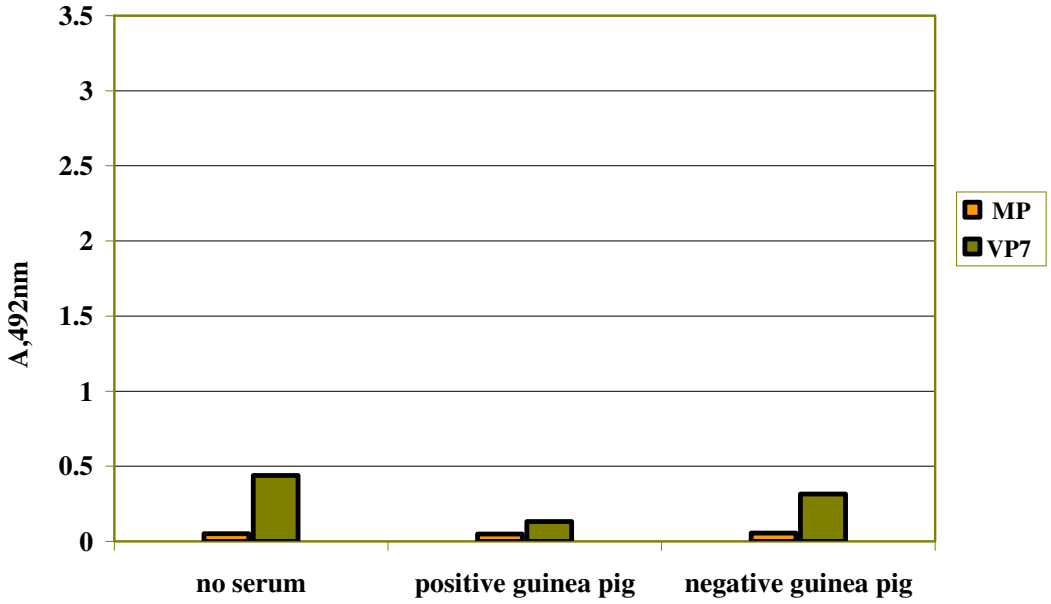


Figure 3.9: Inhibition ELISA of the scFv H2 tested for binding to recombinant VP7st4 with guinea pig serum. VP7st4 (40 µg/ml) and MP were coated directly on Polysorp ELISA wells. Anti-BTV serum was allowed to react with VP7st4 followed by VP7st4-specific scFv H2. Bound scFv was detected using an anti-c-*myc* MAb (clone 9E10).

The positive test serum reduced the signal produced by the scFv A1 by 13% (Figure 3.10). The uninhibited signal was high and the scFv could have been present at saturation levels making it difficult to inhibit the signal. Therefore, a dilution curve was prepared to determine a concentration of A1 that might result in improved inhibition. From the dilution curve the 1:3 dilution was chosen since this concentration of the scFv was on the downward slope but the signal was still high enough to be reduced (Figure 3.11A, arrow). The inhibition increased to 30% under these conditions (Figure 3.11B). Since the scFv H2

gave a weak signal and A1 was only reduced by 30% they were not ideal for an inhibition ELISA. Different panning strategies were therefore followed to select more scFvs to test.

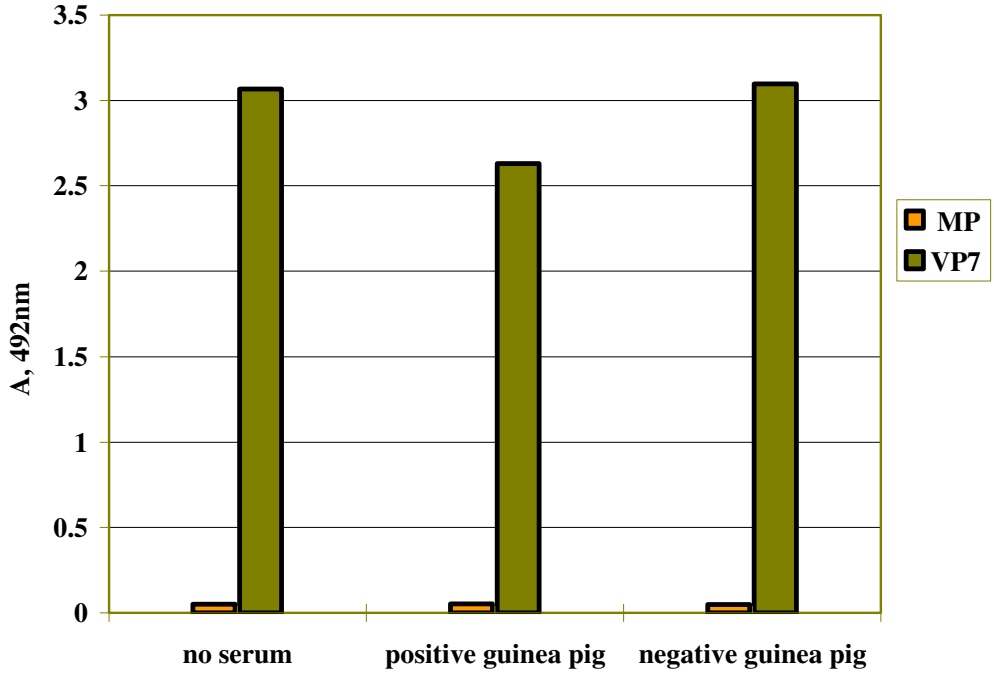


Figure 3.10: Inhibition ELISA of scFv A1 tested for binding to recombinant VP7st4 using guinea pig serum. VP7st4 (40 µg/ml) and MP were coated directly on Polysorp ELISA wells. Anti-BTV serum was allowed to react with VP7st4 followed by VP7st4-specific scFv H2. Bound scFv was detected using an anti-c-*myc* MAb (clone 9E10).

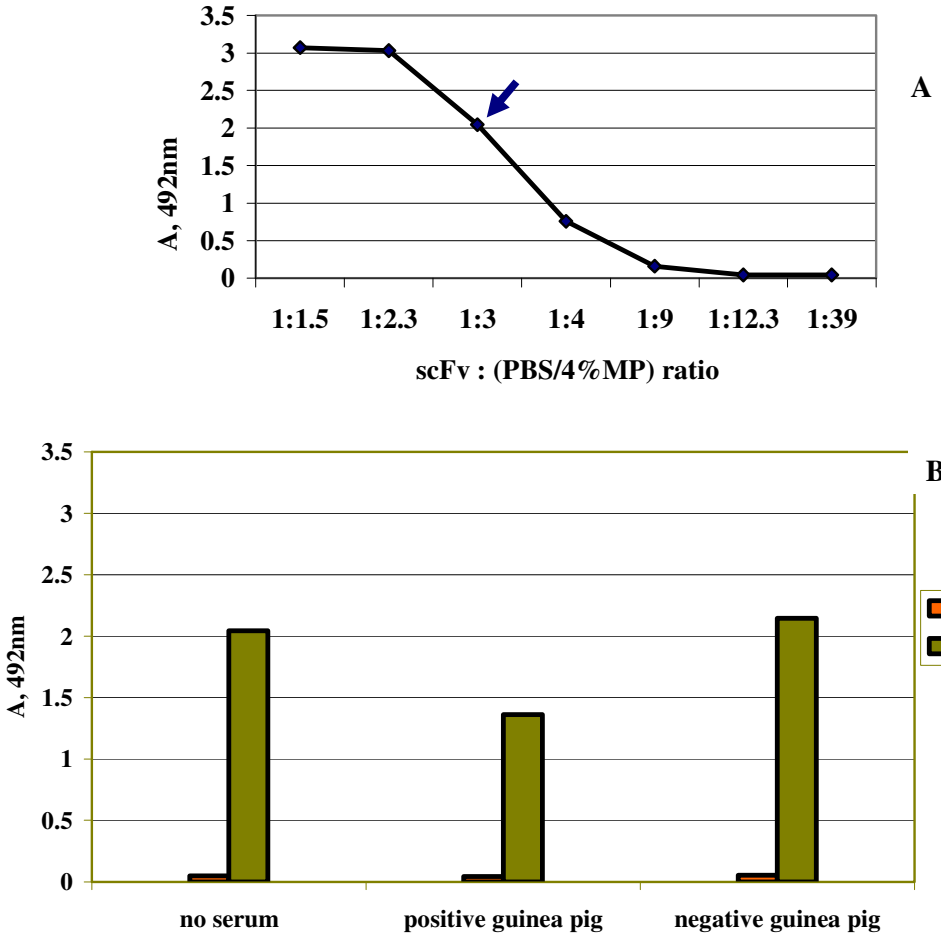


Figure 3.11: A dilution curve (A) and an inhibition ELISA (B) of the scFv A1 tested for binding to recombinant VP7st4 directly adsorbed in Polysorp ELISA wells. ScFv A1 was diluted in MP/PBS and a dilution of 1:3 was used.

3.3.4. Selection of scFvs against VP7st4 following different strategies

Previously, the *Nkuku*® library was panned against BTV serotype 10 virus particles (Van Wyngaardt *et al*., 2004). Initially, selection round two and four outputs of this panning were screened against recombinant VP7st4. These were focused sub-libraries since the previous panning was against BTV whole virus particles. In theory, the antibodies against VP7 would be against the natural epitopes. Following four rounds of selection, no enrichment of clones specific for VP7st4 was obtained and this strategy was discontinued.

Another strategy was then followed. Panning was done in His-Grab™ nickel coated wells instead of the Polysorp ELISA plates. The recombinant VP7st4 had a poly-histidine tag and could be trapped via the tag in these wells. During panning, a mixture of anti-BTV guinea pig and rabbit serum was added after the phages were bound to VP7st4. The

antiserum was allowed to compete for binding to VP7st4 with the phage-displayed scFvs thus releasing the phages. This step was followed by a normal elution with TEA to release the remaining bound antibodies. Phages were then rescued and re-amplified for further rounds of selection. Four consecutive rounds of panning were performed. The pools of phages were tested in a polyclonal ELISA and enrichment was observed after rounds three and four (Figure 3.12). Single clones selected from selection round four of both elution strategies were analysed in the phage and soluble scFv formats (Figure 3.13 and 3.14). Clones released with antiserum produced low ELISA signals. Several clones from TEA elution reacted weakly with recombinant VP7st4 in the phage displayed scFv format with low ELISA signals (Figure 3.14A). Most of these clones reacted strongly with VP7st4 as soluble scFvs (Figure 3.14B). No MP binders were isolated (not shown).

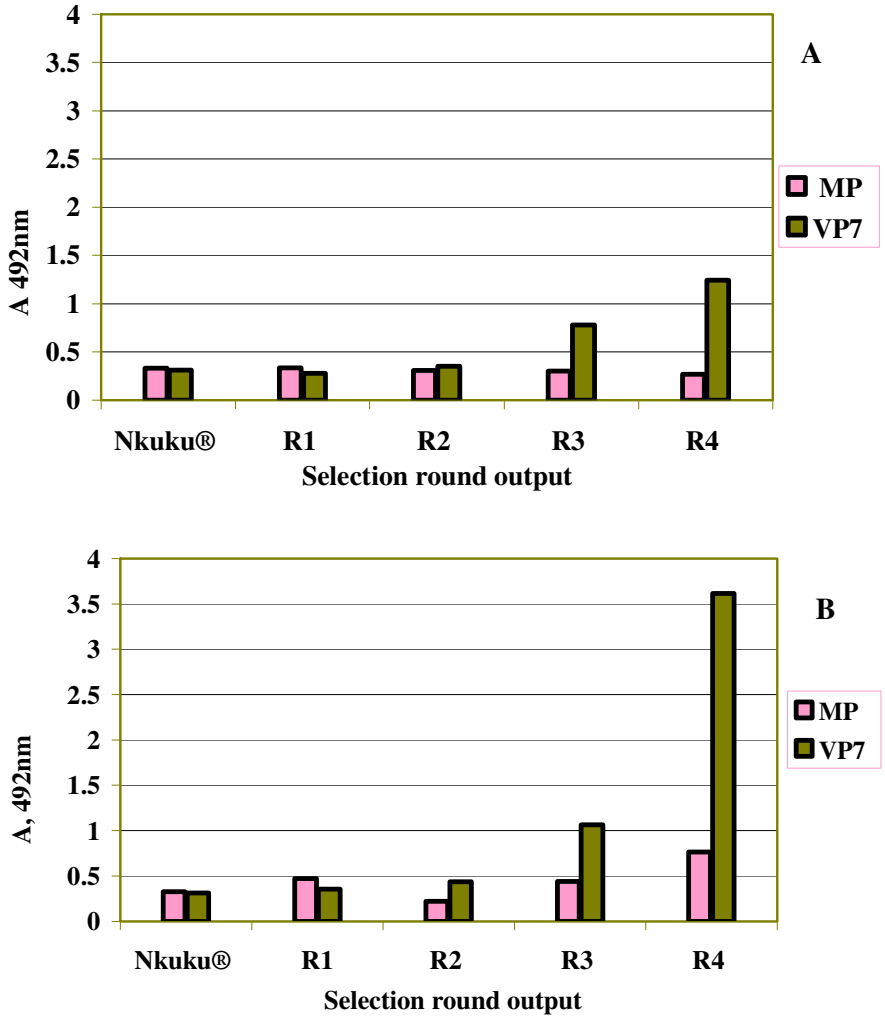


Figure 3.12: Polyclonal ELISA results of phage pools obtained after each round of panning reacting with VP7st4 after competition with anti-BTV guinea pig and rabbit serum (A) and after elution with TEA (B).

Four clones obtained from release with anti-BTV serum were sequenced. Three had the same sequence (designated SA12) and one had no insert (Figure 3.15). Nineteen clones obtained from TEA-elution were also sequenced and three unique sequences were found. Six had the same sequence, designated TC9. Seven more had the same sequence (designated TD12) and one was unique. This one was designated TA8. The remaining two had no inserts. Therefore, in addition to scFvs A1 and H2, four more anti-VP7 scFvs were obtained. The deduced amino acid sequences of the new clones differed from each other and also from the earlier A1, H2 as well as the original F10.

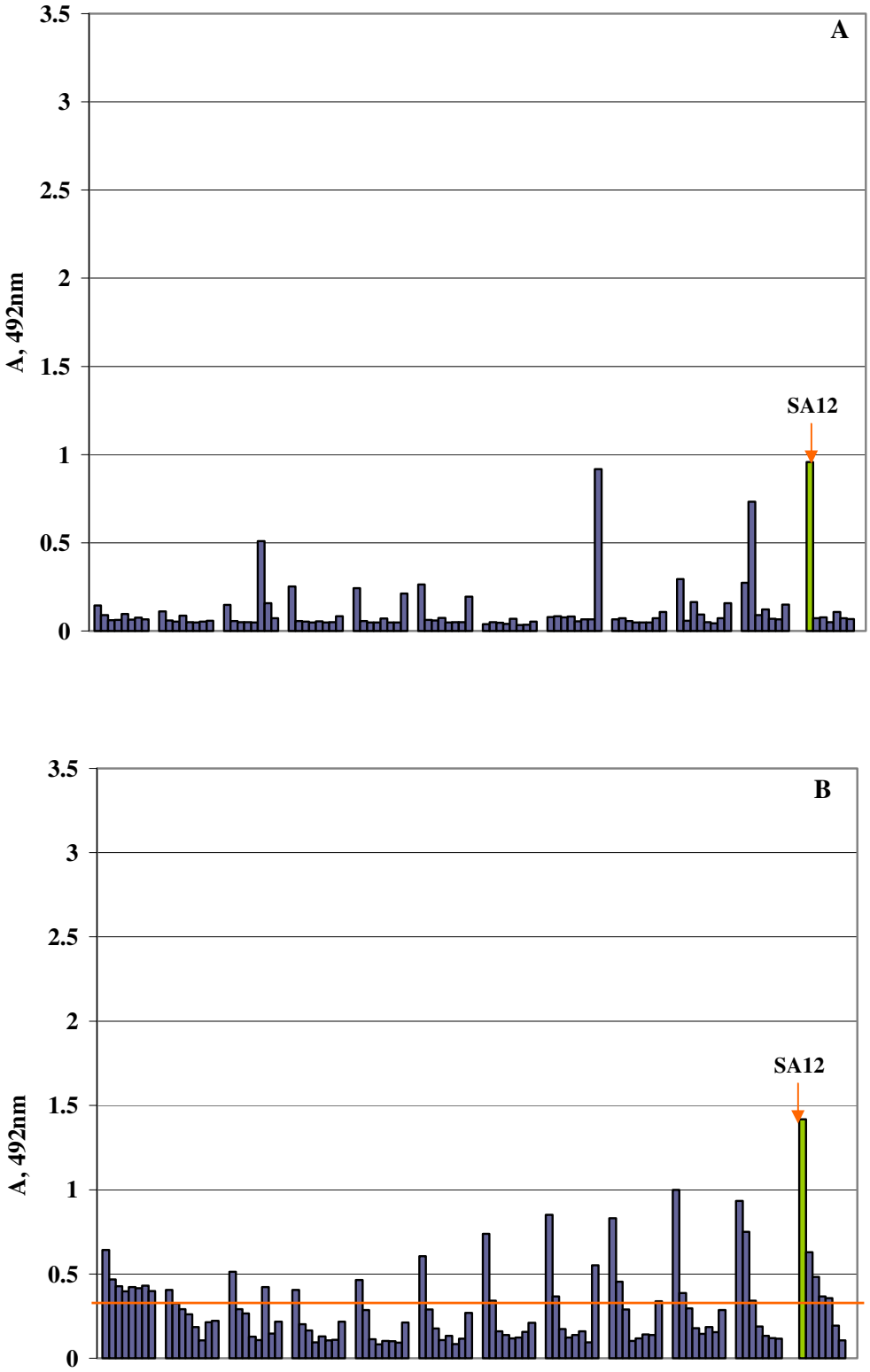


Figure 3.13: ELISA of individual phage displayed clones (A) and soluble antibody fragments (B) against VP7st4 from selection round four after the phages were allowed to compete for binding with a mixture of an anti-BTV rabbit and guinea pig serum. Clones having an ELISA absorbance of above 0.4 were sequenced.

