PRELIMINARY STUDIES ON THE IDENTIFICATION OF VACCINAL MIMOTOPES OF BOVINE VIRAL DIARRHOEA VIRUS USING COMBINATORIAL PEPTIDE LIBRARIES

A thesis submitted to the University of London

For the Degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I certify that:

i) The thesis being submitted for examination is my own account of my own research;
ii) My research has been conducted ethically
iii) The data and results presented here are the genuine data and results actually obtained by me during the conduct of the research;
iv) Where I have drawn on the work, ideas and result of others this has been appropriately acknowledged in the thesis;
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vi) The greater portion of the work described in the thesis has been undertaken subsequent to my registration for the higher degree from which I am submitting for examination;
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Abstract of Thesis

Title of thesis:

Preliminary studies on the identification of vaccinal mimotopes of bovine viral diarrhoea virus using combinatorial peptide libraries

Bovine Viral Diarrhoea Virus (BVDV) can cause acute fatal hemorrhagic disease and fatal mucosal disease. Neutralising antibodies that are directed to the major envelope glycoprotein of BDVD E2 are the main protective mediators for BVDV infections. The aim of this study was to screen combinatorial peptide libraries with neutralising BVDV E2 monoclonal antibodies to facilitate the development of a mimotope-based marker vaccine against BVDV.

In the first part of the present study, a range of neutralising BVDV E2 monoclonal antibodies (Wb166, Wb214, Wb163, and Wb158) was used to select binding ligands (peptides) by using a 15-mer phage-display library. Four peptides (A1-1, A2-1, A2-2, and A2-3) were selected with the mAbWb166. However, they were not considered to be mimotopes because they did not compete with BVDV by a competitive-inhibition ELISA and a neutralisation-inhibition test with the selecting mAb. An 8-mer solid phase library was then screened to select binding ligands to neutralising mAbs (Wb166 and Wb214). The peptide Wb166-P1 selected by the mAbWb166 showed a high sequence homology to the BVDV E2, whereas peptides selected by the mAb Wb214 did not. Since testing the reactivity of these peptides in a SPOT scan assay did not confirm their binding to the selector mAbs and the first peptide Wb214-P1 failed to induce cross-reactive antibodies to BVDV in mice, the planned immunisation of mice with more peptides was cancelled.

The neutralizing mAb Wb166 used for mimotope selection with both a 15-mer phage-display and an 8-mer solid phase libraries had high relative affinity to the BVDV. This ~~was~~ generated the idea that high antibody affinity could be problematic for mimotope selection in using phage libraries. In order to validate our methods and to show the effect of antibody affinity on mimotope selection, a low and a high affinity 2-4 Dinitrophenol (DNP) mAbs (DNP Pα47 and DNP Pα41) were tested by a range of linear and constrained types of phage-display libraries. The low affinity DNP mAb

DNP Pα47 selected bound peptides on phage with each library whereas the high affinity mAb gave no result.

The most antigenic 8-mer regions of two 15-mer potential mimotopes of DNP, DA15-1 and DA15-2, were mapped to either amino or to the carboxyl terminus using a SPOT scan assay. This implies that both flexible ends of mimotopes can be antigenic when displayed on phage. Thus, combinatorial libraries and a SPOT scan ELISA can be used for the selection of the immune reactive regions of mimotopes.

A new BVDV E2 mAb157 that neutralised to a wide range of BVDV-1 isolates was then successfully used to select two 8-mer peptides from an 8-mer solid phase library. The reactivity of two 8-mer peptides (157A1 and 157A2) was then confirmed by a SPOT scan assay. Peptides selected by the mAbs (Wb166 and Wb214) had not bound to their selector mAbs in a similar assay (see above). Future work with the second set of 8-mer peptides 157A1 and 157A2 including competition assays and immunisation studies in mice was not possible within the time of this work.

This study highlighted the importance of using a large range of combinatorial peptide libraries to select discontinuous neutralising epitopes on the E2 of BVDV. Novel aspects of this study were the use of phage-display peptide libraries to select DNP-specific mimotopes from low affinity DNP mAb and the use of an 8-mer solid phase library for selecting potential mimotopes of BVDV E2. In addition, the applicability of a SPOT scan assay for confirmation of the reactivity of the potential mimotopes was also shown.

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LIST OF ABBREVIATIONS

%	Percentage
μ	Mikron
μl	Microlitre
μg	Microgram
A	Alanine
Ag	Antigen
AI	Affinity index
ASD	Average structural Dissimilarity
ATCC	American type culture collection
B-cell	B lymphocyte cells
BDV	Border disease Virus
Bp	Base pair
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
C	Core protein
CBRs	Critical binding residues
CO_2	Carbon dioxide
CI	Confidence interval
Cp	Cytopathic
cpe	Cytopathic effect
CSFV	Classical swine fever virus
C	Cysteine
CTL	Cytotoxic T-lymphocyte
D	Aspartic acid
DMF	N, N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	2-4 dinitrophenol
dsRNA	Double stranded RNA
E^{rns}	Envelope protein with RNase activity
E	Glutamic acid
E2	The major immunodominant glycoprotein
EDTA	Ethylenediaminotetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FCA	Freund's complete adjuvant
F	Phenylalanine
FCS	Foetal calf serum
FCI	Freund's complete adjuvant
FITC	Fluorescein Isothiocynate
FIV	Feline immunodeficiency virus
FMDV	Foot and mouth disease Virus
G	Glycine
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCL	Hydrochloric acid
HCV	Hepatitis c virus
H_2O_2	Hydrogen peroxide

H_2SO_4	Sulphuric acid
H	Histidine
HPLC	High performance liquid chromatography
HIV	Human immunodeficiency virus
HVR	Hypervariable region
I	Isoleucine
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IgG	Immunoglobulin G
(HGV/GBV-C)	Hepatitis G virus/GB- virus C
IBR	Infectious rhinotracheitis virus
IPTG	Isopropyl-thio-β-D-galactosidase
K	Lysine
Kb	Kilobases
kDa	Kilo Dalton
KLH	Keyhole lympet hemocyanin
L or l	Litre
L	Leucine
LB	Luria Bertani
M	Molar
M	Methionine
mAb	monoclonal antibody
MDBK	Madin-Darby-Bovine kidney
Mg	Milligram
Min	Minute
ml	Milliliter
m.o.i	Multiplicity of infection
MV	Measles virus
MW	Molecular weight
mRNA	Messenger RNA
N	Asparagine
NaCl	Sodium chloride
NADL	National animal disease laboratory
$NaHCO_3$	Sodium hydrogen carbonate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
Ncp	non-cytopathic
No.	Number
nm	Nanometer
NPS	Normal pig serum
NMP	1-Methyl 2-pyrrolidinone
NMS	Normal mouse sera
NS	Non-structural protein
NTR	Non-translated region
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PFU	Plaque forming unit
Poly A	Poly tail adenosine
Poly U	Poly tail üridine
P	Proline

PRP	Pseudorabies virus
Q	Glutamine
R	Arginine
RF	Replicative form
Rpm	rate per minute
RNA	Ribonucleic acid
S	Serine
SD	Standard deviation
SDS-PAGE	Sodium doecyl sulphate polycrylamide
SDW	Sterile distilled water
YFV	Yellow Fewer Virus
T	Threonine
TFA	Trifluoroacetic acid
TBS	Tris buffer saline
T-cell	T-lymphocyte cells
$TCID_{50}$	50% Tissue culture infectious dose
TFA	Trifluoraacetic acid
Th-cell	T lymphocyte helper cells
TU	Transducing unit
UK	United Kingdom
USA	United States of America
V	Valine
Y	Tyrosine

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To Mustafa Kemal Atatürk

Peace at home and peace at the world

The founder of Turkish Republic

To my family

Chapter 1. General Introduction

In this chapter, BVDV vaccination and the necessity for the development of a better vaccine will be discussed and the aims and objectives of the thesis on the development of a mimotope-based vaccine will be given.

1.1 Bovine Viral Diarrhoea virus (BVDV) infections

Today Bovine Viral Diarrhoea virus (BVDV) is considered to be one of the most important pathogens of cattle that cause significant economic losses worldwide (Baker, 1995; Nettleton and Entrican, 1995; Hou, 1999). Two genotypes of BVDV have been recognised (BVDV-1 and BVDV-2) based on serological and genetic relatedness (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994; Deregt and Loewen, 1995; Ridpath *et al.*, 2000). Both BVDV-1 and BVDV-2 viruses can exhibit two different biotypes namely, non-cytopathic (ncp) and cytopathic (cp), according to their lytic effect in infected cells. The ncp BVDV is the most common in nature (Baker, 1987; Meyers and Thiel, 1996; Ridpath *et al.*, 2000).

Both genotypes of BVDV cause a similar range of disease manifestations. These include subclinical or mild infections, enteritis, immunosuppression, foetal infections (reproductive disorders, abortions, congenital defects etc), and persistent infections, haemorrhagic and systemic diseases such as fatal mucosal disease (MD) (Baker 1995; Moening and Palegeman, 1992; Bolin, 1995a; Fritzemeier *et al.*, 1997; Fulton *et al.*, 2003; Brock, 2003). More than 90% of all BVDV infections are caused by ncp BVDV, and all the devastating syndromes including the acute hemorrhagic disease and persistent infections are the result of ncp BVDV infections (Meyers and Thiel, 1996). In addition, certain ncp strains of the BVDV-2 are associated with outbreaks of severe acute haemorrhagic syndrome (HS) characterised by high morbidity and high mortalities were first reported in North America, Canada (Pellerin *et al.*, 1994; Ridpath *at al.*, 1994) and recently also detected in Japan and Brazil (Nagai *et al.*, 2001; Flores *et al.*, 2002) also in Europe (Vilcek *et al.*, 2002; Evermann and Ridpath, 2002; Wakeley *et al.*, 2004). A severe outbreak of BVDV-2 infection was reported in Holland in association with the use of contaminated live bovine herpes virus type I (BHV-1) vaccine against bovine infectious rhinotracheitis (Barkema *et al.*, 2001).

The ncp BVDV infections have the biggest impact if they occur in breeding season or during the pregnancy. The most important outcome of this is the birth of persistently infected (PI) calves. The PI animals that survive are immunotolerant to persisting virus, shed a large amount of virus, and are the key to the spread of BVDV (Brownlie. 1990a, b; Brownlie, 1991; Lindberg, 2003). The ncp BVDV as the parent virus from which cp BVDV arise following homologous or heterologous recombination in the genomic region encoding the NS2-3 nonstructural protein in ncp BVDV RNA in PI animals (Meyers and Thiel, 1996). The recombination event results in the cleavage of large NS2-3 protein of ncp virus into two smaller proteins namely, NS2 and NS3 **(Figure 1.2).** The NS3 protein is considered to be a molecular marker for cp BVDV and causes lytic effect in cells. These PI animals later develop a deadly MD by superinfection with homologous ncp BVDV (Tautz *et al.*, 1998; Tautz and Thiel, 2003). Thus, the rational of any control measure should be the prevention of intrauterine infection by identifying and eliminating the PI animals and stopping the infection of foetus. The latter can be achieved by immunising animals before pregnancy.

A number of European countries including, Denmark, Sweden, Norway, Slovenia and Shetland have been undertaking BVDV eradication programmes that are based on the identification and the elimination of PI animals (Greiser-Wilke *et al.*, 2003; Lindberg 2003; Sandvik, 2004). Although the identification and elimination of PI animals is the most ideal, vaccination is still the essential control strategy in most countries

1.2. Classification and diversity of BVDV

1.2.1. Classification of BVDV

BVDV is a member of the genus Pestivirus in the family *Flaviviridae*. The virus family contains three genera: *Pestivirus, Flavivirus,* and *Hepacivirus*. *Hepatitis G virus/ GB-virus C* (HGV/ GBV-C) is classified within the family *Flaviviridae* but has not been assigned to a genus **(Figure 1.1)**. The genus *pestivirus* official nomenclature currently includes the four accepted species namely; *Bovine viral diarrhoea virus-1* (BVDV-1), BVDV-2, *Classical swine fever virus* (CSFV) and *Ovine Border disease virus* (BDV) and one tentative fifth species isolated from a giraffe (Heinz *et al.*, 2000; Becher *et al.*, 2003). BVDV-1, BVDV-2, and BDV are able to cross species barriers to infect a wide range of hosts within the *Artiodactyla* (Becher *et al.*, 1999). However, the natural host range of CSFV is restricted to domestic pigs and wild boars.

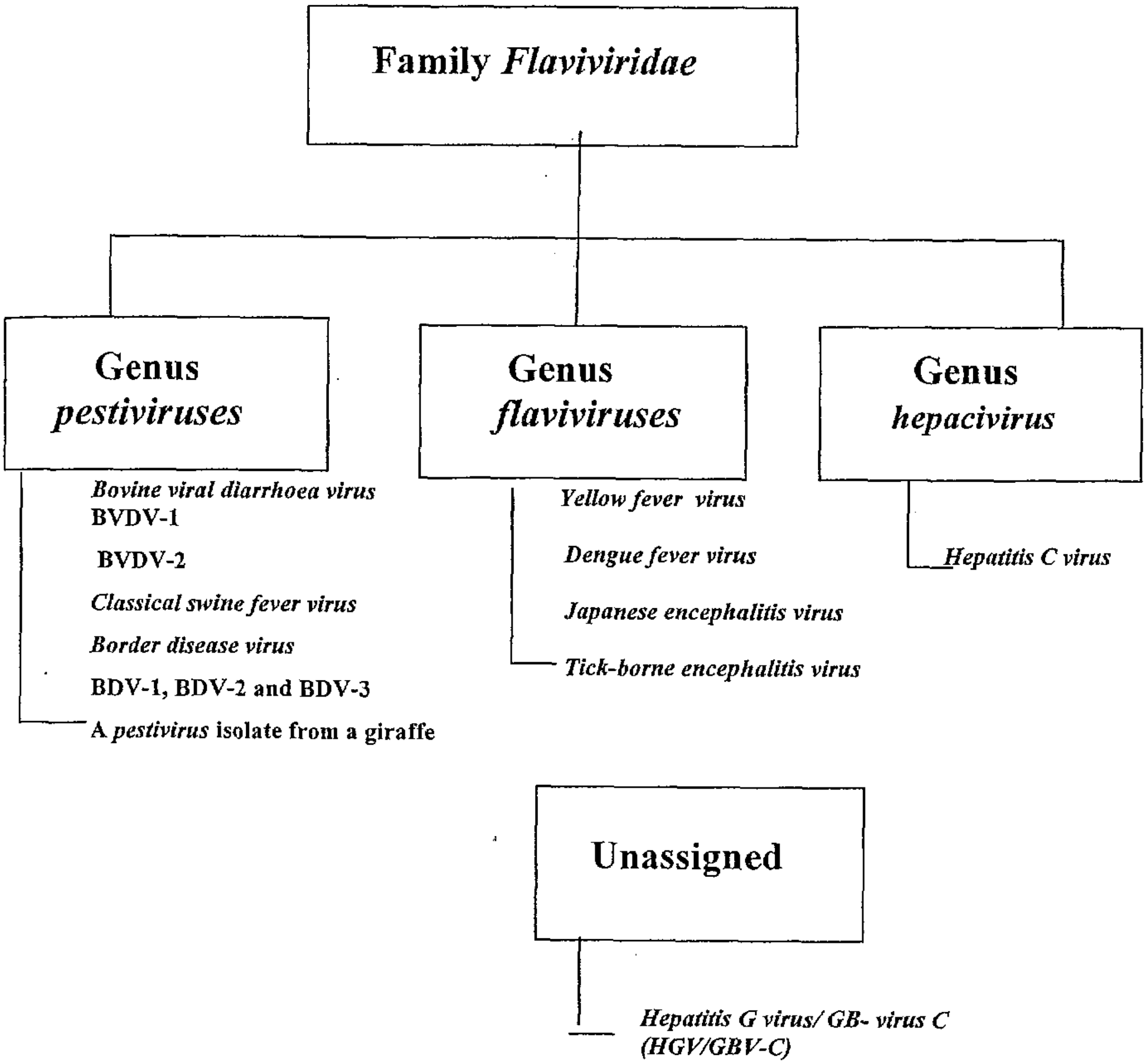


Figure 1.1. Schematic representation of BVDV classification within the family of *Flaviviridae*

As with other members of the family *Flaviviridae*, the BVDV genome consists of a positive sense single-stranded (ss) nonpolyadenylated RNA molecule of approximately 12.5 kb. It contains one large open reading frame (ORF) that is flanked by 5'and 3' nontranslated regions (NTRs) (Collett *et al.*, 1988; Deng and Brock, 1992; Collett, 1992; Meyers and Thiel, 1996). BVDV virions are enveloped and spherical in shape approximately 40-60 nm in diameter (Rice, 1996). In the virus encoded-polyprotein, the viral protein cleavage products in BVDV are arranged in the following order (from the N to the C terminus): N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3 (NS2, NS3), NS4A, NS4B, NS5A and NS5B **(Figure 1.2)** (Meyers and Thiel, 1996). The abbreviation N^{pro} refers to an N-terminal autoprotease and E^{rns} (ribonuclease soluble) refers to a structural protein with ribonuclease activity. In addition to the RNA genome and lipid blayers, BVDV virions are composed of four structural proteins, namely a basic nucleocapsid protein C (p14), and envelope glycoproteins, E^{rns} (gp48), E1 (gp25), and E2 (gp53) (Meyers and Thiel, 1996).

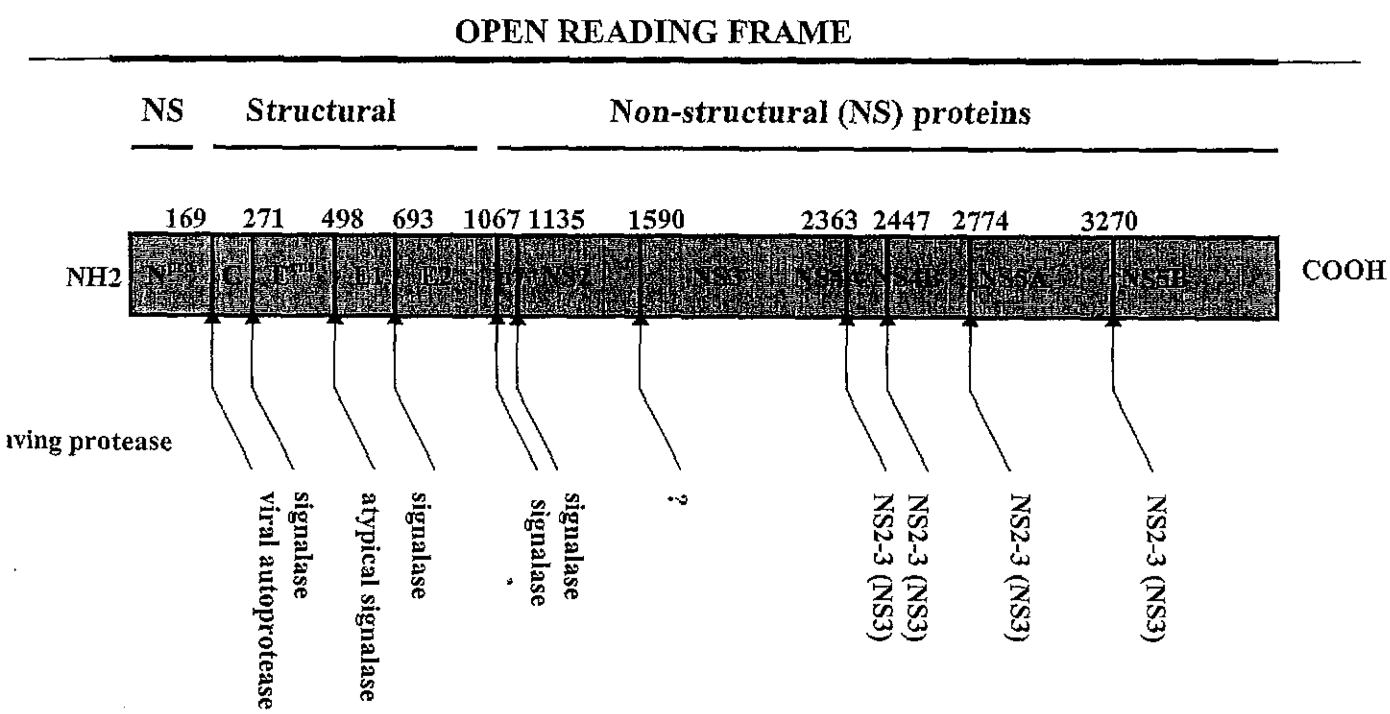


Figure 1.2. Genome organisation of Bovine Viral Diarrhoea Virus (NADL strain)

Structural proteins are located towards the 5' end of the genome whilst the 3'end codes for the non-structural proteins (apart from the first protein N^{pro} is non-structural). N the case of non-cytophatic BVDV NS2-3 is not cleaved whereas in cytopathic BVDV both NS2 and NS3 are produced (Collett, 1992; Rumenaph *et al.*, 1993; Elbers *et al.*, 1996; Mendez *et al.*, 1998; Tautz *et al.*, 1998; Kümmerer *et al.*, 2000).

1.2.2. BVDV diversity

One of the most important features of BVDV is the nature of the genetic and antigenic diversity of BVDV strains (Paton, 1995; Hamers *et al.*, 2001; Fulton *et al.*, 2003). Different regions of pestiviral genome have been used to search for genetic diversity of BVDV including variable fragment of the 5'NTR (Ridpath *et al.*, 1994; Paton, 1995; Vilcek *et al.*, 2003), N^{pro} and E2 (Becher *et al.*, 1999; Vilcek *et al.*, 2001). The homology between the sequences in the 5'NTR of BVDV-1 and BVDV-2 isolates is approximately 75% (Donis, 1995). The nucleotide and amino acid sequences of E2 of BVDV-2 viruses have 60% homology with BVDV-1 (NADL) but 90 % homology with a subgroup of sheep border disease viruses (BDV) (Tijsen *et al.*, 1995; Van Rijn *et al.*, 1997).

Genetic typing of pestiviruses based on N^{pro} and E2 regions revealed the presence of five different genetic groups or subgenotypes for BVDV-1 (1a-1e) (Becher *et al.*, 1999). BVDV-1a and BVDV-1b include the majority of classical strains such as NADL, SD-1, Oregon (C24V), Singer, Osloss and NY1 (Becher *et al.*, 1999; Giangaspero *et al.*, 2001). More recent extensive genetic studies of BVDV-1 isolates obtained from Europe and other countries around the world carried out by Vilcek *et al.*, (2001; 2004) indicated at least 11 genetic groups within the BVDV-1 (BVDV-1a to BVDV-1k).

Despite the high degree of heterogenicity of BVDV-1 within the genotype level, it has been suggested that subgenotypes are tend to be herd specific and different subgenotypes predominating different localities (Couvreur *et al.*, 2002; Vilcek *et al.*, 2003; Lindberg, 2003). For instance, while in Austria five subgenotypes of BVDV-1 (b, d, f, g, h) circulate and the subgenotype BVDV-1f is predominant, in Germany subgenotypes BVDV-1b and BVDV-1d are predominant (Vilcek *et al.*, 2003). The BVDV-1a is predominant in UK (Vilcek *et al.*, 2001) and widely distributed in the USA (Ridpath *et al.*, 1994) and Canada (Pellerin *et al.*, 1994) but is rare or absent in countries of continental Europe. BVDV-1b and BVDV-2 are prevalent in continental Europe and in the USA (Vilcek *et al.*, 1999; Couvreur *et al.*, 2003). Genetic typing of BVDV isolates in Slovakia indicated that subgenotypes BVDV-1d and BVDV-1f were predominant (Toplak *et al.*, 2004). BVDV isolates collected in India were typed as BVDV-1b (Mishra *et al.*, 2004) and predominant subgenotype in Australia is BVDV-1c

(Mahony *et al.*, 2005). It can be assumed that certain subtype(s) of BVDV-1 strains predominate in a particular region or environment unless new isolates are introduced possibly by the use of modified-live vaccines or the trade of animals from other regions (Vilcek *et al.*, 2003: Lindberg, 2003).

BVDV-2 isolates can be subdivided in to four subtypes namely; BVDV-2a, BVDV-2b, BVDV-2c and BVDV-2d based on palindromic nucleotide substitutions at V1, V2 and V3 variable loci at the 5'NTRs and pylogenetic analyses. Subtypes BVDV-2a and BVDV-2c have been isolated only from cattle. Subtype BVDV-2b have been isolated from both sheep and cattle. Subtype BVDV-2d has been isolated only from sheep. Subtypes BVDV-2a and BVDV-2b included both low and high virulent isolates, but subtypes BVDV-2c and BVDV-2d represented only high virulent isolates (Giangaspero *et al.*, 2001: Giangaspero and Harasawa, 2004).

Genetic typing of European pestiviruses has revealed that BVDV-2 is much less prevalent than BVDV-1 (Vilcek *et al.*, 2001; Vilcek *et al.*, 2003). In UK, a low virulent isolate of BVDV-2 was detected in cattle in a few farms (Wakeley *et al.*, 2004). BVDV-2 has also been sporadically detected in several European countries including Belgium (Couvreur *et al.*, 2003), France (Vilcek *et al.*, 2001), Italy (Pratelli *et al.*, 2001: Giangaspero *et al.*, 2001), Germany (Wolfmeyer *et al.*, 1997), Slovakia (Vilcek *et al.*, 2002), Austria (Vilcek *et al.*, 2003).

The genetic diversity between BVDV-1 and BVDV-2 also correlates with the extensive antigenic differences of the E2 glycoprotein resulting in contributing a distant antigenic group based on neutralisation susceptible epitopes (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994; Pottigeter, 1995; Ridpath, 2003). This is an important concern for the protective efficacy of current vaccines and the development of new generation of vaccines (see section 1.4.2).

1.3. The immune response to BVDV

The immune response to BVDV is generated by its envelope glycoproteins (E^{rns}, E1, and E2) and non-structural proteins (NS2-3 and NS3) of BVDV following natural infections or live-attenuated vaccines in cattle. BVDV induces both B-cell and T-cell responses (Bolin, 1993; Potgieter, 1995; Beer *et al.*, 1997; Chase *et al.*, 2004).

1.3.1. BVDV humoral immunity

The BVDV major glycosylated envelope glycoprotein E2 is the main target for inducing neutralising antibodies following natural infections and or immunisation with live-attenuated and inactivated vaccines and its precipitation by bovine serum correlates with the detection of neutralising antibody (Bolin and Ridpath, 1990; Bolin, 1993; Toth *et al.*, 1999). However, it is not considered to be a major T-cell antigen (Kimman *et al.*, 1993).

The E^{rns} glycoprotein is a highly glycoslated, and induces significant levels of antibodies with limited neutralising activity in animals (Weiland *et al.*, 1992; Donis, 1995). Following natural infections or vaccination with live-attenuated or inactivated BVDV vaccines elicit antibodies in calves to E^{rns} and E1 that can be detected by immunoprecipitation. Glycoprotein E1 is found in virions covalently linked to E2 by disulphide bonds. Antibody responses to E1 are lower than those to E2 (Chase *et al.*, 2004).

The non-structural NS2-3 / NS3 protein is the immunodominant antigen and responsible for the production of high titer non-neutralising antibodies during natural BVDV infections and vaccination with live-attenuated vaccines in cattle (Bolin, 1993; Potgieter, 1995). Antibodies generated against BVDV NS2-3/ NS3 are cross-reactive with CSFV NS2-3 and similarly antibodies against CSFV NS2-3 cross-react with BVDV NS2-3. A similar situation also applies for BDV suggesting immune cross-reactivity. Some of the epitopes on NS3 are not conformation dependent and many of them are conserved among pestiviruses (Wenswoort *et al.*, 1989; Paton *et al.*, 1991b; Bolin *et al.*, 1993; Brown *et al.*, 2002). Because of its intracellular nature, cattle vaccinated with inactivated whole virus vaccines produce negligible antibody response against to this non-structural viral protein (Potgieter, 1995).

1.3.2. BVDV Cell-mediated immune response

The effect of BVDV on thymic and follicular T-lymphocytes depends on the virulence of the virus and varies from a mild to severe lymphopenia (Bodersen *et al.*, 1999: Archambault *et al.*, 2000).

The depletion of bovine $CD4^+$ cells that resulted in increased period of virus shedding, whereas the depletion of $CD8^+$ did not (Howard *et al.*, 1992). Conversely, $CD8^+$ depletion increased the period of RSV shedding (Taylor *et al.*, 1995). Infections with either cp or ncp BVDV induce strong $CD4^+$ T-cell response that recognise epitopes on a range of structural and non-structural proteins including capsid protein (C), E2, N^{pro}, and NS3 (Collen and Morrison, 2000; Collen *et al.*, 2002).

The $CD4^+$ mediated T cell response occurs quicker with a cp BVDV infection than ncp BVDV infection (Collen and Morrison, 2000) and ncp viruses prefentially produce a $CD4^+$ mediated Th2 response giving rise to high level of antibody response in cattle (Rhodes *et al.*, 1999).

$CD8^+$ mediated cytotoxic T-cell response play a part in the immune response during acute BVDV infection. Proliferating $CD8^+$ cells produce IL-2 and IFN-γ indicating a Th1 type memory response in BVDV seropositive cattle. In addition, an MHC restricted cytotoxic activity of BVDV-restimulated lymphocytes in cattle immunised and infected with BVDV was demonstrated *in vitro* (Beer *et al.*, 1997).

1.3.3. Mechanism of protection for BVDV infections

Despite the involvement of cell-mediated immune responses in BVDV infections, systemic BVDV neutralising antibodies directed to E2 induced by following natural infections and vaccinations are still considered the main protective mediators for BVDV infections (Brownlie *et al.*, 1995; Bolin and Ridpath., 1996; van Oirchot, 1999). Such neutralising antibody mediated protection is suggested to be more effective against homologous challenge strains following vaccination (Bolin and Ridpath, 1996; Bruschke *et al.*, 1999).

Passive transfer of neutralising antibodies in colostrums can give protection against BVDV infections (Howard *et al.*, 1989; Ridpath *et al.*, 2003). For instance, Ridpath *et al.*, (2003) showed that calves fed by colostrums taken from cows immunised with an inactivated vaccine containing both BVDV-1 and BVDV-2 during their pregnancy were protected against challenge inoculation with a highly virulent BVDV-2 isolate (1373) as compared to calves fed by milk replacer that developed a high body temperature, diarrhoea, anorexia.

The protective mechanism of BVDV antigens can be more fairly understood by comparing analogous antigens of CSFV. Vaccination of pigs with a recombinant pseudorabies virus or vaccinia virus expressing the equivalent glycoprotein of CSFV-E2 induced a protective neutralising antibody response when animals were challenged with virulent CSFV (van Zijl *et al.*, 1991; Rumenaph *et al.*, 1991). In addition, vaccination with baculovirus expressed subunit vaccines containing CSFV-E2 (Hulst *et al.*, 1993; Moorman *et al.*, 2000) or even the separate antigenic units of E2 induced protective immunity in pigs against CSFV challenge (van Rijn *et al.*, 1996). From these data, it can be concluded that the immune response induced by only E2 of CSFV is sufficient for protection. Because of their close relationship, E2 of BVDV is the most likely to be a main mediator for protection and subject for the development of a novel vaccine (see section 1.4.3).

In addition to E2-mediated protection, the second equivalent glycoprotein E^{rns} of CSFV was shown to give protection against virulent virus challenge following delivery of live virus vector (Konig *et al.*, 1995). This may be suggestive for the possible involvement of cell-mediated immune response generated by E^{rns} because no neutralising antibodies were detected.

1.4. Control of BVDV infections through vaccination

Vaccines have been still primarily developed to prevent diseases as a result of infection (Ada, 2001). The main objective of vaccination against BVDV infections is to give cross protection against common subgenotypes of BVDV-1 (BVDV-1a and BVDV-1b) and BVDV-2 to prevent transplacental infections of foetus and thus prevent the establishment of further persistent infections (Brownlie *et al.*, 1995; Patel *et al.*, 2002;

Greiser-Wilke *et al.*, 2003). Since the emergence of virulent strains of BVDV-2 in Holland through a contaminated vaccine (Berkema *et al.*, 2001) and the existence of BVDV-2 isolates in several European countries including UK (Wakeley *et al.*, 2004), BVDV-2 may also become threat for Europe. Thus, it is also important to protect cattle against severe postnatal infections by BVDV-2.

1.4.1. BVDV vaccines: modified live and inactivated

There are both live-attenuated and inactivated BVDV vaccines (Bolin, 1995b; van Oirschot *et al.*, 1999; 2001; Kelling, 2004). Because of antigenic diversity of both BVDV-1 and BVDV-2, live attenuated and inactivated BVDV vaccines containing strains of both genotypes have been developed and now available (Kovacs *et al.*, 2003: Fairbanks *et al.*, 2004). The live vaccines are based on the attenuated strains and generally induce a broader and long lasting immune response than inactivated vaccines due to the involvement of cell-mediated immune response (Bolin, 1995; Van Oirschot, 1999; 2001). The inactivated BVDV vaccines are obviously safe and they are formulated in an adjuvant to induce adequate immunity (Howard *et al.*, 1994; Brownlie *et al.*, 1995). The duration of immunity induced by inactivated BVDV vaccines tend to be shorter and antibody response against different strains may not be adequate as compared to live vaccines, possibly due to a poor cell-mediated immune response (Potgieter, 1995; Bolin, 1995b).

1.4.2. Antigenic diversity and protective efficacy of BVDV vaccines

The main problem for BVDV vaccine development and vaccine efficacy is the existence of great antigenic diversity of BVDV. BVDV-1 strains were consistently shown to be antigenically distinct from BVDV-2 based on sero-neutralisation assays with polyclonal or monoclonal antibodies (Pellerin *et al.*, 1994; Nagai *et al.*, 1998; Dereget *et al.*, 1998a, b; Ridpath *et al.*, 2000). Crouvreur *et al.*, (2002) indicated that both genotypes of BVDV are consistent with sero-neutralisation groups: strains from one genotype are only weakly cross-reactive to strains from the other genotypes. However, in some cases the antigenic differences between BVDV-1 isolates of different subgenotypes can be as different as those found between BVDV-1 and BVDV-2 (Fulton *et al.*, 2003).

It was shown that vaccination of cattle with either live-attenuated or inactivated BVDV-1 vaccines was able to induce heterotypic antibody response that neutralised a wide range of European and American isolates of both BVDV-1 and BVDV-2 (Dean and Leyh, 1999; Hamers *et al.*, 2002: Patel *et al.*, 2005). This raises the most important question of whether or not cross-reactive antibody response elicited by these vaccines could give protection against strains of both genotypes (BVDV-1 and BVDV-2) and subgenotypes within them, particularly BVDV-1a and BVDV-1b. In this section, the protective efficacy of current BVDV vaccines will be evaluated for mainly preventing transplacental infections towards fetal protection.

There have been two encouraging reports for the efficacy of two licensed inactivated BVDV vaccines for preventing transplacental infections of bovine foetus. Firstly, a study carried out by Brownlie *et al.*, (1995) clearly demonstrated the protective efficacy of the first commercially UK-licensed inactivated vaccine (Bovidec) containing a single ncp strain (Pe515ncCI) in all tested cattle for transmitting to challenge virus to their foetus. Secondly, Patel *et al.*, (2002) recently showed the protective efficacy of an EU-licensed inactivated vaccine (Bovilis) based on a broadly immunogenic cp strain (C86) for preventing infection of foetuses against natural BVDV infection for a period of up to six months. Both vaccine strains (Bovidec and Bovilis) belong to BVDV-1a, which is common in England and Wales (Vilcek *et al.,* 1999). However, no data was available for the transplacental protective efficacy of these vaccines against other subgenotypes of BVDV-1 and BVDV-2.

It is suggested that proper vaccination with BVDV-1 vaccines could protect or at least partially protective against clinical disease due to BVDV-2 infections (Ridpath *et al.*, 2000; Makoschey *et al.*, 2001; Fairbanks *et al.*, 2003). The satisfactory protective efficacies of live-attenuated BVDV-1 type vaccines against BVDV-2 challenge were reported (Cortese *et al.*, 1998; Dean and Leyh, 1999; Shimazaki *et al.*, 2001; Fairbanks *et al.*, 2003). Fairbanks *et al.*, (2003) demonstrated the protective efficacy of a live attenuated BVDV-1a (singer) vaccine against clinical disease in calves challenged with virulent BVDV-2 (890 strain). They observed that BVDV-2 associated clinical disease (e.g. leucopoenia and fever) was significantly lower in vaccinated calves as compared to unvaccinated control calves. Makoschey *et al.*, (2001) showed protective efficacy of

Bovilis against the clinical effects of BVDV-2 including respiratory disease, leucopenia, diarrhoea with erosions and haemorrhages along the gastro-intestinal tract.

Frey *et al.,* (2002) reported a study showing cross-protective efficacy of a two-step vaccination protocol with BVDV-1 vaccines against transplacental infections of BVDV-2. In their study, protection was afforded by vaccination of dams with an inactivated BVDV-1 vaccine followed by boosting with a live-attenuated BVDV-1 vaccine. Vaccinated dams were challenged intranasally between 30 and 120 days of pregnancy with a mixture of BVDV-1 and BVDV-2. All vaccinated animals gave birth to clinically healthy, seronegative (precolostral) and BVDV-free calves. Dean *et al.*, (2003) recently reported the protective efficacy of a live-attenuated vaccine derived from the BVDV-WRL strain belonging to BVDV-1b for preventing infection of foetuses from infection with virulent BVDV-1a strain (7443). In that study, no persistent infection was detected 92% of calves born to dams vaccinated prior to breeding.

Kovacs *et al.*, (2003) showed a good fetal protective efficacy of a commercial multivalent live attenuated BVDV vaccine containing BVDV-1 and BVDV-2 (Breed-Back FP 10, Boehringer Ingelheim Vetmedica Inc.) against challenge of BVDV-1 and BVDV-2. The BVDV components of this vaccine consist of two cp BVDV strains (BVDV-1a singer and BVDV-2 296). Both challenge viruses were heterologous to vaccine strains, highly virulent and well characterised. Vaccination gave protection from foetal infection in 91% of dams challenged with BVDV-1 and in 100% dams challenged with BVDV-2.

Fairbanks *et al.*, (2004) also recently showed a high degree of protective efficacy of a multivalent BVDV vaccine containing the same antigens as Kovacs. The vaccinated animals prior to breeding were 100% protected against following intranasal challenge with a heterologous BVDV-1 and 95% protected against a heterologous BVDV-2. The challenge viruses were known to induce a high degree of fetal infection and only mild to moderate clinical signs in the dam.

In both Kovac's and Fairbank's study, two vaccines containing the same BVDV strains of BVDV-1 and BVDV-2 gave high and broad fetal protection against four heterologous challenge strains of both genotypes. The use of both genotypes in live-attenuated BVDV vaccines seems to be more effective for cross protection. However, this strategy can be problematic, since the virus content in live-attenuated vaccines has been shown to cause transplacental infections and infection of foetuses leading to all outcomes known from natural field infections of BVDV (van Oirschot *et al.,* 1998). Furthermore, the use of live-attenuated vaccines may also trigger the induction of MD by RNA recombination with persisting virus (Ridpath and Bolin, 1995; Becher *et al.*, 2001). Therefore, the development of a better and safe vaccine is still needed.