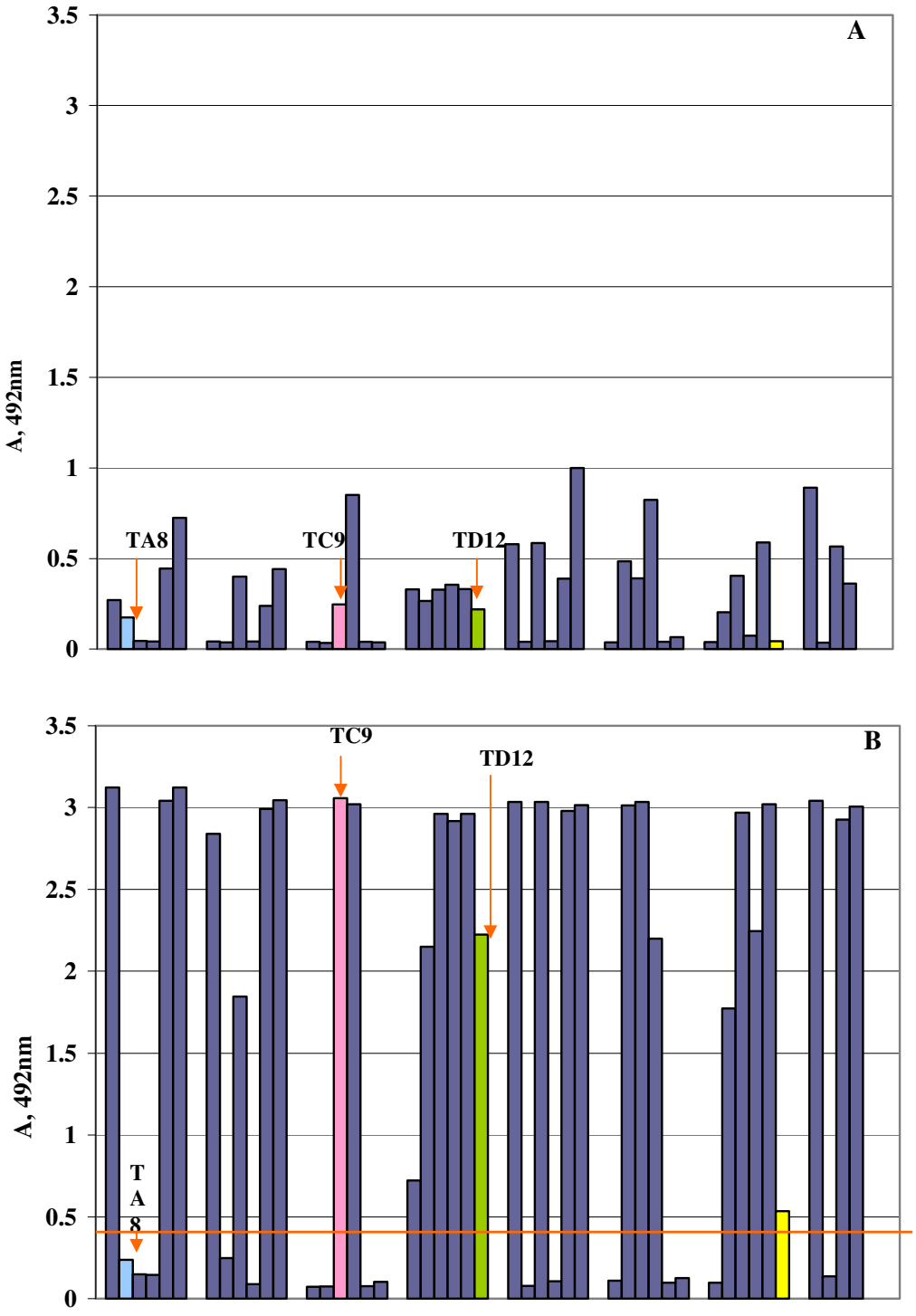


Figure 3.14: ELISA of individual phage displayed clones (A) and soluble antibody fragments (B) against VP7st4 from selection round four after TEA elution of selection round four. Only 48 clones were tested and most of the clones having an ELISA absorbance of above 0.4 and only two clones of the weak binders were sequenced. Yellow bar represent a clone which was negative in a phage format but positive as a soluble scFv.

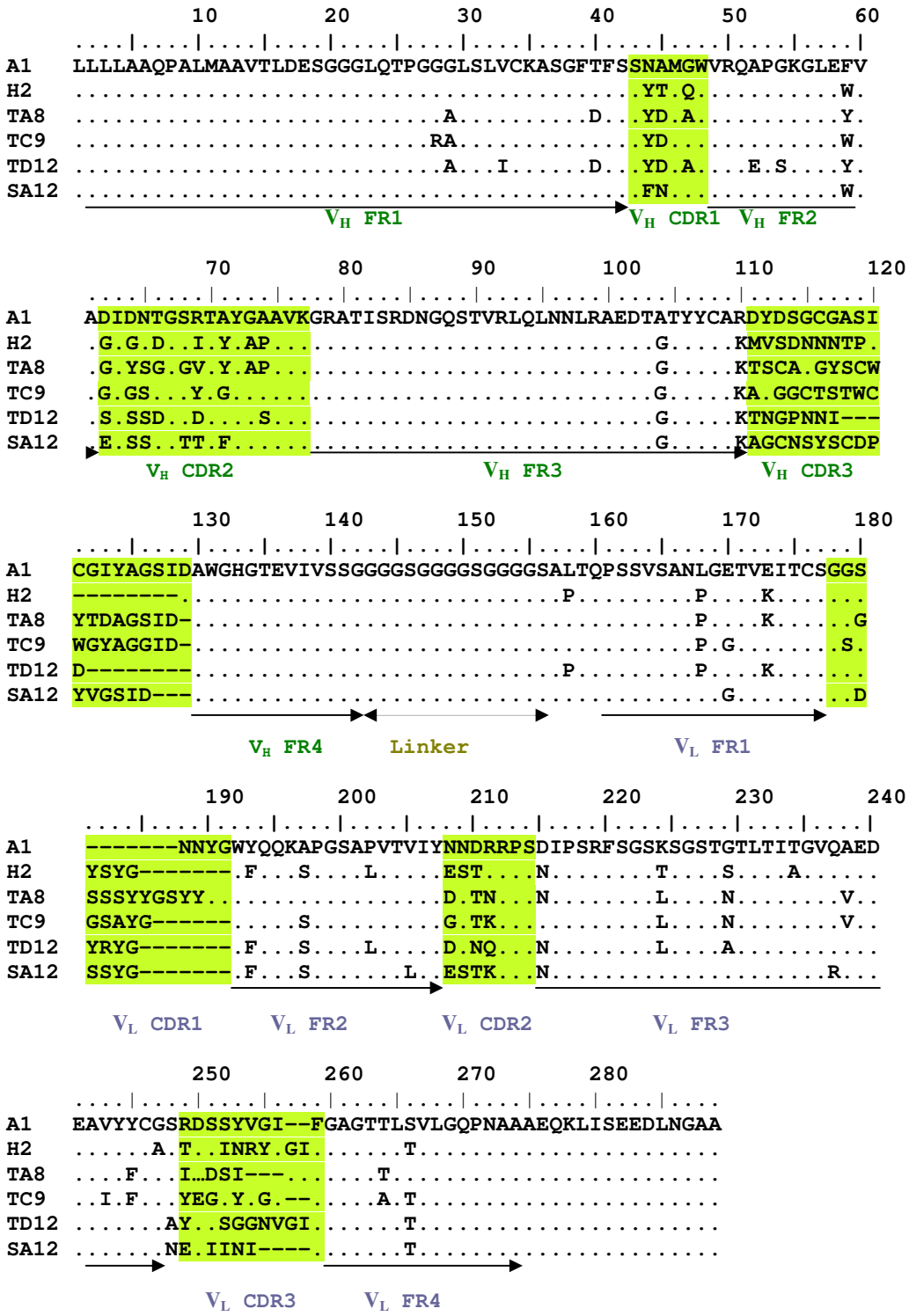


Figure 3.15: Deduced amino acid sequences of scFvs A1, H2, TA8, TC9 and TD12 from TEA eluted phages and SA12 from phages which were obtained after competition with anti-BTV serum. "." represents identical amino acids and "-" represents gaps in the alignment. The framework regions 1 to 4 (FR1 to FR4) and the complimentary determining regions 1 to 3 (CDR1 to CDR3) are indicated. CDRs of the heavy and the light chains are shaded in green. The V_H and V_L are joined by a 15 amino acid linker $(Gly_4Ser)_3$.

The recombinant VP7st4 had been expressed as a fusion protein with thioredoxin to facilitate soluble expression. To make sure that the scFvs recognised VP7st4, and not the thioredoxin, they were tested for binding to the 109 residue long fusion partner alone. MP was included as a negative control (Figure 3.16). TA8 was omitted since its signal in ELISA was too low (not shown). None of the clones reacted with thioredoxin alone.

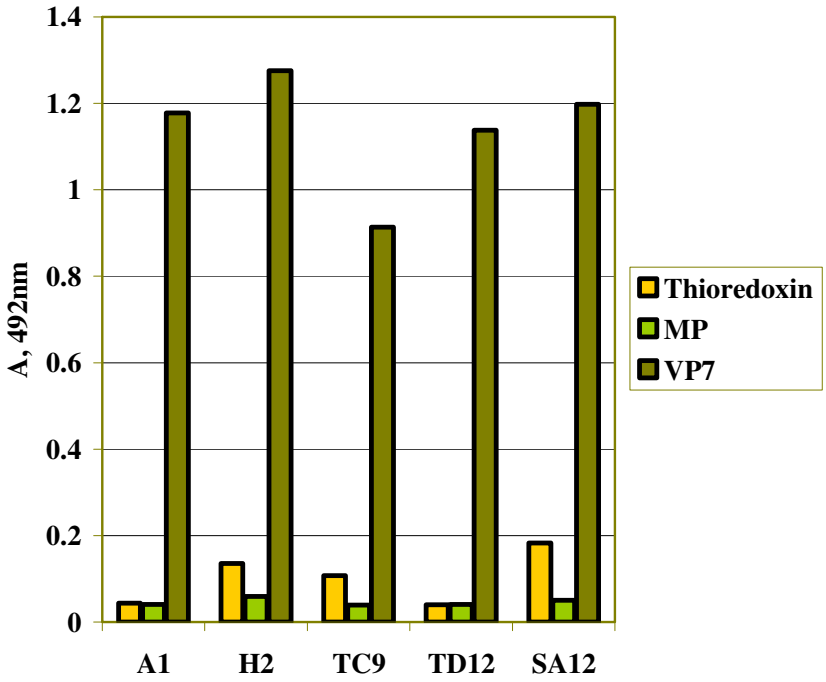
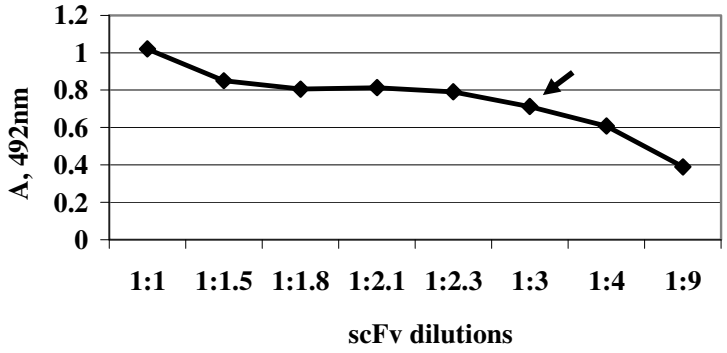


Figure 3.16: ELISA to test if affinity purified scFvs react with recombinant VP7st4 and thioredoxin. MP was used as a negative control instead of VP7st4 and thioredoxin.

Since all the scFvs were specific for VP7st4, the newly selected scFvs were also tested in an inhibition ELISA. Dilution curves were used to determine the concentration at which the scFvs were not present at saturated levels (Figure 3.17). Anti-BTV guinea pig, rabbit and sheep sera were tested for their ability to inhibit the binding of the scFvs. The inhibition ELISAs were performed at the optimal dilutions for each scFv. ScFvs TC9, TD12 and SA12 were used at dilutions 1:64, 1:16 and 1:9 respectively. With the guinea pig serum, 24% inhibition was obtained for TD12, 9% for SA12 and no inhibition was obtained for scFv TC9 (Figure 3.18). No inhibition was obtained with the rabbit and sheep sera.



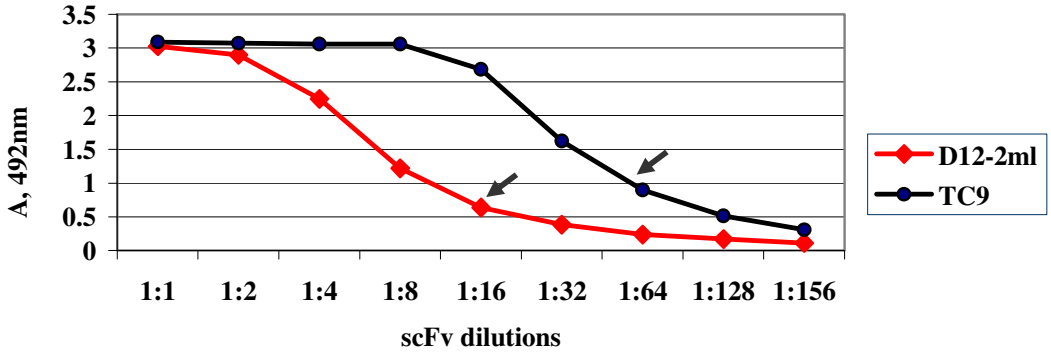


Figure 3.17: Dilution curves of scFvs SA12 (A), TC9 and TD12 (B). ScFvs were diluted in MP/0.1%T and tested for binding to recombinant VP7st4 directly adsorbed in Polysorp ELISA wells. An anti-c-*myc* mouse MAb (clone 9E10) was used for detection of bound scFvs.

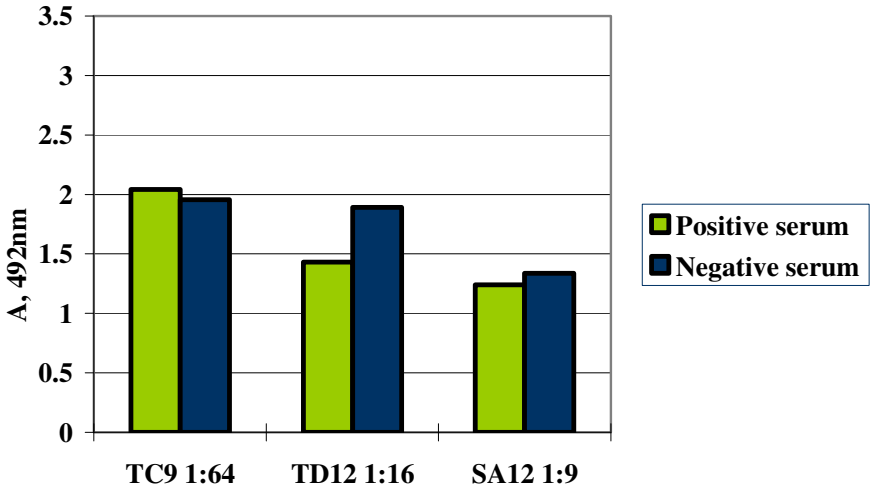


Figure 3.18: ScFvs TC9, TD12 and SA12 tested for binding to recombinant VP7st4 in an inhibition ELISA using a positive and negative guinea pig anti-BTV serum. Dilutions used are indicated after scFv name.

Polyclonal anti-BTV chicken IgY was also tested for its ability to inhibit the binding of scFvs to recombinant VP7st4. The scFvs used in this experiment were isolated from the periplasmic fraction and were thus highly concentrated compared to those in the

supernatant. Optimum dilutions for each scFv first had to be determined (Figure 3.19A). The anti-BTV chicken IgY inhibited the binding of the scFvs from 30% (TC9) up to 87% (SA12; Figure 3.19B). Thus there were more antibodies in the chicken IgY against VP7st4 epitopes similar to those recognised by the scFvs.

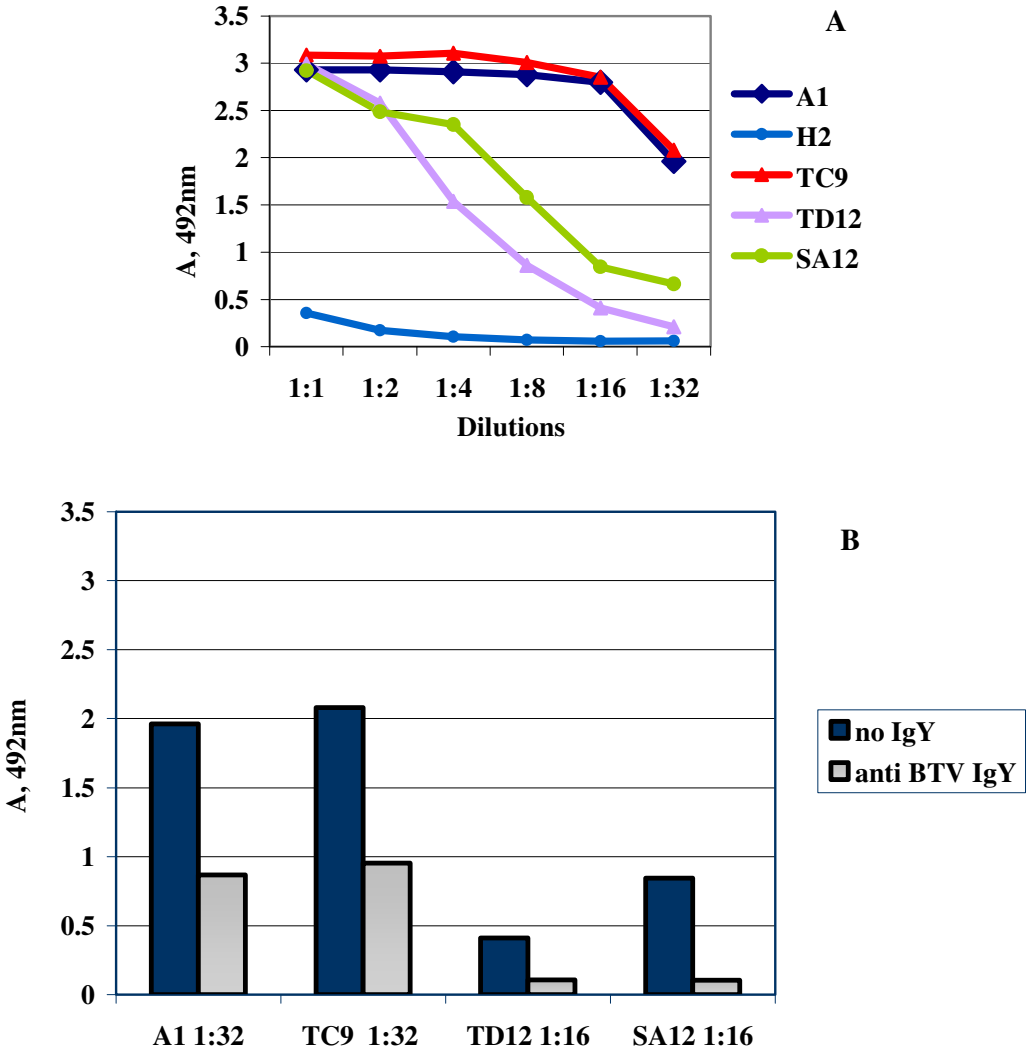


Figure 3.19: Dilution curves of the scFvs (A) and an inhibition test (B) of scFvs A1, TC9, TD12 and SA12 tested for binding to recombinant VP7st4 directly adsorbed in Polysorp ELISA wells. An anti-c-*myc* mouse MAb (clone 9E10) was used for detection of bound scFvs. Dilutions of the scFvs were in MP/PBS and are indicated after scFv name.

3.3.7. Stability of the scFvs

Reagents for use in diagnostics need to be stable. Therefore, all the scFvs selected were stored under different conditions before they were tested for binding in an ELISA (Figure 3.20).

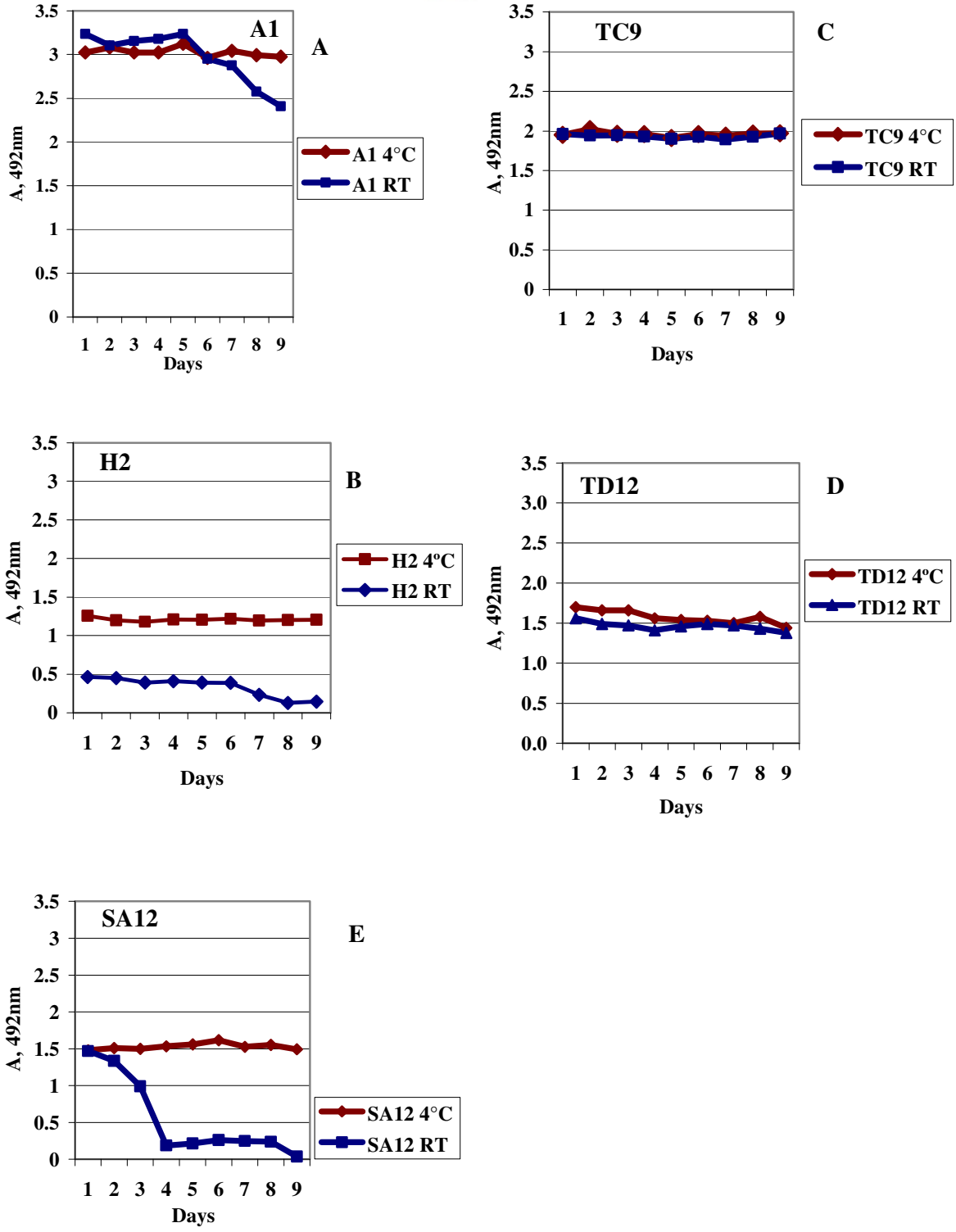


Figure 3.20: ELISA results of scFvs A1 (A), H2 (B), TC9 (C), TD12 (D) and SA12 (E). Samples were stored at 4˚C and RT from which aliquots were taken daily and frozen at -20˚C. After the final aliquots were taken and frozen, all samples were thawed and tested for binding to recombinant VP7st4 in ELISA.

ScFv TC9 retained its binding ability and was stable when stored at 4°C and RT (Figure 3.20C). TD12 was stable at 4°C while its ELISA signal decreased slightly when stored at RT (Figure 3.20D). A1 and SA12 retained their binding ability after storage at 4°C. At RT, the ELISA signal of A1 started decreasing after the sixth day (Figure 3.20A) while that of SA12 decreased after day two (Figure 3.20E). Some samples of the weak binders (e.g. SA12) had bacterial growth which could conceivably result in proteolysis of the antibodies causing a decrease in their ELISA signals (SA12 RT). The scFv H2, also a weak binder, retained its binding ability when stored at 4°C but not at RT (Figure 3.20B). Samples for each scFv were also stored at -20˚C from which aliquots were taken daily after the samples were thawed and tested for stability after several freeze/thaw cycles. The scFvs A1, H2, TC9, TD12 and SA12 retained their binding ability and stable after nine freeze/thaw cycles (Figure 3.21).

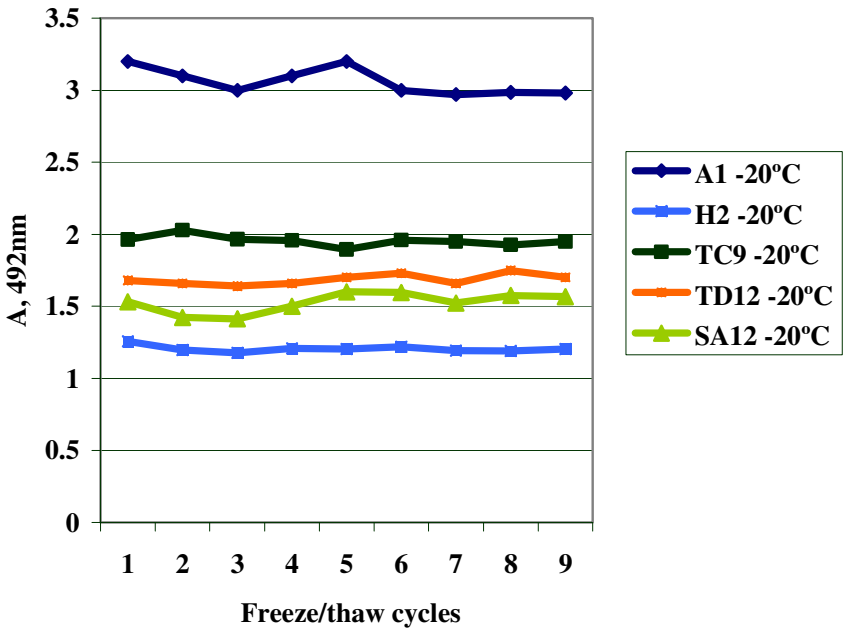


Figure 3.21: ELISA results of scFvs A1, H2, TC9, TD12 and SA12. Samples were stored -20°C and subjected to nine freeze/thaw cycles after which they were tested for binding to recombinant VP7st4.

3.5. DISCUSSION

ScFvs against recombinant VP7st4 were selected from the *Nkuku*® library. In total six new VP7st4-specific binders were obtained by using different selection or panning strategies. ScFvs A1 and H2 were selected from panning against recombinant VP7st4 that

had been directly coated onto Polysorp ELISA plate wells while scFvs SA12, TA8, TC9 and TD12 resulted from panning on VP7st4 captured via its histidine tag in His-Grab™ nickel coated ELISA plate wells. More scFvs were obtained with the second strategy which indicated that the immuno-reactive epitopes of the histidine tag-containing VP7st4 were available when it was trapped. The epitope distortions associated with proteins directly coated on plastic surfaces were thus minimised (Parbosky *et al*., 1996; Qian *et al*., 2000). ScFv SA12 was obtained after bound phages were allowed to compete with hyper-immune serum which was a mixture of an anti-BTV rabbit and guinea pig serum. Clones A1, TC9 and TD12 gave the highest signal in ELISA when tested for binding to the recombinant VP7st4.

The sequencing results showed that the anti-VP7st4 scFvs obtained were different from each other and from the previously selected scFv, F10. They differed from each other in characteristics such as stability under different storage conditions and inhibition of their binding to VP7st4 by anti-BTV serum. Stability is important for diagnostic assay development since robust reagents which can occasionally endure undesired storage conditions are needed. The scFvs were all stable when stored at -20°C, 4°C and after nine freeze/thaw cycles except for H2 which was a weak binder. In most samples which were stored at RT, the scFv integrity was compromised due to bacterial growth.

Although the binding of the scFvs to VP7st4 was inhibited between 50 and 80% by anti-BTV chicken IgY, it is not the target serum for use in developing assays to detect BTV antibodies. No inhibition was obtained with sheep serum that is the target host. Inhibition with guinea pig sera was also not satisfactory. The binding of an antibody A12 to purified BTV-10 particles was inhibited between 44 to 75% using guinea pig serum raised against different BTV serotypes (Van Wyngaardt and Du Plessis, 1998). The low levels of inhibition with guinea pig serum could suggest that VP7st4 was not refolded correctly and therefore the native epitopes were not present. Alternatively, the relevant epitopes could have been folded in such a way that they were not accessible for antibodies in the test serum since these antibodies were generated against VP7st4 in the native form. Even though bound phages were allowed to compete with the antiserum, the level of specific antibodies in the serum could have been low and the competition step did not improve the isolation of readily inhibited scFvs. Therefore, these scFvs may need to be engineered to further improve their binding characteristics and then re-investigated in inhibition assays. The scFv F10 was obtained previously by panning against purified whole BTV virus

particles (Van Wyngaardt *et al*., 2004). In this way, since there are many VP7 molecules on the virus, even when the virus particles coated directly on the plastic surface thereby distorting VP7 to a certain extent, there will still be VP7 molecules not in contact with plastic and hence in the correct conformation. This is an alternative way of obtaining VP7-specific binders that recognise epitopes in the native conformation and are probably more similar to those recognised by the antibodies in the antiserum.

In this study, the scFvs obtained were able to recognise the recombinant VP7st4 directly coated on Polysorp surfaces. This could potentially reduce the steps in the inhibition ELISA. For well characterized diagnostic tests, it is useful to know the region to which antibodies bind. This can be achieved by epitope mapping which will be discussed in Chapter Four.

CHAPTER FOUR

EPITOPE MAPPING

4.1. INTRODUCTION

Epitope mapping refers to the identification of antigenic regions on proteins to which antibodies bind (Wang *et al*., 1995). It plays a key role in vaccine and immunoassay development. There are different ways of mapping epitopes on proteins. For example, overlapping gene-fragments have been cloned into expression vectors resulting in fusion proteins which are then probed with specific MAbs in immunoblot assays (Stanley & Herz, 1987; Böttger *et al*., 1995). Overlapping synthetic peptides have also been used (reviewed by Van Regenmortel, 1989; Böttger *et al*., 1995). Phage display technology offers a cost-effective and time-saving alternative. Epitope libraries consist of many unique peptides displayed on the surface of a filamentous phage. These peptides can be derived from random oligonucleotides (Scott & Smith, 1990; Cwirla *et al*., 1990; Felici *et al*., 1991, Petrenko, 2008) or from target genes (Petersen *et al*., 1995; Wang *et al*., 1995; Fack *et al*., 1997; Blüthner *et al*., 1998; Gupta & Chaudhary, 1999; Bentley *et al*., 2000; Fehrsen *et al*., 2005). The peptides are selected from the library by panning against specific antibodies or other ligands. The binding phages are recovered and their displayed peptides can be identified by sequencing the phage DNA (Parmley & Smith, 1988). Antigenic sites on VP7 of BTV have been mapped using fragmented-gene libraries and specific MAbs (Du Plessis *et al*., 1994; Fehrsen *et al*., 2005).

The aim of this part of the study was to determine the region on VP7st4 to which the newly identified scFvs bind. The strategy is to construct a fragmented-gene library using peptides derived from an authentic VP7st4 sequence. They will be cloned in a phagemid vector resulting in pVIII fusions (Gupta & Chaudhary, 1999). The resulting phage library contains different fragments each representing a part of the gene product. Theoretically, the number of clones required in a library representing a target sequence is calculated using the equation: N=ln(1-P)/ln(1-a/b), where *N* is the number of clones required, *P* is the probability that a target sequence is present (usually taken as 0.99), *a* is the average size of the DNA fragments and *b* is the total size of the gene or genome. One clone in 18 will contain an insert that is cloned in the correct orientation and in the same reading frame as the native protein. Therefore, the size of the library should be 18 times higher than the calculated size (Wang *et al*., 1995; Jacobsson *et al*., 2003). According to this

formula, a library of at least 2.8×10^3 clones is required if the 1700 bp starting gene is fragmented to an average size of 50 bp.

4.2. MATERIALS AND METHODS

4.2.1. Materials

A fragmented-gene phagemid library displaying BTV serotype 1-VP7 peptides fused with pVIII was obtained from Dr. J. Fehrsen, Immunology Section, OVI. The BTV-specific mouse MAb 20F10 was provided by Dr. B. Eaton of the Australian Animal Health Laboratory in Geelong, Victoria, Australia. The phagemid vector CVEPI585042 was obtained from Dr. A. Gupta and Dr. V. K. Chaudhary, Department of Biochemistry, University of Delhi South Campus, India. VP7st4 plasmid DNA was obtained from Dr. C. Potgieter, Virology Section, OVI.

4.2.2. Purification of soluble scFvs

The scFvs were purified using an AminoLink® Plus Immobilization Kit as described by (Roche, Germany) was coupled to the affinity column. Ten ml culture supernatant containing the scFvs was used per purification.

4.2.3. Western blot analysis of purified scFvs reacted with recombinant VP7st4

Western blot analysis was performed as described in Section 2.2.5 except that after recombinant VP7st4 (40 µg/ml) was transferred onto the PVDF membrane; the supernatants containing the scFvs were added and then detected with an anti-c-*myc* MAb (clone 9E10)-HRP diluted 1:1000 in MP/PBS. A substrate solution was added and a photograph of the blot was taken as described in Section 2.2.5. A calibration graph of Log kDa versus Rf values of a known standard (Kaleidoscope Precision) was plotted to enable determination of molecular weights of each scFvs.

4.2.4. Construction of a new fragmented-gene library

4.2.4.1. Amplification of VP7st4 plasmid DNA

The VP7st4 gene was amplified by PCR from a plasmid containing the gene using *Takara Ex Taq* polymerase (Takara, Japan), T7 forward and reverse primers (Invitrogen, USA). The PCR conditions using the *Takara* enzyme were: 94°C for 2 min; 30 cycles of 94°C for 30 s, 43°C for 30 s and 72°C for 30 s; 72°C for 4 min. Following PCR, the amplified product was analysed on a 1% agarose gel by electrophoresis for 1 hr at 100 V and 400 mA.

4.2.4.2. DNase I-fragmentation of VP7st4

A fragmented-gene library was constructed following methods described previously (Du Plessis & Jordaan, 1996; Gupta & Chaudhary, 1999; Fehrsen *et al.*, 2005) with minor modifications. A pilot digestion of DNA fragments was performed by preparing two-fold dilutions from 10 U/ml to 0.039 U/ml of DNase I enzyme in DNase buffer. The dilutions were pre-incubated for 1 h on ice. Simultaneously, 10 µl aliquots of 1.0 µg VP7st4 gene were also pre-incubated in 10×DNase I buffer on ice. The template DNA was digested by adding 3.5 µl of the DNase I dilutions and incubated at 15°C for 10 min. The reaction was stopped with 2 µl of a stop buffer and analysed on a 2.5% agarose gel. Once the digestion conditions were optimised, a dilution which yielded fragments in the range 50-400 bp was used for digesting larger amounts of DNA. The reaction was stopped with a 5 µl of 0.5 M EDTA. The DNA fragments were then extracted with a 50:50 mixture of phenol and chloroform and with chloroform. The DNA was precipitated with 1/10th volume of 3 M sodium acetate and 2 volumes of ice cold ethanol at -70°C for 1 h. The DNA was pelleted at 1320 rpm at 4°C for 30 min, dried and resuspended with 100 µl TE buffer.

4.2.4.3. Blunt-ending and dephosphorylation of the inserts

The DNase-digested fragments were blunt-ended using 5 U of T4 DNA polymerase (Promega)/µg template VP7st4 DNA in the presence of 100 µM dNTPs (Roche) and 10 µl polymerase buffer in a final volume of 100 µl. The reaction was incubated at 37°C for 1 h. Then 2 µl of DNA polymerase I Large (Klenow) fragment (Promega, 9.7 U/µl) was added and incubated for a further 1 h at 37°C. The blunt-ended inserts were dephosphorylated with 5 U antarctic phosphatase enzyme/µg DNA in the presence of the

antarctic phosphatase buffer (New England Biolabs, USA) for 20 min at 37°C and heat inactivated for 20 min at 37°C. The DNA sample was cleaned using Qiaquick Minielute™ Reaction Cleanup Kit (Qiagen). The inserts were ligated into the CVEPI585042 gene VIII phagemid vector.

4.2.4.4. Preparation of gene VIII phagemid vector

The vector was prepared following instructions described in the QIAfilter™ Midi Kit (Qiagen). It was digested with *PmeI* enzyme in the buffer provided with the enzyme (New England Biolabs, USA) at 37°C for 2 h.

4.2.4.5. Ligation of inserts to vector

The digested vector and dephosphorylated blunt-ended inserts were ligated at a ratio of 1:7 (50 ng:350 ng) ON at 16°C in the presence of 1 U of T4 DNA ligase (Roche, Germany), 5 U *PmeI* enzyme, 1× *PmeI* buffer, 100 µg/ml BSA and 100 mM recombinant adenosine 5´-triphosphate (rATP; Promega, USA) in a final volume of 10 µl. A control ligation with no inserts was also prepared.