1.4.3. Novel approaches for BVDV vaccine development

Control and eradication of BVDV could be achieved by systematic vaccination with a new generation of marker vaccine that can differentiate vaccinated and naturally infected animals. Thus, a marker vaccine against BVDV will be accompanied with a companion diagnostic test. The marker vaccine strategy has firstly been developed for pseudorabies virus (Aujesky's disease virus PRV) and also extensively used to control of infectious bovine rhinotracheitis virus (IBR) (van Oirschot *et al.*, 1996; 1999b).

In this section, the novel approaches for the development of a novel BVDV vaccine will be discussed with the examples of the closely related CSFV.

The first potential marker vaccines for CSFV were a live vaccinia virus expressing the structural proteins of CSFV and a gE-deleted live Aujesky's disease virus expressing the immunodominant E2 of CSFV (van Zijl *et al.*, 1991; Rumenaph *et al.*, 1991). However, these promising vaccines were not processed to commercially available due to restrictions of Aujesky's disease free countries. There are currently two licensed CSFV subunit vaccines based on the expression of the E2 of CSFV in baculovirus recombinant system (Lutticken *et al.*, 1998; Moorman *et al.*, 2000) and they can be accompanied with an ELISA that detects antibodies against the E^{rns} protein (Flogel and Niessman, 2001) in infected as compared to vaccinated pigs. However, there is still need to improve the efficacy of these subunit vaccines as compared to the classical live-attenuated CSFV (chinese) vaccine and the detection of antibodies to E^{rns} by ELISA (van Oirschot, 2003a, b).

The synthetic peptide based vaccine approach based on E2 of CSFV has also been evaluated and shown to be promising by Nan Dong *et al.*, (2002; 2005). The two candidate multipeptide-CSFV vaccines based on five overlapping linear peptides (conjugated to BSA) from amino terminal part of B/C region (amino acids 693-777) of CSFV-E2 (Shinmen) strain. The administration of these vaccines either mixed with Freund's or Aluminium hydroxide adjuvant gave protection in pigs challenged with virulent CSFV (Shinmen) (Nan Dong *et al.*, 2002; Nan Dong *et al.*, 2005). Nan Dang and Hua Chen, (2006) recently reported that two peptide vaccines in which peptides from the site A of CSFV-E2 region gave better protection in pigs against lethal challenge as compared to the conventional CSFV vaccine (Chinese). These synthetic peptide based vaccines has also potential for the development of a marker vaccine against CSFV as described above.

Both DNA and recombinant viral vector or subunit novel vaccines for BVDV have also been developed and their protective efficacy tested on an experimental basis. An E2-based subunit approach (recombinant protein expressed in baculovirus infected cells) (Bolin and Ridpath, 1996; Bruschke *et al.*, 1999), live virus vectors expressing E2 of BVDV (Elahi *et al.*, 1999; Toth *et al.*, 1999), plasmid DNA expressing E2 (Nobiron *et al.*, 2001; 2003), and a live vaccine with deletion mutants in 5'NTR (Makoschey *et al.*, 2004) have been applied to design a new generation of BVDV vaccine.

A BVDV-1a DNA vaccine induced neutralising antibody response and cell-mediated immune response in cattle but gave a limited protection against a heterologous BVDV-1b strain (Harpin *et al.*, 1999). Nobiron *et al.*, (2001) showed the enhancement of the Th1 type immune response in mice by coinjection of BVDV-1a DNA vaccine with IL-2 or GM-CSF DNA. By using same approach, the same group later showed a partial protection in cattle that was evidenced by reduced febrile response, reduction of leucopoenia and viraemia against the homologous challenge (Nobiron *et al.*, 2003).

Recombinant baculovirus E2 protein from BVDV-1a (Singer strain) induced neutralising antibody response in calves and gave limited protection against clinical disease with homologous challenge as evidenced by reduction of viral replication and reduction of fever. However, it failed to protect against heterologous BVDV-2 (890 strain) challenge as evidenced by viral replication and dissemination of virus in challenged animals and appearance of clinical symptoms of BVDV-2 infection including high fever, diarrhoea and thrombocytopenia (Bolin and Ridpath, 1996).

Makoschey *et al.*, (2004) studied the potential of a 5'NTR deleted stable mutants of a cp strain of BVDV as a live vaccine. These mutants were able to induce high titre neutralising antibody response and gave complete protection of viremia against challenge with a heterologous BVDV strain.

Despite the promising results were obtained with these experimental BVDV vaccines, the protection afforded by them was not complete against wide-range of BVDV isolates of both genogroups and congenital infections as compared to current live-attenuated and inactivated BVDV vaccines and they are still a basis for their improvement.

Another alternative novel approach that is subject of this thesis is the development of a mimotope-epitope based marker vaccine against BVDV (see section 1.6). In order to develop a mimotope base vaccine against BVDV, protective neutralising epitopes of E2 must be first determined in detail.

1.5. Epitope mapping of BVDV E2

The E2 major envelope glycoprotein, a 53-kDa protein is the most variable immunodominat glycoprotein of BVDV. It harbours neutralising epitopes recognised by host immune system (Paton *et al.,* 1992; Bolin, 1993; Potgieter, 1995; Deregt *et al.,* 1998a, b). The polypeptide length of BVDV-E2 is estimated around 375 to 400 amino acid long and contains three to four consensus glycosylation sites. (Paton *et al.*, 1992; Deregt *et al.*, 1998a, b; Toth *et al.,* 1999; Kweon *et al.,* 1999). It forms homodimers or heterodimers with E1 that are linked by disulphide bonds (Thiel, 1991; Meyers and Thiel, 1996). In addition, The E2 glycoprotein interacts with a cellular receptor bovine CD46 ($CD46_{bov}$ or bovine membrane cofactor protein) as a ligand by facilitating the binding and entry of virus to cells (Maurer *et al.*, 2004).

Paton *et al.,* (1992) mapped epitopes recognised by the Weybridge (wb) panel of E2 specific neutralising mAbs of two strains of BVDV-1a (BVDV-NADL and BVDV-Oregon). Competitive binding studies suggested for clustering of most epitopes on E2.

Their study revealed the existence of two antigenic sites on E2 of BVDV-NADL (BVDV-1a). One of the antigenic sites was suggested to be immunodominant and was also present in BVDV-Oregon. Individual neutralising escape mutants mapped an immunodominant region with broadly cross-reactive neutralising mAbs (Wb166, Wb214 and Wb215) and showed a cluster of three amino acids (71-74) in the N-terminal half of the E2. The second antigenic site affected by an amino acid change at amino acid position 40 of E2 was identified by a strain specific mAb Wb158 (BVDV-NADL) and suggested to be a strain specific epitope. Since the other nucleic acid escape mutants affecting these epitopes were found at distant positions on E2, it can be suggested that both domains are consisted of composite, discontinuous (conformational) epitopes.

Deregt *et al.*, (1998a) also mapped E2 epitopes of two neutralising mAbs (157 and 348) produced against BVDV-DCP isolate (BVDV-1). Both mAbs were bound to the first 192 amino acids of the E2. The mAb 157 was shown to bind and neutralise a range of isolates of BVDV1a (Singer, NADL and Oregon) and BVDV-1b (NY-1 and Hastings) and considered BVDV-1 specific mAb. Except for BVDV-1b (Hastings) isolate, a similar pattern of binding and neutralisation were also observed with the mAb 348. However, the mAb 348 also bound and weakly neutralised BVDV-2 isolates and was considered a cross-reactive mAb for BVDV-1 and BVDV-2. Competitive binding assays revealed that both mAbs (157 and 348) recognised epitopes in the same antigenic site.

Neutralising escape mutant analysis of the mAbs (157 and 348) revealed critical amino acids affecting epitopes recognised by these mAbs. Escape mutants of BVDV-1a (Singer) were selected with both mAbs, mutants of BVDV-2 (BVDV-2-125c) were selected with the mAb 348 and those of BVDV-1b (Hastings) were selected with the mAb157.

Escape mutants selected with the mAb 157 resulted amino acid changes in three amino acids (9, 32 or 72) on E2. Amino acid changes on position 72 also affected the epitope recognised by the mAb 348 (see below). According to Deregt's study, the mAb 157 is likely to recognise a new or unique BVDV-1 genotype specific epitope since the changes at amino acid positions 9 and 32 obtained by escape mutants have not been previously reported by others. The long spatial distance between the amino acid changes observed in the escape mutants was again indicative of a discontinuous epitope.

For the mAb 348, both BVDV-1 and BVDV-2 escape mutants selected with the mAb 348 resulted in changes in three amino acids (71, 72, 74) on E2 affecting its binding and neutralisation. A fourth amino acid change observed with a mutant of BVDV-2 extended this region to position 77 on E2. Thus, the mAb 348 recognised an epitope shared by BVDV-1 and BVDV-2 located in the immunodominant region previously described by Paton *et al.*, (1992) (see above). The short spatial array of amino acid changes was suggestive of a linear part of a discontinuous epitope.

Although Both Paton's and Deregt's studies highlighted the importance of the NH_2 terminal part of E2 containing discontinuous conformational neutralising epitopes, Toth *et al.*, (1999) suggested the presence of an antigenic domain containing neutralising epitope (s) localised within the carboxyl terminal 90 amino acids of the BVDV E2.

Another study carried out by Deregt *et al.*, (1998b) analysed E2 epitopes of BVDV-2 by using BVDV E2 specific mAbs. Unlike BVDV-1 for which conserved neutralising epitopes were located in a single immunodominant antigenic site (Paton *et al.*, 1992; Deregt *et al.*, 1998a), it was shown that BVDV-2 E2 neutralising mAbs were bound to conserved epitopes in three antigenic sites on E2.

The epitopes on E2 of CSFV were extensively mapped to four antigenic domains (A-D) in NH_2-terminal half of the protein (van Rijn *et al.*, 1994). Neutralising epitopes were located in three of these sites (B/C, A) and the site A was higly conserved among more than 90 CSFV isolates (van Rijn *et al.*, 1992, 1993). However, such a conserved site has not been determined for BVDV. Linear peptides from this conserved region were able to induce protective neutralising antibodies when injected to pigs (Nan Dong and Hua Chen, 2006). All CSFV E2 epitopes are suggested to be conformational and are dependent on disulphide bonding between first six cysteine residues. Although these cysteine residues are highly conserved in BVDV, BVDV has two additional cysteine residues. These may involve alternative disulphide bonds and affect the conformational structure of E2 of BVDV. Another difference between CSFV and BVDV is the presence of immunodominant region (71-74) on E2 of BVDV. However, no escape mutant of

CSFV mapped to this region. Because of the existence of a conserved region harbouring neutralising epitopes of CSFV, CSFV vaccines may give better cross protection than BVDV.

Yu *et al.*, (1996) identified a first conserved linear epitope on E2 among pestiviruses. However, this linear epitope is not accessible on the native virus and revealed following denaturation of the E2. By contrast, peptides on the major antigenic loop region of Footh and Mouth Disease (FMDV) VP1 protein induced neutralising antibodies in guinea pigs (Francis *et al.*, 1990).

1.6. Mimotopes for the development of vaccines with regard to BVDV

The development of a mimotope-based peptide vaccine to induce protective neutralising antibodies against BVDV is an alternative approach for the development of a new generation vaccine. This can be facilitated by the identification of epitopes capable of inducing protective neutralising antibodies (B-cell epitopes), epitopes capable of inducing help for antibody production (Th-cell epitopes) and also possibly cytotoxic T-cell sites (CTL epitopes) (Steward *et al.*, 1995; Meola *et al.*, 1995; Chargelegue *et al.*, 1998; Hsu *et al.*, 1999; Meloen *et al.*, 2001; Steward, 2001; Partidos and Steward, 2002: Francis *et al.*, 2003). In this study, the identification of protective B-cell epitopes was the aim.

Epitope mapping studies on E2 of BVDV suggested that neutralising B-cell epitopes located the E2 are discontinuous in nature and an appropriate conformation is likely be essential for their recognition (Paton *et al.*, 1992: Deregt *et al.*, 1998a, b). Apart from X-ray crystollagropraphy, that requires sophisticated and expensive equipments, combinatorial random peptide libraries have also been shown to be promising for identifying mimotopes of discontinuous (conformational) epitopes.

1.6.1. The basis of mimotope concept

Mimotopes can be defined as molecules that can specifically bind to antigen combining site of an antibody, not necessarily identical with the epitope inducing the antibody at the amino acid sequence level, but an acceptable mimic of the essential features of the epitope (Geysen *et al.*, 1986; Steward *et al.*, 1995; Steward, 2001). The definition also includes cases where the natural ligands are not proteinaceous, such as carbohydrate (Sbille *et al.*, 1997a; Phalipon *et al.*, 1997; Pincus *et al.*, 1998; Monzavi-Karbassi *et al.*, 2002), oligonucleotide epitopes (Sbille *et al.*, 1997b) and also haptens (Bottger *et al.*, 1999). Although the term mimotope was originally used to describe an antigenically active peptide having no sequence similarity with the epitope it mimics (Geysen *et al.*, 1986), currently it has a broader meaning. Since the antigenicity of a continuous epitope can be mimicked by a mimotope peptide having little sequence similarity, it also follows that the absence of sequence similarity does not necessarily indicate that the mimotope mimics a discontinuous epitope of the antigen (van Regenmortel 1998).

There are two essential requirements for a real mimotope. Firstly, it mimics the antigenicity by binding the antibody that recognises the native epitope. Secondly, it mimics the immunogenicity by its ability to elicit a humoral immune response against the native epitope that it mimics. The second requirement is crucial if mimotopes are considered for vaccine purpose (Lam *et al.*, 1996).

1.6.2. Combinatorial peptide libraries and mimotope-based viral vaccines

Combinatorial random peptide libraries have been successfully used to identify mimotopes mimicking epitopes of important viral pathogens such as, human hepatitis B virus (Meola *et al.*, 1995; Delmastro *et al.*, 1997; D'Mello et al., 1997; Zhang *et al.*, 2001), hepatitis C virus (HCV) (Tafi *et al.*, 1997; Puntoriero *et al.*, 1998; Petit *et al.*, 2003; Jolivet-Reynaud *et al.*, 2004), human immunodeficiency virus (HIV) (Keller *et al.*, 1993; Scala *et al.*, 1999; Dorgham *et al.*, 2005), measles virus (MV) (Steward *et al.*, 1995); Japanese encephalitis virus (Hirabayashi *et al.*, 1996), and respiratory syncytial virus (RSV) (Chargelegue *et al.*, 1998).

Studies with neutralising mAbs have mostly led to the identification of discontinuous epitopes. However, a small but significant proportion of mAbs recognising discontinuous epitopes on protein antigens are able to bind short linear peptide fragments of the protein (Arnon and Van Regenmortel, 1992; van Regenmortel, 1996;

1998a, b; 1999: 2001a, b). For instance, Heiskanen *et al.*, (1999) reported the identification of binding ligands displaying sequence similarities to the primary sequence of original antigen using phage libraries with neutralising mAbs recognising discontinuous epitopes of puumala hantavirus. There are several convincing examples of the use of random peptide libraries for the identification of neutralising epitopes (Geysen *et al.*, 1986; Luzzago *et al.*, 1993; Steward *et al.*, 1995; Chargelegue *et al.*, 1998; Heiskanen *et al.*, 1999).

Attempts to develop a protective vaccine against HIV-1 have been hindered by difficulties in identifying epitopes capable of inducing broad neutralizing antibody responses. Scala *et al.*, (1999) used sera from HIV-infected individuals to select binding ligands by using random peptide libraries. The isolated peptides were specifically recognized by antibodies from HIV-1-infected individuals and mimicked linear or conformational epitopes generated *in vivo* in infected patients. Furthermore, the selected peptides were able to induce HIV-1 specific neutralising antibodies in mice. This study is a good example to demonstrate the possibility for the identification of HIV-1 specific mimotopes with vaccine potential.

HCV is a major cause worldwide of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. There is still no vaccine against it and the development of an effective vaccine represents a high priority goal. The hypervariable region 1 (HVR1) of the second envelope protein (E2) of HCV contains a principal neutralizing determinant, but it is highly variable among different isolates and it is involved in the escape from host immune response. Thus, an effective vaccine should elicit a cross-reacting humoral response against the majority of viral variants. It was shown that it was possible to induce a broadly cross-reactive immune response in rabbits by immunization with mimotopes of the HVR1, selected from a specialized phage library using sera from HCV infected patients. Importantly, some of the antibodies elicited by mimotopes in rabbits recognised discontinuous epitopes in a manner similar to those induced by the virus in infected patients (Roccasecca *et al.*, 2001).

There have been two elegant studies describing mimotopes that mimic the discontinuous neutralising epitopes of viral proteins of Measles virus (MV) and Respiratory Syncytial virus (RSV). Steward *et al.*, (1995) and Chargelegue *et al.*, (1998) reported the identification of mimotopes mimicking epitopes recognised by a MV Fusion (F) protein (MVF) and a (RSV) F protein specific mAbs by using an 8-mer solid phase epitope library. In both cases, the mimotopes did not bear any amino acid sequence homology with the viral proteins against which the selecting antibodies were directed, merely mimicking the conformation of the epitopes. When used as immunogens, one mimotope from each study induced both virus neutralising and protective antibody responses in mice. In the case of the MV mimotope, this result was surprising, since the mAb used to select the mimotopes was neither neutralising nor protective. The RSV mimotope that mimics the discontinuous epitope of F protein was able to protect mice against viral challenge whereas peptides from the linear sequence of F protein did not. These studies highlight that the use of combinatorial peptide libraries can lead to the development of mimotope based synthetic peptide vaccines against RSV, MV and also other pathogens (HIV and HCV) against which traditional vaccines are not currently available.

1.6.3. A prospect of the development of a mimotope-based vaccine for BVDV

A mimotope (epitope-based) vaccine mimicking neutralising epitopes of BVDV E2 would be a feasible approach for the development of a safe marker vaccine for BVDV. Such a mimotope-based peptide vaccine mimicking protective neutralising epitopes of E2 of BVDV would not induce antibodies against non-structural proteins NS2/3 whereas animals naturally infected with BVDV would develop antibodies against this protein. Tests for the detection of antibodies against BVDV NS2/3 are also available (Paton *et al.*, 1991a, b; Kramps *et al.,* 1999) and therefore they can be combined with a mimotope based marker vaccine to differentiate vaccinated and naturally infected animals for BVDV eradication programme. Once protective and neutralising epitopes are identified, mimotope-based peptide vaccines are safe, stable and easy to produce (Francis *et al.*, 2003). Particularly, if a mixture mimotopes mimicking E2 of both genotypes of BVDV-1 and BVDV-2 can be administrated safely as a multivalent vaccine for BVDV.

In order to identify mimotopes for BVDV E2, combinatorial peptide libraries were used to characterise the neutralising mAbs (Wb166, Wb214 and 157) that bound many isolates of BVDV (Paton *et al.*, 1992; Deregt *et al.*, 1998a) (see section 1.5).

In the next section, general information about combinatorial peptide libraries (phage-display and solid phase libraries) and their application to epitope mapping studies will be described.

1.7. Combinatorial peptide libraries

Combinatorial random peptide libraries consist of a large collection of short variable random peptides that are displayed on the surface of bacteriophage coat proteins (biological libraries) (Scott and Smith, 1990; Cwirla *et al.*, 1990; Smith and Scott, 1993; Smith and Petrenko, 1997) or an insoluble solid phase such as resin beads (solid phase) (Lam *et al.*, 1991; 1996; Hirabayashi *et al.*, 1996). Biological libraries can be classified in two categories, namely phage display random peptide libraries Daniels and Lane, 1996: Smith and Petrenko, 1997; Zwick *et al.,* 1998) and phage display gene-fragment libraries Fack *et al.,* 1997). The main goal in generating random peptide libraries is to create diverse molecular shapes, which can adopt binding properties of a target of interest in order to identify peptide mimics or mimotopes of variety of molecules (Smith and Petrenko, 1997; Irwing *et al.*, 2001; Paschke, 2006). The design of a random peptide library is principally based on generating an enormous collection of peptide sequences containing 6, 8, 20, and 38, etc. amino acid residues. The number of random peptides is associated with the complexity of the library. It is simply calculated as 20^n where n= number of the displayed amino acid residues (Scott and Smith, 1990; Devlin *et al.*, 1990; Lam *et al.*, 1991; Felici *et al.*, 1993; Barbas, 1993; Houghten, 1993; Hirabayashi *et al.*, 1996; Cortese *et al.*, 1996; Wilson and Finley, 1998).

1.7.1. Biological libraries: Phage-display random peptide libraries

A fusion phage is a filamentous bacteriophage vector displaying a foreign peptide on its surface fused to a coat protein (pIII or pVIII) and has a gene for the fusion protein (gIII or gVIII) within its genome (Smith and Scott, 1990; Scott, 1992; Smith, 1993; Scott and

Smith, 1993; Scott and Craig, 1994; Dion and Lane, 1996; Smith and Petrenko, 1997) **(Figure 1.3)**. Filamentous phage particles are infectious and a collection of these particles displaying random peptides creates a phage-display peptide library (Scott, 1992; Smith and Petrenko, 1997: Irwing *et al.,* 2001). Phage libraries have been designed to display random peptides either in a constrained manner with fixed cysteine residues within the peptide sequences, or in an unconstrained (linear) without cysteine residues. (Bonnycastle *et al.*, 1996; Irving *et al.*, 2001).

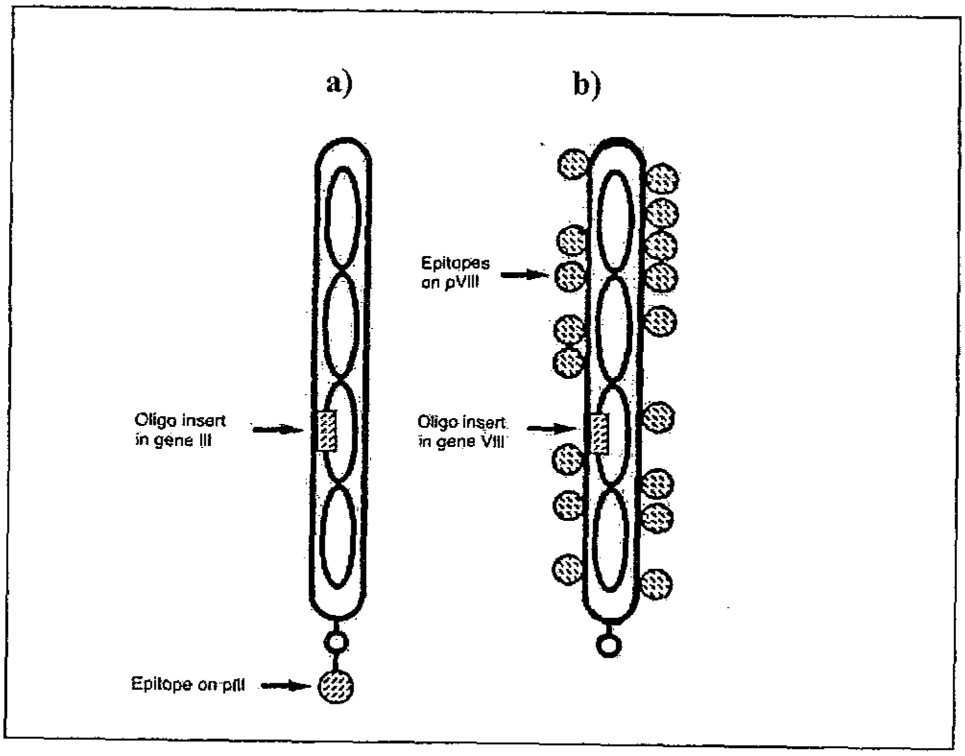


Figure 1.3. Schematic representation of filamentous bacteriophage peptide display strategy

a) Peptide epitopes fused to the minor coat protein pIII b) the major coat protein pVIII. The single-stranded phage DNA genome, which carries an oligonucleotide insert encoding the displayed epitope. Only single copy of pIII is shown, rather than 4-5 copies are present on the phage particle (Adapted from Scott, 1993).

A phage-display library is made by cloning foreign random DNA inserts (oligonucleotides) into the 5' end of the phage coat protein gene that serves as expression vector (Scott and Smith, 1990; Smith and Petrenko, 1997). The foreign peptide sequences are genetically fused to the endogenous amino acids of phage coat protein to create a hybrid "fusion" protein and displayed antigenically outer surface of filamentous phage by generating collections of random peptides as many as 10^9 permutations displayed on phage particles (Smith and Scott. 1993; Smith and Petrenko, 1997; Cabilly, 1999). In addition, the phage vector can also be designed in a way that the fusion protein is found on the phage genome or on a separate genome (Phagemid) (Smith and Petrenko, 1997; Irwing *et al.*, 2001).

The most commonly used phage coat proteins for displaying foreign peptides are pIII minor coat protein and pVIII major capsid protein. The pIII is the longest phage coat protein, 406 amino acid, and known as minor coat protein (Webster, 1996; Cabilly, 1999). The pIII protein can tolerate foreign peptide fusions containing several hundred residues at or near the amino terminus without significantly interruption of its biological function and phage viability (Smith, 1985). Only three to five copies of pIII protein can be expressed at the one terminus of the phage particle thus, foreign peptides are displayed as fusions on pIII surface at low copy number (Smith and Petrenko, 1997). In addition, low copy number of peptide display may also lead selection to high affinity ligands (Burritt *et al.*, 1996).

About 27000 surface accessible copies represent the phage protein of pVIII, the major capsid protein of 50 amino acids (Marvin *et al.*, 1994). The high copy number of pVIII protein makes it attractive candidate for the display of foreign peptides particularly for immunogenic studies (Greenwood *et al.*, 1991; Minekova *et al.*, 1993; Veronese *et al.*, 1994). In addition, peptides displayed on pVIII can also function as ligands for other cellular receptors (Cesareni, 1992; Scott, 1992; Harts *et al.*, 1994). However, foreign peptides consisting of more than 5 amino acids displayed on the pVIII protein interfere with maturation of progeny phage and peptides greater than nine amino acid residues almost entirely interfere phage assembly. Peptide libraries including more than six residues fused to the pVIII protein can be constructed by using specific conditions (e.g phagemid) (Smith and Petrenko, 1997) (see section 1.7.7).

Phage clones whose displayed peptides bind the given ligands such as antibodies tightly are enriched over those that do not bind by means of successive rounds of affinity purification procedure (AP) or biopanning (Parmley and Smith, 1987; Cesareni *et al.*, 1999). The affinity-selected phage clones are cloned and amplified by infecting the fresh bacterial host cells and the peptides responsible for binding are deduced by sequencing of the corresponding coding sequence in the phage DNAs (Smith and Scott, 1993; Smith and Petrenko, 1997; D'Mello *et al.*, 1997).

1.7.2. Gene-fragment phage-display libraries

Gene-fragment phage-display peptide libraries represent an alternative approach to random phage-displayed peptide libraries. It is based on expressing target cDNA fragments that represent an antigenic determinant of interest rather than expressing random peptide sequences on the phage coat proteins. Gene fragment libraries enable to display on the phage surface up to 100 amino acid long peptides in their native structural context (Petersen *et al.,* 1995; Gupta *et al.,* 1999). The libraries are constructed by generating a population of different sizes (100-200 bp) and sequences of cDNA fragments using a randomly cutting endonucleases, such as Dnase I, and cloning these fragments in to the 5' terminal end of gene III by using oligonucleotide linkers (Wang *et al.*, 1995; *Fack* et al., 1995; Petersen *et al.*, 1995; Bluthner *et al.*, 1996).

1.7.3. Solid phase random peptide epitope libraries

Solid phase peptide libraries also represent a novel powerful experimental approach for discovering the ligands of various macromolecule targets. It involves creating a large peptide library consisting of millions of resin beads displaying a single unique peptide with the complete collection representing the universe of possible random peptides roughly equimolar proportion (Lam *et al.*, 1991; 1996; Chargelegue *et al.*, 1998).

This is achieved by using 'split synthesis' method. The first cycle involves distributing a pool of resin beads into separate reaction vessels each with a single amino acid, which is followed coupling reactions to take place and then repooling the beads. This cycle is repeated until the desired length of peptide chain is extended. In this fashion, each bead should contain only a single peptide. The library is also called as one-bead one peptide combinatorial library (Lam *et al.,* 1991; 1996).

The selection procedure is rather straigforward and simple comparing to phage-display libraries. The reactive peptide-beads that interact with a macromolecular target such as a mAb can be identified following being stained by enzymatic reaction on a dye substrate. The reactive stained beads are then removed from the colourless, nonreactive beads remaining in the library and subjected for microsequencing to deduce amino-acid sequences of the reactive peptides. Ideally, a countable number of resin beads are intensely stained and become visible under the naked eye and easily selected under a dissecting microscope comparing to background of colourless nonreactive beads (Lam *et al.*, 1991; 1996; Steward *et al.*, 1995).

1.7.4. Application of combinatorial peptide libraries

Combinatorial random peptide libraries have been tools for the many biological applications including, epitope mapping studies and vaccine development (Scott and Smith, 1990; Cortese *et al.*, 1994; 1995; Bottger *et al.*, 1995; Grihalde *et al.*, 1995; Yao *et al.*, 1995; Irwing *et al.*, 2001; Steward and Chargelegue, 1998), the identification of disease specific epitopes (Cortese *et al.*, 1996; Mennuni *et al.*, 1996; Prezzi *et al.*, 1996; Farilla *et al.*, 2002), the elucidation of protein structure and function (D' Mello *et al.*, 1997, Frenkel *et al.*, 1998), the identification of peptide ligands for drug development (Wrighton *et al.*, 1996; Jefferies, 1998), the selection of tissue and organ specific ligands following *in vivo* administration (Pasqualini and Ruoslathi, 1996; Pasqualini, 1999). In this section, the applicability of combinatorial peptide libraries for epitope mapping and vaccine development with regard to this study will be discussed.

1.7.5. Epitope mapping with combinatorial peptide libraries

Combinatorial peptide libraries have been used as successful simple method for mapping epitopes (Lane and Stephen, 1993; Grihalde *et al.*, 1995; Smith and Petrenko, 1997; Yip and Ward, 1999; Zwick *et al.*, 1998; Irwing *et al.*, 2001). This involves the identification of a consensus sequence obtained from the selection of the specifically bound peptide displayed on phage or beads (solid phase library) to the target and the comparison of this sequence to the original antigen to detect sequence similarities (Cwirla *et al.*, 1990; Scott and Smith, 1990; Stephen *et al.*, 1995; Steward *et al.*, 1995; D'Mello *et al.*, 1997; Gomez-Roman *et al.*, 2002).

Peptides identified by epitope mapping using combinatorial peptide libraries have two important usages. Firstly, they can act as antigenic mimics that serve as specific probes for antibodies that are diagnostic for diseases as much as natural viral proteins such as for equine arteritis virus (EAV) (Iniguez *et al.*, 1998) and for antibodies of lyme disease (LD) (Kouzmitcheva *et al.*, 2001) and for HCV (Tafi *et al.*, 1997). Secondly, one of the most important uses of peptides identified by epitope discovery is their potential to be components of synthetic peptide-epitope based (mimotope) vaccines (Chargelegue *et al.*, 1998; Steward, 2001) (see section 1.6.2).

1.7.5.1. Mapping of continuous (linear) epitopes

It is expected that as long as a short linear peptide exist on the protein antigen that binds to the antibody with an affinity, random peptide libraries probably provide a powerful and easy approach for mapping epitopes compared to other alternative methods (Smith and Petrenko, 1997; Cortese *et al.*, 1994; 1999) that require chemical synthesis of short peptides of the ligand's amino acid sequence (Geysen *et al.*, 1987a; Langeveld *et al.*, 2001).

Phage-display peptide libraries have also been created the opportunity providing valuable information for critical residues responsible for antigen antibody binding. For instance, D'Mello *et al*, (1997) used a 15-mer random phage library to characterise the fine specificity of the mAb 18/7 that was specific for the pre-S1 antigenic determinant of HBV surface antigen (HbsAg). The identified mimotopes possessed a central motif with at least three residues present from the native pre-S1 sequence that contained DPAF (31-34) amino acids and implied their critical role in antibody binding.

1.7.5.2. Mapping of discontinuous (conformational) epitopes

Although combinatorial peptide library approach has been shown to be more applicable to identifying linear epitopes (Irwing *et al.*, 2001), there are also some valuable studies for the identification of discontinuous epitopes by using random phage-display and solid phase libraries (Heiskanen *et al.*, 1999; Chargelegue *et al.*, 1998). Importantly, in most cases the combinatorial library approach did not give a direct sequences information to localise discontinuous epitopes on the native protein, but the identified peptides can mimic features of the ligate binding sites that are called as mimotopes (Steward *et al.*, 1995; Chargelegue *et al.*, 1998: Partidos and Steward, 2002).

Phage-display peptide library approach has been facilitated to map discontinuous epitopes even without the information of their three dimensional structure. For instance, the structure of the hepatitis B virus surface antigen (HBsAg) was investigated by epitope mapping of anti-HBsAg specific mAbs. The comparison of sequences obtained by phage library selection and the native HBsAg revealed the precise location of the binding regions. Three out of four mAbs were found to bind distinct discontinuous epitopes between amino acid residues 101 and 207 of HBsAg. The fourth mAb was found to bind residues 121-124. The sequence information derived from this study was used to map the surface of HBsAg and to derive topological model for the α-carbon trace of the 101-207 region of HBsAg (Chen Jack *et al.*, 1996).

Random peptide libraries have also been useful for defining protein secondary structure information. For instance, a α-helical structure in the C1 domain of the HIV-1 envelope protein gp120 was mapped by using phage peptide libraries with gp120 specific mAbs. The sequence comparison of the identified reactive peptides revealed that a unique and characteristic pattern, a motif, was essential for mAb binding. By using this information, a computer model of the α-helical structure was defined and the contact residues defining the surface of helix were identified (Gershoni *et al.*, 1997; Stern *et al.*, 1997).

1.7.6. Combinatorial epitope libraries used in this study

In this study, two linear and two constrained types four phage libraries and an 8-mer solid phase library were used to identify binding ligands to BVDV mAbs.

The rational of the use of a range of phage-display and an 8-mer solid phase libraries in this study was based on an extensive study carried out by Bonnycastle *et al.*, (1996). Their study was designed to search the interplay amongst the properties of a) the immunogen (protein, peptide or carbohydrate), b) the epitope (linear or discontinuous), c) antibody (monoclonal or polyclonal), and d) phage-displayed peptide (linear or constrained). Bonnycastle *et al.*, (1996), used eleven phage libraries that displayed peptides in different length and structural context together with 17 antibodies including 15 mAbs directed against peptides, linear and discontinuous epitopes on proteins or carbohydrates and two polyclonal antibodies of proteins of known structure. Several important findings were made that are also pertinent to the present study.

1) They found that the reactive peptides were likely to be identified by biopanning of a large range of libraries with antibodies raised against peptides, linear epitopes of proteins, and carbohydrates, but less frequently with antibodies recognising discontinuous epitopes on proteins. 2) Phage libraries having different disulphide bridged constraints gave binding ligands for different mAbs or polyclonal antibodies; however, no subset of libraries was identified for all or most of the binding ligands. 3) Moreover, for a given antibody, it was not common for all libraries to give a consensus sequence; in some cases, different libraries gave different consensus sequences or peptides with no apparent consensus. 4) For most libraries, the tightest-binding peptides varied according to mAbs used for biopanning. 5) It can be concluded that mimotopes can be selected more efficiently by using a large panel of libraries, thus the preference of an antibody for a given library or a range of libraries can not be predicted in advance (Bonnycastle *et al.*, 1996; Irwing *et al.*, 2001).

6) Most of the mAbs recognising discontinuous epitopes tested in the study of Bonnycastle *et al.*, (1996) either isolated weakly binding peptides or did not isolate binding peptides at all. One of the mAb used for screening was b12, a human mAb recognising the $CD4^+$ binding site of the HIV-1 envelope protein gp120, an antibody that neutralises a wide spectrum of HIV-1 primary isolates *in vitro* (Parren *et al.*, 1999). Two phage clones displaying weakly binding peptides were isolated from two constrained phage libraries. Each shared a five-residue consensus within the gp120 protein. Later on, Zwick *et al.*, (2001) used this consensus sequence to make two sublibraries by fixing the sequence and randomising the residues around it. The tightest binding peptide was identified by screening of a sublibrary with mAb b12 and this was shown to be a homodimer bridged by the single cysteine residues on two identical peptide. Furthermore, the screening of a gene fragment library derived from the mAb gp120 with mAb b12 was not successful, providing further evidence that the epitope recognised by this mAb was discontinuous and complex. This example clearly demonstrates the difficulties and one of the most important limitations of phage-display peptide library approach for identifying discontinuous epitopes, and the usefulness of a large range of libraries rather than a single library to identify binding ligands for mAbs recognising discontinuous epitopes. Therefore, two constrained phage libraries used by

Bonnycastle *et al.*, (1996) (see section 1.7.3.3) were obtained and used in this study to select mimotopes for BVDV.

An 8-mer solid phase library where random peptides displayed on resin beads (Lam *et al.,* 1996) was also used in this study. The presentation of random peptides on solid phase such as, beads is more concentrated from those presented on phage. It has also been shown that solid phase library approach is powerful for selecting mimotopes for important viral pathogens such as MV (Steward *et al.,* 1995) and RSV Chargelegue *et al.*, 1998). The solid phase library approach could make some ligands fit antigen-combining sites of BVDV mAbs easier than phage-display library. Furthermore, the use of a solid phase library could exclude the biological limitations of phage-display libraries for selecting binding ligands. Actually, Bonnycastle's study did not include the use of a solid-phase library besides phage libraries.

1.7.7. Description of phage-display and an 8-mer solid phase libraries used in this study

Two different types of phage-display systems and libraries and an 8-mer solid phase epitope library were used in this study. Phage display systems can be classified as types 3, 3+3, 33, 8, 8+8, and 88, depending on the use of pIII or pVIII as the carrier proteins for random peptide display purpose, the number of fusion products, and the vectors employed (Smith, 1993; Smith and Petrenko, 1997).

The first type of phage libraries displaying random linear 15 and 6-mer peptides cloned into phage gene III by using fUSE5 vector based on type 3 system **(Figure 1.4a, b)** (Scott and Smith., 1990; Smith and Petrenko, 1997). The foreign peptides encoded by the DNA inserts are antigenically displayed on each of five copies of pIII protein on the filamentous phage particles (Smith and Petrenko, 1997). Libraries expressing linear random peptides represent a rich source of diverse molecular shape that could interact with binding sites of variety of ligates and could mimic the shape of the different ligands. In addition, in both libraries, the random peptides have a free amino terminus thus allowing the peptide a considerable flexibility much like peptide free in solution (Burritt *et al.*, 1996; Wilson and Finlay, 1998). Similarly, type 8 vector displays peptides on each copy of pVIII protein (Smith and Petrenko, 1997).

The second type of phage libraries were constructed using the phage vector f88.4, which displays random constrained 17 and 10-mer peptides as approximately 250 copies on the N-terminus of the major coat protein pVIII (Bonnycastle *et al.*, 1996). As with pIII display vectors, f88.4 is a derivative of fd-tet (Zacher *et al.*, 1980). However, f88.4 genome bears two genes of VIII, encoding two different types of pVIII protein; one is ordinarily recombinant and used for random peptide display and the other is wild type. In this respect, f88.4 resembles the type 88 vectors (Smith, 1993; Smith and Petrenko, 1997).