4.2.4.6. Electroporation of vector with inserts into competent cells

The ON ligation reaction was added to freshly-thawed electro-competent TG1 cells (3 µl ligation reaction per 50 µl cells for 3 reactions; Stratagene, USA), mixed, transferred into a pre-chilled electroporation cuvette and electroporated using a Gene pulser electroporator (Biorad) set at 1700 V, 25 µF and 200 Ω. The cells were quickly removed from the cuvette and inoculated into 960 µl SOC medium that was pre-warmed at 37°C. The cells were incubated at 37°C with shaking for 1 h and plated on TYE/AG plates which were incubated ON at 30°C.

4.2.4.7. Preparation and purification of phages

The cells were scraped off the plates and glycerol stocks were prepared as before. A portion was inoculated into TY/AG medium to an initial OD_{600nm} of 0.05 and grown to a final OD_{600nm} of 0.5. The phages were infected with M13KO7 helper phage, rescued and PEG-precipitated as described previously (Section 3.2.5).

4.2.4.8. Amplification of inserts of clones in the library by Polymerase Chain Reaction

Single colonies were selected from the TYE/AG plates by transferring the cells from each colony into 20 µl deionised water using a pipette tip. The cells were lysed by boiling at 100°C for 5 min and then immediately chilled on ice followed by a 5 min spin. The supernatant containing the DNA (8.5 µl) was mixed with 12.5 µl of 2×Green master mix (Promega, USA), 2 µl of forward, M13F (5´-GTAAAACGACGGCCAGT-3´) and reverse, M13R (5´-CAGGAAACAGCTATGAC-3´) primers. Polymerase chain reaction conditions were as follows: denaturation at 94°C for 2 min, 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and finally, 72°C for 4 min. The PCR product was analysed on a 1.5% agarose gel for 1 h at 100 V and 400 mA. A 100 bp DNA size marker was included.

4.2.4.9. Sequencing insert of individual phage clones represented in the VP7st4-derived library

The PCR positive clones were grown ON at 30°C in 5 ml TY/AG medium. The DNA was extracted using Qiagen® Miniprep Spin Kit as described by the manufacturer (Qiagen). The M13R primer was used for sequencing (Molecular Biology Section, OVI). The sequences obtained were analysed using the BioEdit version 7.0.4 (Hall, 1999).

4.2.5. Panning of the library

The library was first panned against 20 µg/ml MAb 20F10 directly coated on Polysorp immunotubes. The non-specific sites were blocked with MP for 1 hr at 37°C. The wells were washed thrice with PBS/0.05%T and then with PBS. The VP7st4-derived peptide library which was pre-incubated in MP/PBS/0.05%T was added to the wells and incubated for 1 h. The unbound phages were washed away (Section 3.2.6). The phages were eluted with 1 M glycine-HCl pH 2.2 for 10 min at RT and immediately neutralised with 1 M Tris-HCl pH 9. The phages were used to infect TG1 cells at mid-log phase for 30 min at 37°C in a waterbath. The infected cells were concentrated and plated out on TYE/AG plates followed by an ON incubation at 30°C. A glycerol stock of bacterial cells was prepared as before. The phages were rescued and purified as described in Section

3.2.5. Two selection rounds were performed with the MAb. Single colonies were sequenced as described in Section 4.2.4.9. The library was then panned with the scFvs. The affinity-purified scFvs were either directly coated at 40 µg/ml or trapped with 20 µg/ml of an anti-c-*myc* MAb (clone 9E10) on Polysorp ELISA wells or immunotubes.

4.2.6. ELISA to test the reactivity of purified scFvs to recombinant VP7st4 or baculovirus-expressed VP7st1

To test the reactivity of the affinity-purified selected anti-VP7st4 scFvs with recombinant VP7st4, ELISA plate wells were either coated with the scFvs (20 µg/ml) directly on a plastic surface or by trapping them with 20 µg/ml an anti-c-*myc* MAb (clone 9E10). The non-specific sites were blocked with MP at 37°C for 1 h. The wells were washed thrice with PBS/0.05%T and then with PBS. Recombinant VP7st4 (40 µg/ml) diluted in MP/PBS/0.05%T was added to the wells and incubated for 1 hr at 37°C. A HisDetector™ Ni-HRP conjugate (KPL, USA) diluted 1:1000 in 1% BSA was used for detection of the histidine-tagged VP7st4 followed by an OPD substrate solution as described in Section 3.2.7. To test whether the scFv F10 recognise VP7st4 directly coated on ELISA wells, an anti-c-*myc* MAb (clone 9E10) conjugated to HRP was used for detection.

To test the reactivity of the anti-VP7st4 scFvs to baculovirus-expressed VP7 of BTV 1, ELISA plate wells were coated with 10 µg/ml rabbit anti-BTV IgG as a trapping antibody (Van Wyngaardt *et al*., 2004). A baculovirus-expressed VP7 BTV 1 (obtained from Wouter. Van Wyngaardt but was of a limited supply) diluted 1:100 in MP was added to the wells. The anti-VP7st4 scFvs were added followed by detection with MAb 9E10 conjugated to HRP (Roche). The conditions were as described above.

4.3. RESULTS

4.3.1. Affinity purification of soluble scFvs

ScFvs A1, H2, TC9, TD12 and SA12 were each purified using an anti-c-*myc* affinity column. The gel image of fractions collected throughout the purification of the scFv TD12 is shown as an example (Figure 4.1). The scFv is present in the third and fourth eluted fractions (lanes 6 and 7) and the preparation contained predominantly the purified scFv indicated by a band at 31 kDa. There are, however, additional bands which co-purified with the scFv.

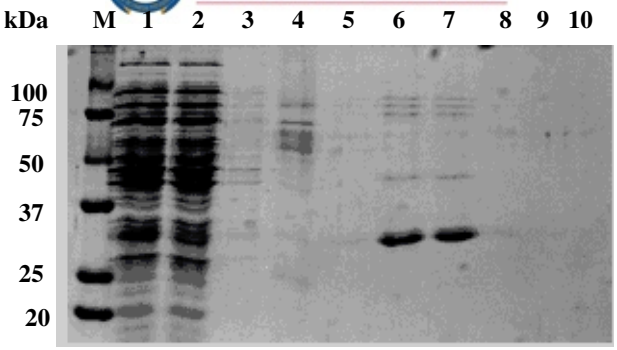


Figure 4.1: Coomassie stained discontinuous 10% SDS-PAGE of scFv TD12 fractions collected during affinity purification. Lane M: Kaleidoscope marker, 1: scFv TD12 after induction with 1 M IPTG, 2: flow-through of the column, 3: wash fraction, 4 to 10: column eluted fraction 1 to 7.

The purified scFvs were tested in an ELISA to identify the fractions which contained the eluted scFvs (Figure 4.2). Most scFvs eluted in fractions two to four (E2, E3 and E4). These fractions were then pooled and dialysed against PBS to remove the elution buffer and rechecked by SDS-PAGE (Figure 4.3).

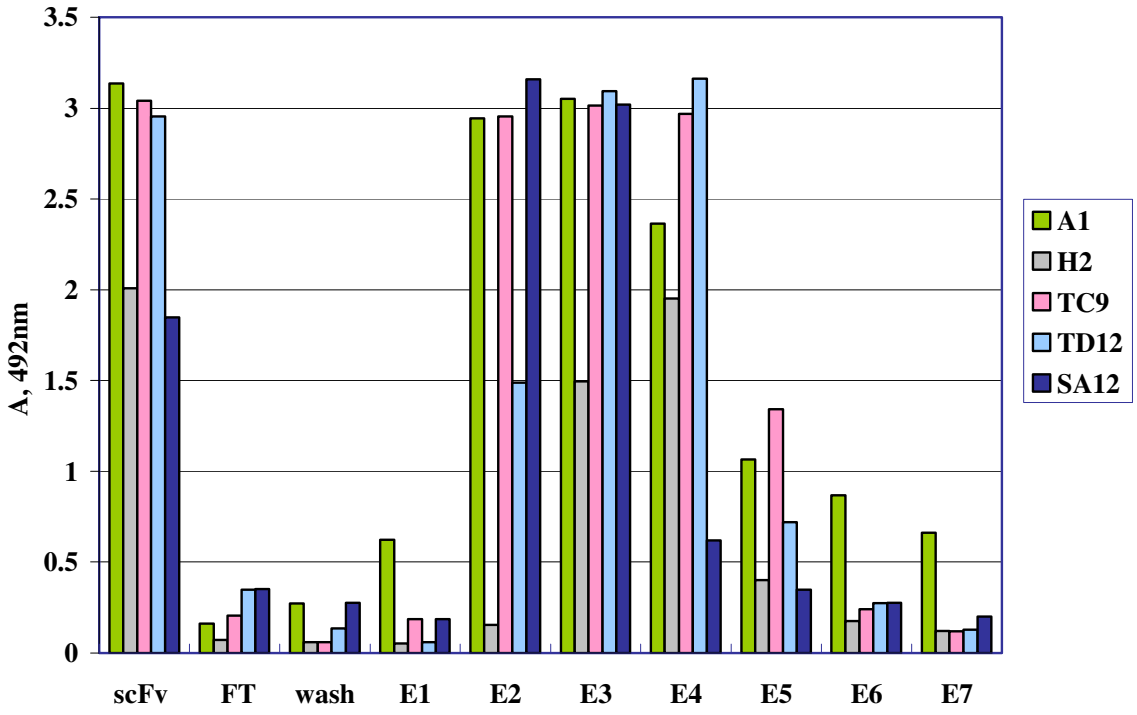


Figure 4.2: ELISA results of scFvs A1, H2, TC9, TD12 and SA12 recognising VP7st4. The culture SN containing unpurified scFvs (scFv), the column flow-through (FT), the wash and the eluted fractions E1 to E8 were tested.

The molecular weight of the scFvs A1, H2, TC9, TD12 and SA12 are shown in Table 4.3. H2 was smaller with a molecular weight of 23 kDa and an additional band which could be a dimer at 48 kDa.

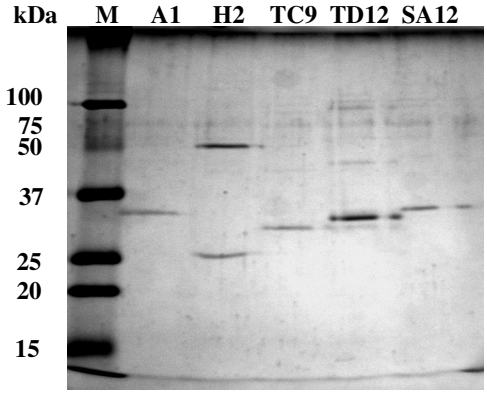


Figure 4.3: Discontinuous 10% SDS-PAGE analysis of the scFvs after dialysis in PBS. Lane M represents a Kaleidoscope Precision marker.

According to the Figure 4.3, the molecular weights of the scFvs were determined as described in Section 4.2.3. The relative mobility (Rf) value is the ratio of the protein migration from the top of its lane and the total migration distance of the tracking dye. A known standard was the Kaleidoscope Precision Marker. The Log Mw values of the standard were plotted against its Rf values. The migrated distances of each scFv as measured from the top of the lanes were also divided by the total migration distance of the dye (60 mm). The Rf values of the scFvs were read from the graph and the corresponding Log Mw values were read and converted to Mw in kDa.

Table 4.1: List the Mw and calculated Log values of the standard, the distance of each protein migrated from the top of its lane as measured on an SDS-PAGE gel and Rf values.

Mw (kDa) of known standard	Log Mw of known standard	Protein migration distance (mm)	Calculated Rf value
20	1.3	49	0.82
25	1.4	44	0.73
37	1.6	33	0.55
50	1.7	26	0.43
75	1.9	19	0.32
100	2.0	14	0.23

Table 4.2: List the distances of each scFv measured from the top of the different lanes, the Rf values, Log Mw values from the graph as well as the calculated Mw in kDa.

ScFv	Protein migration distance (mm)	Rf value	Log Mw read from graph	Mw (kDa)
A1	37	0.62	1.56	36
H2	25	0.42	1.38	24
	44	0.73	1.70	50
TC9	39	0.65	1.49	31
TD12	38	0.63	1.54	35
SA12	36	0.60	1.57	37

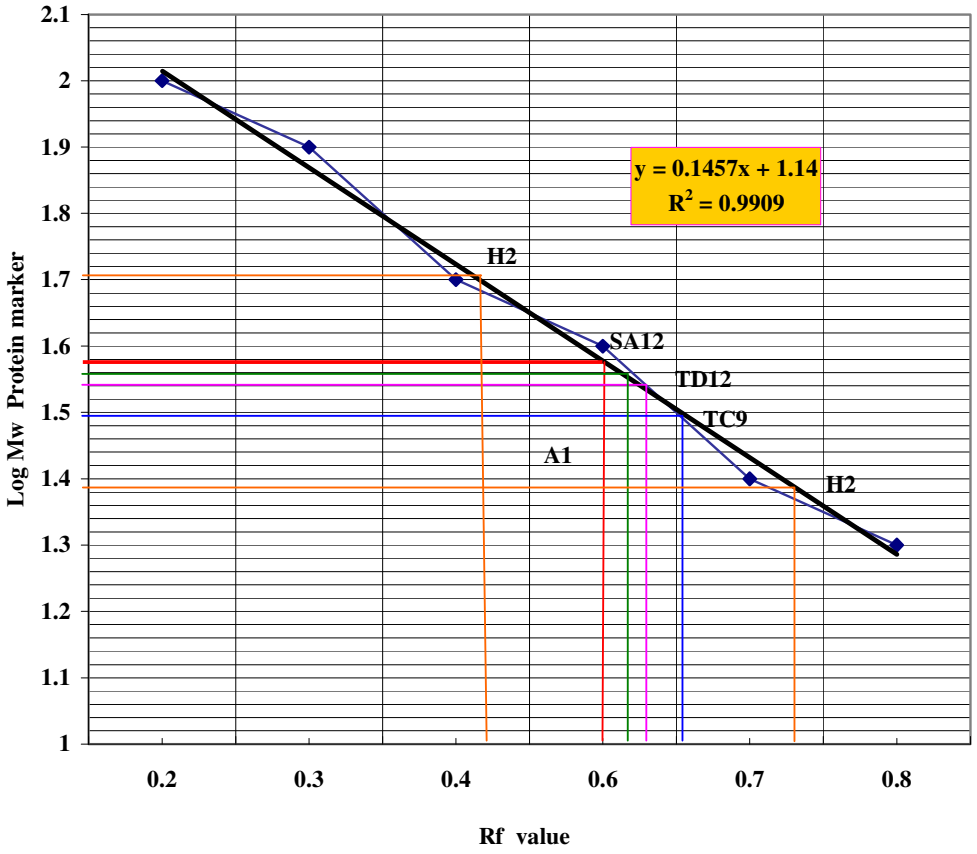


Figure 4.4: The Log Mw values of the known standards are plotted versus relative mobility (Rf) values of each migrated protein band. Known Rf values of scFvs A1, H2, TC9, TD12 and SA12 are read from the x-axis and their corresponding y-axis values representing the Log Mw were used to calculate the Mw of each of the scFvs.

After affinity purification and dialysis, all the scFvs were tested to determine whether they still bound to VP7st4 in an ELISA. They were either directly coated on a plastic surface or trapped with an anti-c-*myc* MAb (clone 9E10; Figure 4.5). The ELISA signals obtained were very low when the scFvs were directly coated except for TD12 which gave an ELISA signal of one (not shown). This is possibly as a result of distortion which can occur when proteins are directly adsorbed on plastic surfaces. The signals were higher when the scFvs were trapped. A1, TC9 and TD12 gave the highest signals. Since the scFvs reacted with the recombinant VP7st4, they were used in panning by trapping with the MAb.

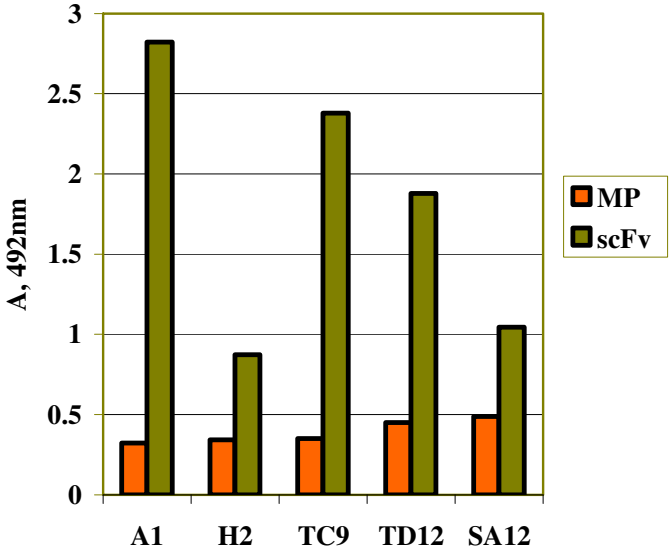


Figure 4.5: ELISA results of purified scFvs reacting with VP7st4. The purified scFvs were trapped with an anti-c-*myc* MAb (clone 9E10). MP was used as a negative control. Recombinant VP7st4 was added to the ELISA plate wells followed by detection with an anti-histidine tag antibody.

The anti-VP7st4 scFvs obtained in this study did not react with a baculovirus-expressed soluble VP7 of BTV 1 (VP7st1) used in the work done by Fehrsen *et al.*, 2005. In addition, the recombinant VP7st4 from *E. coli* that was used in this study was not recognised by the scFv F10 (not shown).

4.3.2. Western blot analysis of purified scFvs reacted with recombinant VP7st4

The scFvs were tested in a western blot to determine whether they reacted with VP7st4 in a denatured form. All scFvs recognised VP7st4 at 50 kDa with the best reaction obtained with TC9 (Figure 4.6, lane 3). The additional bands that the scFvs also recognised could

possibly be degraded VP7st4 or even VP7st4 which was still in the process of being translated (see Figure 2.3).

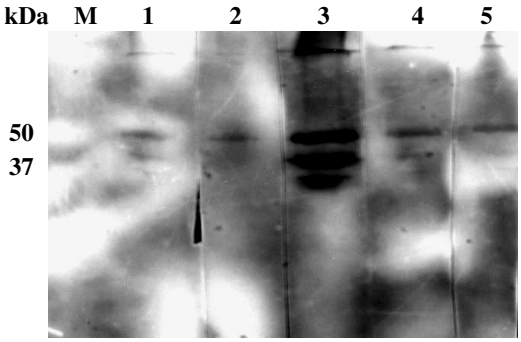


Figure 4.6: Western blot showing binding of scFvs A1, H2, TC9, TD12 and SA12 (lanes 1 to 5) to denatured VP7st4. Lane M: Kaleidoscope precision marker. Bound scFvs were detected with an anti-c-*myc* MAb (clone 9E10)-HRP.

4.3.3. Panning results

Initial screening was done using a VP7st1-derived peptide library that had been constructed previously (Fehrsen *et al.*, 2005). The library was also amplified using different strategies. Panning of the library was done against the anti-VP7st4 scFvs which were adsorbed directly onto polystyrene plates. The positive control did not work and it was decided that a new library be constructed.

4.3.4. Construction of a VP7st4 fragmented-gene library

A fragmented-gene library was constructed by first amplifying the gene from a plasmid clone containing the VP7st4 gene (Figure 4.7, lane 2). An amplified product of 1700 bp was obtained which consisted of 1060 bp VP7st4 gene insert and 640 bp of the vector sequences flanking it.

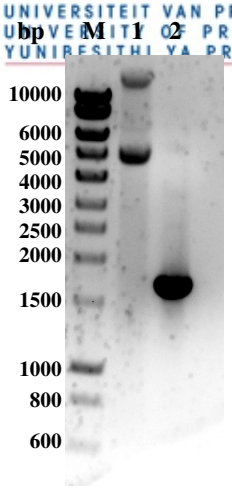


Figure 4.7: A 1% agarose gel showing amplification of VP7st4 gene. Lane M: Hyperladder I DNA size marker; lane 1: VP7st4 plasmid DNA and lane 2: PCR amplified VP7st4.

The amplified DNA was digested with DNase I. A pilot digestion was done using DNase I dilutions from 10 U/ml to 0.625 U/ml. A dilution of 0.3125 U/ml of DNase I enzyme which resulted in the majority of fragments in the range 50-300 bp was then used to scale up by digesting a larger amount of DNA (Figure 4.8, lane 4 and 6).

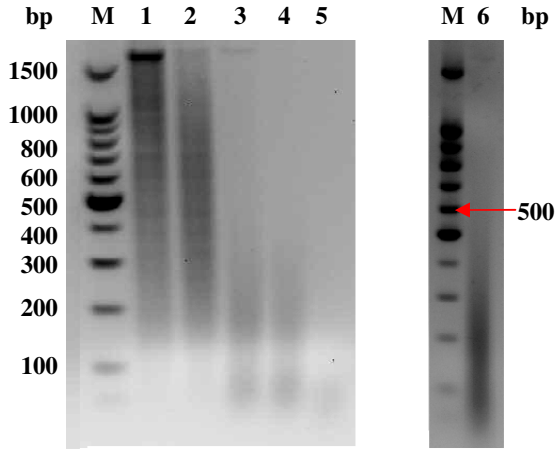


Figure 4.8: DNase I fragmentation of DNA analysed on a 2.5% agarose gel. Lane M: 100 bp DNA ladder; lanes 1 to 5 shows 1.0 µg DNA digested with increasing amounts of DNase I from 0.039 U/ml, 0.078 U/ml, 0.156 U/ml, 0.3125 U/ml to 0.625 U/ml. Lane 6 is the final DNA digested using 0.3125 U/ml DNase I with the majority of fragments in the range 50-300 bp.

The DNA fragments were made blunt-ended prior to ligating into a *PmeI* digested CVEPI585042 gene VIII phagemid vector. The library obtained consisted of 6.5×10^5 clones which is 216 times more than the minimum of 2.8×10^3 required (Section 4.1). The single colonies obtained from titre plates after each selection round were screened by colony PCR. The vector control resulted in amplification of a 400 bp band and clones containing an insert have larger PCR products which migrated more slowly (Figure 4.9).

At least 60% of the clones contained inserts. Therefore, 60% of the library (6.5×10^5) equate to 3.9×10^5 which is still 139 times more than the minimum required.

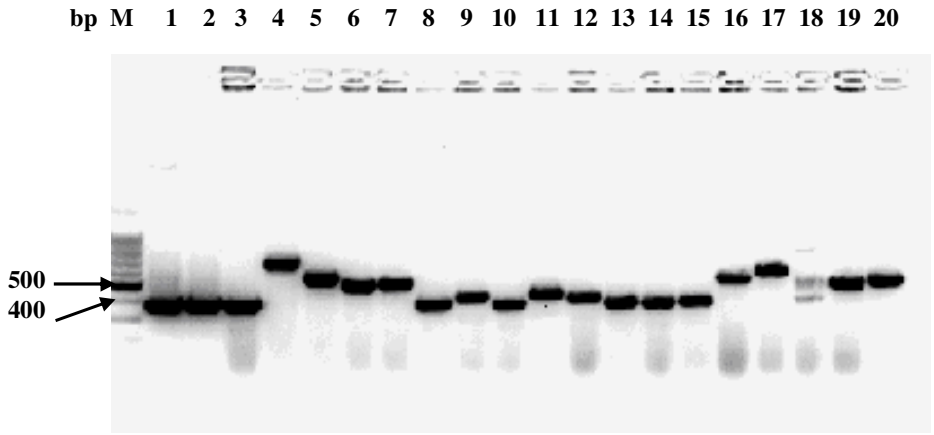


Figure 4.9: Colony PCR results. Lane M: 100 bp DNA ladder, lane 1-2: vector control; lanes 3-20: individual clones randomly picked from titre plates.

The DNA inserts of the clones were sequenced to verify that the library contained useful inserts representing fragments of VP7st4. Of the fourteen that were sequenced, four clones contained DNA inserts matching the nucleotide sequence of VP7st4 and two matched the pET32 vector flanking it. Figure 4.10 depicts where the inserts match the gene. They are spread over the entire region indicating that the library is representative of the whole gene and the flanking regions. These sequences translated into peptides in frame with the fusion partner, protein VIII. Eight of the clones had no inserts. Since the clones in the library contained VP7st4 inserts, a previously characterised antibody was used to pan the library as a control of its quality and usefulness.

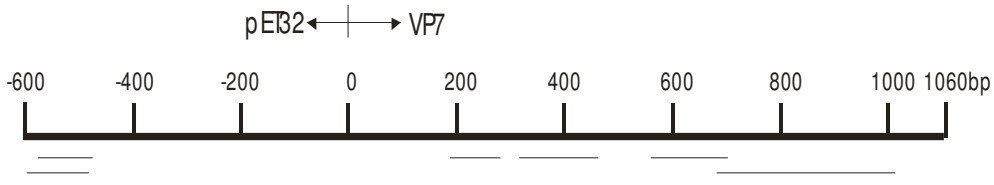


Figure 4.10: Schematic diagram depicting the position of random clones from the VP7st4-derived peptide library in relation to the starting DNA fragment. The lines represent the nucleotide (bp) positions where the sequence of each clone overlaps with the starting fragment.

MAb 20F10 which recognises a linear epitope "QYPALT" (Du Plessis *et al.*, 1994) was used to pan the library. The DNA coding for four individual clones selected from round two was sequenced. Two clones expressed the DNA insert in the correct VP7st4 reading

frame and matched the sequence of VP7st4 at amino acid position 250 to 266 (Figure 4.11). Since it recognised the correct amino acids on VP7st4, the library was considered suitable for panning with the anti-VP7st4 scFvs.

```
Position      250       260
          ......|....|....|....|.......
VP7BTV4   VVFYISMDKTLNQYPALTAEIFNVYSFRD
3 20F10   ------FDKTLNQYPALTAEILN------
4 20F10   ------FDKTLNQYPALTAEILN------
```

Figure 4.11: Deduced amino acid sequences of individual clones from panning the VP7st4-derived peptide library against MAb 20F10 as a positive control. Shaded region show the known epitope recognised by the MAb.

4.3.4.1. Panning of the new library

The library was panned against the scFvs to enrich for peptides specific to each scFv. When the purified scFvs were coated directly on Polysorp ELISA plate wells, very few colonies were obtained which indicated no specific selection. Panning was therefore not continued. Instead, three rounds of panning were performed by trapping the scFvs with an anti-c-*myc* MAb (clone 9E10) as ELISA showed better results (Figure 4.5). The results (Table 4.3) indicate that enrichment for binders had occurred. For example, for scFv A1, the phages released after selection round two was 123 times more than in round one and then there was a slight increase after round three. For scFv H2, there was an increase only after selection round three.

Table 4.3: Selection of clones binding to scFvs A1, H2, TC9, TD12 and SA12.

ScFvs	Output	Round 1	Round 2	Round 3
A1	CFU	7.0×10^4	8.6×10^6	1.7×10^7
	Increase		123×	1.98×
H2	CFU	6.5×10^4	2.0×10^4	1.4×10^7
	Increase		0.31×	700×
TC9	CFU	7.6×10^4	1.5×10^7	6.5×10^7
	Increase		197×	4.33×
TD12	CFU	1.6×10^4	8.0×10^5	1.3×10^7
	Increase		50.0×	16.2×
SA12	CFU	9.9×10^4	1.5×10^7	9.3×10^7
	Increase		151×	6.20×

After three rounds of selection against the scFvs, single clones from each panning were picked and their inserts were sequenced. The nucleotide sequences of each clone matched VP7st4 and the thioredoxin fusion partner flanking it at various positions spanning the entire VP7st4 gene (Figure 4.12).

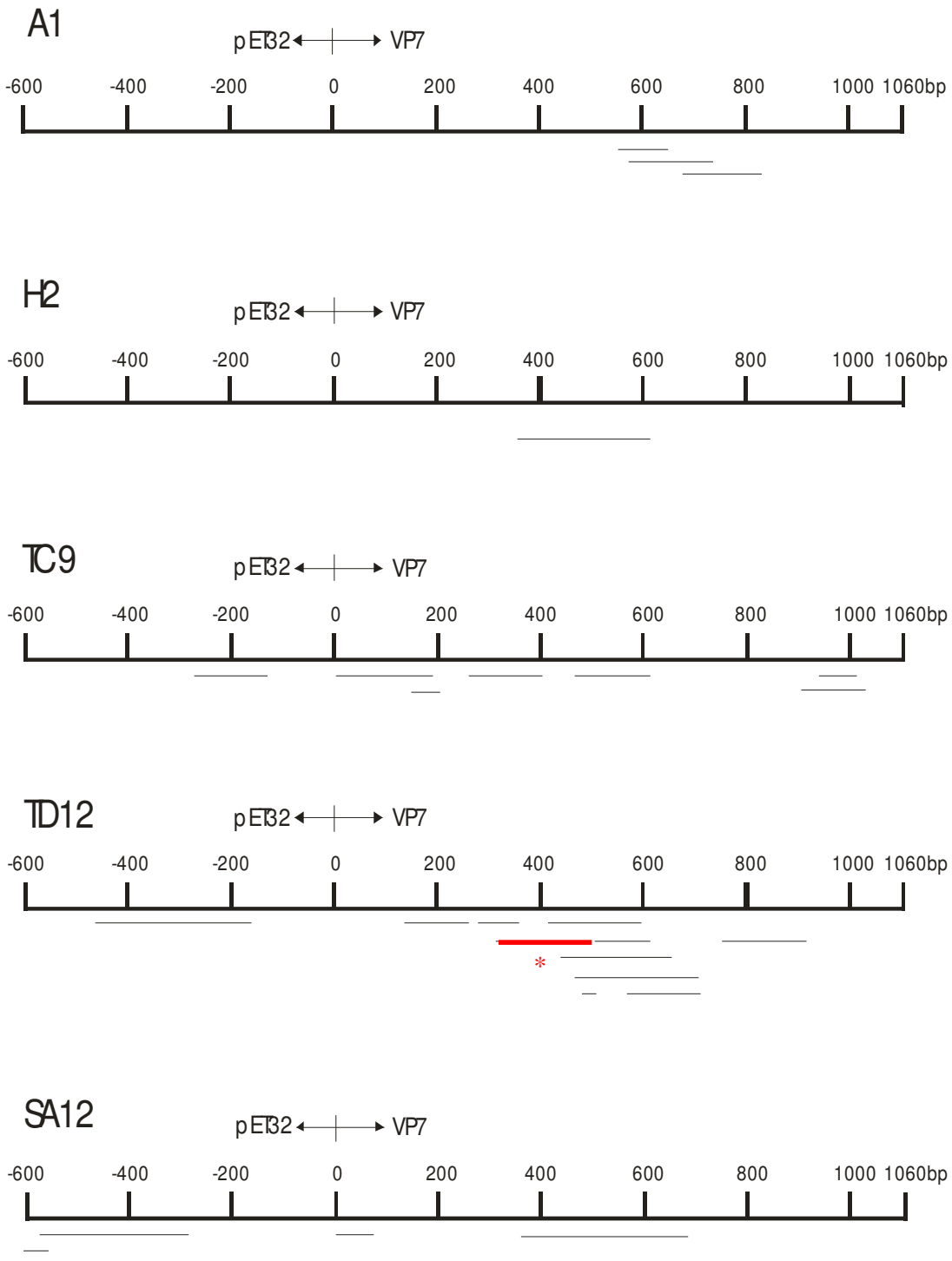


Figure 4.12: Individual clones selected against scFvs A1, H2, TC9, TD12 and SA12 overlapping VP7st4 and pET32 vector sequences flanking it. The values represent the nucleotide (bp) position. "*" below the red line represents duplicate TD12 clone.

Duplicate clones were only obtained with TD12 (*, Figure 4.12). However, after translation the sequences of the clones were not in the same reading frame as protein VIII and in theory no functional VP7st4 protein-VIII fusions should have been produced. This indicates that even though enrichment was observed, no specific selection took place.

4.4. DISCUSSION

The scFvs A1, H2, TC9, TD12 and SA12 were affinity purified and fractions eluted from the affinity column were shown to react with VP7st4 which was adsorbed directly in an ELISA plate (Figure 4.2). The fractions containing the scFvs were pooled and dialysed to remove elution buffer (Figure 4.3). For use in panning, purified scFvs needs to be immobilised to the surface of the ELISA wells in order to select VP7st4-specific peptides. They were shown to bind to VP7st4 when adsorbed directly in ELISA. The ELISA signals, however, were low indicating their sensitivity to plastic adsorption which often leads to epitope distortions (Van Regenmortel, 1989; Qian *et al*., 2000). The ELISA signals were higher when the scFvs were trapped with a MAb (Figure 4.5) and as a result they could be used even without purification. Since the scFvs could trap VP7st4 on the surface of ELISA plate wells, it was assumed that they would be able to trap a portion of VP7st4 displayed on the phage particle.

Initially, control antibodies were used in panning a previously constructed VP7st4-derived peptide library (Fehrsen *et al*., 2005) following different strategies. No satisfactory results were obtained. Peptides in the stored phage preparation could have been hydrolysed. Therefore, it was decided that a new library should be constructed. Libraries which display random fragments of cDNA on phages have been constructed previously. A strategy followed in this study was similar to that described by Gupta and Chaudhary (1996). These authors pointed out that their strategy improved ligation of blunt-ended inserts without using linkers. Other researchers have successfully constructed large fragmented-gene libraries following similar methods (Fehrsen *et al*., 2005).

In order to map epitopes on VP7st4, panning was first performed using a control antibody 20F10. The linear epitope QYPALT recognised by MAb 20F10 was identified previously (Du Plessis *et al*., 1994; Figure 4.11). Thereafter, the library was panned against the anti-VP7st4 scFvs. Three selection rounds were performed with each scFv and enrichment of VP7st4-specific binders was observed (Table 4.3). The sequence of the inserts of

individual clones revealed that no specific peptides were isolated. Therefore, the region on VP7st4 where the scFvs bind could not be identified. The scFvs selected in this study were in all likelihood directed against denatured epitopes of VP7st4. These epitopes might not have been accessible on the VP7st4-derived peptides displayed by the phages of the library or the phage-displayed peptides adopted a conformation not present on the denatured VP7st4. The results revealed that the epitopes on VP7st4 might not have refolded properly into native epitopes. Therefore, it may be advantageous to use either a soluble VP7 or whole BTV particles in panning against native epitopes to overcome the conformational constraints associated with scFvs selected that recognise denatured proteins. Several different conditions were tested to facilitate soluble expression of VP7st4 including lower IPTG concentrations, growth at different temperatures and different *E. coli* strains. However, VP7st4 remained insoluble. Soluble VP7 can be obtained by using heterologous expression systems such as baculovirus but the costs are higher (personal communication, Dr. Potgieter, OVI; Oldfield *et al*., 1990; Chuma *et al*., 1992; Kweon *et al*., 2003; Mecham & Wilson, 2004). In the case of *E. coli* expression, since over 50% of the total insoluble proteins was VP7st4, the use of efficient refolding buffers and additives such as arginine can aid renaturation of the insoluble protein (Babu *et al*., 2008; Lechtken *et al*., 2006; Liu *et al*., 2007). Other authors have used molecular chaperones which improves tprotein folding in the cell (Levy *et al*., 2001; reviewed by Betiku, 2006). A chaperone protein is used as a fusion partner in the novel vector, pCOLD, to facilitate correct folding and increased solubility of proteins (Takara Bio Inc.). This system uses “cold shock” technology which requires the cold shock protein A to express target proteins at low temperatures (Qing *et al*., 2004).

It has been shown previously that antibodies reactive in western blot often recognise continuous epitopes. Since the scFvs selected in this study reacted with the denatured VP7st4 in western blot (Figure 4.6), they were expected to select peptides from the VP7st4-derived peptide library. Zhou *et al*., (2007) reported, however, that positive reactions on western blots do not necessarily indicate the recognition of continuous epitopes. Discontinuous epitopes are thought to be destroyed by electrophoresis under denaturing conditions but still react with the antibody. It is also known that fragmented-gene libraries may not be suitable for mapping discontinuous epitopes while highly effective to map linear epitopes (Du Plessis *et al*., 1994; Fack *et al*., 1997; Cui *et al*., 2003). This is not always the case since the scFv F10 recognised a discontinuous epitope mapped with a fragmented-gene library (Fehrsen *et al*., 2005). The upper domain of VP7

which is exposed on the surface of the virus consists of several loops (Figure 4.13). The loops adopt a conformation to form the native epitopes that are recognised by antibodies in a BTV-infected animal. If these loops are denatured, they will loose their conformation and as a result, the antibodies recognising the native epitopes will not bind. The scFv F10 required the entire upper domain of VP7 of BTV 1 to bind (Fehrsen *et al.*, 2005). Therefore, these native epitopes were absent on recombinant VP7st4. The scFvs selected in this study recognised denatured epitopes on VP7st4 which are not similar to those recognised by antibodies in anti-BTV sera.

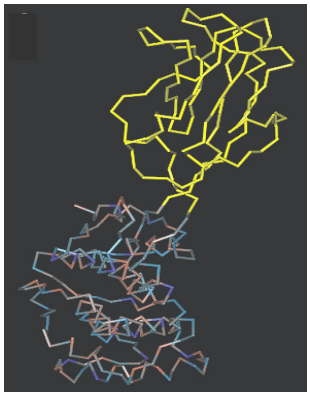


Figure 4.13: Structural diagram of VP7 monomer provided by Dr. J. Fehrsen. The upper domain is shown in yellow. The structure was captured from Cn3D 4.1 (www.ncbi.nlm.nih.gov, protein database accession number 2BTV).