When both pVIII genes are expressed, the recombinant and wild type pVIII coat proteins give a mosaic shape to the phage body (Enshell-Seijffers *et al.*, 1994) as seen in **Figure 1.4c**. It is expected that a vector producing a sparse density of recombinant pVIII-displayed peptides could allow better discrimination between weak and strong binding peptides by antibodies compared to vectors where all copies of pIII have N-terminally fused peptide, or vectors where there is a single copy of pIII-displayed peptide. In addition, these libraries have the potential for constraint by the addition of fixed cysteine (Cys) residues, placed at different sites within a randomised amino acid sequence (Bonnycastle *et al.*, 1996). This could increase the ability of random peptides to adopt stable secondary structures and these peptides could contain higher affinity ligands (Luggazo *et al.*, 1993; McLafferty *et al.*, 1993; Bonnycastle *et al.*, 1996). Similarly, a type '33' vector system bears two genes III, one of which is recombinant (Smith and Petrenko, 1997). This is in contrast to the '8+8' phagemid systems used for pVIII display of peptides and larger proteins (Greenwood *et al.*, 1991) as well as '3+3' system used for pIII display of peptides and larger proteins (Barbas *et al.*, 1993; Martin *et al.*, 1994).

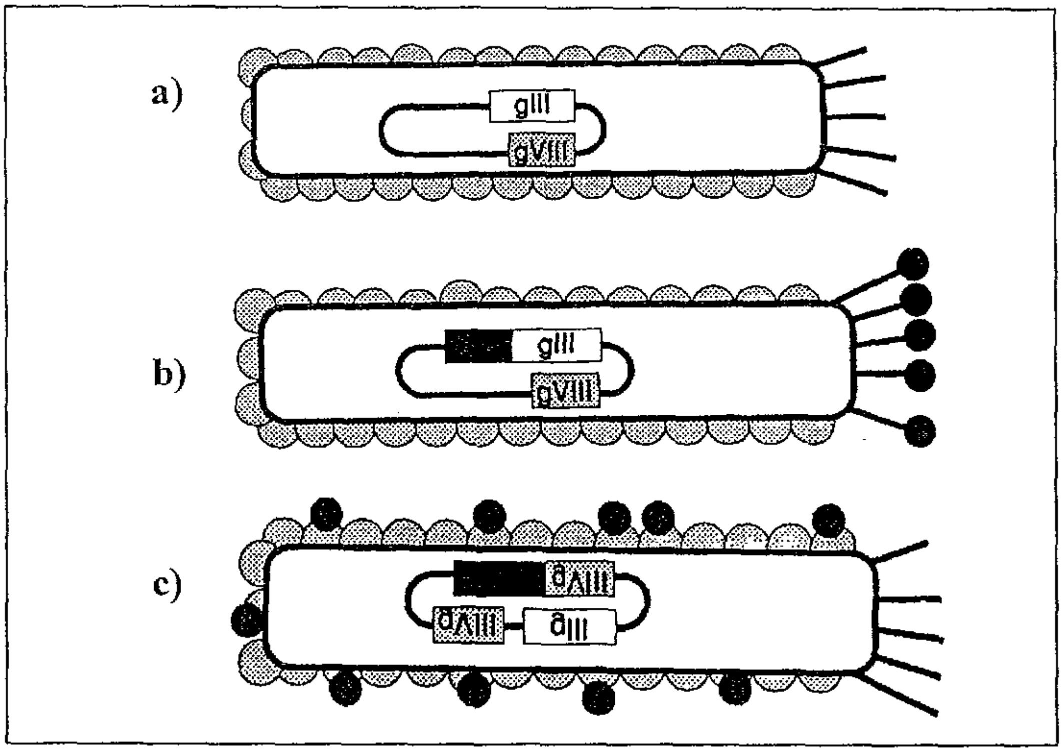


Figure 1.4 (a, b, c). Schematic representation phage-display vectors and phage particles

The black boxes and spheres correspond to foreign DNA inserts and their encoded peptides, respectively. The displayed random peptides are fused to either phage coat protein pIII or pVIII. a) Schematic representation of M13 wild-type phage. b) Schematic representation of pIII display of foreign peptides on a tip of phage particle. c) Schematic representation of recombinant pVIII display of foreign peptides (modified from Armstrong *et al.*, 1996).

The type 8+8 systems differ from a type 88 systems since the two genes of VIII are located on separate genomes. The wild-type version is on a phage that is usually called the 'helper phage', whereas the recombinant version is on a special kind of plasmid called a 'phagemid' (Wilson and Finlay, 1998). The phagemid bears both a plasmid replication origin and phage replication origin and antibiotic resistant gene. The phage replication origin is only effective in the presence of helper phage. Two types of progeny phage virions are thus secreted: virions carrying helper phage DNA and virions carrying plasmid DNA. Both of these virions like type 88 virions are mosaics, whose coats composed of a mixture of recombinant and wild type pVIII proteins. Similarly, type 3+3 and type 6+6 are the similar to the type 8+8 systems, instead of the phagemid carrying an insert bearing recombinant gene III or VI rather than VIII (Smith and Petrenko, 1997).

Both types of phage libraries used in the study require a single entity for their construction and their use in affinity purification is simpler than the use of phagemid systems. In addition, the different peptide display strategies in both libraries have their own advantages that could help for selecting ligands of BVDV E2. Hovewer, the use of an 8-mer solid phase library with the mAb 157 gave the most promising results in the present study.

The description of solid phase libraries and and an 8-mer solid phase library used in this study is given in section 1.7.3.

1.8. The involvement of antibody affinity in selection of mimotopes from phage library

The probability of finding a specific ligand for any given ligate depends on a function of its affinity for the selector ligate such as a mAb and its existence and frequency in the library (Cortese *et al.*, 1996; Bonnycastle *et al.*, 1996).

A phage library includes a small population of phage clones displaying peptides that bind selectively to a selector antibody and considered specific for that antibody. These binding peptides can be further divided into the scarce high affinity binders and much more abundant low affinity binders (Menendez and Scott, 2005). It can be more difficult to select high-affinity binding peptides to antibodies recognising discontinuous (conformational) epitopes than antibodies recognising linear epitopes (Bonnycastle *et al.*, 1996). Bonnycastle *et al.*, (1996) also suggested the use of multivalent peptide display such as pVIII-based constrained type of phage libraries (see 1.7.6) for selecting primary binding peptides for discontinuous epitopes. However, such a multivalent display system in these libraries could be also disadvantageous since phage clones displaying nonbinding peptides and low affinity binding peptides can also be recovered, and they could interfere with the enrichment of phage clones displaying tight binding peptides. In case such interference occur, high affinity binding peptides are best selected by screening of a large panel of libraries for any given mAb or ligate (Bonnycastle *et al.*, 1996) or screening of sublibraries derived from primary binding peptides (see 1.7.6) (Zwick *et al.*, 2001).

Affinity is the strength of binding between the antigen-combining site of an antibody and its interaction with the particular epitope (Janeway *et al.*, 2005). High affinity antibodies bind to epitopes more tightly than low affinity antibodies in a highly specific manner. They are also biologically more effective than low affinity antibodies (Steward and Chargelegue, 1997). This concept was evaluated for the development of a peptide or mimotope based vaccines for FMDV (Steward *et al.*, 1991) and MV (Olszewka *et al.*, 2000). Steward *et al* (1991) suggested that the protection of some but not all cattle by an experimental synthetic peptide vaccine for FMDV correlated with higher affinity antibody production in protected cattle as compared to unprotected cattle. Olszewka *et al.*, (2000) demonstrated the importance of designing a mimotope-based vaccine to MV that was capable of inducing protective high affinity antibody response in mice

High affinity antibodies are valuable substances. Thus, the selection of mimotopes with high affinity mAbs using combinatorial peptide libraries is desirable for designing a mimotope-based vaccine. It is assumed that mimotopes selected with high affinity antibodies will induce valuable high affinity antibody response when they are used as vaccine. On the other hand, it is very likely that high affinity antibodies will be highly selective for their ligands if they are used for combinatorial library selection.

High virus neutralising antibodies are likely to be of high affinity. Bachmann *et al.*, (1997) suggested a correlation of *in vitro* neutralising activity and avidity by evaluating a panel of mAbs directed to the G glycoprotein of Vesicular stomatitis virus (VSV). Kostolansky *et al.*, (2000) also suggested a strong positive correlation between effective affinity and virus neutralising activity using one cross reactive mAb IIB4 recognising an epitope on hemaglutinin of influenza A virus strains of H3 subtype.

1.9. The mAbs of known affinity to DNP was used to test the effect of antibody affinity on selecting mimotopes

Haptens are small organic well-defined molecules of simple structure such as dinitrophenyl (DNP) and phenyl arsonates. They are antigenic but are only able to induce production of anti-hapten antibodies if the molecule is chemically linked to an immunogenic protein carrier. Chemical coupling of a hapten to a large protein is known a carrier that gives hapten-carrier conjugate. The DNP conjugated to a carrier protein, such as bovine serum albumin (BSA) has been used extensively to study antigenicity versus immunogenicity as a model hapten-carrier conjugate system (Janeway *et al.*, 2005).

In order to demonstrate if the antibody affinity influences the frequency of binding ligands using the phage library system, 2-4 Dinitrophenol (DNP) hapten specific mAbs of known affinity were used in the present study.

1.10. The development of a SPOT scan assay for epitope mapping in combination with combinatorial peptide libraries

The synthesis of overlapping peptides covering the primary amino acid sequence of protein antigens has been applied to map B-cell epitopes (Geysen *et al.*, 1987a; Rodda and Tribbick, 1996). The development of the pin system (PEPSCAN) where several hundreds of overlapping peptides covering the entire sequence of protein antigens was made this epitope mapping approach more practical (Geysen *et al.*, 1987; Langeveld *et al.*, 2001). SPOT scan method where peptides are assembled on nitrocellulose membranes (SPOT membrane) instead of polyethylene pins have also been developed for the same purpose (Frank, 1992; Reineke *et al.*, 1999; 2001; Frank, 2002). The reactivity of peptides on the SPOT membranes is then tested by an solid phase ELISA (SPOT scan assay) (Halimi *et al.*, 1996; Chargelegue *et al.*, 1998; Hujer *et al.*, 2004).

Chargelegue *et al.*, (1998) used an 8-mer solid phase library to identify mimotopes of a conserved conformational epitope of the Fusion (F) protein of RSV recognised by the mAb19. Two of the peptides identified from the solid phase library reacted specifically with the selecting mAb when they are presented on solid phase peptides both as resin-bound peptides and as pep tides on nitrocellulose membrane in a SPOT scan assay. Furthermore, the binding of peptides to the mAb19 was greatly improved by doing appropriate amino acid substitution analysis in the SPOT scan assay. It was shown in Chargelegue's study that a SPOT scan assay was the most appropriate way to confirm binding specificity of RSV specific mimotopes that were selected by a solid phase library. This also generated the idea that the presentation of peptides as multiple copies at high density in both a solid phase library and a SPOT scan assay could be critical for the selection RSV mimotopes. Chargelegue *et al.*, (1998) also tested immunogenicity of

RSV mimotopes presented as MAP (Multiple Antigen Presentation) system in mice. Their study revealed that the MAP system was a good way to induce cross-reactive neutralising antibody response to RSV that was protective against challenge in a mice model. In addition, mimotopes presented as MAP system were able to compete with RSV for binding to the selector mAb19. On the other hand, the mimotopes initially synthesised as linear free peptides failed to bind the corresponding antibody and to induce cross-reactive antibody response to F protein of RSV.

From Chargelegue's elegant study, several important consequences can be deduced and used for the present study. 1) The SPOT scan assay can be used to confirm binding specificity of peptides and to map the most antigenic parts of these peptides identified by using combinatorial peptide libraries with mAbs. In addition, the SPOT scan assay should also be used to test the reactivity of peptides selected from phage libraries since peptides synthesised on SPOT system will avoid any possible cross reactivity with the selector mAbs and phage proteins. This was used for potential DNP and BVDV mimotopes in the present study. 2) Once a vaccinal mimotope of a discontinuous epitope has selected from a combinatorial library, it will be possible to improve its reactivity with the selector mAb by making appropriate aminoacid substitution and thus making the conformation of the mimotope similar to that of the epitope. 3) The use of the SPOT scan assay and the MAP system will facilitate the enhancement of antigenicity and immunogencity of mimotopes selected by a solid phase library.

1.11. Aims and objectives of the study

The aim of this study is to define protective neutralising epitopes on E2 glycoprotein for the development of a new generation of mimotope based marker vaccine against BVDV.

Objectives

1) To induce neutralising antibodies to BVDV in mice by mimotopes selected by neutralising mAbs

2) To compare a range of BVDV neutralising mAbs by relative affinity and their ability to select mimotopes

3) To demonstrate the effect of antibody affinity on mimotope selection using low and high affinity mAbs to DNP

4) To make an antigenic map of two 15-mer DNP mimotopes using 8-mer overlapping peptides by SPOT scan assay.

5) To compare a large panel of phage-display and an 8-mer solid phase libraries for the selection of mimotopes for BVDV and to confirm the binding specificity of mimotopes by the SPOT scan assay.

1.12. Layout of the thesis

In the first part of this study, a range of neutralising BVDV E2-specific mAbs was used to select binding ligands by a 15-mer phage library. Four peptides were selected with the mAbWb166. However, their biological activity could not be confirmed by either competitive-inhibition or by a neutralising-inhibition assay. In addition, an 8-mer solid phase epitope library was also used to select binding ligands to neutralising mAbs Wb166 and Wb214. The reactive peptides were selected and three for each mAb sequenced. The peptide Wb214P1 was initially synthesised by coupling a T-helper epitope and used to immunise mice. However, this chimeric peptide failed to induce any form of cross-reactive antibody response with BVDV. Since the reactivity of peptides selected by a 15-mer phage and solid phase libraries were not confirmed by the SPOT scan assay, no more peptides were synthesised for mice immunisation (Chapter 3). These unexpected results were considered to be related to the conformational nature of the epitopes recognised by the neutralising BVDV mAbs and the limitations associated with the combinatorial library system. Another important possibility was related to mimotope selection to the affinity of the mAbs used in this study. The E2 specific BVDV mAbs were tested for their relative affinity (Chapter 4).

In order to trial our techniques and to show the effect of antibody affinity on mimotope selection, a low and a high affinity mAbs to the DNP hapten were tested by a range of available phage libraries (Chapter 5). Two 15-mer mimotopes were then selected to trial the SPOT scan assay for determining the most reactive 8-mer peptides within the 15-mer mimotopes (Chapter 6).

In the last part of the study, one broadly reactive neutralising BVDV-1 mab157 was used in parallel with DNP mAbs (DNP Pα47 and DNP Pα41) with a range of phage-libraries. No binding ligands (mimotopes) of BVDV were selected with the phage-libraries. Finally, an 8-mer solid phase library was used with the BVDV mAb157. The reactivity of two mimotopes was confirmed by a SPOT scan assay (Chapter 7). A plan to synthesise mimotopes for the immunisation of mice was not completed due to lack of the time.

University of Missouri, Columbia, Mo. The constrained type of phage libraries displaying 10 and 17-mer random peptides on pVIII recombinant coat proteins of phages (Bonnycastle *et al.*, 1996) were kindly provided by Jamie Scott, Simon Fraser University, British Columbia, Canada.

2.1.4. Solid phase epitope library

An 8-mer solid phase resin peptide library containing $2.86X10^6$ beads of per g of resin was kindly obtained from Prof. M. Steward, London School of Hygiene Tropical Medicine.

2.2. Cell lines for tissue culture

Two types of cell line namely Madin Darby Bovine Kidney (MDBK) (Madin and Darby, 1958) and Foetal Bovine Lung (FBL) were used during this study. MDBK cells (passage number 150) were obtained from Dr. Martin Fray from the Institute of Animal Health, Compton Laboratory, UK. FBL cells (passage number 6) were also kindly obtained from Dr. Ian Thompson in the Department of Pathology and Infectious Diseases of RVC. MDBK cells were used for preparing BVDV stock, BVDV antigen and testing neutralising activity of BVDV mAbs.

2.3. Mice

Six to eight weeks old, female BALB/c mice were purchased from Charles Rivers, and housed for one week before use in a pathogen free animal facility at the biological unit at the Royal Veterinary College. The animals were kept throughout the experiment in the same unit and were checked for the appearance of any disorder.

2.4. Amplification of phage libraries

To obtain large-scale stocks, phage libraries were amplified based on a protocol by Smith and Scott in 1993. Briefly, a log-phase culture of *Escherichia coli* $K91^R$ that was cultured in 100 ml of terrific broth medium (TB) (Appendix 10.1.2.) was infected by an aliquot of library containing approximately $2X10^8$ clones and shaken slowly at 37 °C for 15 minutes. The culture was poured into a 3 litre baffle flask containing 1 litre Luria-Bertani (LB) (Appendix 10.1.2.) medium supplemented with 18 μg/ml tetracycline and incubated by shaking in a incubator shaker (Innova 4000, New Brunswick Scientific) at 250 rpm overnight at 37°C. The supernatant containing the phage was clarified by pelleting cells at 3000 rpm (MSE, bench centrifuge) at 4 °C for 30 minutes. The sample was clarified by double precipitation with 0.2 volume of PEG solution (BDH Analar) (3.3 M NaCl/ 16.7 % polyethylene glycol 6000) (Appendix 10.1.1.). The phage pellet was finally resuspended in 1 ml of Tris buffered saline (TBS) (50 mM Tris, 150 mM NaCl pH 7.5) (Appendix 10.1.1) containing 0.02% NaN_3 and stored at 4°C. For the amplification of constrained type of libraries, growth medium was supplemented by 1mM isopropyl-1-thio-β-D-galactopypyranoside (IPGT) to maximise expression of random peptides displayed on recombinant pVIII coat protein of phage.

2.5. Biopanning of phage libraries with mAbs

2.5.1. General experimental protocol

Polystyrene beads ¼ inc (Pierce, Rockford, IL) were coated with mAbs in 0.1M carbonate- bicarbonate buffer, pH 9.6, by overnight incubation at 4°C (Parmley and Smith, 1988). The beads were washed with TBS pH 7.5 containing 0.05% Tween 20 (BDL) and then blocked with blocking solution (5% Bovine serum albumin (BSA) (Sigma) in 0.1M bicarbonate buffer) (Appendix 10.1.1.) for 1 hour at 4°C. The beads were placed at –20°C until they were required.

In the first round, two antibody coated beads were incubated with 40μl of epitope library (input phage) in 1 ml reaction mixture TBS containing 1mg/ml BSA at 4C° overnight. The unbound phage clones were removed by washing extensively with TTBS for ten times. The antibody binding phage clones were eluted by incubating the beads with 0.8 ml of elution buffer (0.1 M. HCl adjusted to pH 2.2 with glycine) (Appendix 10.1.1) for 10 min by gentle shaking at room temperature. The eluate was neutralised with 120 μl of 1M Tris-HCl, pH 9.1, and concentrated by using the microcon 30kDa (Amicon) filter

(Millipore Corporation, Bedford, MA) according to the manufacturer's instruction. The concentrated eluate was then amplified by infecting 200 µl of *Escherichia coli* ($K91^R$) cells (cultured in TB medium until the OD_{600} at dilution 1:10 was 0.125-0.250) by shaking at 37°C for 30 minutes. The concentrated eluate and cells were then transferred to 20 ml of LB medium supplemented by 40 µg/ml tetracycline (Sigma) and 100 µg/ml and kanamycin (Sigma) and incubated overnight at 37°C by shaking in an incubator shaker (Innova 4000, New Brunswick Scientific) rotating at 250 rpm.

The phage epitope library that had been used as input for the first round was titrated by serial dilution from 10^{-1} to 10^{-9} and control in TB medium. Similarly, concentrated eluate (output phage) was titrated from 10^{-1} to 10^{-3}. 100 µl of confluent $K91^R$ cells were added to each dilution and mixtures were incubated at 37 C° for 30 min. Then 100 µl of LB medium supplemented with 0.2 µg/ml tetracycline (Sigma) was added to each tube and incubated for 1h. Three ml melted Top agar was added to tubes and then mixtures were poured on to LB agar plates supplemented with 40 µg/ml tetracycline and incubated overnight. Next day, tetracycline resistant colonies on plates (input and output) were counted in order to calculate the yield of biopanning.

The following second and third rounds of the biopanning were performed using a small volume of amplified eluate from the previous rounds. Acid elution steps were used to recover the bound phage from the target mAbs during all three rounds. In the final round of biopanning, the mAb-coated beads were incubated with amplified eluate from 2nd round for 10 minutes. Beads were first incubated with pH 3 elution buffer for 10 minutes by gentle shaking at room temperature and then pH 2.2 Elution buffer. The purpose of stepwise acid elution steps (pH3- pH2.2) combined with a decreased incubation time was to enhance the possibility for recovering the tightest bound phage clones to the mAbs (D'Mello and Howard, 2001).

After the first and second rounds of biopanning, the amplified eluates (phage) were purified by double PEG/NaCl purification procedure. Briefly, purified phage was prepared from 20 ml of overnight cultures of *E.coli* $K91^R$ cells infected with eluates. The cultured cells were then cleared by spinning twice at 2800 rpm (MSE, bench centrifuge) at 4°C for 30 minutes. The amplified phages were isolated by double precipitation with PEG/NaCL. Phage were PEG-precipitated by adding 0.15 volume of

PEG/NaCl (16.7% polyethylene glycol 6000 (w/w)/ 3.3M NaCl) and mixed by inverting the tube 100 times. The mixture was incubated for 4 hours on ice followed by centrifugation at 15000 rpm for 30 minutes at 4°C in the PS-50-A rotor using the Prepspin centrifuge. The pellet was dissolved in 1ml TBS, pH 7.5 and recentrifuged for 5 minutes at 13000 rpm in a microfuge (MSE). The supernatant was again PEG-precipitated overnight at 4°C as before. Finally, pellets were resuspended in 200 μl of TBS containing 0.02% NaN_3 and stored at 4°C.

The progress of biopanning was monitored for each of the three rounds by titrating aliquots of eluted phage that was bound to the mAb (output phage) and input phage by infecting *E. coli* strain $K91^R$ and plating the cells on LB plates supplemented with 40 μg/ml of tetracycline. The titre of the infectious phage was quantified in transducing units (TU) as a result of tetracycline resistant gene since a single infection gives a visible countable tetracycline resistant transductant clone; whereas noninfective phage produced no transductant colonies (Scott and Smith., 1990). The yield obtained each round was estimated as percentage of the input as: Yield % = number of colonies obtained in output/ number of colonies obtained in input x 100 (D'Mello et al., 1997).

2.5.2. Preparation of phage clones for binding assays and sequencing

After final round of all biopanning experiments, individual phage transductant *E. coli* colonies bearing individual phage clones were randomly selected and cultured in 20 ml LB medium supplemented with 40μg/ml tetracycline and 100 μg/ml kanamycin overnight at 37°C by shaking as described in section 2.5.1. The phage clones were purified from the cultures by double PEG/NaCl precipitation protocol as described in section 2.5.1). Pellets were resuspended in 200 μl of TBS, pH 7.5, containing 0.02% NaN_3. A portion of each phage clone was tested by its binding by either simple immunodotblot assay or simple ELISA. The reactive phage clones was then sequenced to deduce amino acid sequences of peptide inserts they display.

2.5.3. Sequencing of phage DNA

The DNA was extracted from the purified phage clones (see above) using the Wizard Miniprep DNA purification kit (Promega) following the manufacture's procedure. A sample of the extracted DNA was run on a 1% agarose minigel for quantitation and to confirm purity. The DNA was stored at -20 °C prior to sequencing. DNA sequencing was performed by the Sanger dideoxy method using an Auto Read™ sequencing kit (Pharmacia) with fluorescence-labelled primer 5'-CCC TAG TTA GCG TTA CG-3' for the phage clones selected by pIII-displayed phage libraries and 5'-CTG AAG AGA AAA AGC primer for the phage clones identified by pVIII based phage libraries. The sequencing reactions were carried out by annealing 1 µl of primer (4 *p*mol/ ml) to 10 µl of 1-2 µg/ml of DNA template for 3 minutes at 65°C. The reaction mixtures were then subjected to a short extension where 4 units of T7 polymerase were added allowed reacting for 10 minutes at 37°C. The extension termination reactions were carried out in a microtitre plate by mixing 3.5 µl of the enzyme-primer-template mixture with 4.25 µl of the four ddNTP (5uM) and dNTP (1uM) with 1 µl of DMSO and 0.25 µl extension buffer. The reaction mixture was incubated at 40 °C for 5 minutes. The reactions were then by adding 4 ηl of stop solution to each well. Finally, the sequencing reactions were denatured at 90°C for 5 minutes just before loading samples on an acrylamide and bis-acrylamide gel, ratio 19:1 (Ultra pure Sequa gel XR, National Diagnostics, UK). Electrophoresis was carried out by using an automated ALF sequencer (Pharmacia). All the sequencing reactions and incubation steps were carried out by using a thermal cycler (Techne PHC-3 Cambridge Ltd, UK).

2.6. Preparation of phage clones with known sequences for binding assays

Following sequencing, phage clones displaying individual peptides were grouped according to their sequence and they were amplified in large volume by infecting 1ml of *Escherichia coli* ($K91^R$) cells cultured in TB medium by shaking at 37°C for 30 minutes. Then, the infected cells were transferred to 250 ml LB medium supplemented by 40 µg/ml tetracycline (Sigma) and 100 µg/ml and kanamycin (Sigma) and incubated overnight at 37°C by shaking in an incubator shaker rotating at 250 rpm. The amplified phage clones were purified by double PEG/NaCl precipitation and their titre of was calculated as TU/ml as described in section 2.5.1.

2.7. Routine subculturing of MDBK and FBL cells

MDBK and FBL cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% foetal calf serum, 100 i.u. benzylepenicillin (Gibco BRL) minimal essential medium (Gibco BRL) and 100μg streptomycin sulphate (Gibco BRL). Cells were cells were grown to confluence in 3-4 days and incubated at 37°C in a 5% CO_2.

For routine subcultures confluent cell monolayers were washed once with PBS (phosphate buffer saline pH 7.5), followed by the addition of trypsin/EDTA for a few minutes. The excess was removed and monolayers were further incubated for 5-10 minutes. The detached cells were resuspended in 10 ml growth medium by gentle pipetting to break any clumps and seeded into medium (80 cm^2 Nunc) or large (175 cm^2 Nunc) flasks containing 25 ml and 50 ml growth medium respectively. Stocks of MDBK and calf lung cells in growth medium containing 20% FCS and 10% dimethyl sulfoxide (DMSO) were labelled with passage number and kept in the liquid nitrogen tank for long-term storage (e.g. one 80 cm^2 flask provided 6 vials for storage). When stocks were taken from liquid nitrogen, cells were rapidly thawed at 37°C, resuspended in 10 ml growth medium containing 20% FCS and spun at 3000 rpm for 10 minutes. The supernatant was removed and the cells were resuspended in 10 ml growth medium and seeded in a small tissue culture flask (25 cm^2).

2.8. Preparation of the BVDV antigen (cell lysate)

BVDV antigen (cell lysate) and mock antigen were prepared for using immunoassays and analysing serum samples by using the method essentially as described by Howard *et al.*, (1985). Briefly, MDBK cells were grown in large tissue culture flasks (Nunc175 cm^2) at a concentration of $10X10^6$ cells per flask. A 24 hours old and 90% confluent cell monolayer was infected with BVDV NADL working stock at low multiplicity (m.o.i 0.1) in 10 ml of maintenance medium for 2 hours at 37°C and 5% CO_2. Control cells for mock antigen were received only maintenance medium. The virus inoculum was removed and 50 ml of maintenance medium was added to each flask and flasks were incubated for 2 days. After observation of 40- 50 % of cytopathic effect under light microscope, the supernatant was removed from flasks and cell sheets were washed once with PBS, pH 7.4. The cell sheets were then scraped off by using cell scrapers (Nunclon) and cells were collected by centrifugation at 500g at 4°C (IEC Centra. 7R refrigerated centrifuge, Life sciences) for 15 minutes at 4°C. The cells were resuspended in 1% non-ionic detergent (Igepal CA-630, Sigma) with constant agitation on a shaker for one hour at room temperature. The cell suspension was cleared by centrifugation as described above. The same protocol was followed for control cells for obtaining mock antigen. The total protein of final content was estimated by using a BCA protein estimation kit (Sigma) according to manufacturer's recommendation. The total protein concentration of BVDV cell lysate and mock cell lysate were standardised to 40µg/ml and stored at -70°C.

2.9. Enzyme Linked Immunosorbent assay (ELISA) for antigen-antibody binding (general protocol)

Three types of ELISA were used throughout this study, namely indirect ELISA, competitive-inhibition ELISA and chequerboard ELISA. These will be specified later. All assays were carried out in 96-well plates (Maxisorb, Nunc or Immulon 2, Dynatech) at 37°C unless otherwise mentioned with reaction volumes of 50µl/well except for the blocking step when 200 µl/well was used. Plates were coated overnight with antigens in the case of phage clones and BVDV cell lysate or mock cell lysate in PBS at 37°C and in the case of DNP_{237} casein conjugate and other protein antigens in 50 mM carbonate-bicarbonate buffer, pH 9.5. All washing steps were done three times with washing buffer (PBS; pH 7.4 containing 0.05% Tween-20 (BDL) (PBS-Tw). The non-specific binding sites were blocked with with PBS, pH 7.4 containing 10% pig serum (Gibco) for 2 hours at 37°C. After washing step, the primary antibodies were diluted in dilution buffer (PBS-Tw 10 % pig serum), added to wells, and incubated overnight at 4 °C or for 3 hours at 37°C. After washing steps, horseradish peroxidase conjugated rabbit anti-mouse IgG (Dako) diluted 1/1000 in dilution buffer was added to wells and plates were incubated at 37°C for 1 hour. After washing plates as before, the substrate, ortho-phenylenediamine (Sigma) diluted in 0.05M phosphate-citrate buffer, pH 5.0 at concentration of 0.4mg ml^{-1} and supplemented with 1.6µl ml^{-1} of 30% H_2O_2 (BDH) was added to all wells and the colour was allowed to develop for 20 minutes at room temperature in the dark. The colour development was stopped by the addition of 25 µl of 2M H_2SO_4 to all wells. The absorbency was measured at 492nm using an ELISA reader (Multiscan plus, version 3.0).

2.10. Peptide synthesis

The 8-mer sequence of BVDV peptide Wb214-P1 was synthesised by using F-moc chemistry by automated solid-phase synthesis (9050 peptide synthesiser: Milligen Division, Millipore, Bedford, Mass). Briefly, F-moc-protected amino acids were converted to the hydroxybenzotriazol-activated esters by treatment with hydroxybenzotriazol diisopropylcarbodiimide in dimethyl foramide (DMF). The subsequent coupling reactions were performed in DMF and F-moc groups were removed by piperidine 20% in DMF followed by series of washes in DMF. After finishing the synthesis, side chain protecting groups were removed and the peptide was cleaved from the resin support by trifluoroacetic acid in the presence of scavengers. The purity of peptide was assessed by analytical high-pressure liquid chromatography (HPLC) and molecular mass of peptide was assessed by mass spectrometry (School of Pharmacy). In addition to this, a chimeric peptide (Th-Wb214-P1) was synthesised by using a Th-helper epitope (288-302: LSEIKGVIVHRLEGV) of measles virus fusion (F) protein (Partidos and Steward., 1990). The chimeric peptide was collinearly synthesised by linking aminoacids of measles virus fusion protein T-helper epitope to the amino terminus of resin supported Wb214-P1 peptide. All peptide synthesis protocols were followed as described above.

2.11. Synthesis of peptides as SPOTs on derivatized cellulose membrane and SPOT scan assay

The DMF (N, N-Dimethylformamide) and NMP (1-Methyl 2-pyrrolidinone) (Sigma) were purchased from manufacturers as peptide synthesis grade with minimal free amine contamination. The methanol, ethanol, dicloromethane (Fluka) and other reagents were of reagent grade. The same protocol was used for SPOT peptide synthesis and the development the SPOT scan assay throughout the thesis.

2.11.1. Assembly of peptides on SPOTs membrane

Peptides were synthesised on a SPOT's pre-activated cellulose membrane by SPOTs method essentially as described by Frank, (1992) and manufacturer's manual (Genosys, Cambridge, United Kingdom). This involves derivatising the hydroxyl constitutes of a commercially available cellulose membrane with 9-fluorenylmethoxycarbonyl-β-alanine (Fmoc-β-Ala) and the subsequent removal of the Fmoc-group. Removal of the Fmoc protecting groups generates free amino functionalities, which after staining with bromophenol blue, appear as distinct blue spots **(Figure 2.1)**. The blue spots represent a modified amino acid that is used as spacer or linker to the membrane thereby peptides are synthesised by coupling amino acids to the spacer (Frank and Overvin, 1996: Reineke *et al.*, 2001). The peptide synthesis on the SPOT's membrane can be done by using a pipeting robot or, as in this case, manual.

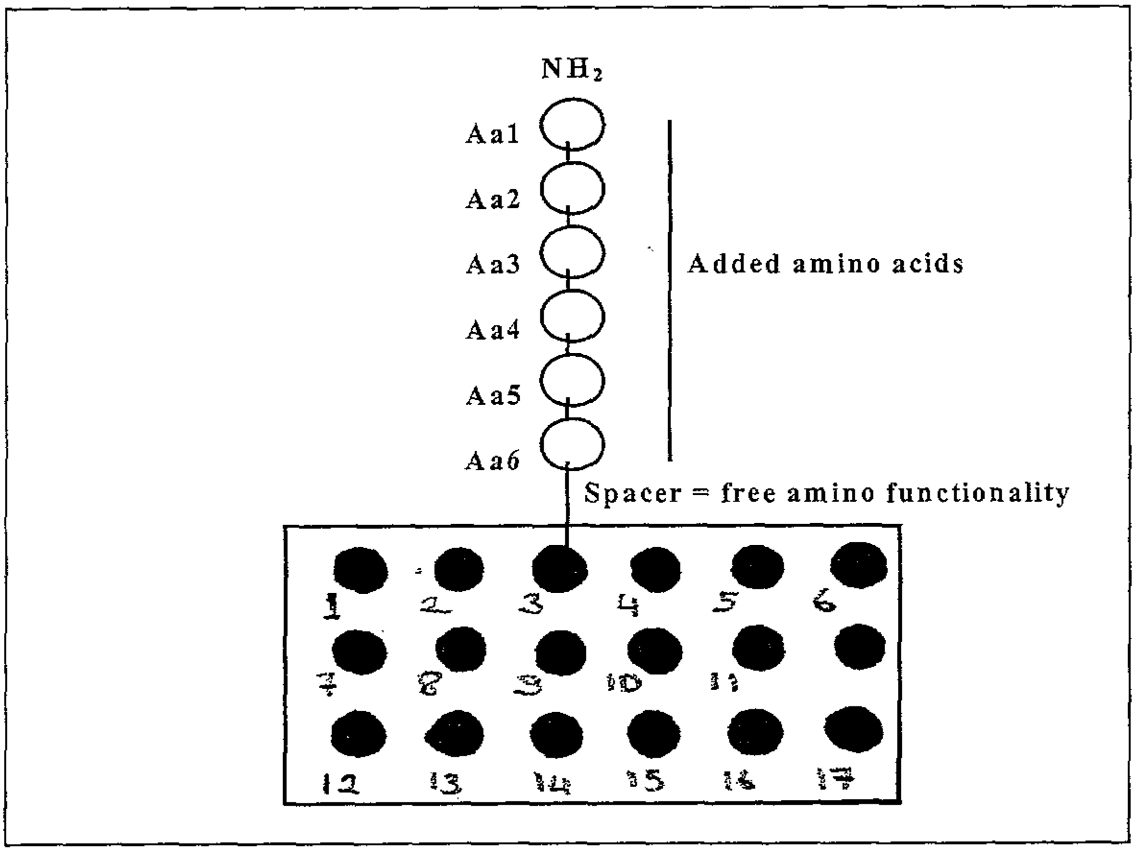


Figure 2.1. Principle of manual SPOT peptide synthesis on a derivatised cellulose membrane

Positions of peptides were marked prior to the first coupling (cycle 1) and 0.9 μl of Fmoc-amino acid derivatives (pentafluorophenyl esters (Opfp) dissolved in NMP were dispensed to these marked areas on the membrane leaving arginine until last. Arginine was dissolved at last, aliquoted, and pipetted out at once. Following 15 minutes incubation, this procedure was repeated and membrane was reincubated for 15 minutes. The same solution of arginine was used for second coupling. The membrane was washed with 20 ml of DMF three times for 2 minutes and all residual amino groups on the membrane were blocked by adding 800 μl of acetic anhydride (Sigma) in last DMF wash by rocking for 15 minutes. The completion of acetylation was observed by removal of the entire remaining blue colour. Following acetylation, the membrane was washed again with DMF by rocking as described above and the Fmoc protecting groups were removed with 20 ml 20% piperidine (Fluka) in DMF followed by a series of washing in DMF in order to generate free amino groups. They were stained as blue spots by adding 1% bromophenol blue (Fluka) solution in 20 ml DMF by rocking for 5 minutes. This step was followed by washing the membrane with methanol three times for 2 minutes. Finally, the membrane was dried with a cool hairdryer. The coupling of amino acids was carried out by repeating the steps described above until the final coupling. The membrane was placed in a plastic bag, sealed and stored at –20 °C at the end of synthesis for each day. After the final cycle, the completed peptides were N-terminally acetylated and then side chain deprotected.

2.11.2. Side-chain deprotection

After finishing synthesis and final acetylation, the protecting groups present on the side-chains of amino acids were removed. Briefly, the membrane was thawed and rinsed in methanol and dried with a cool hair dryer before starting of process. A mixture containing 5 ml of trifluoroacetic acid (Fluka), 5 ml of dichloromethane (Fluka) and 250 μl of triisobutylsilane (Sigma) was made and immediately added to a clean dry polypropylene box containing the SPOT's membrane. The sealed polypropylene box was placed on the rocking table and incubated for 1 hour and the mixture was poured off. The SPOT membrane was washed 20 ml of dichloromethane, DMF and finally methanol three times for 2 minutes. Following the final methanol wash, the SPOT membrane was placed in a folded sheet of Whatman no: 1 chromatography paper and dried with a cool hair dryer and place at -20°C freezer until it was used for antibody binding.

2.11.3. Reactivity of peptides with mAbs in SPOT scan assay

The development of SPOT scan assay was performed as described by Chargelegue *et al.*, (1998). The SPOT's membrane was washed three times with washing buffer (Tris-buffered saline with 0.05% Tween20, pH 8, (TBS-Tween) for 10 minutes and blocked with a casein-based blocking buffer (TBS-Tween with 1% casein) overnight at room temperature. After washing with TBS-Tween, the SPOT membrane was incubated with the mAb at a dilution of 1:100 in blocking buffer at 4°C overnight by rocking. After washing four times for 10 minutes, the membrane was incubated with β-galactosidase sheep anti-mouse conjugate (Genosys) at a dilution of 1:200 in blocking buffer for 2h at room temperature by rocking. Bound enzyme was detected by incubating the membrane with the signal development solution (200 μl of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal 50 μg/ml) (Promega), 100 μl of 1M $MgCl.6H_2O$ and 100 μl of Potassium ferri-ferro cyanide in 10 ml of PBS) for 30 minutes. The SPOT's membrane was then washed with 20 ml of PBS, pH 7.4, twice for 10 minutes. The membrane was then scanned immediately to record the assay. Immediately after probing either with secondary or with primary and secondary antibodies, the SPOT's membrane was regenerated to remove bound antibodies so that reprobing of the membrane was possible with different antibodies. This was achieved by successive washing of the membrane with the following solutions: 20 ml of TBS-Tween (3x10 min), MilliQ water (3x10 min), membrane regeneration solution A (8M urea, 1% (w/v) sodium docecyl sulphate 0.1% (v/v) β-mercapto-ethanol in MilliQ water (3x10 min) and regeneration solution B (50% (v/v) ethanol, 10% glacial acetic acid in MilliQ water) (3x10 minutes). The membrane was then washed with 20 ml of methanol (3X10 min), dried, and stored at -20°C. Before reprobing with another antibody, the membrane was rinsed with methanol.