Recombinant proteins can also be expressed in *E. coli* to identify epitopes as an alternative method to phage display panning. Stanley and Herz (1987) expressed cDNA fragments of human complement component C9 generated by DNase I and screened the resulting recombinants with antibodies to identify epitopes on C9. Discontinuous epitopes and mimotopes have also been reported using phage-displayed random peptide and synthetic libraries (Lugazzo *et al.*, 1993; Böttger *et al.*, 1995; D'Mello *et al.*, 1999; O'Connor *et al.*, 2005). Therefore, scFvs selected in this study still need to be characterised and might be recognising discontinuous epitopes and thus the peptides displayed in the fragmented-gene-library might have been short in length. In the study by Stanley and Herz (1987), antibodies specific for human complement component C9 were detected which had partially discontinuous epitopes because they required longer DNA fragments to express the binding site to which the antibody bind. The phage display method employed in this study to identify VP7st4 antigenic sites may not have been favourable for the anti-VP7st4 scFvs investigated. Other methods described above can be investigated.

CHAPTER FIVE

CONCLUDING DISCUSSION

BTV outbreaks remain a major concern worldwide (Verwoerd & Erasmus, 2004). They have economic implications due to losses from death of affected livestock and because of trade restrictions between BTV-infected and BTV-free countries. Therefore, there is a continuous need for well characterised immunological reagents for use in diagnostics (Van Wyngaardt *et al*., 2004). Recombinant reagents generated by phage display are ideal as they can be cost-effective, specific and their characteristics can be easily engineered (Sapats *et al*., 2003). Development of ELISAs based on these reagents play an important role in the detection of BTV antibodies. Furthermore, the knowledge of epitopes to which recombinant antibodies bind on recombinant proteins enables the development of assays aimed at detecting minor antigenic differences between strains or serotypes of viruses (Fehrsen *et al*., 2005).

In this study, VP7st4 remained insoluble when expressed in *E.coli* Origami™ cells. Six chicken scFvs specific for this recombinant protein were selected from the naïve *Nkuku*® phage display library. The binding of the scFvs A1 and H2 to VP7st4 was partially inhibited in ELISA using anti-BTV serum raised in guinea pigs while no inhibition was obtained for scFvs TC9, TD12 and SA12 binding. The epitopes on VP7st4 to which the scFvs bind could not be identified by phage display using a fragmented-gene library derived from the authentic VP7st4 gene. These findings suggested that the native epitopes on VP7st4 were not refolded properly and as a result the scFvs bound denatured epitopes on VP7st4 which were different to those against which the antibodies in the sera are directed. Therefore, for future investigations, it may be necessary to develop a procedure focused on optimising the yield of soluble VP7st4 by renaturation if *E.coli* is used as an expression host. Lechtken *et al*., (2006) have reported on the expression of a human retinoic acid related orphan receptor (RORα4) in *E.coli* under similar conditions tested as those for the expression of VP7st4. RORα4 was also expressed at different cell densities (OD_{660nm} 0.4-1.2) and by adding glucose or ethanol to the growth media. However, the protein remained insoluble and refolding agents were then used resulting in a high yield of soluble protein (Lechtken *et al*., 2006). The choice of refolding agents for use in facilitating proper refolding of VP7st4 to its native form is therefore critical. It is evident that PBS was not sufficient for this purpose. A refolding buffer containing guanidium chloride, a strong denaturant; salts to prevent ionic interaction and non-specific binding

and other additives were used for RORα4 refolding. L-arginine was also added to improve refolding followed by protein enrichment in PBS (Lechtken *et al*., 2006). It is known that arginine suppresses re-aggregation during refolding of proteins (Liu *et al*., 2007). In another study, Babu and co-workers used a combination of urea and guanidium chloride to solubilise streptokinase followed by refolding in Tris-HCl buffer containing various additives. A protein of high functionality was obtained when it was renatured in 20 mM Tris-HCl at pH 8.0 containing 1% Triton-X-100 and 10% glycerol (Babu *et al*., 2008). The pH also plays an important role as below pH 8.0, some proteins might start to precipitate out of solution. Charbonnier *et al*. (2001) solubilised an outer membrane efflux protein, OprM, of *Pseudomonas aeruginosa* in guanidium chloride and obtained a correctly folded protein by dilution in n-octylpolyoxyethylene. Other authors used molecular chaperones which improves protein folding in the cell (Wang *et al*., 1996; Levy *et al*., 2001; reviewed by Betiku, 2006). Rudolph and Lilie (1996) developed a method for renaturation of inclusion bodies using guanidium chloride at pH 8. The solubilisation step included dithiothreitol which is a reducing agent required for proteins containing cysteine residues as they form interchain disulphide bonds which leads to reduced solubility. The denaturant used is removed by dialysis at low pH 4.5. For VP7st4, the refolding can be monitored by testing it for binding in ELISA with an antibody which is known to recognise a native VP7 (e.g. scFv F10).

Therefore, it is highly recommended to optimise the amount of soluble VP7 since the native epitopes that are important in immunoassays will hopefully be restored. Alternatively, baculovirus expression systems can be used for soluble expressions (personal communication, Dr. Potgieter).

The scFvs were specific to VP7st4, therefore, they can be used in a sandwich ELISA to detect BTV. Van Wyngaardt *et al*., (2004) have shown that African Horse sickness virus (AHSV) and BTV particles trapped from suspension using virus-specific rabbit IgG could be detected using phage-displayed scFvs specific for AHSV and BTV. It has been shown that a few amino acid changes in chicken scFvs affect their binding (personal communication, Dr. Fehrsen, OVI; Wörn & Plückthun, 2001). The scFv H2 was a weak binder and not very stable often giving inconsistent results. Possibly, random mutations can be introduced in the framework or CDRs of this scFv to enhance its binding characteristics (Lantto *et al*., 1991; Finlay *et al*., 2006). Phage display can then be used to re-select clones with higher affinity and stability (Hoogenboom *et al*., 1998). When

mutations were introduced to an anti-digoxin antibody, deletion of the first two residues of the V_H changed its binding affinity to a great extent (Huston *et al*., 1988). Irving and co-workers (1996) used *E. coli* mutator cells to improve the binding activity of scFvs specific for glycophorin A and human erythrocyte membrane.

Since the anti-VP7st4 scFvs reacted in a western blot, alternative methods similar to those described in Section 4.4 (Stanley and Herz (1987) and Böttger *et al*., (1995)) can be adopted for future investigations to identify the regions on VP7st4 to which the scFvs bind. Hydrophilic regions which play a role in the solubility of the protein or small overlapping regions of the protein can be cloned into suitable expression vectors, expressed and then allowed to react with the scFvs on western blots. Yang and co-workers fragmented a gene of a nucleocapsid protein (NP) of an avian influenza virus into overlapping sequences and expressed the fragments in *E. coli*. Two MAbs against the NP and inactivated virus recognised different fragments of the NP in a western blot. Small peptides were then synthesised and the epitope of one MAb could be further located (Yang *et al*., 2008). It is suggested that similar strategies can be adapted to map the antigenic sites on VP7st4.

In conclusion, *E.coli* was used to produce a high yield of recombinant VP7st4 at low cost. Phage display was used to generate recombinant antibodies specific for VP7st4. This study has shown that recombinant antibodies generated *in vitro* are stable and can be produced at ease in a short time frame compared to conventional monoclonal antibodies. Chicken scFvs that retain their activity over long storage periods at various temperatures without precipitating out of solution or being degraded are ideal candidates for immunodiagnostic applications. The scFvs obtained in this study also have the potential to be engineered to improve their specificities for use in developing immunoassays or diagnostic tests. The *Nkuku*® library further demonstrated its ability to serve as an unlimited and sustainable source of highly specific antibodies against many antigens.

6.1. APPENDIX

MEDIA, AGAR PLATES

LB medium

Dissolve 10 g bacto-tryptone, 5 g bacto-yeast and 5 g NaCl in 800 ml deionised water (dH_2O) and adjust to a final volume of 1 L. Autoclave and store at RT.

2×TY medium

Dissolve 5 g NaCl, 16 g tryptone and 10 g yeast extract in 800 ml dH_2O and adjust the volume to 1 L. Autoclave and store at RT.

2×TYE agar plates

Dissolve 15 g agar, 8 g NaCl, 10 g tryptone and 5 g yeast extract in 800 ml dH_2O and adjust the volume to 1 L. Autoclave and cool to 50°C. Add 100 µg/ml ampicillin and 2% glucose, pour plates and store at 4°C.

LB-kanamycin agar plates

Dissolve 5 g agarose, 2.5 g bacto-tryptone, 1.25 g yeast extract and 2.5 g NaCl in dH_2O and adjust volume to 250 ml. Autoclave and cool to 50°C. Add kanamycin to 50 µg/ml, pour plates and store at 4°C.

Molten H-top agar

Dissolve 1 g bacto-tryptone, 0.6 g agar and 0.8 g NaCl in 90 ml H_2O and adjust to a final volume of 100 ml. Autoclave and store at RT.

M9 Minimal medium

Mix 20 ml 5×M9 salts and 200 µl 1 M $MgSO_4$ and filter through a 0.2 µm filter. Add 2 ml of 20% glucose and warm in a water bath. Then add 10 µl of 1 M $CaCl_2$. Add a pre-warmed 1.5 g agarose in 75 ml H_2O (autoclaved) to the M9 mixture and pour plates.

5×M9 salts were prepared by dissolving 64 g $Na_2HPO_4.7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl and 5.0 g NH_4Cl to a final volume of 1 L in dH_2O.

ANTIBIOTICS

Antibiotic	Stock solution	Working concentration
Ampicillin	100 mg/ml	100 µg/ml
Kanamycin	25 mg/ml,	25 µg/ml
Carbenicillin	50 mg/ml	50 µg/ml
Tetracycline	10 mg/ml	12.5 µg/ml

The working stocks of each of these antibiotics were prepared in dH_2O. The solutions were filtered through a 0.2 µm filter and then store in aliquots at -20°C until use.

STOCK SOLUTIONS AND BUFFERS

0.1 M Citrate buffer pH 4.5

0.1 M Tri-sodium citrate (MW=294.10 g/mol): dissolve 14.7 g in H_2O and adjust volume to 500 ml with dH_2O. 0.1 M citric acid monohydrate (MW 210.14 g/mol): dissolve 10.5 g and adjust volume to 500 ml. Add 500 ml or more of 0.1 M Citric acid monohydrate solution into 500 ml of 0.1 M Tri-sodium citrate solution to adjust pH to 4.5. Autoclave and store at 4 °C.

Coomassie brilliant blue stain (0.25% Coomassie, 50% methanol and 10% acetic acid)

Dissolve 2.5 g of Coomassie in 500 ml methanol. Add 100 ml glacial acetic acid and adjust volume to 1 L with dH_2O.

DNase buffer (50 mM Tris pH 7.6, 0.1 mg/ml BSA and 1 mM $MnCl_2$)

Mix 500 µl 1M Tris pH 7.6, 20 µl 50 mg/ml BSA, 10 µl $MnCl_2$ prepared on day of use and dH_2O to a final volume of 10 ml.

OPD ELISA substrate

Dissolve 5 mg OPD tablet in 5 ml 0.1M citrate buffer containing 2.5 µl of hydrogen peroxide.

10×PBS (phosphate buffer saline)

Dissolve 80 g NaCl, 2 g KCl, 26.8 g Na_2HPO_4-$7H_2O$ and 2.4 g KH_2PO_4 in 800 ml dH_2O. Adjust to pH 7.4 with HCl and then to a final volume of 1 L with dH_2O. Autoclave and store at RT.

2×PSB (protein solvent buffer)

Mix 5.5 ml 1 M Tris pH 6.8, 2 ml 10% SDS, 1 ml 60% glycerol, 1 ml 2-mercaptoethanol and 1 ml 0.1% bromophenol blue.

3 M Sodium acetate

Dissolve 20.4 g sodium acetate in 40 ml dH_2O.

Stop buffer (70% glycerol, 75 mM EDTA, 0.3% bromophenol blue)

Mix 7 ml glycerol, 1.5 ml 250 mM EDTA, 30 µl bromophenol blue and 1.47 ml dH_2O.

50×TAE buffer

Dissolve 242 g Tris in 600 ml dH_2O. Add 100 ml 0.5 M Na_2EDTA at pH 8.0 and 57.1 ml glacial acetic acid. Then adjust volume to 1 L with dH_2O. Prepare working solution of 1×TAE from this stock.

Towbin buffer for western blot (25 mM Tris, 200 mM glycine)

Dissolve 4.53 g Tris and 21.63 g glycine in 800 ml dH_2O. Adjust volume to 1 L.

100 mM Triethylamine (TEA)

140 µl TEA in 10 ml dH_2O.

GELS

SDS-PAGE solutions

1 M Tris pH 6.8 (12.12 g /100 ml dH_2O)

1.5 M Tris pH 8.8 (18.18 g/100 ml dH_2O)

10% APS (1 g in 10 ml dH_2O)

Stacking gel (4%)

Mix 1.33 ml acrylamide, 267 µl Rhinohyde, 2.5 ml 1 M Tris pH 6.8, 5.8 ml dH_2O, 100 µl 10% APS and 6.7 µl TEMED to a final volume of 10 ml.

Separating gel (10%)

Mix 5 ml acrylamide, 1 ml Rhinohyde, 3.75 ml 1.5 M Tris pH 8.8, 5.09 ml dH_2O, 150 µl 10% APS and 10 µl TEMED to a final volume of 10 ml. Pour 5 ml of separating gel on SDS-PAGE apparatus and add 1 ml of n-butanol. Allow to set, discard the butanol solution, add 1 ml of stacking solution and insert combs and allow to solidify.

6.2. REFERENCES

Abi-Ghanem, D., Waghela, S. D., Caldwell, D. J., Danforth, H. D., & Berghman, L. R., 2008. Phage display selection and characterization of single-chain recombinant antibodies against *Eimeria tenella* sporozoites. *Veterinary Immunology and Immnuopathology*, 121:58-67.

Afshar, A., Anderson, J., Eaton, B.T., & Gustafson, G. A., 1991. Serological diagnosis of bluetongue by blocking or competitive ELISA by four laboratories. *Journal of Veterinary Diagnostic Investigation*, 3:255-257.

Afshar, A., Eaton, B. T., Wright, P. F., Pearson, J. E., Anderson, J., Jeggo, M. & Trotter, H. C., 1992. Competitive ELISA for serodiagnosis of bluetongue: evaluation of group-specific monoclonal antibodies and expressed VP7 antigen. *Journal of Veterinary Diagnosis and Investigation,* 4:231-237.

Afshar, A., Heckert, R. A., Dulac, G. C., Trotter, H. C., & Myers, D. J., 1995. Application of a competitive ELISA for the detection of bluetongue virus antibodies in llamas and wild ruminants. *Journal of Wildlife Disease*, 31:327-330.

Andris-Widhopf, J., Rader, C., Steinberger, P., Fuller, R., & Barbas III, C. F., 2000. Methods for generation of chicken monoclonal antibody fragments by phage display. *Journal of Immunological Methods*, 242:159-181.

Babu, P. V. C., Srinivas, V. K., Mohan, V. K., & Krishna, E., 2008. Renaturation, purification and characterization of streptokinase expressed as inclusion body in recombinant *E. coli*. *Journal of Chromatography B*, 861:218-226.

Barderas, R., Shochat, S., Martínez-Torrecuadrada, J., Altschuh, D., Meloen, R., & Casal, J. I., 2006. A fast mutagenesis procedure to recover soluble and functional scFvs containing amber stop codons from synthetic and semisynthetic antibody libraries. *Journal of Immunological Methods*, 312:182-189.

Barros, S. C., Ramos, F., Luís, T. M., Vaz, A., Duarte, M., Henriques, M., Cruz, B., & Fevereiro, M., 2007. Molecular epidemiology of bluetongue virus in Portugal during 2004-2006 outbreak. *Veterinary Microbiology*, 124:25-34.

Baylis, M., 2002. The re-emergence of bluetongue. *The Veterinary Journal*, 164:5-6.

Bentley, L., Fehrsen, J., Jordaan, F., Huismans, H. & du Plessis, D. H., 2000. Identification of antigenic regions on VP2 of African horsesickness virus serotype 3 using phage-displayed epitope libraries. *Journal of General Virology*, 81:993-1000.

Bessette, P. H., Åslund, F., Beckwith, J., & Georgiou, G., 1999. Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm. *Proceedings of the National Academy of Sciences*, USA 96:13703-13708.

Betiku, E., 2006. Molecular chaperones involved in heterologous protein folding in *Escherichia coli*. *Biotechnology and Molecular Biology Review*, 1:66-75.

Bhardwaj, D., Singh, S. S., Abrol, S., & Chaudhary, V. K., 1994. Monoclonal antibodies against a minor and the major coat proteins of filamentous phage M13: their application in phage display. *Journal of Immunological Methods*, 179:165-175.

Blüthner, M., Schäfer, C., Schneider, C., & Bautz, F. A., 1998. Identification of major linear epitopes on the sp100 nuclear PBC autoantigen by the gene-fragment phage-display technology. *Autoimmunity*, 29:33-42.

Borden, E. C, Shope, R. E., & Murphy, F. A., 1971. Physicochemical and morphological relationships of some anthropod-borne viruses to bluetongue virus-new taxonomic group physicochemical and serological studies. *Journal of General Virology*, 13:261-271.

Böttger, V., Stasiak, P. C., Harrison, D. L., Mellerick, D. M., & Lane, E. B., 1995. Epitope mapping of monoclonal antibodies to keratin 19 using keratin fragments, synthetic peptides and phage peptide libraries. *European Journal of Biochemistry*, 231:475-485.

Boulanger, P., & Frank, J. F., 1975. Serological methods in the diagnosis of bluetongue. *Australian Veterinary Journal*, 51:185-189.

Bowne, J. G., Luedke, A. J., Foster, N. M., & Joshim, M. M., 1966. Current aspects of bluetongue in cattle. *Journal of the American Veterinary Medical Association*, 148:1177-1180.

Bowne, J. G., Luedke, A. J., & Joshim, M. M., 1967. Bluetongue of sheep and cattle: Past, Present, and Future. *Journal of the American Veterinary Medical Association*, 151:1801-1803.

Breard, E., Hamblin, C., Hammoumi, S., Sailleau, C., Dauphin, G., & Zientara, S., 2004. The epidemiology and diagnosis of bluetongue with particular reference to Corsica. *Research in Veterinary Science*, 77:1-8.

Burton, D. R., 1995. Phage display. *Immunotechnology*, 87-94.