2.12. Screening of an 8-mer solid phase library with BVDV mAbs

Screening of an 8-mer library with BVDV mAbs was performed essentially as described by Steward *et al.*, (1995) and Chargelegue *et al.*, (1998). Briefly, 0.05 g resin beads were weighed out and first blocked with filter sterilised blocking buffer (10 % BSA, 1 % Tween 20 in PBS) at room temperature by shaking for 2 hours. The beads were divided equally five parts in tubes and 500 μl of a range of dilution of mAbs (1:100, 1:500, 1:1000, 1:5000, 1:10000) made in blocking buffer were added to each tube and beads were incubated for 2 hours at room temperature by shaking followed by overnight at 4°C. After that beads were washed 10 times with washing buffer (PBS- Tween 20 0.05 %). The reactive beads with mAbs were identified after the addition of 200 μl of peroxidase-conjugated rabbit anti- mouse IgG (Nordic, Tilburg) diluted 1: 2000 in blocking buffer for 2 hours at 37°C followed by washing with washing buffer and development with 500 μl of DAB substrate (Sigma) per tube for 20 minutes. Finally, the reaction was stopped by addition of 250 μl of 2 M H_2SO_4. Beads were then transferred to the flat-bottomed wells of a 24 well plate (Nunclon) and dark brown coloured ones were selected by capillary tubes by eye. The peroxidase labelled antibody was stripped off the selected beads by incubating them with trifluoroacetic acid for 30 minutes followed by washing with SDW and three beads for each mAb were sent for sequencing.

2.12.1. Sequencing of peptides displayed on beads

Amino acid sequences on selected reactive beads were determined by micro sequencing Dr. Joe Gray, University of Newcastle, and Molecular Biology Unit. Each sequencing cycle was presented by a chromatogram.

Chapter 3. The use of a 15-mer phage-display and an 8-mer solid phase libraries to select mimotopes of BVDV

3.1. Introduction

Virus neutralising antibodies that are important protective mediators for BVDV-1 and BVDV-2 infections are primarily generated to the major envelope glycoprotein E2. Epitopes mapping studies of BVDV E2 with the neutralising mAbs used in this study suggested that epitopes recognised by the neutralising mAbs were discontinuous or conformational (Paton *et al.*, 1992; Deregt *et al.*, 1998a, b) (see chapter 1, section 1.5). The conformational nature of neutralising epitopes of BVDV-E2 makes it very unlikely that they will be identified from analysis of the primary amino acid sequence of E2. Therefore, combinatorial random peptide libraries namely, a 15-mer phage-display and an 8-mer solid phase libraries were used to select binding ligands to BVDV neutralising mAbs.

In the present study, initially a 15-mer phage library was used to select binding ligands to neutralising BVDV E2 mAbs (Wb166, Wb214, Wb163 and Wb158) by biopanning as described by Parmley and Smith, (1988) and D'Mello and Howard, (2001). The phage libraries had already been successfully used to select binding ligands (mimotopes) of several viral antigens with neutralising mAbs including HBV surface antigen (HbsAg) (D'Mello *et al.*, 1997), MV (Deroo *et al.*, 1998); puumala hantavirus (Heiskanen *et al.*, 1997; 1999), murine corona virus (MHV-A59) (Yu *et al.*, 2000), feline immunodeficiency virus (FIV) (D'Mello *et al.*, 1999).

A 15-mer phage library used by D'Mello *et al.*, (1997; 1999) for selecting mimotopes for HBV and FIV was the same one employed here.

D'Mello *et al.*, (1997) used a 15-mer phage library to select mimotopes of a neutralising mAb 18/7 directed to an epitope on pre-S1 region of HBV. The selected mimotopes on phage were able to compete with HBV HBsAg for binding to the selector mAb and able to induce cross-reactive antibody response to the HBsAg in mice. Heiskanen *et al.*, (1997) showed that the mimotope mimicking the neutralising epitope of G2 protein of puumala hantavirus blocked virus infection in cell culture.

The neutralising mAb Wb166 selected phage clones by biopanning of a 15-mer phage library. The specific binding of these clones with the selector mAb was first tested by an immunodotblot assay used for mimotopes of HBV (D'Mello *et al.*, 1997) and FIV (D'Mello *et al.*, 1999). The reactive phage clones were then sequenced to deduce amino acid sequences of their inserts to determine how many different peptides would be potential mimotopes of BVDV E2. Phage clones were then grouped according to amino acid sequences of their inserts. The insert peptides were then compared with sequence of E2 to detect any similarities or localise epitopes recognised by the mAb Wb166 by Proasd program (Padlan, 1977) as used for mimotopes of HBV and FIV (D'Mello *et al.*, 1997; 1999). The phage clones were optimised and tested for their specific reactivity with the mAb in an immunodotblot assay to see if the cross-reactivity observed with the irrelevant clones would be decreased. The specific reactivity of peptides on phage was also tested by a western blot assay as used for mimotopes for plasmodium falciparum (Coley *et al.*, 2001).

The mimotope potential the selected peptides on phage mimicking the BVDV E2 epitope recognised by the selector mAb Wb166 was tested by a competitive-inhibition ELISA as earlier used for mimotopes of HBV (D'Mello *et al.*, 1997) and puumala hantavirus (Heiskanen *et al.*, 1997) and a neutralisation inhibition assay as for puumala hantavirus (Heiskanen *et al.*, 1997). However, the mimotope potential of the peptides were not confirmed by these assays.

To extend the chance of selecting mimotopes of BVDV, an 8-mer solid phase library (Lam *et al.*, 1991) that had been very successful for selecting mimotopes of MV (Steward *et al.*, 1995) and RSV (Chargelegue *et al.*, 1998) was used to select mimotopes of BVDV. The screening of an 8-mer library with mAbs Wb166 and Wb214 selected potential mimotopes of BVDV E2. Their amino acid sequence homology to BVDV E2 was compared by Proasd program.

A SPOT scan assay was used to investigate the specific binding of all peptides selected from an 8-mer solid phase library and a 15-mer phage library. In a Spot scan assay, peptides are synthesised in the same orientation on SPOT's nitrocellulose membrane as synthesised on resin beads in the solid phase library. Thus, the assessment of the reactivity of peptides with the selecting mAbs would be an appropriate way to confirm their binding specificity (Chargelegue *et al.*, 1998).

Chargelegue *et al.*, (1998) used a spot scan assay to further select and improve RSV vaccinal mimotopes selected by screening an 8-mer solid phase library with a neutralising mAb19 to F protein. In their study, they used the SPOT scan assay for reselecting mimotopes of RSV and making the best vaccinal mimotope by doing amino acid replacement studies. It was shown that the presentation of peptides on solid phase in SPOT scan assay with high density was very critical for appropriate antigenic and immunogenic mimicry of the RSV mimotope.

Heiskanen *et al.*, (1999) also used the SPOT scan assay to confirm the binding of 18-mer peptides selected by biopanning of an 18-mer constrained phage library with the neutralising mAbs 5A2, 4G2 and 1C9 recognising discontinuous epitopes of puumala hanta virus (PUU) G1 and G2 envelope glycoproteins respectively. The peptides selected by biopanning reacted with their selector mAbs in the SPOT scan assay and had similarities with the primary amino acid sequences of envelope glycoproteins G1 and G2. The synthesis of amino acid sequences of these glycoproteins as overlapping peptides and probe them with the selector mAbs 4G2 and 1C9 in the SPOT scan assay revealed that the reactive sites corresponded to the sites where they matched with phage-displayed peptides. This study was also a good example for using both phage library technology and the SPOT scan assay for mapping discontinuous epitopes of PUU.

With regard to BVDV, the SPOT scan assay was used to investigate binding of all potential mimotopes peptides selected from an 8-mer solid phase library and a 15-mer phage library in the present study. This may also avoid false positive results or non-specific reactivity obtained during an 8-mer solid phase library selection procedure. Whilst this was ongoing, the first peptide selected by the mAb Wb214 was collinearly synthesised with the T-helper epitope of F protein of MV for mice immunisation studies to trial this technique, as done for MV mimotopes (Steward *et al.*, 1995) and RSV mimotopes (Chargelegue *et al.*, 1998).

3.2. Materials and Methods

3.2.1. BVDV monoclonal antibodies

The neutralising BVDV E2 mAbs (Wb166, Wb214, Wb163 and Wb158) were described in (chapter 1, section 1.5 and chapter 2, section 2.1.1).

3.2.2. Combinatorial peptide libraries

A pIII-based linear 15-mer phage-display and an 8-mer solid phase libraries used in this study were described in (chapter 1, section 1.7.6 and 1.7.7 and chapter 2, section 2.1.3 and 2.1.4).

3.2.3. Biopanning of a 15-mer phage library with BVDV E2 mAbs

Approximately $2X10^{10}$ TU/ml phage clones were incubated with the mAb coated beads (1:100 dilution) and subjected for a three rounds of biopanning as described in (chapter 2, section 2.5.1).

3.2.4. Testing the reactivity of phage clones by immunodot blot

After the final round of biopanning experinents, twenty six phage transductant *E. coli* colonies bearing individual phage clones were randomly selected and cultured in LB medium overnight and then they were purified from the cultures and suspended in 200 µl of TBS buffer (see chapter 2, section 2.5.2 for detail). The specific binding of these phage clones with the mAb Wb166 were analysed by immunodot blot assay. Phage clones displaying peptides specific for MV and FIV were used as unrelated controls. Briefly, 5 µl of each phage clone was blotted onto a nitro-cellulose filter (0.45 µm, Amersham). The non-specific binding sites were blocked with blocking buffer (PBS, pH 7.4 containing 2% skimmed milk powder) for 1 hour at room temperature with gentle shaking. After washing three times with washing buffer (PBS containing 0.05% Tween-20), the membranes were incubated with the mAb Wb166 diluted 1:500 in dilution buffer (PBS+2% skimmed milk powder + 0.05% Tween20) in a sealed plastic bag overnight at 4°C on a shaker. The filters were washed as described above and affinity purified horseradish peroxidase conjugated goat anti-mouse IgG (Jackson) diluted 1:1000 were incubated with membranes for 1 hour at room temperature. The membranes were then washed twice as before and once with PBS only. The bound antibody was detected by incubating the membranes with DAB substrate (one tablet of 3,3'-diaminobenzidine tetrachloride (Sigma) was dissolved in 15 ml of PBS, pH 7.4, followed by adding 30 mg of 4-chloro-1-Naphthol (Sigma) and 15 μl of 30% H_2O_2. The substrate was filtered through 0.4μm syringe filter. The membrane and substrate were rocked on a platform for 15 minutes. Washing the membrane thoroughly with tap water stopped the reaction.

3.2.5. Amino acid sequences of peptide inserts of phage clones and their comparison with BVDV E2

Following immunodot blot assay, all reactive phage clones were sequenced to deduce amino acid sequences of insert peptides displayed on phage by single-strand dideoxy-sequencing using the chain termination method (Sanger *et al.*, 1977). A detailed description of these methods was given in General Materials and Methods (chapter 2, section 2.5.2, 2.5.3).

The homology of peptide sequences was compared with the BVDV (NADL) E2 sequence (Collet *et al.*, 1988) by using the Proasd program. This is based on comparing a short peptide sequence with that of the complete protein sequence. It takes into account the structural dissimilarity between two sequences expressed as the average structural dissimilarity (ASD) value (Sneath, 1966; Padlan, 1977). Sequences with a low ASD score are indicative of the greatest similarity with the compared sequence, whereas those with the highest ASD scores are least similar.

3.2.6. Immunodot blot assays with phage clones of known sequences

Following sequencing, phage clones were grouped according to sequences of insert peptides they display. Phage clones were then amplified in a larger volume and their titre was determined as TU/ml (see chapter 2, Section 2.5.1). Reactivity of these phage clones was tested by using 11 irrelevant control phage clones displaying mimotopes of HBV (D'mello *et al.*, 1997) and portions of unselected a 15-mer and a 6-mer phage libraries.

To asses the reactivity of phage clones more accurately, equal amount of quantified phage clones including control clone were used in immunodot blot assays. Briefly, phage clones were titrated and standardised as $2X10^{10}$ TU/ml. 5 μl of each phage clone including an unrelated control clone was aliquoted in the defined areas on nitro-cellulose membranes by titrating 2 fold starting from 1:2 to 1:512. The membrane was then incubated with the mAb Wb166 at a dilution of 1:500 overnight. The assay was developed as described in section 3.2.4.

3.2.7. Western blotting and immunostaining

SDS-polyacrylamide gel electrophoresis of phage proteins

Phage clones of known sequences were electrophoresed by SDS-PAGE gel according to the method described by Laemmli (1970). The samples were first denatured by mixing 5:1 with 5x loading buffer (500 mM Tris, pH 6.8, 100mM dithiothreitol (Sigma), 2% SDS (BDH), 0.1% Bromophenol blue (Fluka) and 10% glycerol and were boiled for 5 minutes in a water bath. The denatured samples were electrophoresed on a 15% Tris-HCL (Ready Gels from Bio-Rad) and 15 μl of samples were loaded. The gels were cast at room temperature and were initially electrophoresed at 100 volts for 24 minutes until the dye had migrated in to resolving gel and then the voltage was increased to 200 volts for 36 minutes. The running buffer consisted of 25mM Tris base (BDH and 0.1% SDS). A mid range protein (mol wt 14,400- 97,400) standard was used as a molecular weight marker (promega). The gels were removed from the apparatus for electroblotting.

Electroblotting

The mini Trans-Blot cell (Bio-Rad) was used. At the end of electrophoresis, a 0.45μm, supported nitro-cellulose membrane (Bio-Rad) was floated on the surface of deionised water, allowed to become wet from underneath by capillary action and then submerged for at least 5 minutes to displace any trapped air bubbles. Three sheets of Whatman 3MM filter paper cut to the size of the gel were soaked in transfer buffer (39mM glycine, 48mM Tris base, 0.037% SDS, 20% Methanol, pH 9.2 (BDH), placed on the cathode electrode plate, and air bubbles squeezed out with a wooden rod. The gel was then rinsed gently with deionised water and placed carefully on to the 3MM filter paper. The nitro-cellulose membrane was placed on the top of the gel, aligned and air bubbles removed. Another three wet sheets of 3MM filter paper were placed on the top of the nitro-cellulose membrane. Finally, the lid was placed on the top of the stack, graphite side-down, and electroblotting was carried out at 160mA for 1.5 hours at room temperature.

Immunostaining

All the immunostaining steps were carried out in a mini hybridisation oven (Hybaid) at 37°C. The membrane was blocked with blocking buffer of 3% gelatine in Tris-buffered saline with Tween-20 (TBST: 10 mM Tris-HCL pH 8.0, 0.15 M NaCL, 0.05% Tween-20) for 1 hour. The membrane was then cut and the small part of the membrane was incubated with a pIII-phage protein specific mAb (Dente *et al.*, 1994) diluted in 1:100 in a sealed plastic bag that was used as assay positive control. The other part of the membrane was incubated with the mAb Wb166 diluted 1:1000 in blocking buffer in a sealed plastic bag overnight at 4°C. Following washing of both membranes separately, rabbit anti-mouse IgG conjugated to alkaline phophatase at a dilution of 1:5000 was incubated with membranes for 1 hour to detect the antigen-antibody binding. A 5 ml volume of ready to use Western blue™ colour substrate for alkaline phosphatase of BCIP (5-Bromo-4-chloro-3-indolyl-phosphatase)/NBT (nitro blue tetrazolium) colour substrate (promega) was reacted with the blots for 10 minutes. The blots were then washed thoroughly with deionised water, read and stored in a sealed plastic bag at 4°C.

3.2.8. A competitive-inhibition ELISA to confirm binding specificity of peptides on phage

The phage clones and an irrelevant control phage clone displaying peptide to MV were amplified in a large volume and titrated (TU/ml) as described in General Materials and Method (chapter 2, section 2.6). Each clone was adjusted to be $2x10^{11}$ TU/ml. 96 Well ELISA plate (inhibition ELISA plate) were coated with 50 μl of BVDV cell lysate (Howard *et al.*, 1985) at a dilution of 1:100 in PBS (chapter 4, section 4.3.2) at 37°C. Another 96 well plate was first blocked with blocking buffer and incubated for 2 hours at 37°C. The plate was washed three times with washing buffer and dried. Serial two-fold dilutions of inhibitors (Phage clones A1-1, A2-1, A2-2, A2-3 and a control clone, BVDV cell lysate and mock cell lysate) were diluted in blocking buffer and titrated across the plate retaining 25μl to each of duplicate wells. Equal amount of the mAb Wb166 (1: 64000 dilution) (see chapter 4, section 4.3.3) that was known to give 0.6-0.7

solid phase library with the mAb Wb214 was synthesised as linear free peptide by using F-moc chemistry by automated solid-phase synthesis (9050 peptide synthesiser (Milligen Division, Millipore, Bedford, Mass). In addition, a chimeric peptide was synthesised in which a T-helper epitope of the F protein of MV (Partidos and Steward, 1990) was co-linearly synthesised at the amino terminus of resin supported Wb214-P1 peptide (23-mer). The detailed description of peptide synthesis was given in General Materials and Methods (chapter 2, section 2.10).

3.2.12. Immunisation of mice with chimeric (Th-Wb214 P1) peptide

Groups of four Balb/c mice were immunised intraperitoneally with 50 μg of the Th-Wb214-P1 chimeric peptide diluted in SDW and emulsified with Freund's complete adjuvant (FCA) (1:1) (200 μl for each mice). A control group was immunised with FCA with diluent. Immunised mice were bled before injection and 2 weeks after injection, and the boosted after 3 weeks with the same dose of peptide in Freund's incomplete adjuvant (FIC). Mice were immunised at day 0 and 21 and blood samples were collected at day 0, 14, and 28.

3.2.12.1 ELISA for the detection of anti-peptide and anti-chimeric antibodies in mice sera

Each group of sera was pooled and tested for anti-peptide reactivity to the mimotope and the chimeric peptide. The presence of anti-mimotope and anti-chimeric peptide antibodies was assessed by a solid-phase ELISA. Wells were coated with 50 μl of the peptise Wb214-P1 and the chimeric peptide Th-Wb214-P1 at 10μg/ml in sodium carbonate buffer (pH 9.6) overnight at 4°C. The plates were then washed with washing buffer and blocked with 100 μl of blocking buffer for 2 hours at 37°C. After washing, the plates were incubated with serial twofold dilutions of mice sera in the diluting buffer for 2 h at 37°C.

3.2.12.2. ELISA for detecting BVDV E2 antibodies to mice sera

The presence of antibodies to BVDV E2 in mice sera immunised with the Th-Wb214-P1 chimeric peptide was tested by ELISA following one week boosting. Wells of a

OD_{492} nm with BVDV cell lysate was added to wells and the plate was incubated at 37 °C for 2 hours. The mixture was then transferred to the inhibition ELISA plate and the plate was incubated for 2 hours at 37°C.

3.2.9. A neutralisation-inhibition assay to test antigenic mimicry of peptides on phage with BVDV E2

Phage clones (A1, A2-1, A2-2, and A2-3) and an irrelevant control clone to MV were adjusted to be $2x10^{11}$ TU/ml and filter sterilised (0.2 µm filter, Amicon). Serial two fold dilutions of phage clones in the maintenance medium (starting from neat to 1:2048) were made in wells of 96 flat bottom plates (Nunclon) retaining 25 µl in each of the duplicate wells. Equal amount of the mAb Wb166 that had a neutralising titre of 1:512000 was diluted 1:256000 and then mixed with the phage clones. The plate was incubated at 37 °C and 5% CO_2 for 1 hour.

50 µl of BVDV NADL giving 100 $TCID_{50}$ were aliquoted to wells, excluding the cell control wells, and the plate was further incubated for another 1 hour. MDBK cells adjusted to $2x10^4$ (cells /per well) were added and the plates were incubated up to 5 days at 37 °C and 5% CO_2 until a complete cytopathic effect was observed in the virus control wells. Plates were fixed with 10% formalin in PBS (pH 7.4) and stained with crystal violet.

3.2.10. Screening of an 8-mer solid phase library with BVDV E2 mAbs (Wb166 and Wb214)

A portion of 8-mer solid phase epitope library (0.05 g) was screened with the BVDV E2 mAbs Wb166 and Wb214. Three beads, which stained dark brown at the lowest mAb concentration (1:500) for each mAb, were finally selected and sent for microsequencing (University of Newcastle, Molecular Biology Unit). The detailed description of an 8-mer solid phase library selection procedure was given in General Materials and Methods (chapter 2, section 2.12 and 2.12.1).

3.2.11. Synthesis of Wb214-P1 and chimeric Th-Wb214-P1 peptides

The potential mimotope peptide Wb214-P1 that was selected by screening an 8-mer microtiter plates were coated with BVDV cell lysate and mock cell (control) lysate at a dilution of 1:100 at 37°C for overnight. The plates were then washed with washing buffer and blocked with 100 µl of blocking buffer for 2 hours at 37°C. After washing, the plates were incubated with 50 µl of serial twofold dilutions of mice sera starting from 1:25 to 1:800 in the blocking buffer for 2 h at 37°C. ELISA was developed as described in General Materials and Methods (Chapter 2, Section 2.9).

3.2.12.3. A Neutralisation assay for detecting neutralising antibodies in mice sera

The presence of neutralising antibodies in mice immunised with the Th-Wb214-P1 chimeric peptide was tested following boosting in a microtiter neutralisation assay Briefly, serial 2-fold dilutions of mice sera was diluted across microtiter plates starting from 1:10 to 1:12800 and duplicate wells were used for each dilution series and 50 µl of diluted sera was retained in each well. The neutralising E2 specific BVDV mAb Wb163 (1:1000 dilution) was used as assay positive control. An equal volume of BVDV NADL that was diluted to give 100 $TCID_{50}$/0.05 ml was aliquoted to each well except for cell control. The plates were incubated for 1 hour at 37°C and 5% CO_2. Finally, 50 µl of a suspension of MDBK cells at $2X10^4$ cells/ each well suspended in maintenance medium was added and the plates were incubated up to 4 days at 37°C and 5% CO_2 until complete cytopathic effect (cpe) was observed in virus control wells. Plates were fixed with 10% formalin in PBS (pH 7.4) and stained with crystal violet.

3.2.13. A SPOT scan assay for testing the peptides selected by an 8-mer solid phase and a 15-mer phage libraries

Synthesising peptides on SPOT's membrane

Peptides selected by screening an 8-mer solid phase library with mAbs Wb214 and Wb166 and peptides selected by biopanning of a 15-mer phage library with the mAb Wb166 were synthesised on SPOT's activated nitrocellulose membrane (Frank, 1992) by the SPOT's method (Genosys, Cambridge, UK). An 8-mer peptide QLDPAFGA from HBV pre-S1 region of HBsAg comprising residues 29-36 (D'Mello *et al.*, 1997) was synthesised as an irrelevant control peptide (CP1). In addition, a 6-mer peptide CAHYID (CP2) representing an epitope of the extracellular domain of human epidermal growth factor receptor (EGF-R) and the mAb specific for this domain (c-erb-

1/EGF-R) were employed as assay positive control (Parker *et al.*, 1984). The detailed description spot peptide synthesis is given in General Materials and Methods (chapter 2, section 2.11.1 and 2.11.2).

Testing the reactivity of peptides with mAbs by SPOT scan assay

The reactivity of peptides with BVDV mAbs (Wb166 and Wb214) and control peptides with the control mAb EGF-R were tested by a solid phase ELISA (SPOT scan assay) (Frank and Overvin, 1996). The detailed description of the SPOT scan assay development protocol is given in General Materials and Methods (chapter 2, section 2.11.3).

Conjugate control assay

The reactivity of all peptides (No: 1-17) was first tested with the sheep anti-mouse β-galactosidase conjugate to detect any non-specific binding. Following conjugate control assay, the membrane was regenerated and the reactivity of peptides with the mAbs was tested. Briefly, the mAbs diluted in 1:100 and incubated with the SPOT membrane overnight. After testing the peptides with each mAb, the membrane was regenerated for reusing. Brief description of testing peptides with mAbs is given below:

Testing the reactivity of peptides with the mAb Wb166 and control peptides with mAb EGF-R

The membrane was then regenerated and the reactivity of peptides (No: 1-15) was tested with the mAb Wb166 and control peptides (No: 16-17) with the control mAb to EGF-R (assay control) at the same time using the same batch of reagents.

Testing the reactivity of peptides with the mAb Wb214

After regenerating the membrane, peptides (No: 1-15) were tested with the mAb Wb214.

Assessment of the reactivity of peptides

The strength of the reaction between peptides and mAbs were assessed by comparing visually the development of blue colour intensity that was expressed as arbitrary units (AU) (+, ++, +++, ++++, etc) (Chargelegue *et al.*, 1998).

3.3. Results

3.3.1. Biopanning of a 15-mer phage library with BVDV E2 mAbs

A three round biopanning was carried to select binding ligands to E2 specific neutralising mAbs Wb166, Wb214, Wb163 and Wb158 for several trials. The only mAb Wb166 selected phage clones that were amplified for subsequent rounds. In the first experiment, no colony formation was observed in the plates during the first round, but amplification of the bulk-eluted phage was successful and the experiment was continued for subsequent (second and third) rounds (**Table 3.1**). The ratio of yields between second and third rounds was 9. The experiment was repeated using a freshly amplified of new batch of the same library. This time phage colony formation was observed for each round in output. The ratio of yields between second and fist round of biopanning was 24, which was indicative of enrichment of the bound phage clones although no subsequent enrichment was obtained in the third round (**Table 3.1**). Four phage clones from the first and 22 from the second experiment collected from the plates and propagated for the future work (sequencing and binding assays).

Table 3.1. Yields obtained from biopanning of a 15-mer phage library with the mAb Wb166.

		1st experiment			2nd experiment		
Rounds	**Incubation time**	**Input (I) TU**	**Output (A) TU**	**% Yield**	**Input (I) TU**	**Output (A) TU**	**% Yield**
1st	Overnight	2×10^9	0^a	0	8×10^9	4.5×10^2	0.0000056
2nd	4 hours	2×10^9	9×10^2	0.004	5×10^{10}	6.7×10^4	0.000134
3rd	10 mins	1×10^9	3.6×10^3	0.036	4×10^{10}	$5.5X10^3$	0.0000137

The percentages of yields for the three rounds of biopanning were determined by dividing the number of eluted phage (output) by the number of input phage times 100. Increases in percentage (%) of yield with the mAb Wb166 between rounds were 9 for the first experiment and 23.92 for the second experiment.
a) No clones observed.

3.3.2. Testing the reactivity of phage clones by immunodot blot

In order to confirm binding specificity of peptides displayed on phage with the mAb Wb166, an immunodotblot and a Western blot assays were used.

The neutralising BVDV E2 mAb Wb166 was first examined for reactivity with all 26 phage clones obtained by biopanning. It can be seen from the **Figure 3.1 (a, b)** that reactivity was observed with all phage-clones including the unrelated phage clones. This was first considered to due to cross-reactivity between two unrelated phage clones and the mAb Wb166.

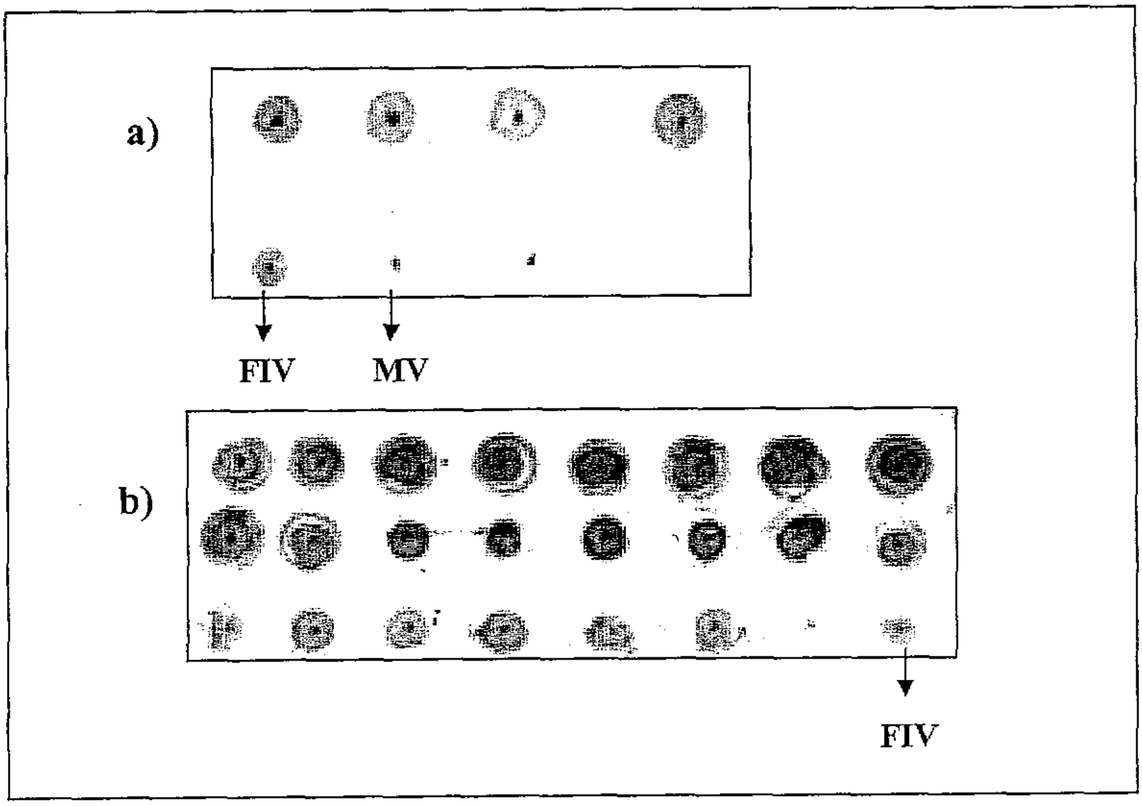


Figure 3.1 (a, b). Testing the reactivity of phage clones by immunodot blot

a) Four phage clones obtained from the first biopanning b) 22 phage clones obtained from the second repeat of the same experiment. Measles (MV) and FIV phage clones were used as unrelated clones. The phage clones were blotted on to a nitrocellulose filter. Reactivity of phage clones was tested with the mAb Wb166 at a dilution of 1:500.

3.3.3. Amino acid sequences of peptide inserts of phage clones and their comparison of BVDV E2

The sequencing of four phage clones selected in the final round of the first biopanning gave an identical sequence and it was designated as A1-1 SRFALVLCAWLSVAF. The sequencing of 22 phage clones selected in the second and third rounds of the second repeat of the biopanning gave three different peptides and they were designated as A2-1 SRLYGPFLYYWAVAE, A2-2 SFSGSFSHIPFSSVE and A2-3 NRLFVSTTSIGNRYV. The sequences of these peptides and their comparison with BVDV-E2 (NADL) (~690-1090 amino acids) compared by proasd program **(Table 3.2)**. The lowest ASD score (55.01) was observed between the A2-3 NRLFVSTTSIGNRY and the BVDVE2 region SILVVVVALLGGRY that is located within the carboxyl end of BVDV-E2.

Table 3.2. Sequences of peptides and their comparison with the E2 region of BVDV (NADL)

Peptide sequences	BVDV (NADL) E2 region	Sequences	ASD score
A1-1 **SRFALVLCAWLSVAF**	1033-1046	AESILVVVALLGG	62.49
A2-1 **SRLYGPFLYYWAVAE**	1042-1053	ALLGGRYVLWLLVY	69.51
	942-705	GLDDCKPEFSYAIA	70.27
	880-893	TCVPGVQLLYKGGS	70.34
A2-2 **SFSGSFHIPFSSVE**	825-838	GVTGTVCTSFNMD	67.01
	874-887	LLGGNWTCVPGDQL	68.80
	830-843	VSCTSFNMDTLATT	71.31
A2-3 **NRLFVSTTSIGNRYV**	**1035-1048**	**SILVVVVALLGGRY**	**55.01**
	986-999	ACTFNYTKTLKNKK	67.53

Similarity between the phage-display peptides and the E2 region of BVDV NADL was compared by proasd program and results were expressed as ASD score. The peptide showing the lowest ASD score with the BVDV E2 were in bold and underlined.

3.3.4. Immunodot blot assays with phage clones of known sequences

Testing the reactivity of phage clones (A1-1, A2-1, A2-2 and A2-3) with the mAb Wb166 by using 11 irrelevant clones of HBV (No: 5 to15) and unselected a 15 and a 6-mer phage libraries (No: 16-17) showed that the reactivity was observed with all the phage clones (**Figure 3.2a**). An immunodot blot experiment was then set up following titrating and standardising the amount of the test and an irrelevant MV clone. Titration of phage clones might be expected to reduce non-specific reactivity with the irrelevant clone to the mAb Wb166. It can be seen in **Figure 3.2b** that the reactivity of phage clones and the control MV clone was not markedly affected. These results indicate that there is a cross reactivity between the mAb Wb166 and one or more phage proteins.

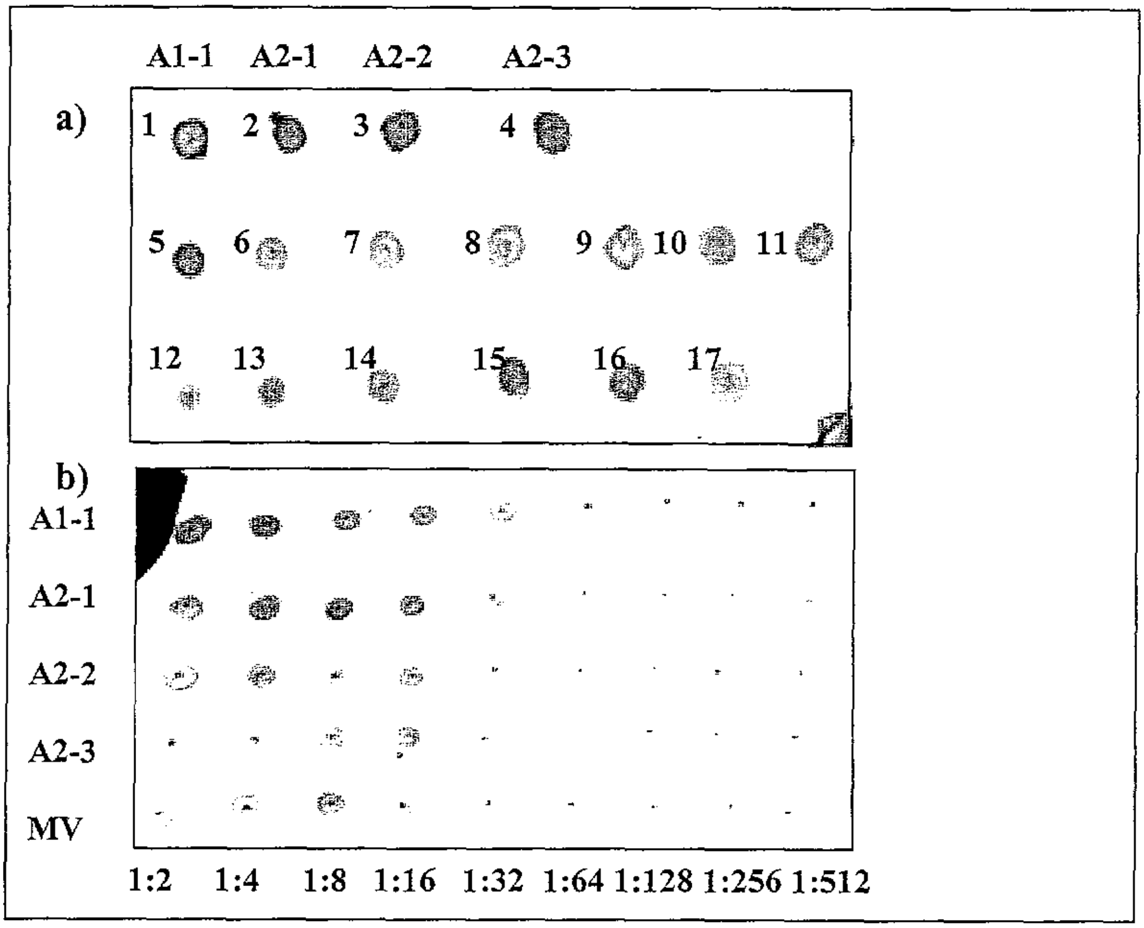


Figure 3.2 (a, b). Immunodot blot assays with phage clones of known sequences with the mAb Wb166

a) Reactivity of phage clones A1-1, A2-1, A2-2 and A2-3 (No: 1-4) with the mAb Wb166 was tested by using a number of irrelevant control phage clones (No: 5-15) and a 15-mer and a 6-mer unselected phage libraries (No: 16-17) respectively. **b)** Phage clones were titrated and standardised, as $2X10^{10}$ TU/ml. 5 μl of each clone was aliquoted in the defined areas on nitro-cellulose membrane by titrating 2 fold starting from 1:2 to 1:512. The membrane was incubated with the mAb Wb166 at a dilution of 1:500 overnight. Phage clone displaying an irrelevant peptide to MV was used as unrelated control.

3.3.5. Reactivity of phage clones with the mAb Wb166 by western blot assay

In order to further investigate the binding specificity of the phage clones (A1-1, A2-1, A2-2, and A2-3), all clones were run on a SDS-PAGE gel, protein samples were transferred to a nitrocellulose filter and probed with the mAb Wb166.

The objective of this experiment was to demonstrate if there was any additional specific reactivity between the phage displayed peptides and mAb Wb166 rather than only to the normal phage coat proteins as suggested by the immunodot blot experiments. The BVDV infected cell lysate and mock cell lysate were used as positive and negative controls respectively (see chapter 4, section 4.3.3). In addition, a mAb specific for the pIII phage minor coat protein (Dente *et al.*, 1994) was also used as assay positive control and probed with clone A1-1.

No reactivity was observed between mAb Wb166 and phage proteins, particularly the pIII protein containing the random insert peptides, whereas a specific reactivity was detected with the pIII specific mAb and pIII protein of clone A1-1 **(Figure 3.3.)**. A reactivity was also observed between the BVDV cell lysate and mAb Wb166 as seen in lanes 3 and 4. The estimated size of the major protein band (53-55 kDa) was suggestive of the E2 of BVDV. Some other minor bands were observed with the mock-infected control cell lysate.

The lack of the reactivity between the phage clones displaying peptides and the mAb Wb166 could be due to either the denaturation of the peptides during western blotting or peptides on pIII protein were not specific binders (see section 3.3.4).

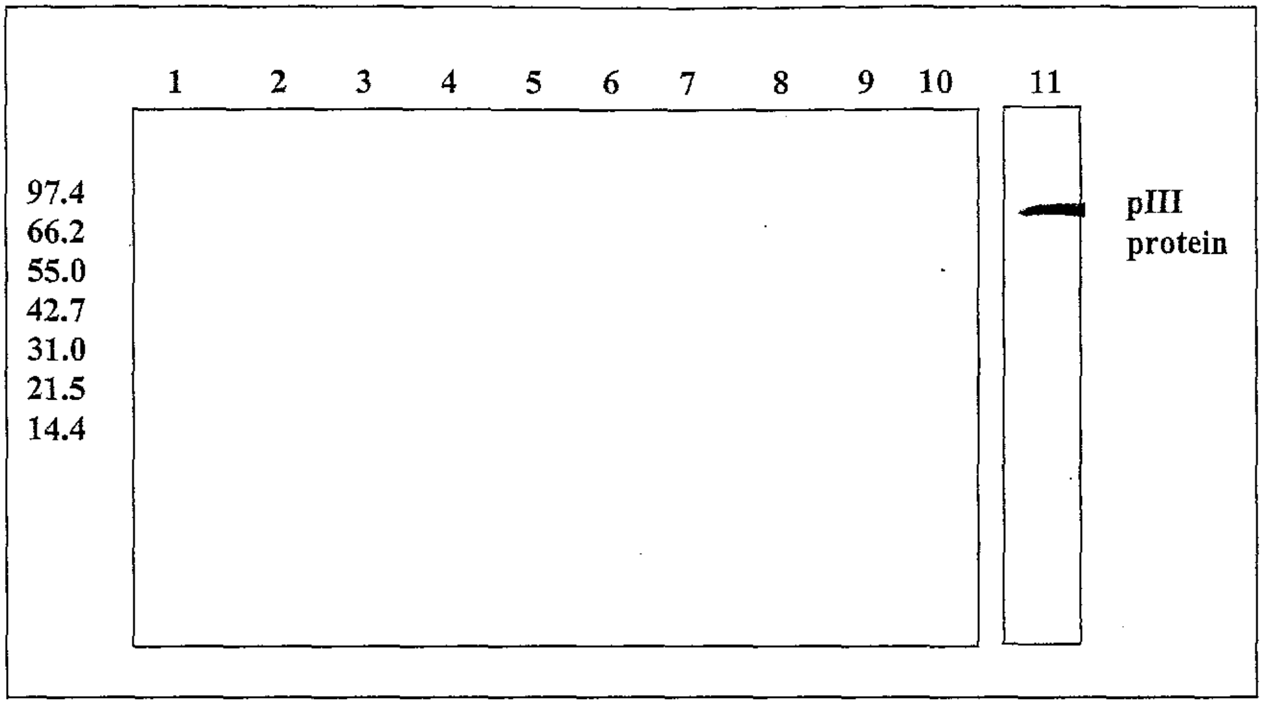


Figure 3.3. The reactivity of the peptides displayed on pIII protein of phage clones with mAb Wb166 by western blot assay

The reactivity of phage display peptides was tested by immuno-blotting using mAb Wb166 at a 1/500 dilution. The concentrations of BVDV cell lysate and mock cell lysate were used in 1:100 dilution and phage clones were $2x10^{10}$ TU/ml.

Lane 1: Molecular weight marker
Lane 2: Mock cell lysate
Lane 3: BVDV cell lysate
Lane 4: BVDV cell lysate
Lane 5: A1-1 phage clone
Lane 6: A2-1 phage clone
Lane 7: A2-2 phage clone
Lane 8. A2-3 phage clone
Lane 9. Measles clone (control)
Lane 10: Emty
Lane 11: A1-1 phage clone (developed by anti-pIII antibody)

3.3.6. A competitive-inhibition ELISA to test binding specificity of peptides on phage

To examine whether or not the selected phage clones (A1-1, A2-1, A2-2 and A2-3) were antigenic mimics (mimotopes) of the BVDV E2 epitope recognised by the mAb Wb166, a competitive-inhibition ELISA and a neutralisation-inhibition assays were performed as described in section (3.2.8 and 3.2.9.). In competitive-inhibition ELISA, phage clones were failed to inhibit the binding of the mAb Wb166 to the BVDV cell lysate (**Figure 3.4**).

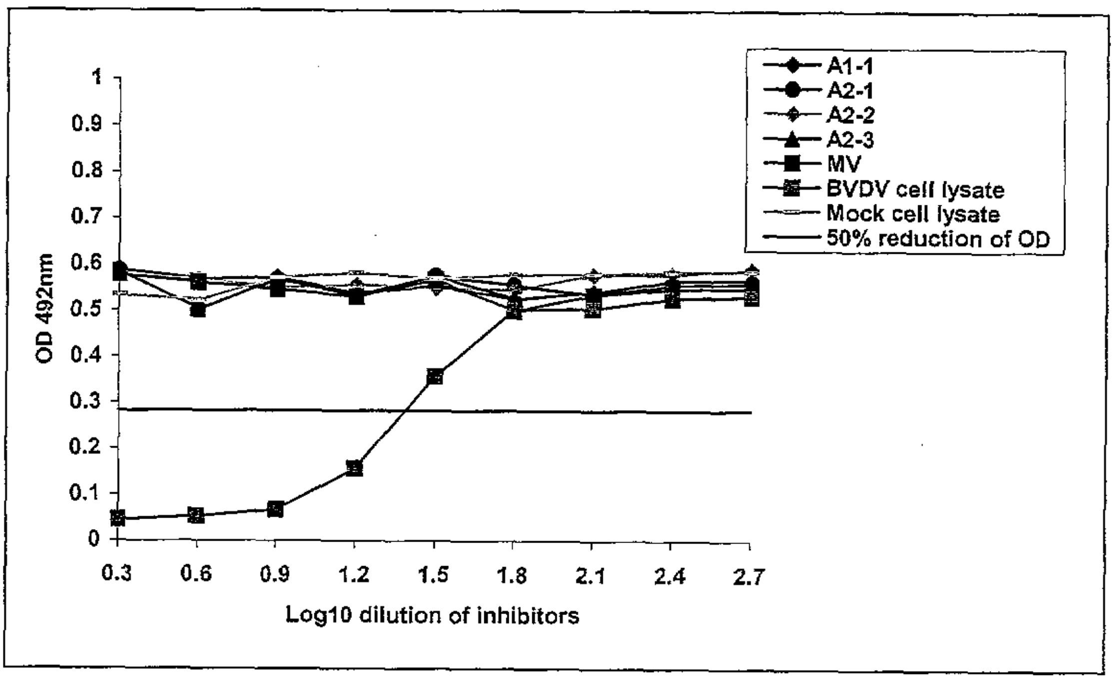


Figure 3.4. A competitive-inhibition ELISA testing the antigenic mimicry of peptides on phage with BVDV E2

1:64000 dilution of the mAb Wb166 was preincubated with 2-fold diluted inhibitors (A1-1, A2-1, A2-2, A2-3 and an irrelevant control clone to MV) starting from $2x10^{10}$ -$7.8x10^{7}$ TU/ml. The mixtures were then transferred to ELISA plate precoated with 0.4 μg/ml BVDV cell lysate (1:100 dilution). Inhibition was assessed by a decrease in the optical density (y-axis) with an increase in inhibitor concentration (x-axis). An irrelevant phage clone to MV was used as control. BVDV cell lysate and mock cell lysate were used as assay positive and negative controls respectively.

3.3.7. A neutralisation-inhibition assay to test antigenic mimicry of peptides on phage with BVDV E2

A neutralisation-inhibition assay was performed to see if phage clones (A1-1, A2-1, A2-2 and A2-3) could compete with the native form of BVDV E2 for binding to the mAb Wb166. The phage was toxic to cells up to a dilution of 1:8 and did not inhibit neutralisation (e.g. allow virus replication) at other dilutions **(Figure 3.5)**.

The next experiment was to switch to an 8-mer solid phase library to select binding ligands to the mAb Wb166 and Wb214.

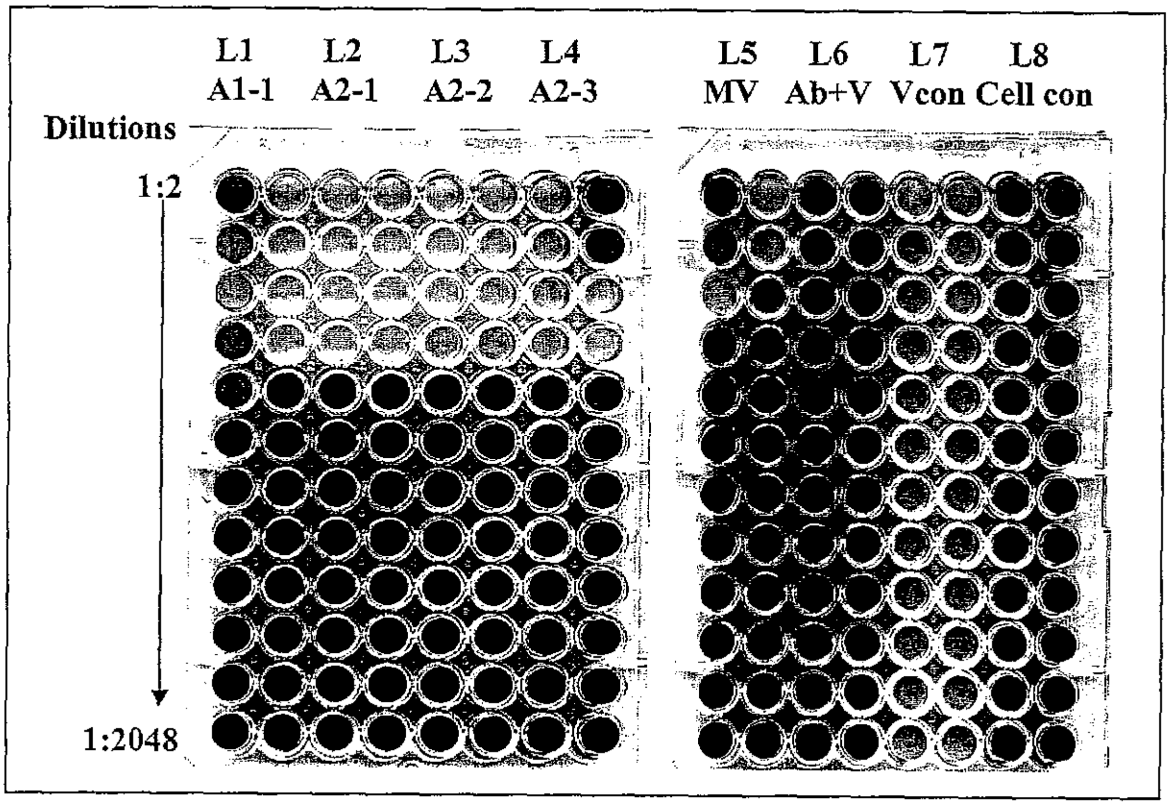


Figure 3.5. A neutralisation-inhibition assay testing the antigenic mimicry of peptides on phage with BVDV.E2

Neutralisation inhibition assay was performed by using phage clones (A1-1, A2-1, A2-2, and A2-3) and unrelated control clone for MV as inhibitors). Their concentration was adjusted to be 2×10^{10} TU/ ml and diluted 2 fold starting from the neat to 1:2048 down the microtiter plates and pre-incubated with 1:256000 dilution of mAb Wb166. Equal volume of BVDV NADL virus strain, which contains 100 $TCID_{50}$, was added to each well excluding cell control. Plates were incubated for five days until a complete cytopathic effect was observed in the virus control.

L1: A1-1, L2: A2-1, L3: A2-2, L4: A2-3, L5: control clone, L6: Antibody control (no inhibitor Ab+virus), L7: Virus control (Vcon) (no antibody), L8: cell control (cell con)#

3.3.8. Screening of an 8-mer solid phase library with the BVDV-E2 mAbs (Wb166 and Wb214)

Since the inconclusive results obtained using a 15-mer phage library with the mAb Wb166, an 8-mer solid phase library was used to select binding ligands to the mAbs Wb166 and Wb214.

3.3.8.1. Selection of reactive peptides with the mAbs

A countable number (~20) of reactive beads displaying peptides were seen using both mAbs at a dilution of 1:500. The three darkest stained beds for each mAb were picked up and sent for micro sequencing to deduce their amino acid sequences.

3.3.8.2. Amino acid sequences of the peptides and their comparison with the BVDV E2

According to whether they were selected by mAb Wb166 or Wb214, amino acid sequence analysis of the peptides revealed the following sequences: DVVDLYE and XEENDFQ (where X = Q or H) for the mAb Wb166 and AKKHIHVD and YMMVHTHA for the mAb Wb214 **(Table 3.3)**. Although Solid phase library displays 8-mer peptides, two of the identified peptides with the Wb166 mAb revealed 7 amino acids only. The first amino acid of the Wb166 P3 was not clear and suggested to be Q (glutamine) or H (histidine) according to the microsequencing results. Therefore, this peptide was designated in both forms as Wb166-P3 and Wb166-P3.1 **(Table 3.3)**.

In order to examine the presence of any homology between these peptides and BVDV-E2, the proasd program was used as described earlier (section 3.3.2). This analysis identified several domains with various ASD scores from 35 to 66 for peptides selected by the mAb166 and from 57 to 65 for peptides selected by the mAbWb214 **(Table 3.3)**. One peptide Wb166-P2 (**DVV**DLYE) sequence showed a high similarity to the E2 protein (**DVV**EMND) corresponding to 783-789 amino acids of BVDV, which is located within amino terminal part of BVDV E2 (first 100 amino acids) **(Table 3.3)**.

Table 3.3. Amino acid sequence comparison of peptides with the E2 region of the BVDV (NADL)

Peptide sequence	BVDV-E2 region	Sequences	ASD scores
Wb166-P1 QPPAPDID	814-821	NGPAFQMV	61.29
	957-964	QVIAMDTK	61.21
	1068-1075	QYGSGEVV	61.17
Wb166-P2 DVVDLYE	783-789	**DVVEMND**	**34.57**
	786-792	EMNDNFE	57.86
Wb166-P3 QEENDFQ	786-792	EMNDNFE	57.86
	785-791	VEMNDNF	59.19
	788-794	NDNFEFG	60.10
	781-787	QEDVVEM	63.48
Wb166-P3.1HEENDFQ	785-791	VEMNDNF	59.37
	786-792	EMNDNFE	63.04
	788-794	NDNFEFG	66.29
Wb214-P1 AKKHIHVD	N.S.	N.S	NR.
Wb214P2 YMMVHTHA	818-825	FQMVCPIG	57.31
	758-765	YHILVVHP	64.15
	973-980	YEIISSEG	65.54

Amino acid sequences peptides selected by screening an 8-mer solid phase library with BVDV E2 mAbs Wb166 and Wb214 were compared with BVDV E2 region (693-1093 amino acids of the ORF) (Collett *et al.*, 1988) by Proasd program. Results were expressed as ASD score.
NS. No similar sequence on BVDV E2 region
NR: *no result*

3.3.9. Reactivity of peptides selected by an 8-mer solid phase and a 15-mer phage library by a SPOT scan assay

Conjugate control assay

When SPOT peptides were first tested for their reactivity in conjugate control assay, a non-specific reactivity was observed with A2-2 NRLFVSTTSIGNRV with (++++) AU. The same peptide was reactive with the mAb Wb166 in the test run expressed as (+++) AU **(Figure 3.6)**.

Assay control

The positive control peptide CP2 (CAHYID) represented by Spot No (17) showed a strong reactivity with the mAb EGF-R expressed as (+++++) AU indicating a successful peptide synthesis and assay procedure **(Figure 3.6)**. This control experiment was performed at the same time with the test experiment with the peptides and the mAb Wb166 on the same day by using the same preparation of reagents.

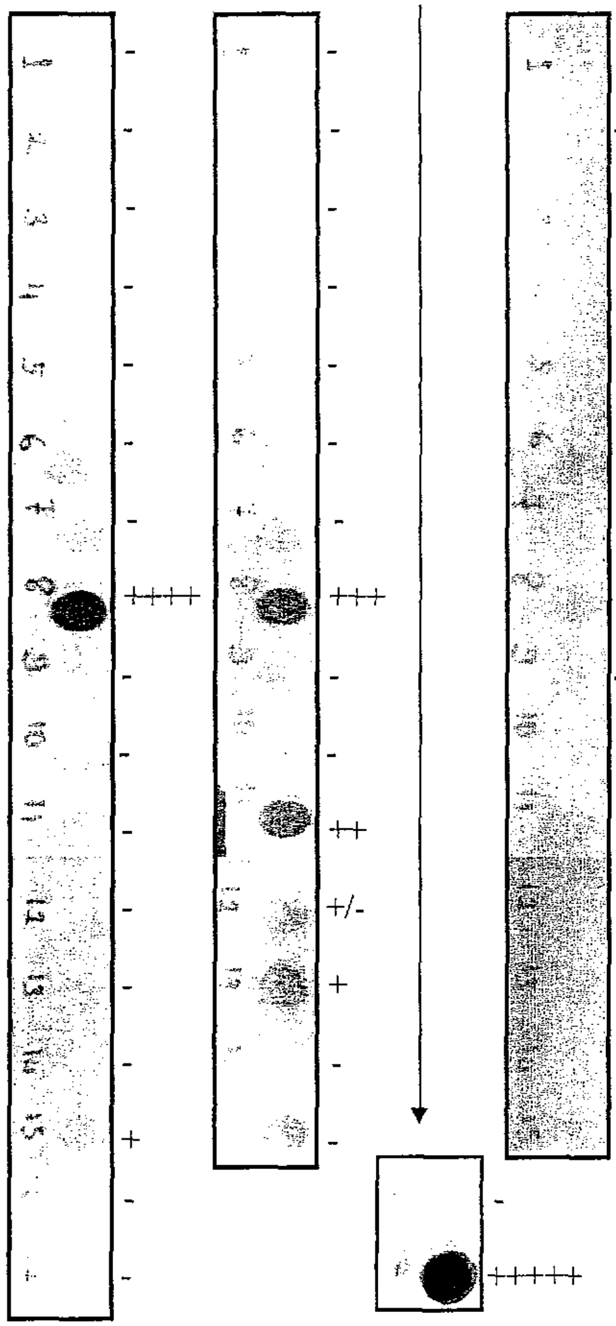
Testing reactivity of peptides with the mAb Wb166

Peptides selected by screening an 8-mer solid phase library with the mAb Wb166 did not show any reactivity with the selector mAb in the SPOT scan assay. However, there was some reactivity with peptides Wb214-P1 (+/-) AU and Wb214-P2 (+) AU selected by the mAb Wb214 with the mAb Wb166 **(Figure 3.6)**. No reactivity was observed between peptides (A1-1, A2-1, A2-2 and A2-3) selected by a 15-mer phage library and the mAb Wb166 **(Figure 3.6)**.

Testing the reactivity of peptides with the Wb214

Up on reacting with the mAb Wb214, no reactivity was observed with peptides Wb214-P1 (AKKHIHVD) and Wb214-P2 (YMMVHTHA) and peptides selected with the mAb Wb166 **(Figure 3.6)**.

No reactivity was observed with an unrelated peptide CP1 with test mAbs Wb166, Wb214 and control mAb EGF-R **(Figure 3.6)**.



SPOT No. Sequences	Conjugate only	Wb166	EGF-R	Wb214
1. Wb166 P1 QPPAPDDID	-	-		-
2. Wb166 P2 DVVDLYE	-	-		-
3. Wb166 P2.1 DVVEMNDN	-	-		-
4. Wb166 P3 QEENDFQ	-	-		-
5. Wb166 P3.1 HEENDFQ	-	-		-
6. A1-1 SRFALVLCAWLSVAF	-	-		-
7. A2-1 SRLYGPFLYYWAVA	-	-		-
8. A2-2 NRLFVSTTSIGNRYV	++++	+++		-
9. A2-3 SFSGSFSHIPFSSVE	-	-		-
10. CP1 QLDFAFGA	-	-		-
11. CP2 CAHYID	-	++		-
12. Wb214 P1 AKKHIHVD	-	+/-		-
13. Wb214 P2 YMMVHTHA	-	+		-
14. CP1 QLDPAFGA	-	-		-
15. CP2 CAHYID	+	-		-
16. CP1 QLDPAFGA	-		-	
17. CP2 CAHYID	-		+++++	

Figure 3.6. Reactivity of peptides selected using an 8-mer solid phase and a 15-mer phage library by a SPOT scan assay

The reactivity of all peptides was first tested with the sheep anti-mouse β-galactosidase conjugate and then BVDV mAbs (Wb166 and Wb214) and control mAb EGR-F. The SPOT membrane was regenerated following each test reactions. The mAbs (Wb166, Wb214 and EGFR/cerb-1) were diluted 1:100 and incubated with the membrane overnight. The binding of antibody to peptides was revealed by the addition of the secondary antibody and signal development allowed for 30 minutes at room temperature.

3.3.10. Immunogenicity of the chimeric peptide Th-Wb214-P1

Whilst the SPOT scan assay was ongoing, the first peptide Wb214-P1 AKKHIHVD selected by the mAb Wb214 was synthesized both as a linear peptide and a chimeric peptide. The chimeric peptide was synthesised whereby a 15-mer peptide LSEIKGVIVHRLEGV representing a T-helper (Th) epitope of the F protein of MV (Partidos and Steward, 1990) was linked to the amino terminus of the peptide Th-Wb214-P1 LSEIKGVIVHRLEGV-AKKHIHVD. The purity of peptides was checked by analytical high-pressure liquid chromatography (HPLC) and the molecular mass of peptide was assessed by mass spectrometry (School of Pharmacy) (results not shown).

The chimeric peptide was used to immunise mice to test mimotope potential of the Wb214-P1 for inducing cross-reactive antibody response to the BVDV.

3.3.10.1. Anti-chimeric and anti-peptide antibody response in BALB/c mice

The final antibody response to the chimeric peptide (Th-Wb214-P1) and the peptide (Wb214-P1) were assessed by indirect ELISA. A high antibody response was observed one week after the second injection **(Figure 3.7)**. The mean antibody titre to potential mimotope peptide Wb214-P1 was $10^{3.2}$ and to the chimeric peptide Th-Wb214-P1 was $10^{4.2}$. This represents a good immune response to the peptide Wb214-P1. The negative group did not develop a specific antibody response.

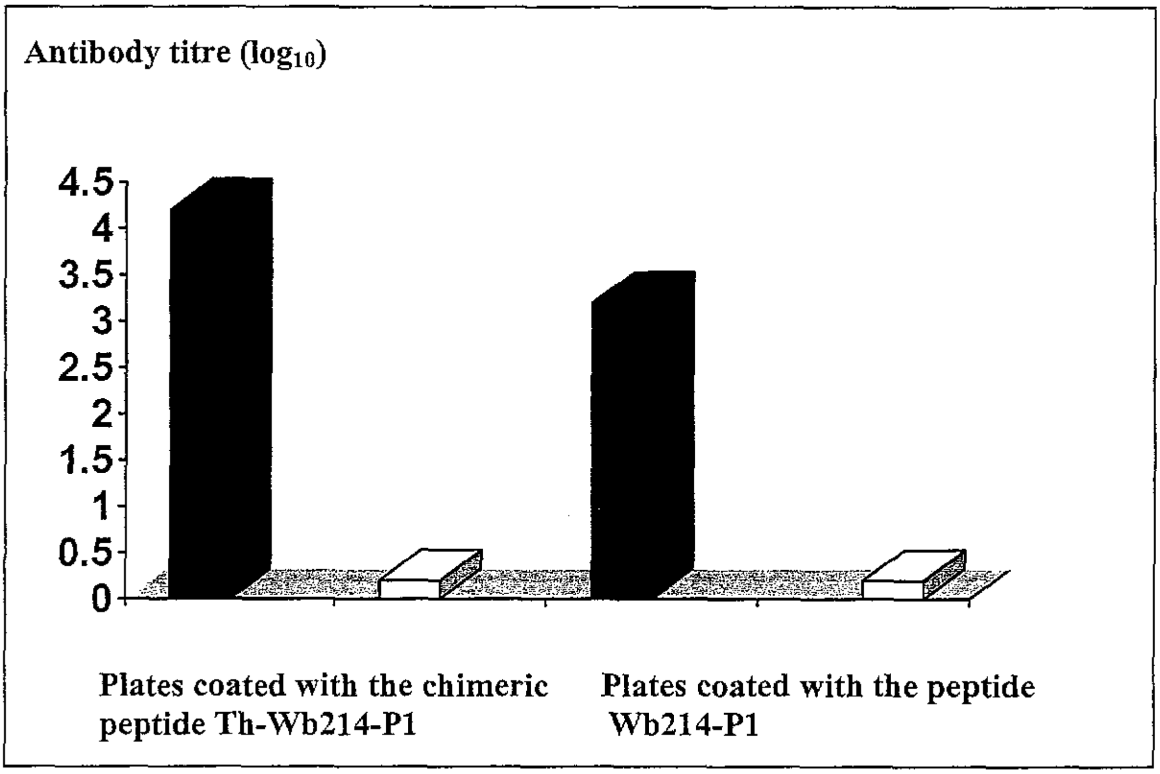


Figure 3.7. Anti-chimeric and anti-peptide antibody response in BALB/c mice.

Mice were immunised with the chimeric peptide (Th-Wb214 P1) in FCA and boosted after 3 weeks of immunisation with the same dose of peptide in IFA. Sera was taken after 1 week of boosting and tested for the presence of antibodies against both chimeric peptide and mimotope by ELISA. Results are expressed as the reciprocal of the log_{10} dilution of the group of four mice serum of 0.1 at OD_{492} nm. 0.1 is the mean OD + 3 SD of the highest concentration of prebleed sera with OD492nm values of 0.070, 0.064, 0.065 and 0.074. The antibody titre obtained to the chimeric and mimotope peptides was of log_{10} 4.2 and log_{10} 3.2 respectively.

■ Antibody response to chimeric peptide, ■ Antibody response to mimotope

Antibody response in control group mice

3.3.10.2. Recognition of the anti-chimeric antibodies to the BVDV cell lysate by ELISA

The above immune sera induced by the chimeric peptide Th-Wb214-P1 were tested for its reactivity to the BVD cell lysate (see chapter 4, section 4.3.3). The mean antibody response of the mice immunised with the chimeric peptide did not differ from the age-matched negative control group by an unpaired student t test (GraphPad InStat version 3, San Diego) on BVDV cell lysate **(Figure 3.8)** or mock-infected cell lysate (result not shown).

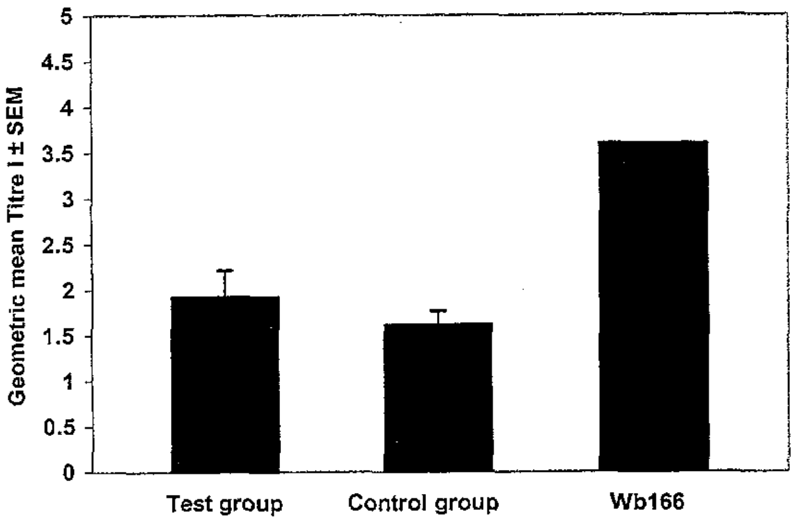


Figure 3.8. Recognition of the anti-chimeric antibodies to the BVDV cell lysate by ELISA

The reactivity of anti-chimeric Th-Wb214-P1 antibodies with BVDV cell lysate was tested by ELISA The mAb Wb166 was used as assay positive control. The results are presented as geometric mean tire ± SEM and assay was done in duplicate.

3.3.10.3. Detection of neutralising antibodies to BVDV in mice sera induced by Th-Wb214-P1 chimeric peptide

Immune sera induced by the chimeric peptide Th-Wb214-P1 were tested for the presence of neutralising antibodies to BVDV in a microtiter neutralisation test. This would be the most appropriate way to confirm biological activity of the Wb214-P1 peptide mimicking the epitope recognised by mAb Wb214. Immune sera obtained from each of four mice following six weeks of the second injection was tested for the presence of neutralising antibodies to BVDV. Immune sera of control group was pooled and used as negative control. A neutralising BVDV E2 mAb Wb163 was used as assay positive control. Neutralising activity was not observed any of the test mice sera at 1:20 dilution. Neutralisation was observed with the control BVDV E2 specific mAb Wb163 at 1:1000 dilution **(Figure 3.9).**

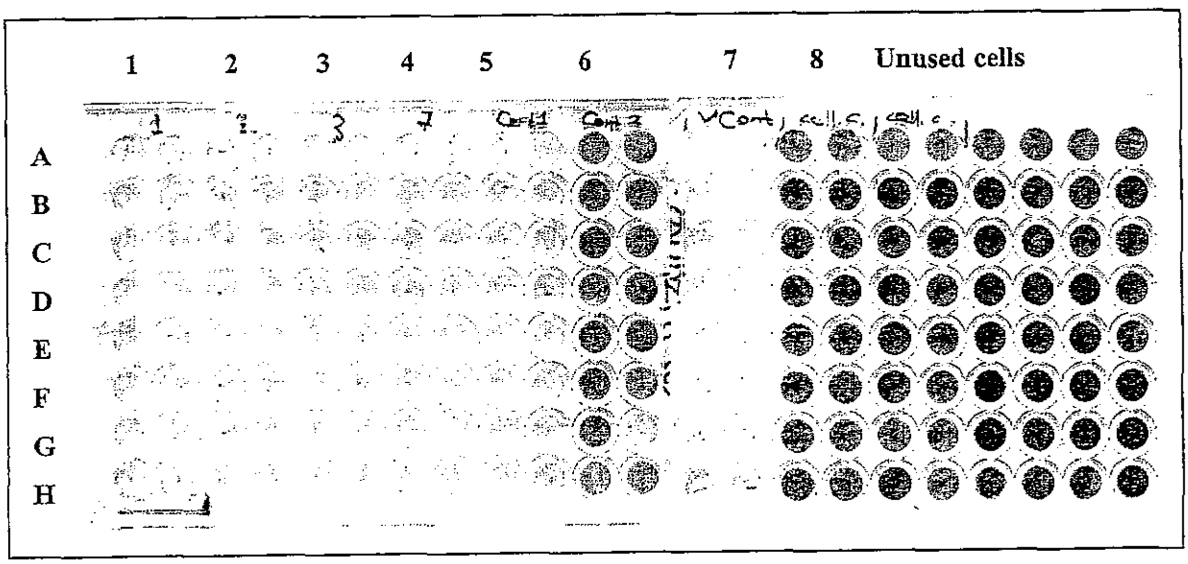


Figure 3.9. Detection of neutralising antibodies to BVDV in mice sera induced by Th-Wb214-P1 chimeric peptide

Mice sera immunised with the Th-Wb214P1 chimeric peptide was tested the presence of neutralising antibodies to the BVDV following boosting by a microtiter neutralising assay. The neutralising E2 specific BVDV mAb Wb163 (10^{-3} dilution) was used as assay positive control.

Columns:

1- 4: Mice sera immunised with the Th-Wb214 P1 chimeric peptide
5: Control mice sera
6: Positive control mAb Wb163
7: BVDV NADL virus control
8: Cell control

3.4. Discussion

Following three rounds of biopanning of a 15-mer phage library with the mAb Wb166, four potential mimotope peptides were selected. The comparison of these peptides with the BVDV E2 (Collet *et al.*, 1988) by proasd program were inconclusive although some similarity was observed between the A2-3 NRLFVSTTSIGN*RY*V and the part of the BVDV E2 SILVVVVALLGGRY (ASD score 55.1) that was located within the carboxyl part of the E2 **(Table 3.2)**. Despite the amino-terminal part of E2 containing neutralising epitopes (Paton *et al.*, 1992: Deregt *et al.*, 1998a, b), Toth *et al.*, (1999) suggested the presence of an antigenic domain containing neutralising epitopes within the carboxyl end of BVDV E2. Although these peptides did not show a high sequence similarity with the BVDV E2 (e.g. ASD scores less than 50), this is not unusual for discontinuous epitopes since only limited identity or no identity is usually found between the sequence of a mimotope and that of the corresponding epitope (Felici *et al.*, 1993; Luzzago *et al.*, 1993; Steward *et al.*, 1995; Chargelegue *et al.*, 1998).

The binding specificity of the peptides with the mAb Wb166 was tested by means of immunodot blot and western blot assays. The mAb Wb166 did not distinguish between the peptides displayed on phage and control phage clones displaying irrelevant peptides. This was suggestive for a cross reactivity between the some phage protein(s) with the selector mAb Wb166 **(Figure 3.1a, b and 3.2a)**. The filamentous phage particles consist of a number of structural proteins. Some part of these protein structures may adopt a conformation recognised by the mAb Wb166. Following titration and optimisation of phage clones, it was observed that the degree of cross reactivity with all phage clones decreased with increasing dilutions of phage **(Figure 3.2b)**.

The possible specific reactivity of phage-display peptides to the mAb Wb166 was also tested by western blot assay. By this way, it could be possible to exclude non-specific binding observed with other phage protein(s) as shown in immunodot blot assay **(Figure 3.3)**. The lack of the reactivity with the peptides displayed on test clones and mAb Wb166 could be due to the denaturaturation of the peptides during the SDS-PAGE procedure and transference of proteins to the nitrocellulose membrane. It should also be noted that the density of peptides displayed on pIII protein might not be sufficient for detection in this assay. A reaction between BVDV cell lysate containing E2 of BVDV with the mAb Wb166 was observed although the epitope recognised by this mAb is suggested to be conformation dependent. This could be due to some refolding of E2 native structure that occurred during the blotting procedure. Coley *et al.*, (2001) demonstrated the specific reactivity of phage-display mimotopes to plasmodium falciparum identified by biopanning of a 15-mer library with the mAb 5G8 directed to a linear epitope within the apical membrane antigen-1 (AMA1) in western blot assay.

The biological mimicry of the potential peptide mimotopes on phage was tested by means of competitive-inhibition ELISA and a neutralisation-inhibition assay. In the competitive-inhibition ELISA, peptides on phage were not able to block the binding of the mAb Wb166 to the BVDV cell lysate **(Figure 3.4)**. A similar outcome was also observed in the neutralisation inhibition assay **(Figure 3.5)**. From these results, it can be assumed that the selected peptides are not antigenic mimics of the epitope recognised by the mAb Wb166. It could also be possible that the peptides displayed on pIII protein of phage were not able to compete with the antigenicity of the native BVDV E2 epitope because of their lower density (e.g. 3 to 5 copies of peptides on pIII of each pahage clone) (Smith and Petrenko, 1997) as compared to the E2 protein in the BVDV cell-lysate and BVDV itself where several hundred glycoprotein spikes are displayed on each virus. In addition, the presentation and the density of the peptides displayed on pIII protein of phage may not be able to present them in a way to promote a high affinity reaction, as is the antibody reaction, as is the case with the native E2. For this reason, an 8-mer solid phase library where peptides are presented in higher density was used to select BVDV mimotopes later.

D'Mello *et al.*, (1997) selected a number of mimotopes sharing a core motif (DX1X2F) within the amino acids 28-35 of the pre-S1 region of HBV by biopanning of a 15-mer library with the mAb 18/7. These mimotopes on phage were shown to be reactive in an immunodot blot assay and were able to block the binding of HbsAg to the selector mAb in a competitive-inhibition ELISA. In addition, mimotopes on phage were able to induce cross-reactive antibody response to HBsAg of HBV in mice. However, such techniques did not work with the mAbs to BVDV in this study.

A portion of phage library selecting binding ligands of any given ligate such as a mAb may contain a small number of phage clones displaying peptides that bind selectively to the antigen-combining site of a given antibody (Scott and Smith, 1990: Smith and Petrenko, 1997). These are specific binders and may be further divided in to scarce high affinity binders and much more common low affinity binders and non-binders. The phage libraries undoubtedly contain a number of phage clones displaying peptides that may bind to constant regions of the antibody and bind to the natural antibodies in the mouse ascetic fluid or bind to other components of the screening system such as the solid phase (beads or plastic) (Adey *et al.*, 1995) or the capturing molecule (streptavidin etc) (Menendez and Scott, 2005). This might be another explanation for a cross-reactivity between some of the phage protein(s) and the mAb Wb166 as shown in immunodot blot assay (see above). In addition, the majority of phage clones in the library will not bind to the selecting mAb and thus fall within the nonbinding phage group. In some cases, phage library may not contain specific ligands for some mAbs recognising either discontinuous (Irwing *et al.*, 2001) or long linear epitopes (Fack *et al.*, 1997). Thus, this will enable the enrichment of non-specific binders to dominate the biopanning. It should also be noted that nonspecific binding ligands are also isolated in the presence of specific binding ligands.

Another important limitation of phage library application is associated with the inherent bias in the biological system resulting in underpresentation of certain peptide sequences. Some peptides may be toxic to the bacterial cells, susceptible to proteases or incompatible with the maintenance of phage infectivity (Rodi and Makowski., 1999). The biological system may also let some phage-display peptides that have a growth advantage to be non-specifically enriched ones in biopanning. During the each round of amplification procedure, there is a chance that weak or non-specific binders with better growth properties will overgrow specifically binding phage clones (Bruritt *et al.*, 1996). This could also be case for the peptides bound to the mAb Wb166 and amplified during each round of biopanning were either weak or non-specific binders for the selecting mAb since they failed to mimic the epitope recognised by the mAb Wb166 as shown in neutralising-inhibition and competitive-inhibition assays.

To enhance the chance for selecting mimotopes of BVDV, an 8-mer solid phage library was used to screen BVDV mAbs Wb166 and Wb214. The use of a solid phase library would exclude above-mentioned biological limitations with phage library application. The reactive peptides were selected with the both mAbs Wb166 and Wb214. The lowest ASD score, 34.5, was observed between the peptide Wb166-P2 <u>DVV</u>DLYE corresponding to 783-789 aminoacids (<u>DVV</u>EMND) of E2 BVDV that was located within the amino terminal part of E2 (**Table 3.3**). This was indicative as being a linear epitope or a part of the discontinuous epitope recognised by the mAb Wb166. This was in contrast to peptides selected by biopanning of a 15-mer phage library with the mAb Wb166 where the lowest ASD score was 55.1 **(Table 3.2)**.

In order to examine the mimotope potential of these peptides, the peptide Wb214-P1 was initially synthesised and used for mice immunisation. In most cases, the short synthetic peptides, less than 10-15 amino acid long are not by themselves good immunogens even when administered in a potent adjuvant. Therefore, animals receive peptides linked to T-helper molecules (Shaw *et al.*, 1993; Langeveld *et al.*, 1995; Steward *et al.*, 1995) or peptides in the form of multiple antigen presentation (MAP) system (Chargelegue *et al.,* 1998). However, there is still not a general rule for which type of these constructs is the most effective way for peptide immunisation for inducing anti-peptide antibodies cross reactive with viruses or viral antigens (Muller, 1999). In this study, the peptide Wb214-P1 was synthesised as a chimeric peptide (Th-Wb214-P1) whereby a T-helper epitope of fusion protein of MV (Partidos and Steward, 1990) linked to its amino terminus and used for mice immunisation.