Butler, J. E., Spradling, J. E., Suter, M., Dierks, S. E., Heyermann, H., & Peterman, J. H., 1986. The immunochemistry of sandwich ELISAs-I. The binding characteristics of immunoglobulins to monoclonal and polyclonal capture antibodies adsorbed on plastic and their detection by symmetrical and asymmetrical antibody-enzyme conjugates. *Molecular Immunology*, 23:971-982.

Cabilly, S., 1989. Growth at sub-optimal temperatures allow the production of functional, antigen-binding Fab fragments in *Escherichia coli*. Gene, 85:553-557.

Carrió, M. M., & Villaverde, A., 2003. Role of molecular chaperones in inclusion body formation. *FASEB Letters*, 537:215-221.

Castillo, J., Goodson, B., & Winter, J., 2001. T7 displayed peptides as targets for selecting peptide specific scFvs from M13 scFv display libraries. *Journal of Immunological Methods*, 257:117-122.

Charbonnier, F., Köhler, T., Pechère, J. C., & Ducruix, A., 2001. Overexpression, refolding, and purification of the histidine-tagged outer membrane efflux protein OprM of *Pseudomonas aeruginosa*. *Protein Expression and Purification*, 23:121-7.

Chuma, T., Le Blois, H., Sánchez-Vizcaíno, J. M., Diaz-Laviada, M., & Roy, P., 1992. Expression of the major core antigen VP7 of African horsesickness virus by a recombinant baculovirus and its use as a group-specific diagnostic reagent. *Journal of General Virology*, 73:925-931.

Cicchini, C., Ansuini., H., Amicone, L, Alonzi, T., Nicosia, A., Cortese, R., Tripodi, M., & Luzzago, A., 2002. Searching for DNA-protein interactions by lambda phage display. *Journal of Molecular Biology*, 322:697-706.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D., & Winter, G., 1991. Making antibody fragments using phage display libraries. *Nature*, 352:624-8.

Courtney, M., Buchwalder, A., **Tessier, L-H**., **Jaye, M**., **Benavente, A**., **Balland, A**., **Kohli, V**., **Lathe, R**., **Tolstoshev, P., & Lecocq, J-P.,** 1984. High-level production of biologically active human alpha 1-antitrypsin in *Escherichia coli*. *Proceedings of the National Academy of Sciences*, USA 81:669-73.

Cui, X., Nagesha, H. S., & Holmes, I. H., 2003. Mapping of conformational epitopes on capsid protein VP2 of infectious bursal disease virus by fd-tet phage display. *Journal of Virological Methods*, 114:109-112.

Cwirla, S. E., Peters, E. A., Barrett, R. W., & Dower, W. J., 1990. Peptides on phages: a vast library for identifying ligands. *Proceedings of the National Academy of Sciences*, USA 87:6378-6382.

Davies, E. L., Smith, J. S., Birkett, C. R., Manser, J. M., Anderson-Dear, D. V., & Young, J. R., 1995. Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *Journal of Immunological Methods*, 186:125-135.

De Boer, H. A., Comstock, L. J., & Vasser, M., 1983. The *tac* promoter: A functional hybrid derived from the *trp* and *lac* promoters. *Proceedings of the National Academy of Sciences*, USA 80:21.

Dercksen, D., Groot, N. N., Paauwe, R., Backx, A., van Rijn, P., & Vellema, P., 2007. First outbreak of bluetongue in goats in The Netherlands. *Tijdschrift voor diergeneeskunde*, 132:786-790.

Devlin, J. J., Panganiban, L. C., & Devlin, P. E., 1990. Random peptide libraries: a source of specific protein binding molecules. *Science*, 249:404-406.

D'Mello, F., Kairo, S. K., Howard, C. R., & Partidos, C. D., 1999. Mapping the specificity of an anti-feline immunodeficiency virus monoclonal antibody using a filamentous phage displayed peptide library. *Vaccine*, 18:371-375.

Du Plessis, D. H., & Jordaan, F., 1996. Phage libraries displaying random peptides derived from a target sequence. In: Kay, B. K., Winter, J., & McCafferty, J., eds. Phage Display of Peptides and Proteins, A Laboratory Manual: United States, California, Academic Press, Inc., 141-150.

Du Plessis, D. H., Romito, M., & Jordaan, F., 1995. Identification of an antigenic peptide specific for bluetongue virus using phage display expression NS1 sequences. *Immunotechnology*, 1:221-230.

Du Plessis, D. H., Wang, L-F., Jordaan, F. A., & Eaton, B. T., 1994. Fine mapping of a continuous epitope on VP7 of bluetongue virus using overlapping synthetic peptides and a random epitope library. *Virology*, 198:346.

Du Toit, R. M., 1944. The transmission of bluetongue and horse-sickness by *Culicoides*. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 19:7-16.

Eaton, B. T., Gould, A. R., Hyatt, A. T., Coupar, B. E., Martin, J. R., & White, J. R., 1991. A bluetongue serogroup-reactive epitope in the amino terminal half of the major core protein VP7 is accessible on the surface of bluetongue virus particles. *Virology,* 180:687-696.

Els, H. J., & Verwoed, D. M., 1969. Morphology of bluetongue virus. *Virology*, 38:213-219.

Erasmus, B. J., 1975. The epizootiology of bluetongue: The African situation. *Australian Veterinary Journal*, 51:196-198.

Fack, F., Hügle-Dörr, B., Song, D., Queitsch, I., Petersen, G., & Bautz, E. K. F., 1997. Epitope mapping by phage display: random versus gene-fragment libraries. *Journal of Immunological Methods*, 206:43-52.

Fehrsen, J. & Du Plessis, D. H., 1999. Cross-reactive epitope mimics in a fragmented-genome phage display library derived from the rickettsia, *Cowdria ruminantium*. *Immunotechnology*, 4:175-184.

Fehrsen, J., Van Wyngaardt, W., Mashau, C., Potgieter, A. C., Chaudhary, V. K., Gupta, A., Jordaan, F. A., & Du Plessis, D. H., 2005. Serogroup-reactive and type-specific detection of bluetongue virus antibodies using chicken scFvs in inhibition ELISA. *Journal of Virological Methods*, 129:31-39.

Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R., & Cesareni, G., 1991. Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *Journal of Molecular Biology*, 222:301-310.

Finlay, W. J. J., Shaw, I., Reilly, J. P., & Kane, M., 2006. Generation of high-affinity chicken single-chain Fv antibody fragments for measurements of the *Pseudonitzschia pungens* toxin domoic acid. *Applied and Environmental Microbiology*, 72:3343-3349.

Frangione, B., Milstein, C., & Franklin, E. C., 1968. Intrachain disulphide bridges in immunologlobulin G heavy chains. *The Biochemical Journal*. 106:15-21.

Foord, A. J., Muller, J. D., Yu, M., Wand, L-F., & Heine H. G., 2007. Production and application of recombinant antibodies to foot-and-mouth disease virus non-structural protein 3ABC. *Journal of Immunological Methods*, 321:142-151.

Forrer, P., & Jaussi, R., 1998. High-level expression of soluble heterologous proteins in the cytoplasm of *Escherichia coli* by fusion to the bacteriophage Lambda head protein D. *Gene*, 224:45-52.

Foster, N. M., & Luedke, A. J., 1968. The direct assay of bluetongue virus by intravascular inoculation of embryonating chicken eggs. *American Journal of Veterinary Research*, 29(3):749-753.

Gambles, R. M., 1949. Bluetongue of sheep in Cyprus. *Journal of Comparative Pathology*, 59:176-190.

Georgiou, G., Telford, J. N., Shuler, M. L., & Wilson, D. B., 1986. Localization of inclusion bodies in *Escherichia coli* overproducing β-lactamase or alkaline phosphatase. *Applied and Environmental Microbiology*, 52:1157-1161.

Georgiou, G., & Valax, P., 1996. Expression of correctly folded proteins in *Escherichia coli*. *Current Opinion in Biotechnology*, 7:190-197.

Gibbs, E. P. J., & Greiner, E. C., 1994. The epidemiology of bluetongue. *Comparative Immunology and Microbiology of Infectious diseases*, 17:207-220.

Goeddel, D. V., Kleid, D. G., Bolivar, F., Heyneker, H. L., Yansura, D. G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K., & Riggs, A. D., 1979. Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proceedings of the National Academy of Sciences*, 76:106-110.

Grimes, J., Basak, A. K., Roy, P., & Stuart, D., 1995. The crystal structure of bluetongue virus VP7. *Nature*, 373:167-170.

Grimes, J. M., Jakana, J., Ghosh, M., Basak, A. K., Roy, P., Chiu, W., Stuart, D. I., & Prasad, B. V. V., 1997. An atomic model of the outer layer of the bluetongue virus core derived from X-ray chrystallography and electron cryomicroscopy. *Structure*, 5:885-895.

Grimes, J. M., Burrroughs, J. N., Gouet, P., Diprose, J. M., Malby, R., Zientaras, Mertens P. P. C., & Stuart, D. L., 1998. The atomic structure of the bluetongue virus core. *Nature*, 395:470-477.

Gupta, S., & Chaudhary, V. K., 1999. Simplified gene-fragment phage display system for epitope mapping. *Biotechniques*, 27:328-334.

Hall, T. A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acid Symposium Series*, 41:95-98.

Harmsen, M. M., & De Haard, H. J., 2007. Properties, production, and applications of camelid single-domain antibody fragments. *Applied Microbiology and Biotechnology*, 77:13-22.

Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., & Winter, G., 1991. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acid Research*, 19:4133-4137.

Hoogenboom, H. R., de Bruïne, A. P., Hufton, S. E., Hoet, R. M., Arends, J-W., & Roovers, R. C., 1998. Antibody phage display technology and its applications. *Immunotechnology*, 4:1-20.

Hourrigan, J. L., & Klingsporn, A. L., 1975. Epizootiology of bluetongue: the situation in the United States of America. *Australian Veterinary Journal*, 51:203-208.

Hourrigan, J. L., & Klingsporn, D. V. M., 1975a. Bluetongue: the disease in cattle. *Australian Veterinary Journal*, 51:170-174.

Huismans, H., & Erasmus, B. J., 1981. Identification of serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort Journal of Veterinary Research,* 48:51-58.

Hust, M., Maiss, E., Jacobsen, H-J., & Reinard, T., 2002. The production of a genus-specific recombinant antibody (scFv) using a recombinant potyvirus protease. *Journal of Virological Methods*, 106:225-233.

Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M-S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., & Oppermann, H., 1988. Protein engineering of antiboding binding sites: recovery of specific activity in an aniti-dogoxin single-chain Fv analoque produced in *Escherichia coli*. *Proceedings of the National Academy of Sciences*, USA 85:5879-5883.

Hutcheon, D., 1881. Fever of epizootic catarrh, Report College of Veterinary Surgeons, London.

Itakura, K., Hirose, K. T., Crea, R., Riggs, A. D., Heyneker, H. L., Bolivar, F., & Boyer, H. W., 1977. Expression in *Escherichia coli* of a chemically synthesized gene for the hormone sormatostain. *Science*, 198:1056-1063.

Irving, R. A., Kortt, A. A, & Hudson, P. J., 1996. Affinity maturation of recombinant antibodies using *E. coli* mutator cells. *Immunotechnology*, 2:127-143.

Irving, M.B., Pan, O., & Scott, J. K., 2001. Random-peptide libraries and antigen-fragment libraries for epitope mapping and the development of vaccines and diagnostics. *Current Opinion in Chemical Biology*, 5:314-324.

Jacobsson, K., Rosander, A., Bjerketorp, J., & Frykberg, L., 2003. Shotgun phage display-selection for bacterial receptions or other exported proteins. *Biological Proceedings Online*, 5:123-135.

Jellis, C. L., Cradick, T. J., Rennert, P., Salinas, P., Boyd, J., Amirault, T., & Gray, G. S., 1993. Defining critical residues in the epitope for HIV-neutralizing monoclonal antibody using phage display and peptide array technologies. *Gene*, 137:63-68.

Jonasson, P., Liljeqvist, S., Nygren, P. A., & Ståhl, S., 2002. Genetic design for facilitated production and recovery of recombinant proteins in *Escherichia coli*. *Biotechnology and Applied Biochemistry*, 35:91-105.

Jung, S., Honegger, A., & Plückthun, A., 1999. Selection for improved protein stability by phage display. *Journal of Molecular Biology,* 294:163-180.

Karu, A. E., Bell, C. W., & Chin, T. E., 1995. Recombinant antibody technology. *National Research Council, Institute of Laboratory Animal Resources Journal*, 37:1-14.

Kashiwazaki, Y., Na, Y. N., Tanimura, N., & Imada, T., 2004. A solid-phase blocking ELISA for detection of antibodies to Nipah virus. *Journal of Virological Methods*, 121:259-261.

Kay, B. K., Adey, N. B., He, Y. S., Manfredi, J. P., Mataragnon, A. H., & Fowlkes, D. M., 1993. An M13 phage library displaying random 38-amino-acid peptides as a source of novel sequences with affinity to selected targets. *Gene*, 128:59-65.

Kiefhaber, T., Rudolph, R., Kohler, H-H., & Buchner, J., 1991. Protein aggregation *in vitro* and *in vivo*: a quantitative model of the kinetic competition between folding and aggregation. *Biotechnology*, 9:825-829.

Köhler, G., & Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256:495-497.

Kowalik, T. F., & Li, J. K., 1991. Bluetongue virus evolution: sequence analyses of the genomic S1 segments and major core protein VP7. *Virology*, 181:749-755.

Kowalik, T. F., Li, J. K.-K., Chuang, R. Y., Doi, R. H., & Osburn, B. I., 1990. The complete nucleotide and deduced amino acid sequence of the gene encoding the major inner capsid protein, VP7 of US bluetongue virus serotype 17. *Nucleic Acids Research*, 18 (17):5302.

Kweon, C. H., Kwon, B. J., Ko, Y. J., & Kenichi, S., 2003. Development of competitive ELISA for serodiagnosis on African horsesickness virus using baculovirus expressed VP7 and monoclonal antibody. *Journal of Virological Methods*, 113:13-18.

Lantto, J., Jirholt, P., Barrios, Y., & Ohlin, M., 2002. Chain Shuffling to modify properties of recombinant immunoglobulins. Methods in Molecular Biology, 178:303-316.

Larsson, A., Wejåker, P-E., Forsberg, P-O., & Lindahl, T., 1992. Chicken antibodies: a tool to avoid interference by complement activation in ELISA. *Journal of Immunological Methods*, 156:79-83.

Le Blois, H., & Roy, P., 1993. A single point mutation in the VP7 major core protein of bluetongue virus prevents the formation of core like particles. *Journal of Virology*, 353-359.

Lechtken, A., Zündorf, I., Dingermann, T., Firla, B., & Steinhilber, D., 1996. Overexpression, refolding and purification of polyhistidine-tagged human retinoic acid related to orphan receptor RORα4. *Protein Expression and Purification*, 49:114-120.

Lee, Y-C., Leu, S-J. C., Hu, C-J., Shih, N-Y., Huang, I-J., Wu, H-H., Hsieh, W-S., Chiang, B-L., Chiu, W. T., & Yang, Y-Y., 2007. Chicken single-chain variable fragments against SARS-CoV spike protein. *Journal of Virological Methods*, 146:104-111.

Levy, R., Weiss, R., Chen, G., Iverson, B. L., & Georgiou, G., 2001. Production of correctly folded Fab antibody fragment in the cytoplasm of *Escherichia coli* trxB gor mutants via the coexpression of molecular chaperones. *Protein Expression and Purification*, 23:338-347.

Li, Y., Kilpatrick, J., & Whitelam, G. C., 2000. Sheep monoclonal antibody fragments generated using a phage display system. *Journal of Immunological Methods*, 236:133-146.

Liu, Y. D., Li, J. J., Wang, F. W., Chen, J., Li, P., & Su, Z.G., 2007. A newly proposed mechanism for arginine-assisted protein refolding-not inhibiting soluble oligomers although promoting a correct structure. *Protein Expression and Purification*, 51:235-242.

Lilie, H., Schwarz, E., & Rudolph, R., 1998. Advances in refolding of proteins produced in *E. coli*. *Current Opinion in Biotechnology*, 9:497-501.

Lunt, R. A., White, J. R., & Blacksell, S. D., 1988. Evaluation of a monoclonal antibody blocking ELISA for the detection of group-specific antibodies to bluetongue virus in experimental field sera. *Journal of General Virology*, 69:2729-2740.

Luzzago, A., Felici, F., Tramontano, A., Pessi, A., & Cortese, R., 1993. Mimicking of discontinuous epitopes by phage-display peptides, I. Epitope mapping of human H ferritin using a phage library of constrained peptides. *Gene*, 128:51-57.

Makrides, S. C., 1996. Strategies for achieving high-level expression of genes in *Escherichia coli. Microbiological Reviews*, 60:512-38.

Malys, N., Chang, D-Y., Baumann, R. G., Xie, D., & Black, L. W., 2002. A bipartite bacteriophage T4 SOC and HOC randomised peptide display library: Detection and analysis of phage T4 terminase (gp17) and late σ factor (gp55) interaction. *Journal of Molecular Biology*, 319:289-304.

Markland, W., Roberts, B. L., Saxena, M. J., Guterman, S. K., & Ladner, R. C., 1991. Design, construction & function of a multicopy display vector using fusions to the major coat protein of bluetongue M13. *Gene,* 109:13.

Marks, J. D., Griffiths, A. D., Malmqvist, M., Clackson, T. P., Bye, J. M., & Winter, G., 1992. By-passing immunization: building high affinity human antibodies by chain shuffling. *Biotechnology*, 10:779-783.

Marston, F. A. O., 1986. The purification of eukaryotic polypeptides synthesized in *Escherichia coli*, *Biochemical Journal*, 240:1-12.

Martyn, J. C., Gould, R., & Eaton, B. T., 1991. High level expression of the major core protein VP7 and the non-structural protein NS3 of bluetongue virus in yeast: use of expressed VP7 as a diagnostic, group-reactive antigen in a blocking ELISA. *Virus Research,* 18:165-178.

Marvin, D., A., & Hohn, B., 1969. Filamentous bacterial viruses. *Bacteriological Reviews*, 33:172-209.

Matsuda, H., Mitsuda, H., Nakamura, N., Furusawa, S., Mohri, S., & Kitamoto, T., 1999. A chicken monoclonal antibody with specificity for the N-terminal of human prion protein. *FEMS Immunology and Medical Microbiology*, 23:189-194.

Matsushita, K., Horiuchi, H., Furusawa, S., Horiuchi, M., & Shinagawa, M., 1998. Chicken monoclonal antibodies against synthetic bovine prion protein. *Journal of Veterinary Medical Science*, 60:777-779.

McCafferty, J., Griffiths, A. D., Winter, G., & Chiswell, D. J., 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*, 348:552-554.