Immunisation of mice showed an antibody response directed to both peptides, with antibodies directed to the potential mimotope peptide Wb214-P1 **(Figure 3.7)** but these antibodies were either not cross-reactive with BVDV cell lysate or not neutralising to BVDV **(Figure 3.8 and 3.9)**. The possible reason(s) for these results could be due to the nature of immunogen or immunisation strategy. The chimeric peptide immunogen may not have included all the essential parts of the complete discontinuous epitope on E2, which are recognised by the mAb Wb214 for appropriate immunogenic mimicry. Such a lack of the antibody response was also shown with mimotopes of a neurotropic murine corona virus (MHV-A59) selected by biopanning of a large range of phage libraries with the mAb 7-10A recognising a discontinuous epitope of the S glycoprotein. It is generally accepted that antibodies cross reacting with the original antigen will only be obtained if the conformation of the peptide immunogen(s) resemble or mimic that of the corresponding epitope of the virus or viral antigen (van Regenmortel, 2001). This immunogen is termed as a true mimotope (Lam *et al.*, 1996).

The BVDV cell lysate that was used in ELISA represented an impure source of the BVDV antigen and commonly used for detecting BVDV antibodies (Howard *et al.*, 1985; 1989; Paton *et al.*, 1998). It may not be able to distinguish cross-reactive BVDV antibodies in mice sera induced by the chimeric peptide from those to normal bovine cell components. In addition, the antibody response induced by the chimeric peptide might not have been sufficient for detecting it in neutralisation assay. However, the recombinant E2 protein that was successfully used to screen antibody response to BVDV E2 DNA vaccine (Nobiron *et al.*, 2002) was not obtainable during the time of this ELISA.

Another possibility could be due to the presentation and immunisation strategy of the chimeric peptide. Chargelegue *et al* (1998) reported that multiple antigen presentation (MAP) system was essential for RSV mimotopes for inducing cross-reactive and protective neutralising antibody responses to RSV since no RSV antibodies obtained with immunisation of mimotopes as linear peptides. In addition, since peptides are generally poor immunogens as compared to intact virus or viral antigens (van Regenmortel, 1996), further injections and longer time might have been necessary for obtaining anti-BVDV antibodies. For instance, D'Mello *et al.*, (1997) reported that cross-reactive anti-HBsAg antibodies with HBV mimotopes were obtained following boosting twice. In this study, mice were not kept for longer than four weeks due to the economic reasons.

During the immunisation study, a SPOT scan assay was used to test binding specificity of peptides selected by an 8-mer solid phase library and a 15-mer phage-display library. However, the reactivity of peptides Wb214-P1 and Wb214-P2 with the selector mAb Wb214 was not confirmed in a SPOT scan assay although a weak cross-reactivity was observed between these peptides and the mAb Wb166 as described below.

Testing the reactivity of peptides with the mAb Wb*166 in* a SPOT scan assay, a reactivity was observed with the peptide A2-3 NRLFVSTTSIGNRVY selected by a 15-mer phage library **(Figure 3.6)**. However, a stronger non-specific reaction was also observed with this peptide in the conjugate control assay. It has been reported that occasionally, some SPOT peptides may give a non-specific reactivity (blue) when the membrane is incubated with secondary antibody only. Typically, this reactivity is caused by a non-specific binding of the antibody-enzyme conjugate to peptides that contain combinations of basic amino acids such as lysine, arginine and histidine (SPOT's Method Manual Genosys, Cambridge, UK). Some weak reactivity was also observed between the peptides Wb214-P1 and Wb214-P2 selected by the mAb Wb214 with the mAb Wb166 suggesting a cross reactivity between two mAbs. The epitope-mapping studies with these mAbs suggested the clustering of epitopes in a single antigenic domain and the presence of an immunodominant region on E2 of BVDV (Paton *et al.*, 1992). This could explain a cross reactivity of peptides selected with the mAb Wb214 and the mAbWb166. Peptides identified by screening an 8-mer solid phase library with the mAb Wb166 were not shown reactivity with the same mAb in the SPOT scan assay. Surprisingly, the peptide Wb166-P2 **DVV**DLYE showed the highest similarity to the E2 region of BVDV **DVV**EMND (see above and **Table 3.4)** was not reactive with the mAb Wb166 in the SPOT scan assay **(Figure 3.6).**

Chargelegue *et al.*, (1998) selected five reactive peptides from an 8-mer solid phase library with the mAb19 recognising a conformational epitope of F protein of RSV. However, when the reactivity of peptides with the selector mAb was tested in a SPOT scan assay, only one peptide gave a strong reactivity with the mAb and one peptide did not give any reactivity. They then used the SPOT scan assay to improve the binding of the most reactive peptide by doing amino acid substitution analysis that showed some substitutions resulted in improved reactivity with the mAb. In Chargelegue's study, the SPOT scan assay was successfully used to further selection and improvement of the mimotopes of RSV. The improved mimotope when presented as MAP system was able to induce anti-RSV specific protective neutralising and antibodies in mice. It can be assumed from Chargelegue's study that the high-density peptide presentation on both solid phase library selection and the SPOT scan assay were critical for RSV mimotopes. In addition, multiple copies of peptides in MAP system in high density was also critical for antigenic and immunogenic mimicry of the RSV mimotope since no binding and cross-reactive antibody response obtained with the linear form of RSV mimotopes (Chargelegue *et al.*, 1998).

The negative binding of peptides in the SPOT scan assay by the mAbs Wb166 and Wb214 was another indication that the selected peptides were not specific BVDV mimotopes **(Figure 3.6)**. This would also explain why one of the peptide Wb214-P1 did not induce anti-BVDV antibodies in mice. In this study, only three beads for each mAb were sent for sequencing because of the cost of sequencing. It would be useful to sequence more peptides for each mAb. Since the solid phase library contained only a portion of all the theoretical number of 8-mer peptides ($2.6x10^{10}$), the SPOT scan assay can be used for further selection of mimotopes by doing amino acid substitution analysis as suggested by Chargelegue *et al.*, (1998). In this manner the putative cross-reactivity observed between peptides Wb214-P1 and Wb214-P2 with the mAb Wb166 might have been improved and these peptides could perhaps be made reactive with the mAb Wb214 in the SPOT scan assay.

In this study, a range of peptides selected by a 15-mer phage-display and an 8-mer solid phase libraries with BVDV mAbs had been tested using standard assays without conclusive results. The next question was whether the BVDV E2 mAbs directed to discontinuous neutralising epitopes on E2 used were not suitable for this kind of study and one possible reason would also be their relative affinity. In the next chapter, the relative affinity of BVDV mAbs was examined.

Chapter 4. The estimation of relative affinities of neutralising BVDV mAbs

4.1. Introduction

To estimate the relative affinities of the neutralising BVDV mAbs (Wb166, Wb214, Wb158, and Wb163), an ELISA-based elution assay with urea was used. This method is because chaotropes, such as diethylamine (DEA), thiocyanate, or urea, break noncovalent bonds between antigen and antibody (Steward and Chargelegue, 1997). It is expected that the low affinity antibodies can be easily eluted from the antigen in the presence of chaotropes, whereas higher affinity antibodies are more difficult to elute (Olszewska *et al.*, 2000).

The use of urea for estimating relative antibody affinity was introduced by Hedman and Seppala, (1988). It is based on the elution of antibodies bound to the antigen by urea in a solid phase ELISA. The urea-based elution has been applied to test the antibody avidity for evaluating MV vaccine efficacy in ELISAs (de Souza *et al.*, 1997), measuring antibody affinity in patients with RSV infection (Meurman *et al.*, 1992) and serodiagnosis of Hepatitis C virus infection (Kanno and Kazuyama, 2002).

In this study, the resistance to urea elution was utilised to estimate the relative affinity of each neutralising mAb. The relative affinity was expressed as a percentage of affinity index (i.e % residual OD after 8M urea) (de Souza *et al.*, 1997).

4.2. Materials and Methods

4.2.1. Neutralising activities of BVDV E2 mAbs

BVDV E2 mAbs (Wb166, Wb214, Wb158, Wb163 and Wb215) were described in chapter 2 (section 2.1.1). A microtiter neutralisation test was performed essentially as described by Howard *et al.*, (1987). The above mAbs were already known to have varying neutralising titres (Paton *et al.*, 1992). A bovine antibody (2359) to BVDV was also used as positive control. Briefly, serial two-fold dilutions of mAbs were made in maintenance medium in 96-well flat-bottom tissue culture plates (Nunclon). Duplicate wells were used for each dilution series and 50 μl of each dilution of antibody were retained in the wells. An equal volume of BVDV NADL that was diluted to give 100 $TCID_{50}$/0.05 ml was aliquoted to each well. Virus and cell controls were included in each assay. The plates were incubated for 1 hour at 37°C and 5% CO_2. Finally, 50 μl of a suspension of MDBK cells at $2X10^4$ cells/well suspended in maintenance medium was added and the plates were incubated up to 4 days at 37°C and 5% CO_2 until a complete cytopathic (cpe) was observed in the virus control wells. Neutralisation was quantal and was the complete loss of viral cpe. Any well with foci of virus was scored as not containing neutralising antibody. Duplicate wells were used and 50% end point titre was determined by the Kärber formula (Log ID_{50} = L-d (S-0.5), where L= negative log lowest dilution of serum, d= difference between log dilution steps, S= sum of proportions of wells showing neutralisation.

4.2.2. Estimation of the working dilution of BVDV cell lysate for use in the relative affinity ELISA

BVDV cell lysate and mock cell lysate were prepared from infected and mock-infected MDBK cells as described in General Materials and Methods (Chapter 2, Section 2.8). The total protein contents of BVDV infected and mock-infected cell lysate was estimated by using Micro BCA protein assay reagent kit according to manufacturer's instructions (Pierce) to 40μg/ml. A microtitre ELISA plate was coated with a 2-fold dilution of the BVDV cell lysate and the mock cell lysate starting from the dilution of 1:25 to 1:3200 in PBS in duplicate rows. The plate was incubated overnight at 37°C. The plate was washed and blocked with 100 μl blocking buffer to each well (10% pig sera in PBS-Tw) by incubating 2 hours at 37°C and then washed again. Normal mouse serum at 1: 16000, mAb Wb166 to BVDV at 1: 16000, and mAb Wh303 at 1:16000 were added to different wells and plate was incubated overnight at 4°C. Antibody binding was detected as described in General Materials and Methods (Chapter 2, Section, 2.9).

4.2.3. Estimation of the dilutions of mAbs to be used in assay for relative affinity

Microtiter ELISA plates were coated with the BVDV cell lysate at a concentration of 0.4 μg/ml in PBS (1:100 dilution) at 4°C overnight. Following washing and blocking, the mAbs Wb166, Wb214, Wb158, Wb163, Wb215 and normal mouse sera were titrated two-fold dilutions starting from $10^{3.9}$ to $10^{6.3}$. The dilution of each mAb to give an OD in the range 0.5 to 0.7 was used later in the relative affinity assay.

4.2.4. The estimation of relative affinity of neutralising BVDV E2 mAbs by ELISA

The relative affinities of the E2- specific mAbs were assessed by an ELISA based affinity elution assay. In preliminary assays, a pre-treatment of BVDV cell lysate with 8M urea did not reduce the binding of mAb Wb166 (result not shown). Selected dilution of mAbs (Wb166, Wb214, Wb158 and Wb163 to give ODs of 0.5-0.7 were incubated with BVDV cell lysate coated plates. After washing off the mAbs with PBS-Tw, serial 2 fold dilutions of urea (8M to 0.06M) in PBS (de Souza *et al.,* 1997; Kano and Kazuyama, 2002) was added to the duplicate wells of the plate. Following 30 minutes of incubation in urea at 37°C, plates were washed and developed with conjugate and OPD substrate. The resistance to elution by the highest concentration (8M) of urea was utilised to estimate the relative affinity of the neutralising mAbs. The affinity index, (AI) expressed as percentage, was calculated by (OD with urea/ OD without urea) x 100 (de Souza *et al*., 1997).

4.3. Results

4.3.1. Neutralising activities of BVDV E2 mAbs

The bovine antiserum (2359) to BVDV that was used as a positive control had a neutralising titre of 10^3 as expected. The BVDV mAbs Wb166, Wb214, Wb158 and Wb163 displayed high neutralising titre of $10^{5.7}$ to 10^4 whereas the mAb Wb215 showed no neutralising activity at 10^2 **(Figure 4.1)**.

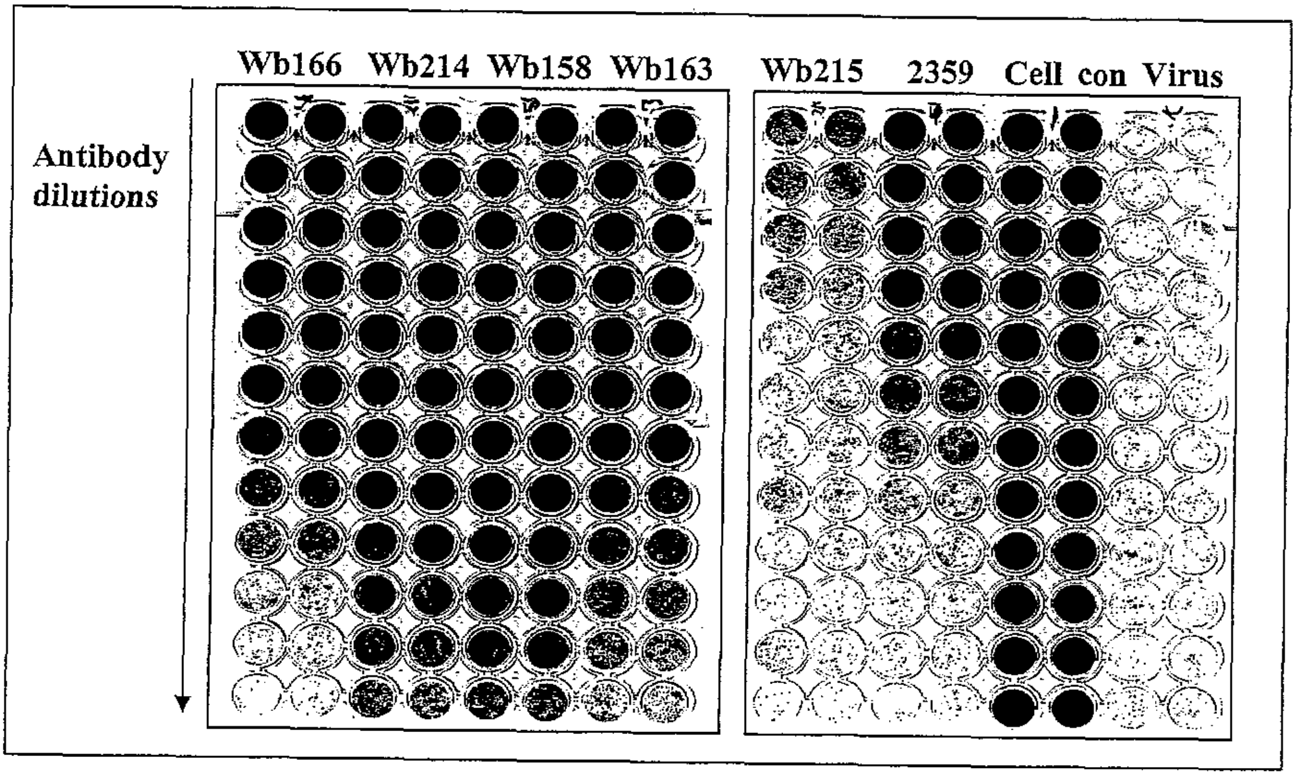


Figure 4.1. Microtiter virus neutralisation to test neutralising titres of BVDV mAbs and bovine antisera to BVDV

Antibodies were titrated 2-fold across the microtiter plates in maintenance medium and equal amount of BVDV NADL that was diluted to give 100 $TCID_{50}$ was added to wells excluding the cell control. The starting dilutions of mAbs were 10^{-4} for Wb166, for 10^{-3} for Wb214, and 10^{-2} for mAbs Wb158, Wb163 and Wb215. A bovine antisera (2359) to BVDV was used as positive control. Following 1-hour incubation, MDBK cells that were adjusted to be $2x10^4$ per/well were added to wells and plates were incubated until complete cytopathic effect was seen in virus control. The cells were than fixed with 20% (v/v) formalin in PBS followed by staining with crystal violet for 1 hour. The neutralising titres of the mAbs were as follows: $10^{5.7}$ for Wb166, $10^{5.3}$ for Wb214, $10^{4.9}$ for Wb158, 10^4 for Wb163 and $<10^2$ for Wb215.

4.3.2. Estimation of the working dilution of BVDV cell lysate for use in the relative affinity ELISA

The binding of mAb Wb166 was related to the concentration of BVDV cell lysate with OD values of 1.6 dropping to 0.2 (**Figure 4.2**). No detectable reactivity was observed with the negative control mAb Wh303 directed against E2 of CSFV (Paton *et al.,* 1995) and NMS (**Figure 4.2**). No binding was observed between control antigen obtained from the mock infected cells and the mAb Wb166 (results not shown). The 10^{-2} dilution of BVDV cell lysate (0.4 μg/ml), which gave OD of 1.0, was used in the relative affinity assay.

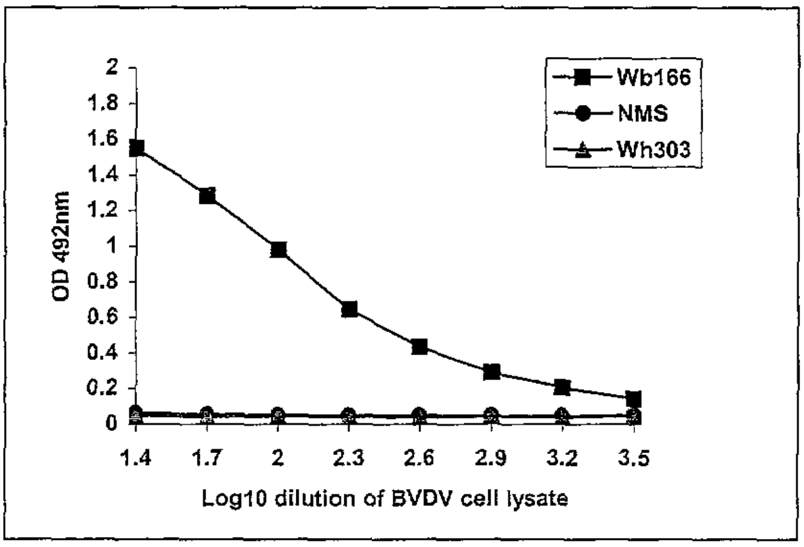


Figure 4.2. Estimation of the working dilution of BVDV cell lysate for use in the relative affinity ELISA

Serial dilutions of cell lysate were titrated against E2 specific neutralising mAb Wb166 (1:16000). The mAb Wh303 to CSFV E2 and NMS were used as negative controls.

4.3.3. Estimation of the dilutions of mAbs to be used in the assays for relative affinity

A standard curve was generated for each mAb **(Figure 4.3)**. The dilution of each mAb to give an OD of 0.5 to 0.7 was selected for use in the relative affinity ELISAs. These dilutions were $10^{5.1}$ for Wb166, $10^{5.4}$ for Wb214, $10^{4.5}$ for Wb158, and $10^{3.9}$ for Wb163. The mAb Wb215 did not achieve an OD of 0.5.

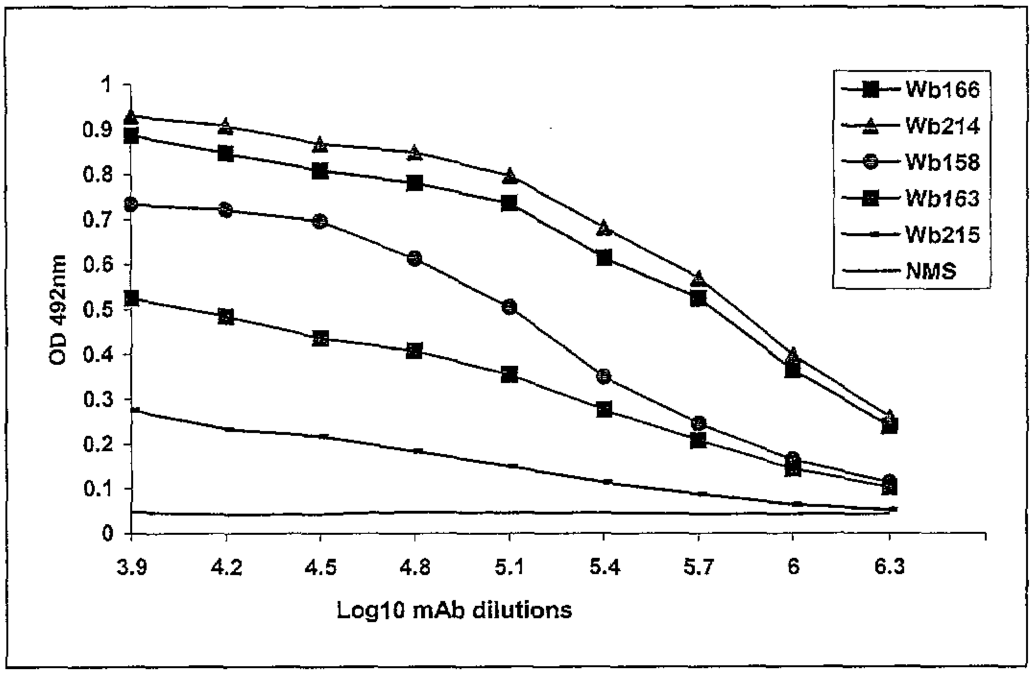


Figure 4.3. Estimation of the dilutions of mAbs to be used in assay for relative affinity

Reactivity of E2 specific BVDV mAbs was tested against BVDV cell lysate. 96 well plates were coated with the BVDV cell lysate and mock cell lysate at a dilution of 1:100 (0.4 µg/ml total protein concentration). Serial two fold dilutions of mAbs were titrated across the plate and reactivity was tested by the addition of secondary antibody followed by addition of the substrate. A normal mouse serum was used as a negative control.

4.3.4. The estimation of relative affinity of neutralising BVDV E2 mAbs by ELISA

The relative affinity of neutralising the mAbs (Wb166, Wb214, Wb158 and Wb163) against BVDV cell lysate was tested by an ELISA-based urea elution assay. The procedure involved exposing wells containing the mAbs bound to BVDV cell lysate to increasing concentrations of urea (0-8M) **(Figure 4.4)**. Urea had little effect on elution until its molarity reached 4M. The affinity indexes (AI) of the mAbs Wb166, Wb214, Wb158 and Wb163 with 8M urea were 66%, 17%, 33% and 18% respectively.

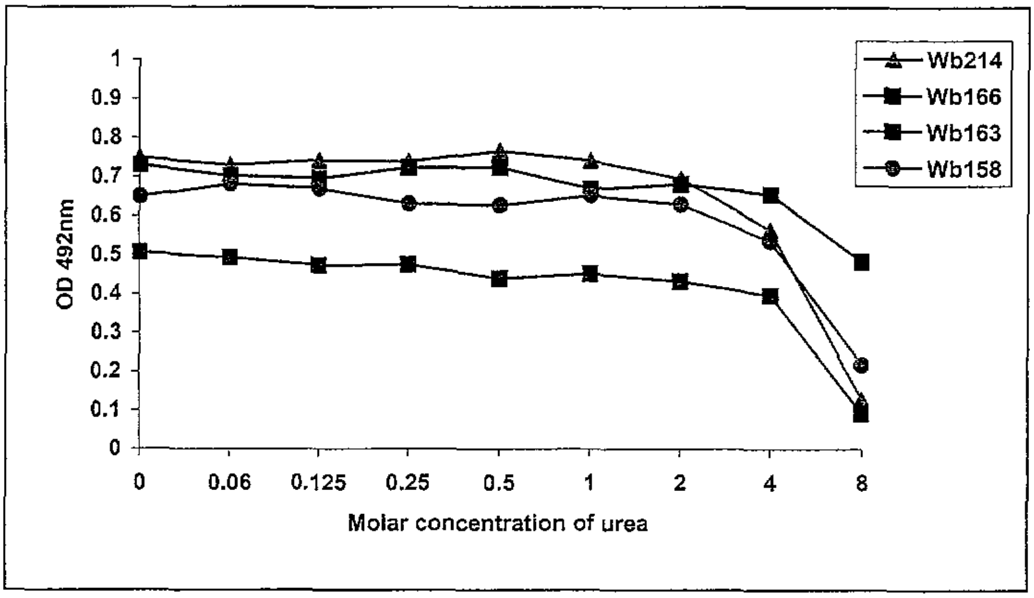


Figure 4.4. The estimation of relative affinity of neutralising BVDV E2 mAbs by ELISA

ELISA plates were coated with the BVDV cell lysate at a dilution of 1:100. The dilution of mAbs that gave 0.5-0.7 OD_{492} nm value with BVDV cell lysate were incubated. Then, urea was added to duplicate wells in concentrations ranging from 0 to 8 molar and incubated for 30 minutes. AI was calculated by OD with 8M/ OD without urea X 100. Binding of mAbs Wb166, Wb214, Wb163, and Wb158 to BVDV cell lysate in the absence of urea were OD_{492} 0.749, 0.731, 0.506, and 0.649. % AIs of mAbs were 66% for Wb166, 17% for Wb214, 33% for Wb158 and 18% for Wb163.

4.4. Discussion

4.4.1. A comparison of the neutralising titres of mAbs tested in present study and Paton *et al* (1992)

The neutralising activity of four mAbs (Wb166, Wb214, Wb158, and Wb163) to BVDV NADL was assessed. All four mAbs had neutralising activity to BVDV with the mAb WB166 showing the greatest neutralising titre **(Figure 4.1)**. The mAb Wb163 that was raised against BVDV Oregon (C24V) was initially reported to have neutralising antibody titre of less than 35 to BVDV NADL compared with 420 to BVDV Oregon (Paton *et al.*, 1992). In contrast, in the present study the same antibody was found to have neutralising titre of 10,000 to NADL (section 3.3.1). The contradiction between the neutralising titre of mAb Wb163 published by Paton *et al* (1992) and presented in this study could relate to the variability of the sequence shown between NADL viruses in different laboratories **(Table 4.1)**. The present virus was not sequenced but had a similar passage history to that produced one year earlier in the same laboratory (Iqbal, 2001) but by separate plaque purification. Iqbal's NADL isolate shares a Valine (V) at position 79 with Oregon whereas Paton's NADL isolate has glutamic acid (E) at position 79 **(Table 4.1)**.

Table 4.1. Neutralisation of the mAb Wb163 with NADLs and role of amino acid 79

Virus Strains	Viruses used by researchers	Amino acid positions 53	79	264	History of NADL in laboratories	Neutralisation
Oregon	Paton *et al.*, (1992)	L	V	V		+
NADL	Collett *et al.*, (1988)	L	V	V		ND
NADL	Paton *et al.*, (1992)	F	E	V	Two plaque purification and 12 passages	–
NADL	Iqbal, (2001)	F	<u>V</u>	A	Three plaque purification and <7 passages	ND
NADL	Present	ND	ND	ND	Three plaque purification and <7 passages	+

ND= not done

To confirm that the replacement of V to E at position 79 in Paton's NADL might influence neutralisation, the present virus would need to be sequenced and Iqbal's NADL would need to be used in neutralisation test with the mAb Wb163.

Paton *et al.,* (1992) was unable to achieve escape mutants of Oregon using Wb163. The neutralising titre of Wb163 with Oregon was 420. The present work achieved a neutralising titre of 10^4 for the mAb Wb163 with NADL. Thus, the present stock of NADL might be more effective than Paton's stock for selecting mutants and determining whether the position 79 has a role in neutralisation.

The amino acid change on E2 at position 264 of NADL where V was replaced with A (Iqbal, 2001) as compared to NADL Collett *et al.*, (1988) and Oregon Paton *et al.*, (1992) has not been reported before **(Table 4.1).** This suggests that mutations at different regions of E2 are continuing sources of antigenic variation and perhaps vaccine failures.

4.4.2. The estimating relative affinities of neutralising mAbs

In order to see if there is a correlation between neutralising titers and relative affinities of mAbs (Wb166, Wb214, Wb158 and Wb163), an ELISA-based urea elution assay was used. The highest AI of 66% was observed with the mAb Wb166 that had the highest neutralising titre was suggestive that the relative affinity of the mAb Wb166 was higher than mAbs Wb214, Wb158 and Wb163 **(Figure 4.4)**. There was not such great difference between the relative affinities of the mAbs Wb214, Wb163 and Wb158 (AIs17%-33%).

The nature of discontinuous epitopes recognised by neutralising BVDV mAbs might limit their applicability for selecting mimotopes using combinatorial peptide libraries. In addition, the mAb Wb166 that was used with both a 15-mer and an 8-mer solid phase libraries for BVDV mimotope selection had the highest relative affinity. This could be another reason for the failure of selecting BVDV mimotopes (chapter 3). It is known that high affinity antibodies bind to antigens tighter than low affinity antibodies and high affinity interaction between both counterparts is highly specific (Steward and Chargelegue, 1997). It is likely that high affinity mAbs require highly specific ligands for binding and they will be highly selective for their ligands. These ligands may not be present in a 15-mer phage display and an 8-mer solid phase libraries used in the present study (see chapter 3). Therefore, the chance for selecting binding ligands to the mAbs directed to discontinuous epitopes with high affinity would be very much diminished as compared to low or moderate affinity mAbs from combinatorial peptide libraries. Alternatively, if a peptide on phage was selected and bound to the mAb with a high affinity, it might not be eluted by acid elution in biopanning.

In the next chapter, the possible effect of antibody affinity on mimotope selection was assessed by using a low affinity and a high affinity mAbs to DNP hapten.

Chapter 5. The use of low and high affinity monoclonal antibodies to select mimotopes of DNP

5.1. Introduction

In the previous chapter, it was shown that the BVDV E2-neutralising mAb Wb166 bind to the BVDV with relatively high affinity. This might explain the failure to identify binding ligands to this mAb using combinatorial epitope libraries. The mAb Wb166 was probably very selective for its ligands, which may be not present in the libraries tested (see chapter 3).

To investigate the possible effect of antibody affinity for selecting mimotopes, two mAbs (DNP Pα47 and DNP Pα41) with known affinity to DNP were used. The affinity values of the mAbs DNP Pα47 and DNP Pα41 based on equilibrium dialysis using DNP-lysine were 0.91 and 4.30 M^{-1} respectively (Stanley *et al,* 1983). DNP is a simple small organic well-defined hapten molecule with aromatic shape **(Figure 5.1)**. It is likely to have fewer epitopes than the five to six conformational neutralisation epitopes on BVDV E2 (Paton *et al.*, 1992: Deregt *et al.*, 1998a, b, Toth *et al.*, 1999). Because of the simple structure of DNP, small peptides from phage libraries are likely to bind to antigen-combining sites of DNP mAbs.

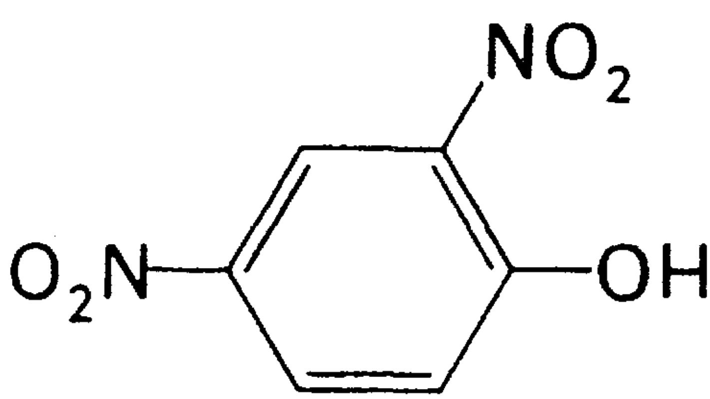


Figure 5.1. Chemical structure of 2-4 Dinitrophenol (DNP)

In this chapter, the selection of peptides bound by low and high affinity DNP mAbs were compared. This would also be a novel comparative study for testing applicability of phage libraries for selecting mimotopes of non-protein epitopes (DNP) and mimotopes of BVDV. DNP was used to confirm the mimotope selection methods and assays with BVDV were working and whether affinity an issue in mimotope selection as a model. Peptide mimotopes of DNP have not been selected before and so the work is novel. It will be discussed relative to the effect of mAb affinity on the selection of mimotopes of the fluorescein hapten (Bottger *et al.*, 1999).

The preference of any combinatorial peptide library for any given ligate cannot be predicted in advance as suggested by Bonnycastle *et al.*, (1996) (see chapter 1, section 1.7.6). Thus, the two available pIII-based linear (a 6-mer and a 15-mer) and two pVIII-based constrained (a 17-mer and a 10-mer) phage libraries were used to increase the chance of the selection of DNP mimotopes. The same libraries and same methods were used to select the mimotopes of BVDV side by side with DNP. The results of BVDV study will be reported later (see chapter 7). The methods used to select the mimotopes of DNP are summarised in a flow diagram **(Figure 5.2)** and will be outlined below.

The isotypes and affinity of DNP mAbs were first confirmed and then they were subjected for biopanning of phage libraries. Results showed that the low affinity mAb DNP Pα47 selected bound phage clones with each phage library whereas the high affinity mAb DNP Pα41 did not select any clones. The selected phage clones were tested for their specific binding to the selector mAb by ELISA. The ELISA positive phage clones were then sequenced (to deduce the amino acid sequences of their inserts) to determine how many different peptides would be potential mimotopes of DNP.

The binding specificity of the phage clones with known sequences to the mAb DNP Pα47 and their mimotope potential for DNP were then tested by ELISA and competitive-inhibition ELISAs. The competitive-inhibition ELISAs used in the present study were as follows:

1) To show whether the phage clones displaying different peptide sequences competed with each other

2) To show that DNP could block mAb binding to phage clones to confirm the peptides on phage shared epitope with DNP

3) To show whether phage clones could block DNP binding to the mAb

Characterisation of DNP mAbs (DNP Pα47 and DNP Pα41)

↓

Isotyping and estimating their relative affinity (avidity)

↙ ↘

Biopanning of phage libraries with the DNP mAbs
(Two linear (15 and 6-mer) and two constrained (17 and 10-mer) libraries

↓

Selection of 8-12 phage clones from the final round of biopanning with each library

ELISA *to test binding of phage clones with selector mAb*

Sequencing of ELISA positive phage clones

↓

Quantitative ELISAs with phage clones of known sequences

↓

Competitive-inhibition ELISAs to test mimotopes of DNP

1) One phage clone inhibiting the mAb binding to a second phage clone

2) DNP inhibiting the mAb binding to phage clones

3) Phage clones inhibiting the mAb binding to DNP

Figure 5.2. Schematic representation of the selection of DNP mimotopes by phage libraries

5.2. Materials and methods

5.2.1. DNP monoclonal antibodies and antigens

Two mAbs (DNP Pα47 and DNP Pα41) to the hapten 2-4-dinitrophenol (DNP) were kindly obtained from Prof. Michael Steward (London School of Hygiene and Tropical Medicine). The production and the purification of these mAbs were described by Stanley *et al.*, (1983). Briefly, the DNP mAbs were purified by affinity chromatography of filtered ascites fluids on columns of p-nitrophenyl (PNP) casein-CNBr activated Sepharose 4B, DNP-lysyl-Sepharose 4B or trinitrophenyl (TNP) aminoethyllaminocarboxy-methyl-Sepharose 4B. The columns were eluted with 0.1 M dinitrophenol and the antibodies separated from the DNP by chromatography (Stanley *et al.*, 1983). These purified mAbs were used in the present study.

The DNP_{237} casein conjugate was kindly obtained from Prof. Michael Steward (London School of Hygiene and Tropical Medicine). The DNP-L-Lysine, Casein and Poly-L-Lysine were obtained from Sigma-Aldrich. The DNP_{237} casein conjugate and DNP-L-Lysine were used as polyvalent and monovalent haptenic inhibitors respectively.

5.2.2. Phage libraries

Phage libraries (linear and constrained) used in the present study were described in chapter 1 (section 1.7.6 and 1.7.7) and in chapter 2 (section 2.1.3). Phage libraries were amplified to have fresh stock before biopanning (see chapter 2, section 2.4).

5.2.3. Characterisation of the DNP mAbs

Isotype determination of the DNP mAbs

Isotyping of the DNP mAbs (DNP Pα47 and DNP Pα41) was performed by using the DNP_{237} casein-conjugate coated plates (0.05 μg/ml) and using isotype-specific conjugates (IgG1, IgG2a, IgG2b, IgG3) (Nordic) by ELISA.

Estimating the relative affinity of DNP mAbs

The relative affinities of the DNP mAbs (DNP Pα47 and DNP Pα41) were determined by an inhibition ELISA as described by Rath *et al.*, (1988). The dilution of antibodies (1:800 for DNP Pα47 and 1:6400 for DNP Pα41) that gave 0.6-0.7 OD_{492} nm value with the DNP_{237} casein conjugate was preincubated with the decreasing concentration of DNP (starting from 1.6 μg/ml to 0.0125 μg/ml) for 2 hours at 37°C. The mixtures were then transferred to ELISA plate precoated with 0.05μg/ml DNP_{237} casein conjugate. Bound antibody was detected by the addition of peroxidase-labelled rabbit anti-mouse conjugate followed by subsequent addition of substrate. Absorbancies were measured at an OD of 492nm. Affinity of mAbs was estimated from the concentration of inhibitor (DNP) causing 50% decrease of OD (Rath *et al.*, 1988).