McCormack, W. T., Hurley, E. A., & Thompson, C. B., 1993. Germ line maintenance of the pseudogene donor pool for somatic immunoglobulin gene conversion in chickens. *Molecular and Cellular Biology*, 13:821-830.

Mecham, J. O., & Wilson, W. C., 2004. Antigen capture competitive enzyme-linked immunosorbent assays using baculovirus-expressed antigens for diagnosis of bluetongue virus and epizootic hemorrhagic disease virus. *Journal of Clinical Microbiology*, 42:518-523.

Mellor, P. S., & Wittmann, E. J., 2002. Bluetongue virus in the Mediterranean Basin 1998-2001. *The Veterinary Journal*, 164:20-37.

Mertens, P. P. C., & Diprose, J., 2004. The bluetongue virus core: a nano-scale transcription machine. *Virus Research*, 101:29-43.

Mergulhão, F. J. M., Summers, D. K., & Monteiro, G. A., 2005. Recombinant protein secretion in *Escherichia coli*. *Biotechnology advances*, 23:177-202.

Messens, J., & Collet, J-F., 2006. Pathways of disulfide bon formation in *Escherichia coli*. *The International Journal of Biochemistry & Cell Biology*, 38:1050-1062.

Murby, M., Uhlén, M., & Ståhl, S., 1996. Upstream strategies to minimize proteolytic degradation upon recombinant production in *Escherichia coli*. *Protein Expression and Purification*, 7:129-136.

Murphy, F. A., Borden, E. C., Shope, R. E., & Harrison, A., 1971. Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus-a new taxonomic group. Electron microscopic studies. *Journal of General Virology*, 13:273-88.

Nagesha, H. S., Wang, L. F., Shiell, B., Beddome, G., White, J. R., & Irving, R. A., 2001. A single chain Fv antibody displayed on phage surface recognizes conformational group-specific epitope of bluetongue virus. *Journal of Virological Methods,* 91:203-207.

Nakamura, N., Shuyama, A., Hojyo, S., Shimokawa, M., Miyamoto, K., Kawashima, T., Aosasa, M., Horiuchi, H., Furusawa, S., & Matsuda, H., 2004. Establishment of a chicken monoclonal antibody panel against mammalian prion protein. *Journal of Veterinary Medical Science*, 66:807-814.

Naresh, A., & Prasad, G., 1995. Relative superiority of c-ELISA for detection of bluetongue virus antibodies. *Indian Journal of Experimental Biology*, 33:880-882.

Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D., & Winter, G., 1994. Antibody fragments from a "single pot" phage display library as immunochemical reagents. *The EMBO Journal*, 13:692-698.

O'Brien, P. M., Aitken, R., O'Neil, B. W., & Campo, M. S., 1999. Generation of bovine mAbs by phage display. *Proceedings of the National Academy of Sciences*, USA 96:640-645.

O'Connell, D., Becerril, B., Roy-Burman, A., Daws, M., & Marks, J. D., 2002. Phage versus phagemid libraries for generation of human monoclonal antibodies. *Journal of Molecular Biology*, 321:49-56.

O'Connor, K. H., Königs, C., Rowley, M. J., Irving, J. A., Wijeyewickrema, L. C., Pustowka, A., Dietrich, U., & Mackay, I.R., 2005. Requirement of multiple phage displayed peptide libraries for optimal mapping of a conformational antibody epitope on CCR5. *Journal of Immunological Methods*, 299:21-35.

Oldfield, S. J., Adachi, A., Urakawa, T., Hirasawa, T., & Roy, P., 1990. Purification and characterization of the major group-specific core antigen VP7 of bluetongue virus synthesized by a recombinant baculovirus. *Journal of General Virology*, 71:2649-2656.

Parbosky, L. R., Dunn, K. E., Gibbs, C. S., & Dougherty, J. P., 1996. A nickel chelate microtiter plate assay for six histidine-containing proteins. *Analytical Biochemistry*, 234:60-65.

Park, S. G., Lee, J. S., Je, E. Y, Kim, I. J., Chung, J. H., & Choi, I. H., 2000. Affinity maturation of natural antibody using a chain shuffling technique and the expression of recombinant antibodies in *Escherichia coli*. *Biochemical and Biophysics Research Communication*, 275:553-557.

Parmley, S. F., & Smith, G. P., 1988. Antibody selectable filamentous fd phage vectors: affinity purification of target genes. *Gene*, 73:305-318.

Parvari, R., Avivi, A., Lentner, F., Ziv, E., Tel-Or, S., Burstein, Y., & Schechter, I., 1988. Chicken immunoglobulin γ-heavy chains: limited VH gene repertoire, combinatorial diversification by D gene segments and evolution of the heavy chain locus. *The EMBO Journal*, 7:739-744.

Perry, L. J., & Wetzel, R., 1986. Unpaired cysteine-54 interferes with the ability of an engineered disulfide to stabilize T4 lysozyme. *Biochemistry*, 25:733-739.

Petersen, G., Song, D., Hügle-Dörr, B., Oldenburg, I., & Bautz, E. K. F., 1995, Mapping of linear epitopes recognised by monoclonal antibodies with gene-fragment phage display libraries, *Molecular General Genetics*, 249:425-431.

Petrenko, V. A., 2008. Landscape phage as a molecular recognition interface for detection devices. *Microelectronics Journal*, 39:202-207.

Pigiet, V. P., & Schuster, B. J., 1986. Thioredoxin-catalyzed refolding of disulphide-containing proteins. *Proceedings of the National Academy of Sciences*. USA 83:7643-7647.

Porter, R. R., 1959. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. *The Biochemical Journal*, 73:119-126.

Price, D. A., & Hardy, W. T., 1954. Isolation of bluetongue virus from Texas sheep: Culicoides shown to be a vector. *Journal of the American Veterinary Medical Association*, 124:255-258

Qian, W., Yao, D., Yu, F., Xu, B., Zhou, R., Bao, X., & Zuhong L., 2000. Immobilization of antibodies on ultraflat polystyrene surfaces. *Clinical Chemistry*, 46:1456-1463.

Qing, G., Ma, L. C., Khorchid, A., Swapna, G. V., Mal, T. K., Takayama, M. M., Xia, B., Phadtare, S., Ke, H., Acton, T., Montelione, G. T., Ikura, M., & Inouye, M., 2004. Cold-shock induced high-yield protein production in *Escherichia coli*. *Nature Biotechnology*, 22:887-882.

Reddington, J. J., Reddington, G. M., & MacLachlan, N. J., 1991. A competitive ELISA for detection of antibodies to the group antigen of bluetongue virus. *Journal of Veterinary Diagnostic Investigation*, 3:144-147.

Reynaud, C-A., Anquez, V., Dahan, A., & Weill, J-C., 1985. A single rearrangement event generates most of the chicken immunoglobulin light chain diversity. *Cell*, 40:283-291.

Reynaud, C-A., Anquez, V., Grimal, H., & Weill, J-C., 1987. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell*, 48:379-388.

Reynaud, C-A., Dahan, A., Anquez, V., & Weill, J-C., 1989. Somatic hyperconversion diversifies the single V_H gene of the chicken with a high incidence in the D region. *Cell*, 59:171-183.

Ridder, R., Schmitz, R., Legay, F., & Gram, H., 1995. Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast Pichia pastoris. *Biotechnology*, 13:255-260.

Rinas, U., Tsai, L. B., Lyons, D., Fox, G. M., Stearns G., Fieschko, J., Fenton, D., & Bailey, J. E., 1992. Cysteine to serine substitutions in basic fibroblast growth factor: effect on inclusion body formation and proteolytic susceptibility during *in vitro* refolding. *BioTechnology*, 10:435-440.

Roy, P., 1989. Bluetongue virus genetics and genome structure. *Virus Research*, 13:79-206.

Rudolph, R., & Lilie, H., 1996. In vitro folding of inclusion body proteins. *The FASEB Journal*, 10:49-54.

Russel, M., 1991. Filamentous phage assembly. *Molecular Microbiology*, 5:1607-1613.

Sapats, S. I., Trinidad, L., Gould, G., Heine, H. G., Van Den Berg, T. P., Eterradossi, N., Jackwood, D., Parede, L., Toquin, D., & Ignjatovic, J., 2006. Chicken recombinant antibodies specific for every virulent infectious bursal disease virus. *Archives of Virology*, 151:1551-1566.

Sapats, S. I., Heine, H. G., Trinidad, L., Gould, G., Foord, A. J., Doolan, S. G., Prowse, S., & Ignjatovic, J., 2003. Generation of chicken single chain antibody variable fragments (scFv) that differentiate and neutralize infectious bursal disease virus (IBDV). *Archives of Virology*, 148:497-515.

Scott, J. L., 1998 ed., Part IV: Bluetongue and Epizootic Hemorrhagic Disease, Foreign Animal Diseases “The Gray Book”.

Scott, J. K., & Smith, G. P., 1990. Searching for peptide ligands with an epitope display. *Science*, 249:386-390.

Sellers, R. F., 1984. Bluetongue in Africa, the Mediterranean region and near East-disease, virus and vectors. *Preventive Veterinary Medicine,* 2:371-378.

Sheets, M., D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J. C., & Marks, J. D., 1998. Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human

single-chain antibodies to protect antigens. *Proceedings of the National Academy of Sciences*, USA 95:6157-6162.

Shimamoto, T., Nishibori, N., Aosasa, M., Horiuchi, H., FururSawa, S., & Matsuda H., 2005. Stable production of recombinant chicken antibody in CHO-K1 cell line. *Biologicals*, 33:169-174.

Shirano, Y., & Shibata, D., 1990. Low temperature cultivation of *Escherichia coli* carrying a rice lipoxygenase L-2 cDNA produces a soluble and active enzyme at a high level. *FEBS*, 271:128-130.

Smith, G. P., 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens of the virion surface. *Science*, 228:1315-1317.

Smith, G. P., 1992. Cloning in fUSE vectors. February 10, 1992 ed. Division of Biological Sciences, University of Missouri, CO.

Sørensen, H. P., & Mortensen, K. K., 2005. Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli. *Microbial cell factories*, 4:1.

Spieker-Polet, H., Sethupathi, P., Yam, P-C., & Knight, K. L., 1995. Rabbit monoclonal antibodies: generating a fusion partner to produce rabbit-rabbit hybridomas. *Proceedings of the National Academy of Sciences*, 92:9348-9352.

Spreull, J., 1905. Malarial Catarrhal Fever (Bluetongue) of sheep in South Africa, *Journal of Comparative Pathology*, 18:321-337.

Stanley, K. K., & Herz, J., 1987. Topological mapping of compliment component C9 by recombinant DNA techniques suggest a novel mechanism for its insertion into target membranes. *The EMBO Journal*, 6:1951-1957.

Stewart, E. J., Åslund, F., & Beckwith, J., 1998. Disulfide bond formation in the *Escherichia coli* cytoplasm: an *in vivo* reversal fro the thioredoxins. *The EMBO Journal*, 17:5543-5550.

Studdert, M. J., Pangborn, J., & Addison, R. B., 1966. Bluetongue virus structure. *Virology*, 29:509-511.

Tan, G. H., Yusoff, K., Seow, H. F., & Tan W. S., 2007. A phage-displayed single chain variable fragment that interacts with hepatitis B core antigen: library construction, selection and diagnosis. *Journal of Clinical Virology*, 38:49-56.

Tanha, J., Dubuc, G., Hirama, T., Narang, S. A., & MacKenzie, C. R., 2002. Selection of phage display of llama conventional V_H fragments with heavy chain antibody V_HH properties. *Journal of Immunological Methods*, 263:97-109.

Theiler, A., 1906. Bluetongue in sheep. Annual Report of the Director of Agriculture, Transvaal for 1904-1905, 110-121.

Thomas, J. G., & Baneyx, F., 1996. Protein misfolding and inclusion body formation in recombinant *Escherichia coli* cells overexpressing heat-shock proteins. *The Journal of Biological Chemistry*, 271:11141-11147.

Thompson, C. B., & Neiman, P. E., 1987. Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. *Cell*, 48:369-378.

Tsurushita, N., Park, M., Pakabunto, K., Ong, K., Avdalovic, A., Fu, H., Jia, A., Vásquez, M., & Kumar, S., 2004. Humanization of a chicken anti-IL-12 monoclonal antibody. *Journal of Immunological Methods*, 295:9-19.

Van den Berg, B., Ellis, R. J., & Dobson, C. M., 1999. Effects of macromolecular crowding on protein folding and aggregation. *The EMBO Journal*, 18:6927-6933.

Van Dijk, A. A., 1993. Development of recombinant vaccines against bluetongue. *Biotechnology Advances*, 11:1-12.

Van Regenmortel, M. H. V., 1989. Structural and functional approaches to the study of protein antigenicity. *Immunology Today*, 10:266-171.

Van Wyngaardt, W., & Du Plessis, D. H., 1998, Selection of an scFv phage antibody that recognizes bluetongue virus from a large synthetic library and its use in ELISAs to detect viral antigen and antibodies. *Onderstepoort Journal of Veterinary Research*, 65:125-131.

Van Wyngaardt, W., Malatji, T., Mashau, C., Fehrsen, J., Jordaan, F., Miltiadou, D., & Du Plessis, D. H., 2004. A large semi-synthetic single-chain Fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnology*, 4:6.

Vasina, J. A., & Baneyx, F., 1997. Expression of aggregation-prone recombinant proteins at low temperatures: a comparative study of the *Escherichia coli cspA* and *tac* promoter systems. *Protein Expression and purification*, 9:211-218.

Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J., & Johnson, K. S., 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunised phage display library. *Nature Biotechnology*, 14:309-314.

Verma, R., **Boleti, E., & George, A. J. T.,** 1998. Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. *Journal of Immunological Methods*, 216:165-181.

Verwoerd, D. W., 1970. Diplornaviruses: A newly recognized group of double-stranded RNA viruses. *Progress in Medical Virology*, 12:192-210.

Verwoerd, D. W., Louw, H., & Oellermann, R. A., 1970a. Characterization of bluetongue virus ribonucleic acid. *Journal of Virology*, 5:1-7.

Verwoerd, D. W., Els, H. J., De Villiers, E. M., & Huismans, H., 1972. Structure of the bluetongue virus capsid. *Journal of Virology*, 10:783-794.

Verwoerd, D. W., & Erasmus, B. J., 1994. Bluetongue. In: Coetzer, J. A. W., Thomson, G.R., Tustin, R. C., eds. Infectious Diseases of Livestock with Special Reference to Southern Africa: Cape Town, Oxford University Press, 443-459.

Verwoerd, D. W., & Erasmus, B. J., 2004. Bluetongue. In: Coetzer, J. A. W., Tustin, R. C., eds. Infectious Diseases of Livestock, 2nd ed, Cape Town: South Africa, Oxford University Press, 2:1201-1220.

Wang, L-F., Du Plessis, D. H., White, J. R., Hyatt, A. D. & Eaton, B. T., 1995. Use of gene-targeted phage display random epitope library to map an antigenic determinant on the bluetongue virus outer capsid protein VP5. *Journal of Immunological Methods*, 178:1-12.

Wang, L-F., Kattenbelt, J. A., Gould, A. R., Pritchard, L. I., Crameri, G. S., & Eaton, B. T., 1994. Major core protein VP7 of Australian bluetongue virus serotype 15: sequence and antigenicity divergence from other BTV serotypes. *Journal of General Virology*, 75:2421-2425.

Wang, X., Campoli, M., Ko, E., Luo, W., & Ferrone, S., 2004. Enhancement of scFv fragment reactivity with target antigens in binding assays following mixing with anti-tag monoclonal antibodies. *Journal of Immunological Methods*, 294:23-25.

Webster, R. E., 1996. Biology of the filamentous bacteriophages. In: Kay, B. K., Winter, J., & McCafferty, J., eds. Phage display of peptides and proteins, A laboratory manual: United States, California, Academic Press, Inc., 1-16.

Wieland, W. H., Orzáez, D., Lammers, A., Parmentier, H. K., & Schots, A., 2006. Display and selection of chicken IgA Fab fragments. *Veterinary Immunology and Immunopathology,* 110:129-140.

Williams, D. C., Van Frank, R. M., Muth, W. L., & Burnett, J. P., 1982. Cytoplasmic inclusion bodies in *Escherichia coli* producing biosynthetic human insulin proteins. *Science*, 215:687-688.

Wilson, D. R., & Finlay, B. B., 1998. Phage display: applications, innovations, and issues in phage and host biology. *Canadian Journal of Microbiology*, 44:313-329.

Winter, G., Griffiths, A. D., Hawkins, R. E., & Hoogenboom, H. R., 1994. Making antibodies by phage display technology. *Annual Review of Immunology*, 12:433-55.

Wörn, A., & Plückthun, A., 2001. Stability engineering of antibody single-chain Fv fragments. *Journal of Molecular Biology*, 305:989-1010.

Xiong, S., Wang, Y. F., Ren, X. R., Li, B., Zhang, M. Y., Luo, Y., Zhang, L., Xie, Q. L., & Su, K. Y., 2005. Solubility of disulfide-bonded proteins in the cytoplasm of *Escherichia coli* and its "oxidizing" mutant. *World Journal of Gastroenterology*, 11:1077-1082.

Xiong, S., Zhang, M.Y., Qian, C. W., Ran, Y. C., Wang, Y. F., Ren, X. R., Su, K. Y., & Yu, Z. Y., 2003. Influence of the reductase deficient *Escherichia coli* on the solubility of recombinant proteins produced in it. *Sheng Wu Gong Cheng Xue Bao*, 19:686-691.

Yamanaka, H. I., Inoue, T., & Ikeda-Tanaka, O., 1996. Chicken monoclonal antibody isolated by a phage display system. *The Journal of Immunology*, 157:1156-1162.

Yang, M., Berhane, Y., Salo, T., Li, M., Hole, K., & Clavijo, A., 2008. Development and application of monoclonal antibodies against avian influenza virus nucleoprotein. *Journal of Virological Methods*, 147:265-274.

Yu, Y., Fukusho, A., Ritter, D. G., & Roy, P., 1988. Complete sequence of the group-reactive antigen VP7 gene of bluetongue virus. *Nucleic Acids Research,* 16:1620.

Zhou, Y-H., Chen, Z., Purcell, R. H., & Emerson, S. U., 2007. Positive reactions on western blots do not necessarily indicate the epitopes on antigens are continuous. *Immunology and Cell Biology*, 85:73-78.

Other references

Institute of Molecular and Cell Biology, Accessed on 18 January 2008, website: http://www.imcb.a-star.edu.sg/antibodiestalk/DLtalks.pdf.

University of Carlifonia, Microbiology and Immunology, Accessed on 15 July 2008, website: http://www.pathmicro.med.sc.edu/mayer/stru-4jpg.