5.2.4. Biopanning of phage libraries with DNP mAbs

Polystyrene beads coated with the DNP mAbs (DNP Pα47 and DNP Pα41) at a concentration of 30 μg/ml and incubated with approximately $2x10^{10}$ TU/ml phage libraries for biopanning (see chapter 2, section 2.5.1). The schematic presentation of biopanning procedure is shown in **Figure 5.3.**

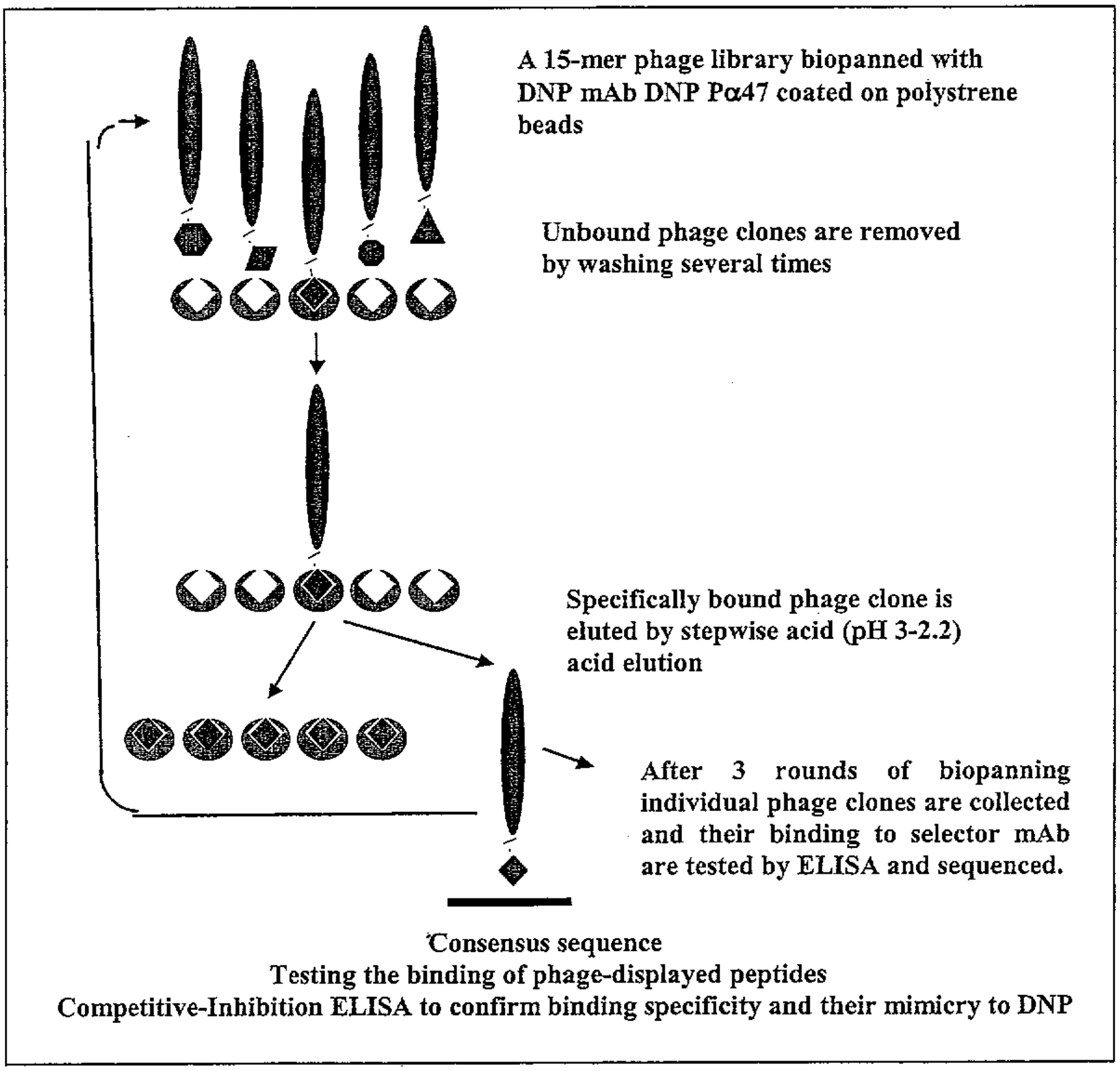


Figure 5.3. Schematic representation of selecting mimotopes of DNP by biopanning of a 15-mer library with mAb DNP Pα47

5.2.5. Simple ELISA to test binding of the selected phage clones with mAb

After the final round of biopanning, 8-12 phage transductant *E. coli* colonies bearing individual phage clones for each phage library (6, 15, 10 and 17-mer) were randomly selected and cultured in LB medium overnight. Phage clones were purified from the cultures and suspended with 200 μl of TBS buffer (see chapter 2, section 2.5.2). The binding of phage clones to the selector mAb at a dilution of 1:200 was tested by ELISA. Briefly, duplicate wells of ELISA plates were coated with 50μl of phage clones diluted at 1:10 in TBS at 37°C. The phage clones displaying 15-mer mimotopes for HCV (K1) (L El-Attar, 2000) and HBV (PC4) (D'Mello *et al.*, 1997) were used as irrelevant control phage. A non-related mAb to Tacaribe virus was used as irrelevant mAb. The reactivity was measured as 2x the mean OD obtained for the irrelevant mAb when reacted with all phage clones. Phage clones with OD value above this was considered positive reactivity.

5.2.6. Amino acid sequences of peptide inserts of phage clones

ELISA positive phage clones were then sequenced by single-strand dideoxy-sequencing using the chain termination method (Sanger *et al.*, 1977). A detailed description of these methods was given in General Materials and Methods (Chapter 2, Section 2.5.3).

5.2.7. ELISA assays with phage clones of known sequences

Following sequencing, phage clones were grouped according to sequences of insert peptides they display. They were amplified in a larger volume and their titre was determined as TU/ml (see chapter 2, section 2.6). To asses the reactivity of phage clones more accurately, equal amount of quantified phage clones selected by each library including control clones were used as antigen in ELISA. In the present study, peptides on phage clones were used to test for binding and competitive-inhibition assays.

5.2.7.1. Quantitative ELISA to test the binding of phage clones with the selecting mAb

96 well ELISA plates were coated with 50μl of phage clones at a concentration of $1.25X10^8$ TU/ml for DA15-1 and DA15-2 and $4X10^8$ TU/ml for DA6-1 and DA6-2 and $2X10^9$ TU/ml for DA17-1 and DA17-2 and DA10-1. Two fold dilutions of the mAbs (DNP Pα47 and DNP Pα41) starting from 1:200 to 1:3200 were added to wells and incubated overnight at 4°C. In addition to irrelevant phage clones K1 and PC4 as described in section (5.2.5) and an empty phage clone f88.4 (Bonnycastle *et al.*, 1996) was also used as irrelevant control clones. ELISA was developed as described in General Materials and Methods (Chapter 2, Section 2.9).

5.2.8. Competitive-inhibition ELISAs to test the mimotopes of DNP

To confirm binding specificity of the selected phage clones with the selector mAb DNP Pα47 and testing their antigenic mimicry with the DNP, competitive-inhibition ELISAs were performed. Duplicate wells were used for each dilution.

Before doing any competition assay, the limiting dilution of mAb and each antigen (phage clone or DNP) had to be established.

5.2.8.1. To determine the limiting dilutions of antigen and antibodies for use in competitive-inhibition ELISAs

The limiting concentrations of the phage clones and DNP_{237} casein conjugate and the optimal dilution of the mAb for using in competitive-inhibition ELISAs were estimated by checkerboard titration. Briefly, 96-well ELISA plates were coated with a two fold dilutions of the phage clones diluted in TBS and incubated at 37°C overnight. Two fold dilutions of the mAb DNP Pα47 starting from 1:200 dilution to 1:3200 was added to wells of each horizontal row A-H. The wells of the row 12 received dilution buffer only to give a background reading. Rabbit anti-mouse IgG (Dako) horseradish peroxidase conjugate diluted 1:1000 in ELISA dilution buffer were added to wells and incubated for 1 hour. The optimal concentration of phage clones and the mAb was taken as values giving OD_{492}=0.6-0.7. The concentrations of inhibitors and the dilutions of antibodies used for competitive-inhibition ELISAs are given in Table 5.4. Similarly, a checkerboard titration was also done by optimising working concentration of the DNP_{237} casein-conjugate to the mAb DNP Pα47 and DNP Pα41 for estimating the relative affinities of these mAbs (see section 5.2.3).

5.2.8.2. Competitive-inhibition ELISAs

All competitive-inhibition ELISAs were followed the same procedure. Briefly, in one ELISA plate, the mAb was preincubated with dilutions of inhibitors (phage clones or DNP) for 2 h at 37°C. The DNP was used as inhibitor in both polyvalent (DNP_{237} casein conjugate) and monovalent (DNP-L-Lysine) forms. The mixtures were then transferred to a second plate coated with relevant antigens (phage clones or DNP_{237} casein (see text for figures). The inhibition reactions were assayed for 50% reduction of OD value with increased dilution of inhibitors as compared to irrelevant inhibitors. The 50% reduction of OD value was calculated as mean OD obtained with direct binding of the mAb to the corresponding phage clone or DNP_{237} casein conjugate. This was indicated on each graph. Results were plotted as OD versus dilution of inhibitors.

Three types of competitive-inhibition ELISAs were used. Brief descriptions of them were given below.

5.2.8.2.1. One phage clone inhibiting mAb binding to the second phage clone

96-well ELISA plates were first blocked with blocking solution. Decreasing two fold concentration of both homologous and heterologous phage clones (inhibitors) (from $2x10^9$ TU/ml to $3.90x10^6$ TU/ml) were preincubated with a fixed dilution of the mAb DNP Pα47 (1:200) for 2 hours at 37°C. The mixture was then transferred to ELISA plates precoated with phage clones ($1x10^8$ TU/ml for DA15-1, DA15-2 and $4x10^8$ TU/ml for DA6-1, DA6-2). OD_{492} values were measured for each dilution of inhibitors. Results were plotted as OD versus concentration of phage clones.

5.2.8.2.2. DNP inhibiting mAb binding to phage clones displaying 15, 6 and 17-mer peptides (DA15-1, DA15-2, DA6-1, DA6-2 and DA17-1)

ELISA plates were first blocked with blocking solution. Decreasing concentration of the inhibitors (DNP_{237} casein conjugate, DNP-L-Lysine, casein and Poly-L-Lysine) was preincubated with a fixed dilution of the mAb DNP Pα47 in blocked ELISA plates. The mixtures were then transferred to ELISA plates precoated with phage clones ($1x10^8$ TU/ml for DA15-1 and DA15-2 and $4x10^8$ TU/ml for DA6-1 and DA6-2.

Decreasing ten-fold concentration of inhibitors (DNP_{237} casein conjugate, DNP-L-Lysine, casein and Poly-L-Lysine) was preincubated with a fixed dilution of the mAb DNP Pα47 (1:800) for two hours at 37°C. The mixture was then transferred to an ELISA plate precoated with the DA17-1 at a concentration of $2x10^9$ TU/ml. OD_{492} values were measured for each dilution of inhibitors.

5.2.8.2.3a) Phage clones (DA15-1 and DA15-2) inhibiting mAb binding to DNP

An ELISA plate was blocked with blocking solution Decreasing two fold concentrations of phage clones displaying two 15-mer peptides (DA15-1, DA15-2) from $4x10^9$ TU/ml to $6x10^7$ TU/ml were preincubated with the mAb DNP Pα47 at a dilution of 1:800 for 2 hours at 37°C. The mixtures were then transferred to an ELISA plate precoated with DNP_{237} casein conjugate at a concentration of 0.05 μg/ml. OD_{492} values were measured for each dilution of inhibitors.

5.2.8.2.3b) Phage clones (DA17-1, DA17-2 and DA10-1) inhibiting mAb binding to DNP

Phage clones (inhibitors) were titrated 10 fold from $5x10^{11}$ to $5x10^4$ TU/ml and preincubated with the mAb DNP Pα47 at a dilution of 1/800 for 2 hours at 37°C. The mixture was then transferred to ELISA plates precoated with DNP_{237} casein conjugate at a concentration of 0.05 μg/ml. Inhibition of binding was observed by a decrease in the optical density (y-axis) with concentration of inhibitor (x-axis). The statistical analysis of inhibition, OD versus $\log_{10}$ inhibitor, was achieved by linear regression (GraphPad Prism™ version 4, San Diego).

5.3. Results

5.3.1. Characterisation of the DNP mAbs

Isotype determination of the DNP mAbs

The isotypes of the DNP mAbs Pα47 and Pα41 were determined by ELISA. The isotypes of the DNP mAbs DNP Pα47 and DNP Pα41 were IgG1 and IgG2a respectively (**Figure 5.4**).

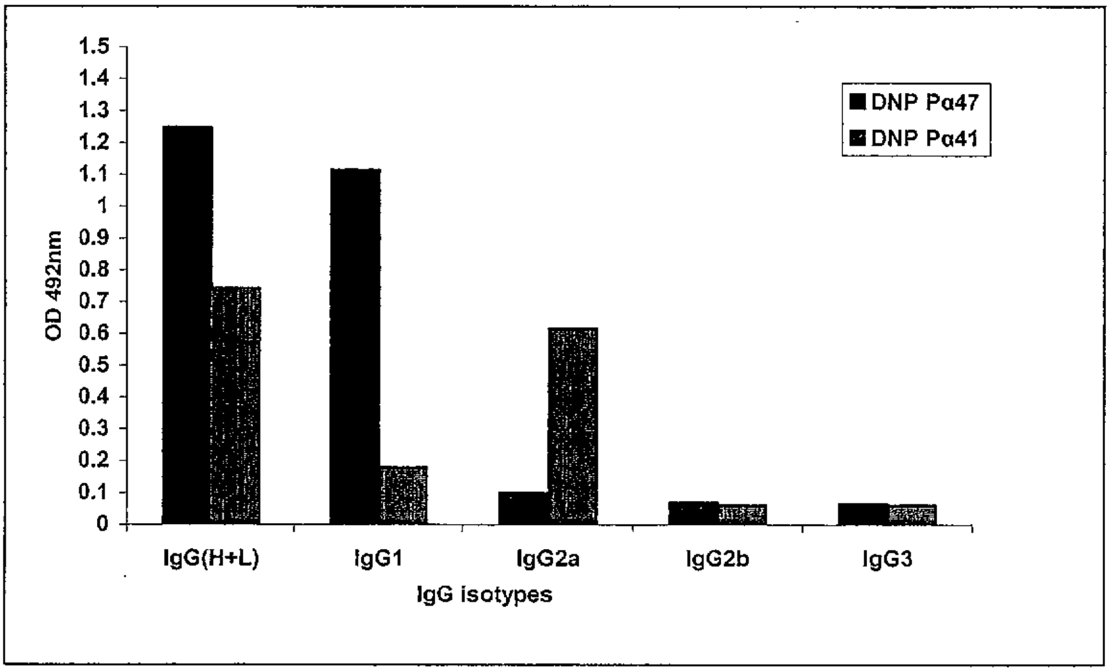


Figure 5.4. Determination of isotypes of the DNP mAbs by ELISA

Microtiter plates were coated with the DNP_{237} casein conjugate at a concentration of 0.05 μg/ml and incubated with a fixed dilution of mAbs. The isotypes of antibodies were determined using different peroxidase-labelled secondary antibodies (IgG H+L, IgG1, IgG2a, IgG2b, IgG3).

Estimating the relative affinity of DNP mAbs

The relative affinities of the mAbs DNP Pα47 and DNP Pα41 were estimated by inhibition ELISA. The DNP_{237}-casein conjugate was used as inhibitor. It was found that the concentration of the DNP_{237} casein conjugate required for a 50 % reduction in initial absorbency was greater for the mAb DNP Pα47 (0.09 μg/ml) compared to the DNP Pα41 (0.019 μg/ml) **(Figure 5.5).** Thus, the affinity of mAb DNP Pα47 was lower than that of the mAb DNP Pα41.

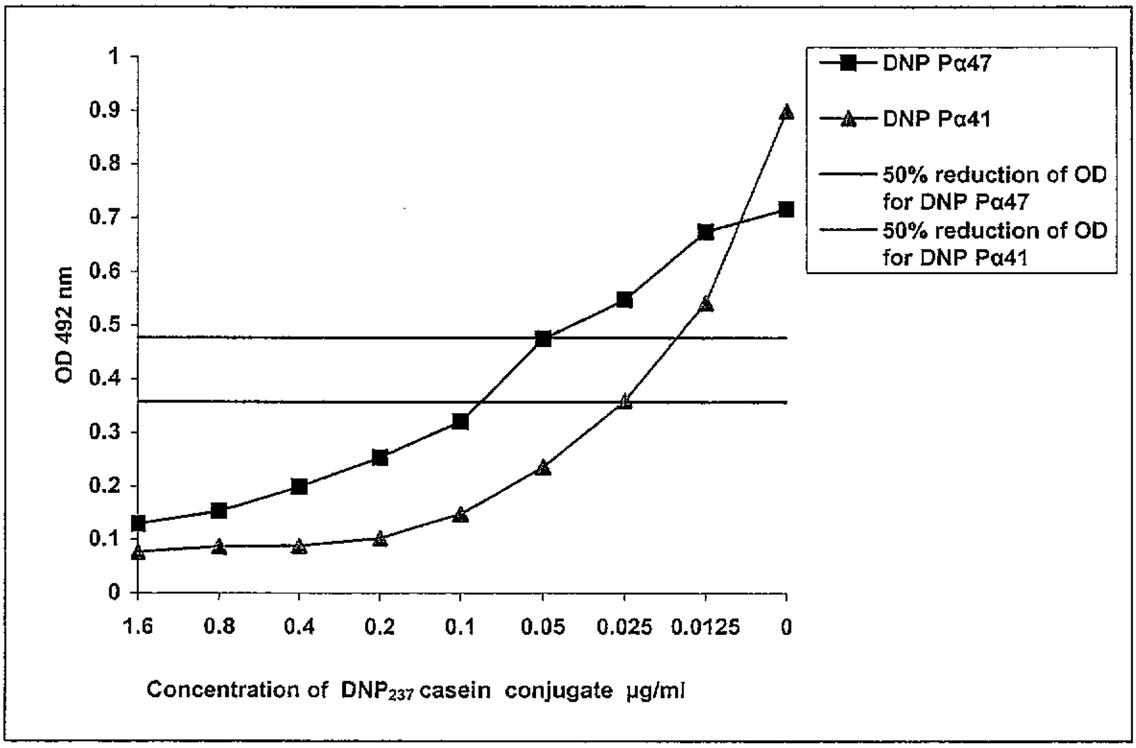


Figure 5.5. Estimating the relative affinity of the DNP mAbs

ELISA plate were precoated with the DNP_{237}casein-conjugate at a concentration of 0.05 μg/ml. The ilutions of mAbs that gave ODs of 0.6-0.7 were 1:800 for DNP Pα47 and 1:6400 for DNP Pα47 were pre-incubated with the decreasing concentrations of DNP_{237} casein conjugate (0 to 1.6μg/ml) before transferring to plate precoated with DNP_{237}casein-conjugate (0.05 μg/ml). Inhibition of binding was observed by a decrease in optical density (y) axis with increasing in inhibitor concentration (x-axis). The estimated concentration of inhibitor causing 50% inhibition of initial absorbencies for the mAbs DNP Pα47 and DNP Pα41 was 0.09 μg/ml and 0.019 μg/ml respectively.

5.3.2. Biopanning of phage libraries with DNP mAbs

The DNP mAbs (DNP Pα47 and DNP Pα41) were used to screen two pIII-based linear (15,- 6-mer) and two pVIII-based constrained (17,-10-mer) phage libraries. Three rounds of biopanning with each phage library resulted in enrichment of eluted phage clones bound to the low affinity mAb but none bound to the high affinity mAb. The yields (recovery) (Phage eluted output/ phage input x 100) were calculated as a percentage of input phage for the three rounds **(Table 5.1 and 5.2)**. The increase of the percentage of recovered phage clones between the second and first rounds of biopanning was indicative for the amplification of specifically bound peptides displayed on phage clones to the mAb DNP Pα47. As illustrated in **Table 5.1,** the percentage yield with 2nd rounds of biopanning was higher than with the 1st round (>9625 and >160) for pIII based a 15-mer and a 6-mer phage libraries and (>1132 and >25) for pVIII based constrained a17-mer and a 10-mer phage libraries respectively **(Table 5.2)**.

Table 5.1. Biopanning of pIII-based (a 15-mer and a 6-mer) linear phage libraries with DNP mAbs

15-mer library

		Low affinity DNP Pα47			High affinity DNP Pα41		
Round	**Incubation time**	**Input (I) TU**	**Output (A) TU**	**%Yield**	**Input (I) TU**	**Output (A) TU**	**% Yield**
1st	Overnight	1.6×10^9	3×10^3	0.000187	1.6×10^9	1.25×10^3	0.000156
2nd	4 hours	3×10^8	5.4×10^6	1.8	2×10^7	0	0
3rd	10 minutes	1×10^9	3.2×10^6	0.32	n.d		

6-mer library

		Low affinity DNP Pα47			High affinity DNP Pα41		
Round	**Incubation time**	**Input (I) TU**	**Output (A) TU**	**%Yield**	**Input (I) TU**	**Output (A) TU**	**% Yield**
1st	Overnight	8×10^8	1.5×10^3	0.000187	8×10^8	1.25×10^3	0.000156
2nd	4 hours	3.7×10^7	3×10^4	0.0081	2×10^7	0	0
3rd	10 minutes	1×10^8	1.68×10^5	0.168	n.d		

Yields were obtained by biopanning of pIII-based (a 15-mer and a 6-mer) phage libraries with mAbs DNP Pα47 and DNP Pα41. Increases in % of yield between rounds 1st and 2nd were 9625 for the 15-mer library and 160 for the 6-mer library.

Table 5.2. Biopanning of pVIII-based (a 17-mer and a 10-mer) constrained phage libraries with DNP mAbs

17-mer library

		Low affinity DNP Pα47			High affinity DNP Pα41		
Round	**Incubation time**	**Input (I) TU**	**Output (A) TU**	**%Yield**	**Input (I) TU**	**Output (I) TU**	**% Yield**
1st	Overnight	5×10^8	7.95×10^3	0.00159	5×10^8	3×10^2	0.00006
2nd	4 hours	2×10^7	3.6×10^5	1.8	4×10^7	0	0
3rd	10 minutes	9×10^6	8.1×10^4	0.90	n.d		

10-mer library

		Low affinity DNP Pα47		
Round	**Incubation time**	**Input (I) TU**	**Output (A) TU**	**%Yield**
1st	Overnight	9.6×10^8	9.8×10^4	0.0102
2nd	4 hours	7×10^7	1.8×10^5	0.25
3rd	10 minutes	1×10^8	9.8×10^4	0.098

Yields were obtained by biopanning of pVIII-based (a 17-mer and a 10-mer) phage libraries with mAbs DNP Pα47 and DNP Pα41. Increases in % yields between rounds 1st and 2nd were 1132 for the 17-mer library and 25 for the 10-mer library.

5.3.3. Simple ELISA to test binding of the selected phage clones with mAb DNP Pα47

8-12 Individual phage clones (total 38 clones) randomly collected in the final round of biopanning from each library were preliminary tested for their binding to the selector mAb DNP Pα47 by ELISA. To ensure the validity of the assay, one of two irrelevant phage clones (K1 or PC4) was introduced in each run. All phage clones had OD values to the selector mAb higher than 2x the mean OD with the irrelevant mAb to Tacaribe virus. In contrast none of the irrelevant phage clones bound to mAb DNP Pα47 (**Figure 5.6**).

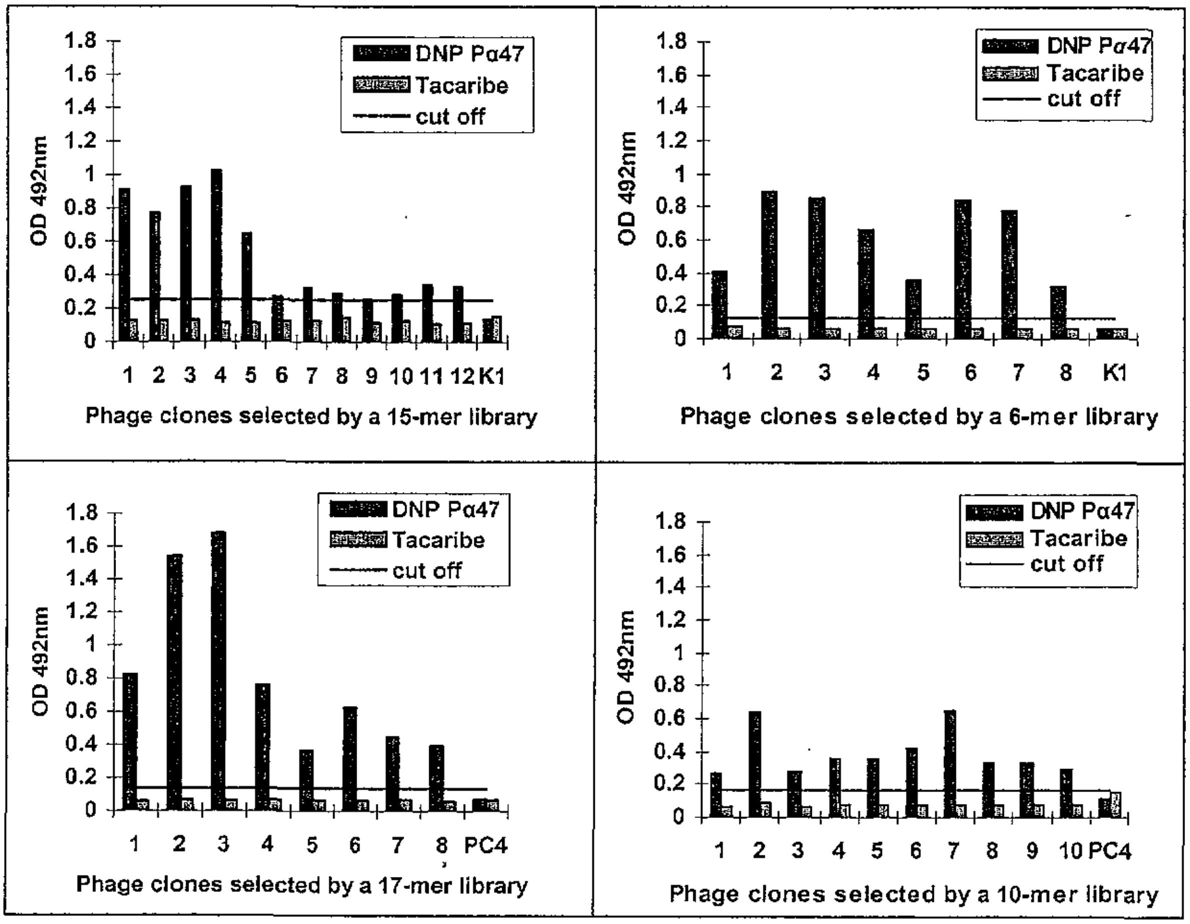


Figure 5.6. Simple ELISA to test binding of the selected phage clones with mAb DNP Pα47

Phage clones diluted 1:10 were attached to microwells and the mAb to DNP and Tacaribe used at a dilution of 1:200 in an ELISA. The cut off value was twice the mean OD value of the mAb to Tacaribe with the phage clones. The OD values ranged from 1.026 to 0.256 for clones selected by a 15-mer and ranged from 0.890 to 0.316 for clones selected by a 6-mer linear phage libraries. The OD values ranged from 1.681 to 0.366 for phage clones selected by a 17-mer and ranged from 0.657 to 0.265 clones selected by a 10-mer constrained phage libraries. The cut off values for the 15-mer, 6-mer, 17-mer and 10-mer libraries were 0.253, 0.131, 0.133 and 0.166 respectively.

5.3.4. Amino acid sequences of peptide inserts of phage clones

All ELISA positive phage clones were sequenced to determine how many different peptides had been selected. Phage clones selected by each library were grouped according to amino acid sequences of their inserts.

5.3.4.1 Amino acid sequence of peptides selected by pIII-based linear phage libraries

Sequencing of 12 phage clones selected by a 15-mer library and 8 phage clones selected by a 6-mer library gave two distinct 15-mer and two distinct 6-mer peptides. The 15-mer peptides and phage clones displaying these peptides were referred as DA15-1 LFDAVRPGPVGSVIT and DA15-2 DWDSACLDGNPRCLD. The 6-mer peptides and the respective phage clones displaying these sequences referred as DA6-1 MLDGTR and DA6-2 MVLAVK **(Table 5.3)**.

5.3.4.2. Amino acid sequence of peptides selected by pVIII based constrained phage libraries

Similarly, sequencing of 8 individual phage clones selected by a 17-mer constrained phage library and 10 phage clones selected by a 10-mer constrained phage library gave two distinct 17-mer sequences and one 10-mer sequence. The 17-mer peptides and phage clones displaying these peptides were referred to as DA17-1 RPSTRCDLPWLYPSSCY, DA17-2 QPPMTKQESVSVCRGCN and the 10-mer peptide and the phage clone displaying this peptide referred to as DA10-1 NCHPMDMACL **(Table 5.3)**.

5.3.4.3. Analysis of amino acid sequences of peptides

By examining the resulting 15-mer sequence, DA15-1 LFDAVRPGPVGSVIT, it was found that the peptide consisted of amino acids having nonpolar, aliphatic side chains (L, A, 3V, 2P, 2G, I), charged side chains (R, D), polar uncharged and hydrophilic side chains (S, T) and an aromatic side chain (F). The peptide is rich for hydrophobic nature of amino acids (L, F, 3V, I, 2P, A). Similarly, it was found that the peptide sequence of DA15-2 DWDSACLDGNPRCLD consisted of the amino acids having nonpolar aliphatic side chains (A, L, G, P), charged side chains (R, D), polar uncharged and hydrophilic side chains (C, N) and an aromatic side chain amino acid (W). The sequence also rich for hydrophobic amino acids (W, A, 2C, 2L, P).

By examining 6-mer peptide sequences of DA6-1 MLDGTR and DA6-2 MVLAVK, it was found that these sequences generally contained amino acids having nonpolar, aliphatic side chain (L, G, A, V) and charged side chains (R, K) and hydrophobic amino acids (A, V, M, L).

By examining the 17-mer peptide of DA17-1 RPSTRCDLPWLYPSSCY, it was observed that it contains amino acids having nonpolar aliphatic side chains (3P, 2L), polar, uncharged side chains (3S, T, 2C), and aromatic side chains (2Y, W) and charged side chains (2R, D). The sequence contains hydrophobic aminoacids (2P, 2Y 2C, 2L, W).

By examining the sequence of DA17-2 QPPMTKQESVSVCRGCN, it was observed that the sequence contains amino acids having nonpolar aliphatic side chains (2P, 2V, G), polar uncharged side chains (M, 2C, T, N, 2Q), charged side chains (R, K, E). It contains hydrophobic amino acids (2P, M, 2V, 2C).

The examination of a 10-mer peptide DA10-1 NCHPMDMACL showed that it contains amino acids having polar, aliphatic side chains (L, P, A), polar, uncharged side chains (2C, 2M, N), charged side chains (D, R, H) and hydrophobic amino acids (2C, P, A, 2M, L).

Table 5.3. Nucleotide and amino acid sequences of peptides selected by phage libraries

Peptides	Nucleotide and amino acid sequences of peptides (NH_2-COOH)
DA15-1	CGT AAT CAC ACT ACC AAC AGG ACC AGG ACG AAC AGC ATC AAA CAG L F D A V R P G P V G S V I T
DA15-2	GAT TGG GAT TCT GCT TGT CTT GAT GGT AAT CCT CGG TGT CTT GAT D W D S A C L D G N P R C L D
DA6-1	ATG CTG GAT GGT ACT CGT M L D G T R
DA6-2	ATG GTG TTG GCG GTG AAG M V L A V K
DA17-1	CGT CCG TCT ACT AGG TGT GAT CTG CCT TGG CTT TAT CCT AGT TCG TGT TAT R P S T R C D L P W L Y P S S C Y
DA17-2	CAG CCT CCG ATG ACT AAG CAG GAG AGT GTG AGT GTT TGT CGG GGG TGT AAT Q P P M T K Q E S V S V C R G C N
DA10-1	AAT TGT CAT CCT ATG GAT ATG GCG TGT CGT N C H P M D M A C L

Sequencing of 12 individual phage clones selected by a 15-mer phage library and 8 clones selected by a 6-mer library gave two distinct 15-mer (DA15-1, DA15-2) and two distinct 6-mer (DA6-1, DA6-2) peptide sequences. Sequencing of 8 individual phage clones selected by a 17-mer phage library and 10 phage clones selected by a 10-mer phage library gave two distinct 17-mer (DA17-1, DA17-2) and one 10-mer (DA10-1) peptide sequences. Hydrophobic amino acids in these sequences were shown in red.

5.3.5. ELISA assays with phage clones of known sequences

To confirm the binding specificity of the phage clones more accurately and to test the antigenic mimicry of peptides with DNP, ELISA and competitive-inhibition ELISAs were performed.

5.3.5.1. Quantitative ELISA to test the phage clones with the selecting mAb DNP Pα47

At the 1:200 initial dilution of the mAb DNP Pα47, all phage clones DA15-1, DA15-2, DA6-1, DA6-2, DA17-1, DA17-2 and DA10-1 were bound by the mAb DNP Pα47. Their OD was at least 8 times that to the irrelevant phage clones (K1 or PC4) and decreased to background, as the mAb was diluted 16-fold (1:3200) (see text for figures). No binding was observed with the high affinity mAb DNP Pα41 and an irrelevant mAb to Tacaribe virus **(Figure 5.7 and 5.8)**.

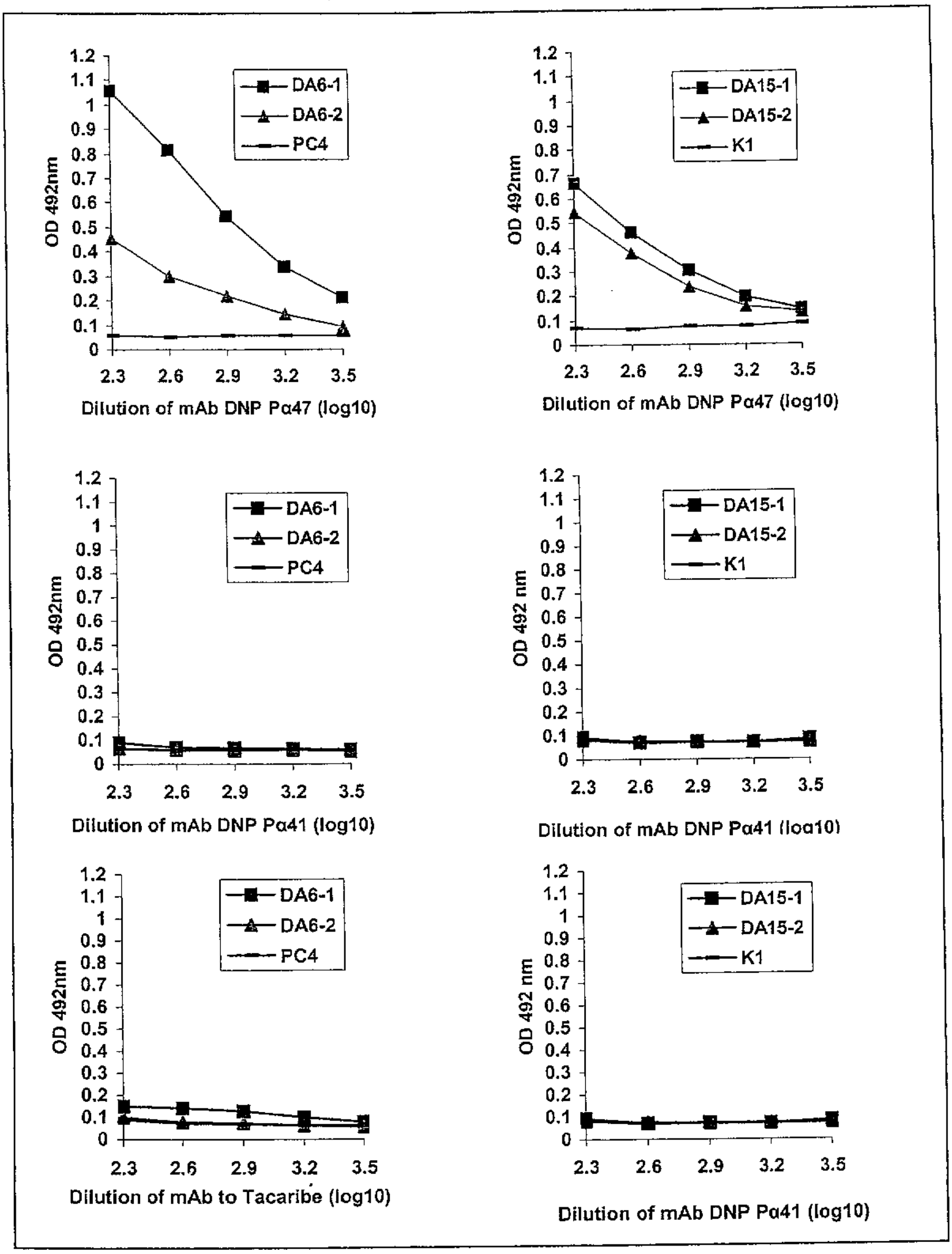


Figure 5.7. Reactivity of phage clones of known sequences selected by pIII-based linear (15 and 6-mer) libraries with DNP mAbs

Reactivity of phage clones DA15-1, DA15-2 and DA6-1, DA6-2 was tested with DNP mAbs (DNP Pα47, DNP Pα41). The mAb DNP Pα47 (i.e 1:200 dilution) showed strong binding to DA15-1, DA15-2 and DA6-1, DA6-2 with OD_{492nm} 0.666, 0.545, 1.055 and 0.452 compared to irrelevant phage-clones of K1 and PC4 with OD_{492nm} 0.070 and 0.060 respectively. No binding was observed between phage clones and the a high affinity mAb DNP Pα41 and an irrelevant mAb to the Tacaribe virus.

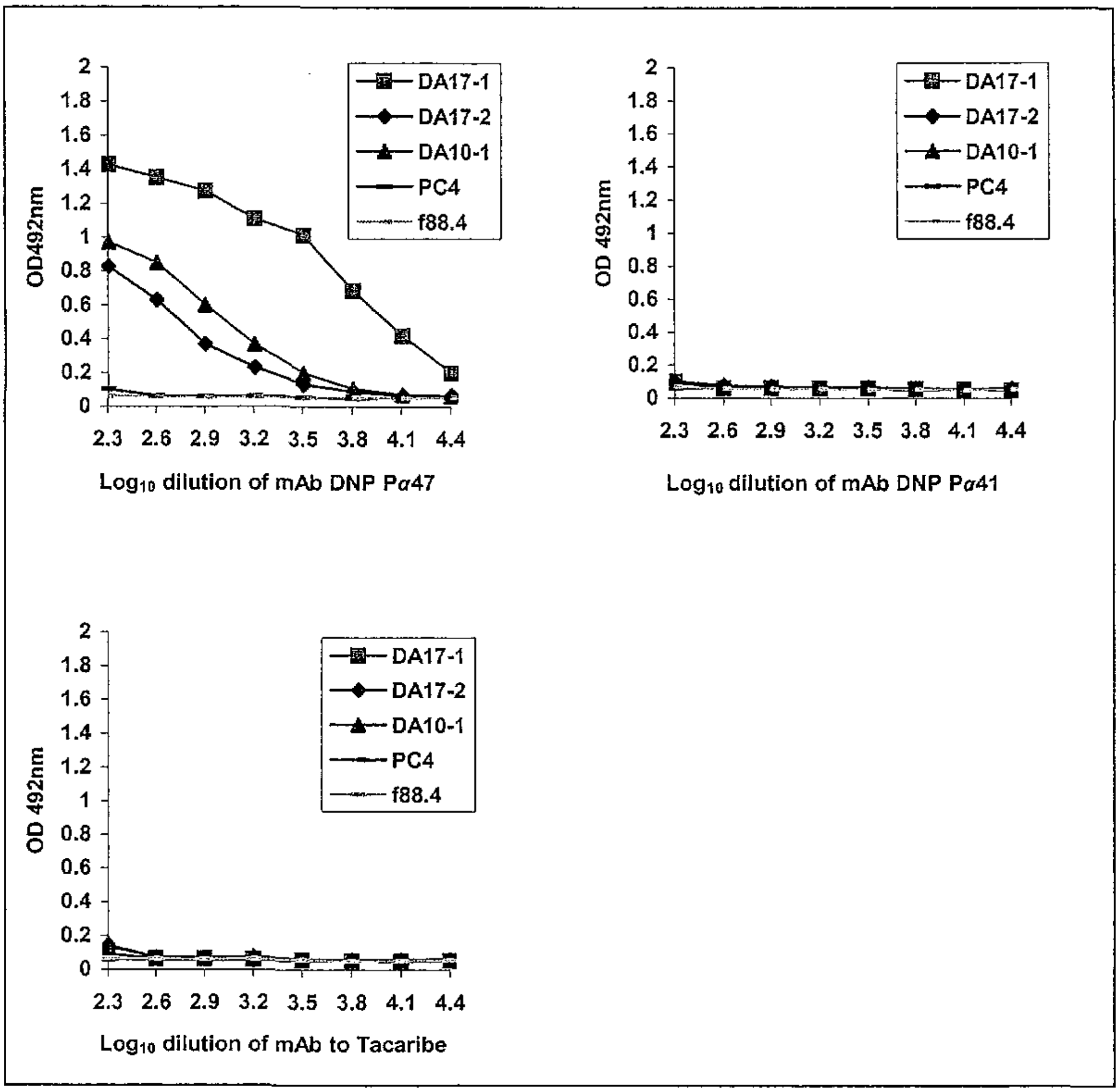


Figure 5.8. Reactivity of phage clones of known sequences selected by pVIII-based constrained (17 and 10-mer) libraries with DNP mAbs

Reactivity of phage clones DA17-1, DA17-2 and DA10-1 was tested with DNP mAbs (DNP Pα47, DNP Pα41). The mAb DNP Pα47 (i.e 1:200 dilution) showed strong binding binding to DA17-1, DA17-2 and DA10-1 with OD_{492nm} 1.425, 0.828, 0.970 compared to irrelevant phage clone (PC4) of HBV and empty phage clone with f88.4 with OD_{492nm} 0.103 and 0.062 respectively. No binding was observed between phage clones and the high affinity DNP mAb DNP Pα41 and an irrelevant antibody to the Tacaribe virus.

5.3.6. Competitive-inhibition ELISAs to test the mimotopes of DNP

5.3.6.1. To determine the limiting dilutions of antigens and antibody for use in competitive-inhibition ELISAs

Limiting dilution of mAb DNP Pα47 and concentration of inhibitors and coating antigens were estimated by checkerboard titration. The starting and final concentration of inhibitors and dilution of the mAb used in competitive-inhibition ELISA are listed in **Table 5.4**.

Table 5.4. Limiting concentration of antigen and antibodies used in competitive-inhibition ELISAs

Concentration of coating antigens	Dilution of mAb (OD_{492}=0.6-0.7	Starting and final concentration of inhibitors
DA15-1, DA15-2: $1x10^8$ TU/ml	DNP Pα47 1:200	DA15-1, DA15-2: $2x10^9$-$3.9x10^6$ TU/ml DNP_{237} casein: 0.8-0.025 μg/ml DNP-L-Lysine: 100-3.125 μg/ml
DA6-1, DA6-2: $2x1x10^8$ TU/ml	DNP Pα47 1:200	DA6-1, DA6-2: $1x10^9$-$1.56x10^7$ TU/ml DNP_{237} casein: 0.8-0.025 μg/ml DNP-L-Lysine: 250-7.81 μg/ml
DA17-1: $2x10^9$ TU/ml	DNP Pα47: 1:800	DNP_{237} casein: 40-0.0004 μg/ml DNP-L-Lysine: 1-0.00001mg/ml DA17-1: $2x10^{10}$-$2x10^3$ TU/ml
DNP_{237}casein: 0.05 μg/ml	DNP Pα47: 1:800	DA15-1, DA15-2: $2x10^9$-$3.9x10^6$ TU/ml DA17-1, DA17-2: $5x10^{10}$-$5x10^3$ TU/ml DA10-1: $5x10^{10}$-$5x10^3$ TU/ml DNP_{237} casein: 40-0.0004 μg/ml

5.3.6.2. Competitive-inhibition ELISAs

The results of each competitive-inhibition experiment are presented as a graph **(Figure 5.9-5.14)**. For ease of interpretation, they have been summarised as inhibition or no inhibition on a table **(Table 5.5)**. The positive inhibition obtained with diluted inhibitors was correlated with 50% reduction of OD value as compared with irrelevant inhibitors. The inhibition reactions were dilution dependent; a linear increase of binding (OD value) was observed as the inhibitors were progressively diluted and an excess of unbound antibody become available.

Table 5.5. Competitive-inhibition ELISAs for testing binding of phage clones

Inhibitors	Coating antigens					
	DA15-1	DA15-2	DA6-1	DA6-2	DA17-1	$DNP_{237casein}$
DA15-1	+	+				-
DA15-2	+	+				-
K1	-	-				-
DA6-1			+	+		
DA6-2			+	+		
PC4			-	-		
$DNP_{237casein}$	+	+	+	+	+	+
Casein	-	-	-	-	-	
DNP-L-Lysine	+	+	+	+	+	
Poly-L-Lysine	-	-	-	-	-	
DA17-1					+	+
DA17-2						+
DA10-1						+
17-mer library						-

All diluted inhibitors were preincubated with the mAb DNP Pα47 before transferring ELISA plates coated with antigens (phage clones or DNP_{237} casein conjugate). The same batches of phage clones and irrelevant control clones were used for competitive-inhibition ELISAs. Phage clones displaying mimotopes for HCV (K1) and HBV (PC4) and unselected 17-mer library were used as irrelevant controls.

Blank means experiments not done.
+: positive inhibition: 50% decrease of OD at the starting dilution of inhibitor.
-: negative inhibition: less than 10% decrease in OD at the starting dilution of inhibitor

Competitive-inhibition ELISAs to test mimotope potential of peptides on phage were as follows:

5.3.6.2.1. One phage clone inhibiting mAb binding to the second phage clone

Each pair of peptides selected by a 15-mer and a 6-mer linear phage libraries (DA15-1, DA15-2 and DA6-1, DA6-2) blocked each other for binding to the mAb although they had different amino acid sequences **(Figure 5.9 and Table 5.5)**.

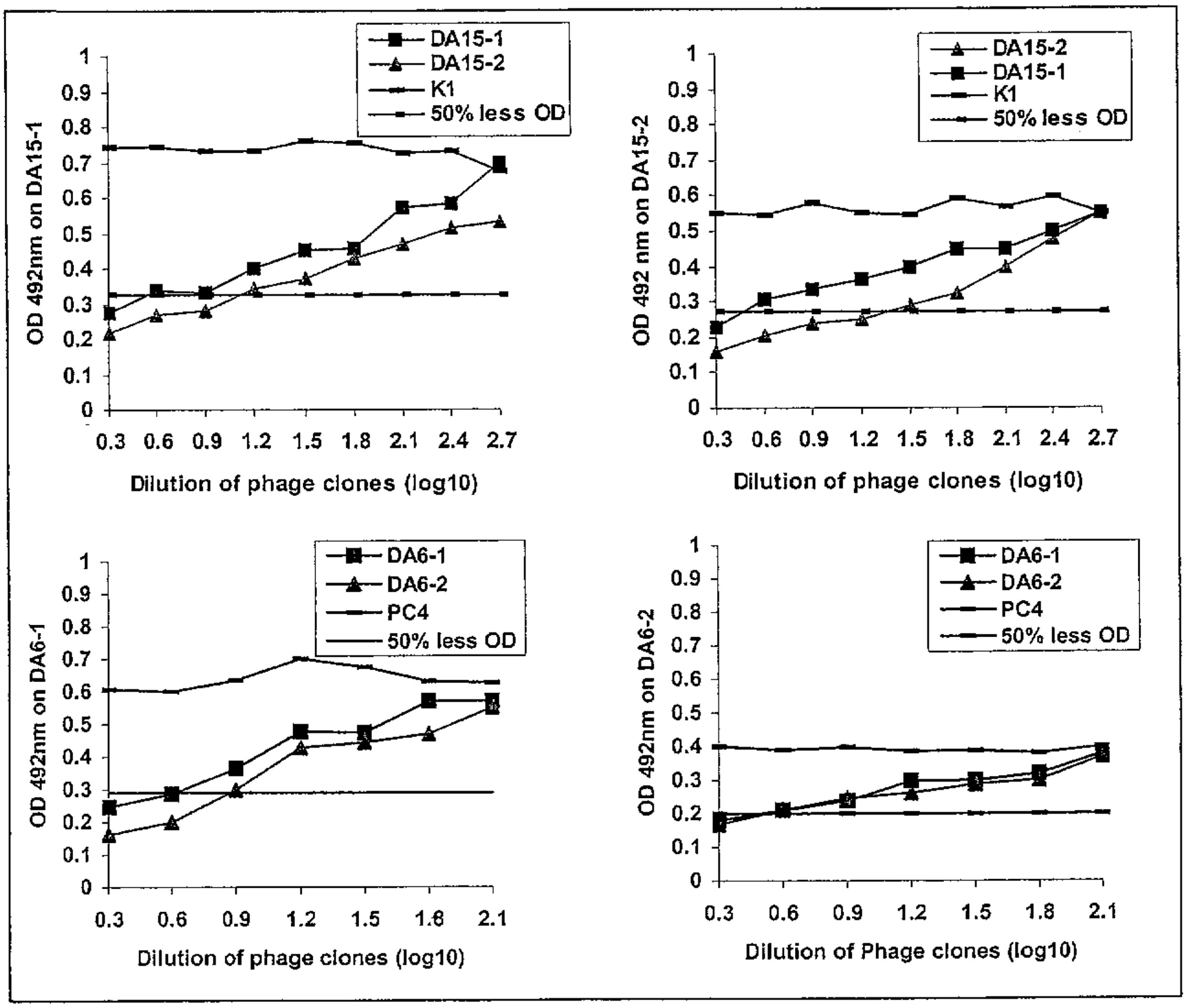


Figure 5.9. One phage clone inhibiting mAb binding to a second phage clone

A 1:200 dilution of the mAb DNP Pα47 was preincubated with the two fold diluted phage clones (inhibitors) (DA15-1, DA15-2 and DA6-1, DA6-2) and irrelevant phage clones (PC4 or K1) before transferring ELISA plates coated with the relevant phage clones (antigens). Binding of the mAb DNP Pα47 to each pair of reactive phage clones was inhibited by homologous and heterologous clones in a concentration dependant manner. Inhibition of binding was observed by a decrease in optical density (y-axis) with increasing inhibitor concentration (x-axis). No inhibition was observed by irrelevant control peptides.

5.3.6.2.2. DNP inhibiting mAb binding to phage clones displaying 15, 6 and 17-mer peptides (DA15-1, DA15-2 and DA6-1, DA6-2 and DA17-1)

The ability of peptides on phage (DA15-1, DA15-2 and DA6-1, DA6-2 and DA17-1) to bind to the antigen-binding site of the mAb DNP Pα47 was tested in a competitive-inhibition ELISA. The DNP_{237} casein conjugate and DNP-L-Lysine inhibited the binding of the mAb to the peptides on phage **(Table 5.5 and Figure 5.10, 5.11 and 5.12)**.

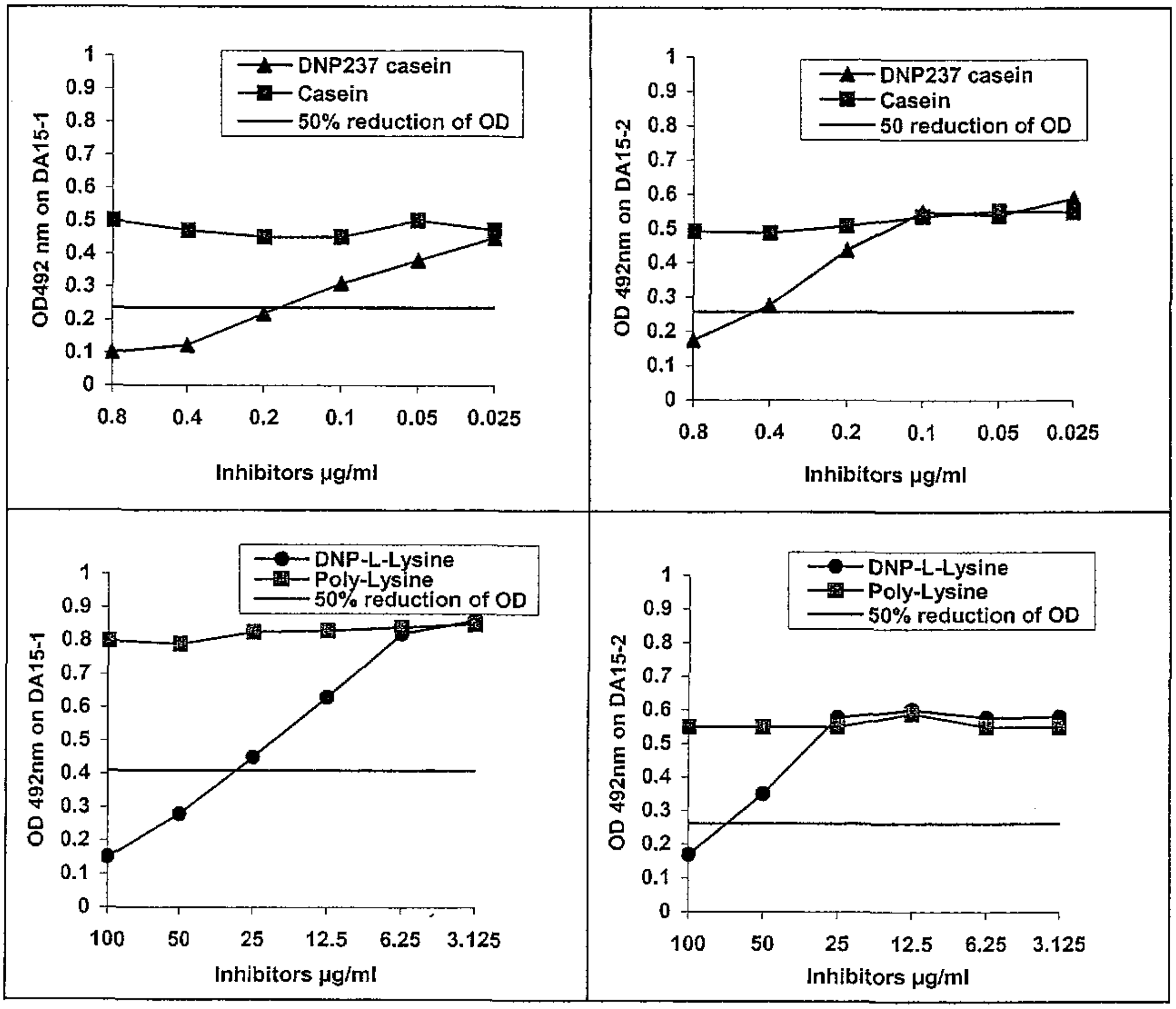


Figure 5.10. DNP inhibiting mAb binding to phage clones (DA15-1 and DA15-2)

A 1:200 dilution of the mAb DNP Pα47 was preincubated with the decreasing concentrations of the inhibitors (DNP_{237} casein conjugate and DNP-L-Lysine) before transferring to ELISA plates coated with phage clones DA15-1, DA15-2 at a concentration of $2.x10^8$ TU/ml. Inhibition of binding between phage clones and the mAb was observed by the inhibitors. No inhibition was observed with casein and poly-L-lysine.

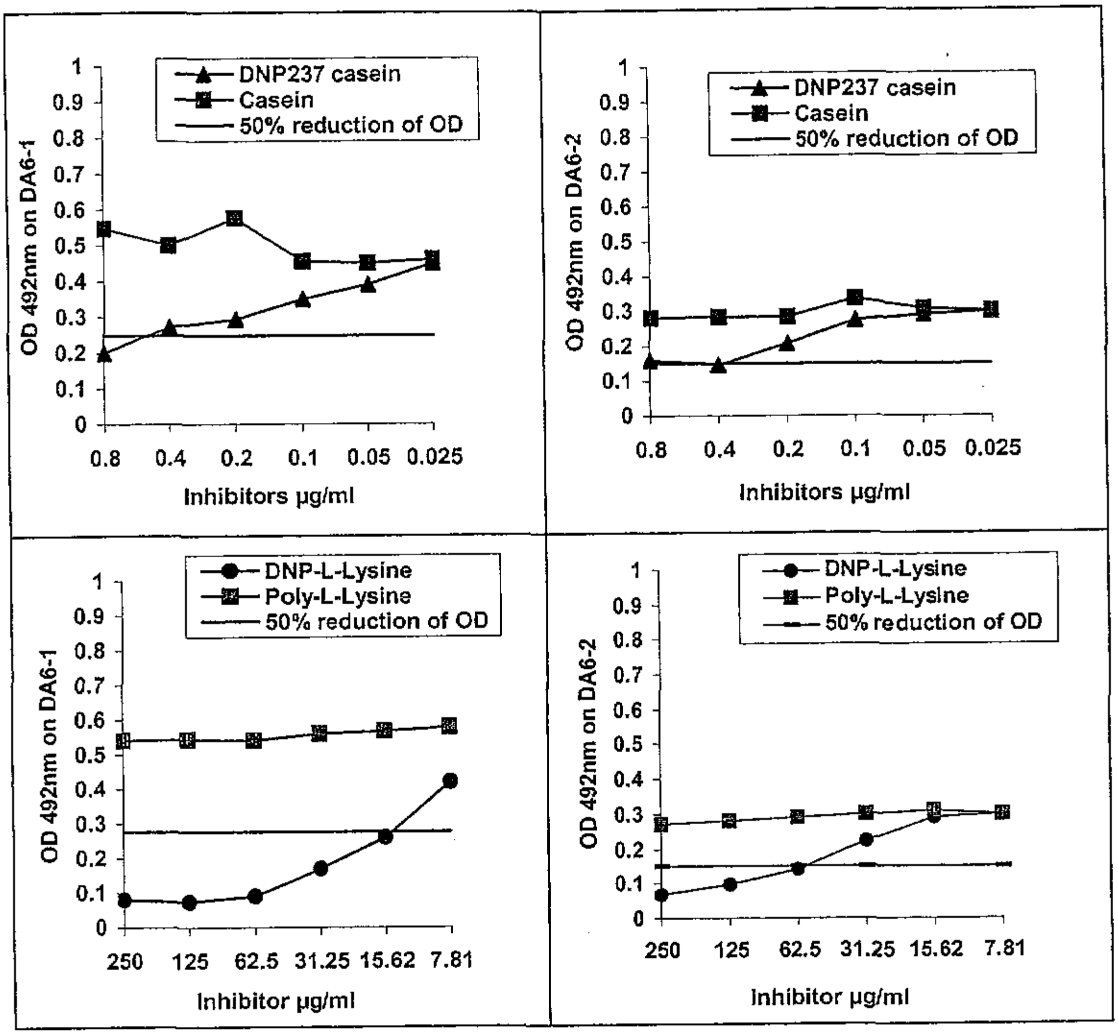


Figure 5.11. DNP inhibiting mAb binding to phage clones (DA6-1 and DA6-2)

A 1:200 dilution of the mAb DNP Pα47 was preincubated with the decreasing concentrations of the inhibitors (DNP_{237} casein conjugate and DNP-L-Lysine) before transferring to ELISA plates coated with phage clones DA6-1 and DA6-2 at a concentration of $4x10^8$ TU/ml. Inhibition of binding between phage clones and the mAb was observed by the inhibitors. No inhibition was observed with casein and Poly-L-Lysine.

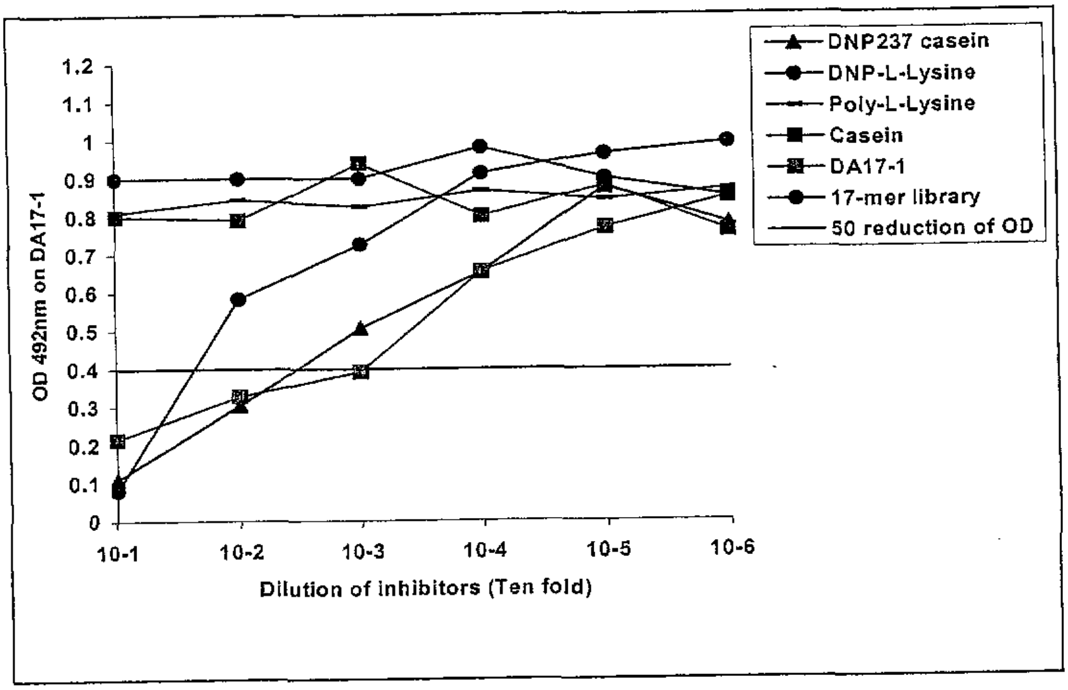


Figure 5.12. DNP inhibiting mAb binding to phage clone DA17-1

Ten fold-diluted inhitors (DNP_{237} casein and DNP-L-Lysine) were first preincubated with *1/800 dilution* of the mAb DNP Pα47 and added to the plates precoated with the phage clone DA17-1 at a concentration of $2X10^{10}$ TU/ml. Inhibition of binding was observed by a decrease in the optical density (y-axis) with increasing in inhibitor concentration (x-axis). Poly-L-Lysine and caseine were used as negative control. DA17-1 was used as known inhibitor. The starting concentration of DNP_{237} and DNP-L-Lysine were 40 μg/ml and 1 mg/ml respectively.

5.3.6.2.3a. Phage clones (DA15-1 and DA15-2) inhibiting mAb binding to DNP

The two 15-mer peptides displayed on phage were unable to block DNP binding to the mAb in a competitive-inhibition ELISA (**Table 5.5 and Figure 5.13**).

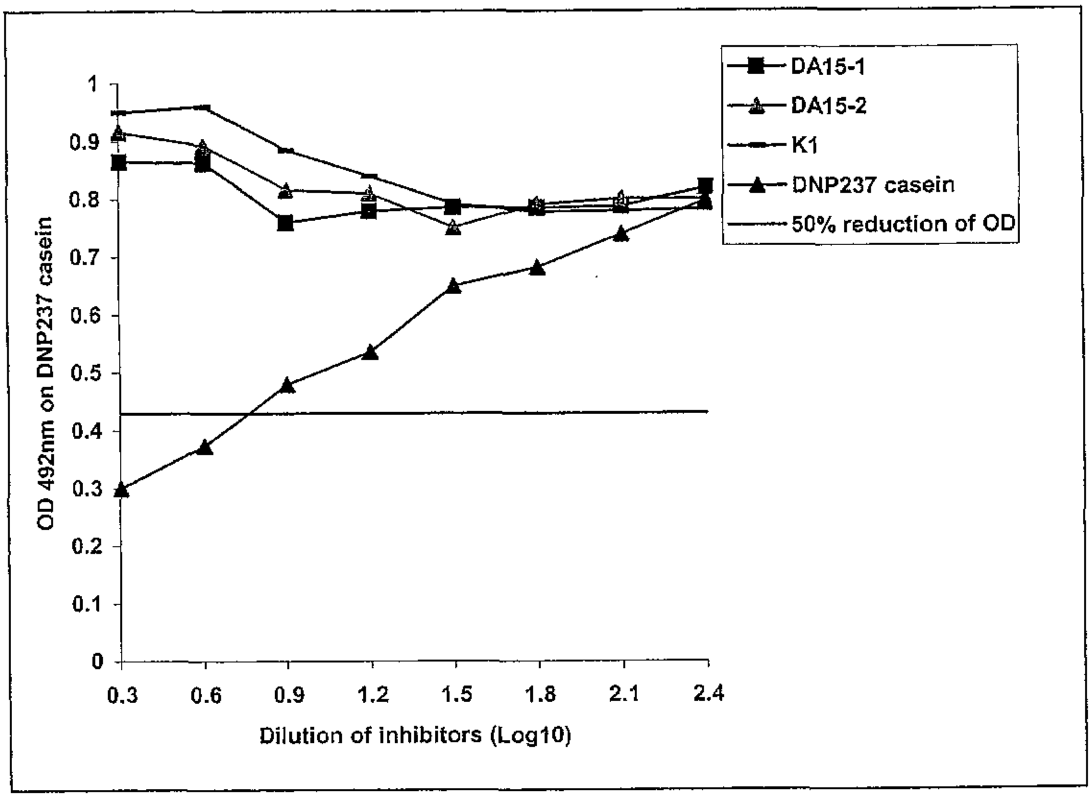


Figure 5.13. Phage clones (DA15-1 and DA15-2) inhibiting mAb binding to DNP

A 1:800 dilution of the mAb DNP Pα47 was preincubated with 2-fold titrated inhibitors (DA15-1 and DA15-2 and an irrelevant control clone K1) starting from $4X10^9$ TU/ml to $6X10^7$ TU/ml. The mixtures were then transferred to an ELISA plate precoated with 0.05 μg/ml of DNP_{237} casein-conjugate. No inhibition was observed by phage clones. The DNP_{237} casein conjugate was used as known inhibitor.

5.3.6.2.3b. Phage clones (DA17-1, DA17-2 and DA10-1) inhibiting mAb binding to DNP

DA10-1, DA17-1 and DA17-2, selected by 10 and 17-mer constrained libraries, were able to block DNP binding to its mAb in a concentration dependent manner **(Figure 5.14 and Table 5.5)**. DA17-1 and the DNP_{237} conjugated to casein caused a 50% reduction in OD and their slopes of their inhibition curves were no different **(Fig 5.14)**. DA 17-2 and DA10-1, by contrast, did not reach a 50% reduction in OD; their inhibition curves were nearly identical but significantly shallower than those of DA17-1 and DNP_{237} **(Table 5.6)**. The unselected 17-mer library did not block the binding.

Statistical analysis of the confidence limits of the lines of regression for each inhibition curve showed that DA17-1 and DNP_{237} formed one group that was different or marginally different to the second group that comprised DA17-2 and DA10-1 **(Table 5.6)**.

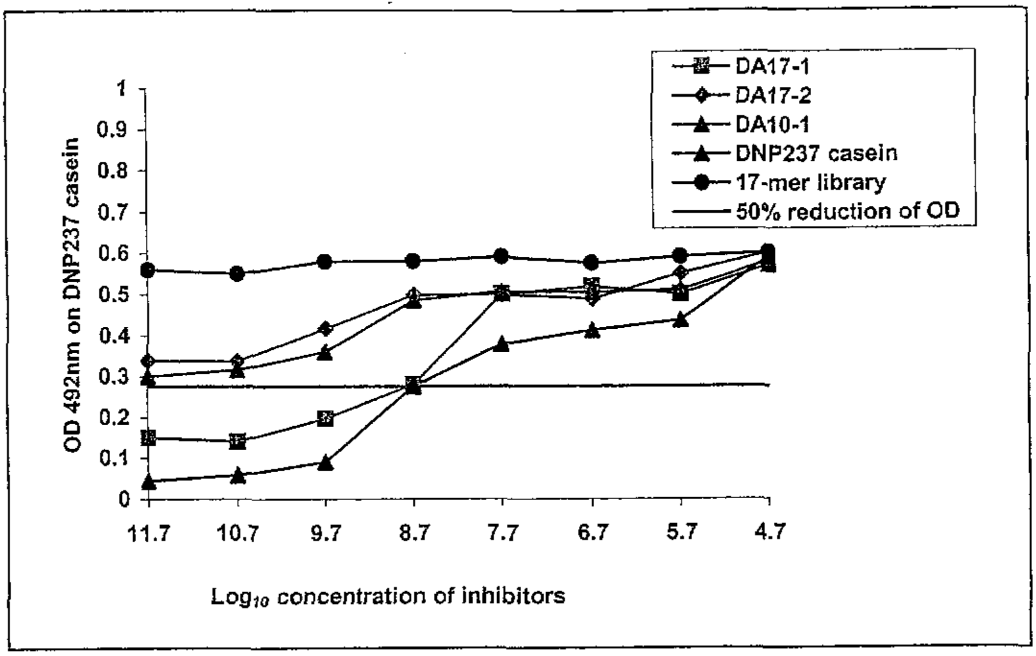


Figure 5.14. Phage clones (DA10-1 and DA17-1, DA17-2) inhibiting mAb binding to DNP

1:800 dilution of the mAb DNP Pα47 was preincubated with 10-fold titrated inhibitors (DA17-1, DA17-2 and DA10-1) *starting from* $5X10^{11}$ TU/ml to $5X10^{4}$ TU/ml before transferring to ELISA plates precoated with 0.05 μg/ml DNP_{237} casein conjugate. A same amount unselected 17-mer library was used as irrelevant control. Inhibition of binding between DNP_{237} casein conjugate and the mAb was observed by the inhibitors in a concentration dependant manner ($p<0.05$ apart from control). The DNP_{237} casein conjugate was also used as known inhibitor.

Table 5.6. Statistical analysis of phage clones (DA10-1 and DA17-1, DA17-2) inhibiting mAb binding to DNP

Inhibitors	Best fit value of slope	95% confidence interval of each slope		Group
DNP_{237} casein	0.066	0.039	0.093	a
DA17-1	0.067	0.042	0.092	a
DA17-2	0.027	0.012	0.042	b
DA10-1	0.034	0.016	0.056	b
17-mer library	0.0026	0.003	0.002	c

Differences in CI of slope are indicated by separate letters for each group. Thus,

b differs from a, CI's > 90 or 95%
c differs from a or b, CI's > 95%

5.4. Discussion

This chapter describes peptide mimotopes of a routinely used hapten DNP as seen by a low affinity mAb DNP Pα47. The affinity of the mAb DNP Pα47 was lower than that of the mAb DNP Pα41 in an inhibition ELISA **(Figure 5.5)**. This was in accordance with an extensive study carried out by Stanley *et al.*, (1983) where they compared several techniques to test antibody affinity by utilising nine IgG1 DNP mAbs including mAbs DNP Pα47 and DNP Pα41. However, the mAb DNP Pα41 was found to be IgG2a in contrast to Stanley's study where it was reported to be IgG1 **(Figure 5.4)**.

5.4.1. The effect of antibody affinity for selecting potential mimotopes of DNP

The selection of phage clones displaying potential mimotopes of DNP was successful only with the low affinity mAb to DNP but not with the high affinity mAb to DNP using the same libraries and standard techniques in biopanning **(Table 5.1 and 5.2 and Figure 5.6)**. Phage clones selected by the low affinity DNP mAb were not bound by the high affinity DNP mAb **(Figure 5.7 and 5.8)**.

The possible explanation for the successful outcome with the low affinity mAb could be explained by the antibody binding mechanism of haptens. Antibodies against haptens have very small binding sites to accommodate small haptens. Antibody binding to hapten molecules usually involves the insertion of the hapten into a deep cavity or pocket between the complementary determining regions (CDR) of the antibody (Brunger *et al.*, 1991). In the case of the high affinity DNP mAbs, peptides may have difficulty to fitting into the small deep cavity because they are too big and require a wide shallow binding sites (Foote, 2003). However, the binding site of low affinity DNP mAbs may probably have shallower sites that could accommodate peptides as they bound to DNP hapten since they do not bury the hapten as well as high affinity mAbs. In addition, it is also possible that the high affinity mAb had more of a cavity that buried the DNP better but excluded peptides for the reasons of steric hindrance (Scott, J personal communication 2004). To what extend this was linked to antibody affinity and not to chance would have to be investigated using at least three mAbs of low and high affinity in a much larger study. This was suggested as future work (chapter 8, section 8.4.4).

The neutralising BVDV E2 mAbs used earlier in this thesis had high relative affinity against BVDV cell lysate and this may limit their use for peptide mimotope selection (see chapter 4, section 4.3.4).

5.4.2. Analysis of amino acid sequences of potential mimotope peptides

The sequencing of phage clones revealed a total of seven peptides recognised by the low affinity mAb **(Table 5.3)**. Despite the sequences of seven peptides being different, they all contained high proportion of hydrophobic and most of them also contain aromatic amino acids (section 5.3.4 and Table 5.3). Because of the hydrophobic and aromatic nature of DNP (Edebo and Richardson, *1985*; Foote, 2003), this could be indicative for the preference of the DNP mAb for selecting these potential mimotope peptides from phage libraries. In general, the peptides also contain charged and hydrophilic amino acids (section 5.3.4) that might contribute to their antigenic features for binding to DNP mAb (Hodges *et al.*, 1988; Geysen *et al.*, 1987b; Getzoff *et al.*, 1987).

In addition to the above hydrophobic amino acids, the second amino acids of DA15-1: LFDAVRPGPVGSVIT and DA15-2: DWDSACLDGNPRCLD were aromatic amino acids (F, W) that might enable the selection of these peptides from the library and enable them to mimic of the shape of DNP **(Figure 5.1)**. The proline residues in both peptides (2P DA15-1 and 1P DA15-1) might also enable them to adopt an antigenic β-turn configuration (Chou and Fasman, 1974). In addition, the two-cysteine residues within the DA15-2 could form a disulphide bond that might give a conformation to the peptide to mimic the ring shape of DNP.

The sequences of DA10-1: NCHPMDMACL, DA17-1: RPSTRCDLPWLYPSSCY and DA17-2: QPPMTKQESVSVCRGCN were also rich in hydrophobic amino acids. Particularly, the DA17-1 contains three hydrophobic aromatic amino acids (2Y, W) and two proline (2P) that might enable it to bind the DNP mAb. In addition, the two-cysteine residues within the DA17-1, DA17-2 and the DA10-1 might enable to form a disulphide bond to mimic DNP.

5.4.3. Testing the mimotope potential of peptides on phage

In competitive-inhibition ELISAs, one of each pair of peptides on phage (DA15-1. DA15-2 and DA6-1 and DA6-2) inhibited the binding of the other peptide from the same library to the mAb, but no inhibition was seen with irrelevant peptides **(Table 5.5 and Figure 5.9)**. This suggests that the reactivity between the mAb and phage clones were indeed specific for the peptides on phage rather than for other phage proteins. Furthermore, the results showed that peptides with different amino acid sequences represented the same epitope recognised by the low affinity mAb. However, this could also be explained by the cross-reactive peptides bound to sites close to but not identical to DNP binding site and each other (in terms of the exact residues on the mAb surface involved) and being excluded by steric hindrance.

The DNP_{237} casein conjugate and the smaller DNP-L-Lysine blocked binding of the mAb to peptides on phage (**Table 5.5** and **Figure 5.10, 5.11 and 5.12)**. In particular, the ability of DNP-L-Lysine to block the binding of the mAb to peptides suggested that peptide-binding site is very close to DNP binding site of the mAb. As DNP is a small molecule, it might only occupy part of the mAb combining site. On the other hand, peptides could probably interact with larger areas that may not include the DNP interaction sites. This was tested by the ability of peptides to inhibit mAb binding to DNP.

The two 17-mer peptides (DA17-1 and DA17-2) and the one 10-mer peptide (DA10-1) but not the two 15-mer peptides (DA15-1 and DA15-2) successfully blocked DNP (DNP_{237} casein) binding to its mAb **(Table 5.5 and Figure 5.13, 5.14).** This indicated that 17 and 10-mer peptides selected by pVIII-based constrained libraries mimicked the DNP and thus they can be considered as mimotopes. However, a second explanation for the failure of the two 15-mer peptides to compete with the DNP_{237} casein conjugate may be the low copies of peptides (3-5) presented on pIII-based library as compared to a high number of peptides (250-300) presented on pVIII-based libraries (Bonnycastle *et al.*, 1996). Furthermore, it can also be seen in **Figure 5.14** that the use of pVIII-based constrained phage libraries enabled the selection of mimotopes of DA17-1, DA17-2 and DA10-1 having different affinity towards the DNP. DA17-1 inhibited binding of the DNP_{237} casein to the mAb better than DA17-2 or DA10-1 **(Figure 5.14)**. The slope of inhibition curves of DA17-1 and DNP_{237} were very close but were different or marginally different from DA17-2 and DA10-1 (GraphPad InStat version 3, San Diego) **(Table 5.6)**. Thus, the DA17-1 was the best inhibitor and best mimotope of DNP and it bound to the mAb DNP Pα47 with the affinity almost similar to the DNP_{237} casein conjugate **(Figure 5.14)**. The presence of three hydrophobic aromatic amino acids (W, 2P) and other hydrophobic amino acids (3P, 2C, and 2L) and two cysteine residues within the DA17-1: PSTRCDLPWLYPSSCY might give better conformation to this mimotope mimicking with DNP better than other mimotopes (see earlier).

The whole issue of whether the mimotope peptides are really mimicking the DNP radical or simply binding in a structurally unrelated way to the same region of the mAb is one which could only be resolved by structural analysis of the binding of DNP and of peptide by such techniques as NMR, X-ray crystallography and co crystallisation that were beyond the scope of this work.

5.4.4 The comparison of the use of linear and constrained phage libraries for selecting DNP mimotopes

It can be suggested that the pVIII-based phage libraries facilitated the selection of better DNP mimotopes. In general, this was in accordance with an extensive study carried out by Bonnycastle *et al.*, (1996). The authors suggested the importance of using a large range of phage libraries to enhance the chance for selecting tightest binding ligands for any given ligate and the usefulness of constrained phage libraries for selecting mimotopes of conformational epitopes. Because the fixed cysteine residues were placed within random peptide sequences in pVIII-based libraries, this could also enhance the chance for the selection of mimotope peptides mimicking the DNP. Furthermore, the display of random peptides in sparse density on pVIII protein (approximately 250 peptides sprinkled among 4000 copies of pVIII protein) might have allowed the better discrimination between weak and strong binding peptides (e.g DA17-1and DA17-1 and DA10-1) **(Figure 5.14)** by the mAb during biopanning as suggested by Bonnycastle *et* *al.*, (1996). Because of this, pVIII-based phage libraries were also biopanned with BVDV mAb157 to select mimotopes of BVDV (see chapter 7).

5.4.5 The comparison DNP mimotope study with regard to other mimotope studies of haptens

This study is the first example for describing mimotopes mimicking DNP by exploiting two linear and two constrained phage libraries. Bottger *et al.*, (1999) selected mimotopes of fluorescein hapten as seen by anti-fluorescein (FITC) hapten mAb using 6 and 12-mer pIII-based linear phage libraries. The fluorescein hapten mimotopes selected by the mAb did not give any reactivity with a second mAb to fluorescein. This was in accordance with the present results since the DNP mimotopes selected with the low affinity mAb did not give a cross-reactivity with the high affinity mAb. Furthermore, Bottger *et al.*, (1999) were not able to select mimotopes from their second mAb. From Bottger's and the present results, it might be assumed that not every mAb is applicable for mimotope selection from phage libraries even with mAbs for simple haptenic molecules. The fluorescein hapten mimotopes had a high proportion of hydrophobic aromatic amino acids that was suggested to be the mAb's preference for hydrophobic structures as the fleurescein hapten is hydrophobic too (Bottger *et al.*, 1999). This was also in accordance with the present results as the potential DNP mimotopes had also high proportion of hydrophobic and aromatic amino acids that might also be related to the hydrophobic nature of DNP as discussed above. However, in Bottger's study, they did not mention or determine the affinity of anti-fluorescein mAbs and they only used two linear phage libraries. Furthermore, their mimotopes were unable to induce antibodies in mice cross-reactive with the fluorescein hapten when they were used as immunogens by conjugating with carrier molecules. In the present study, a plan for testing the immunogenicity of DNP mimotopes was not completed due to the lack of the time. This experiment was suggested for future work with a relevant animal project licence (see chapter 9).

5.4.6 Summary and links to the next two chapters

Biopanning of linear and constrained phage libraries with the low affinity mAb to DNP gave binding peptides on phage. The phage clones selected from a 15-mer and a 6-mer library competed with each other for binding to the selector mAb. Phage clones selected from a 17-mer a 10-mer libraries appeared to be true mimotopes. To prove they were mimotopes, mouse immunisation studies could have been done but a plan for this experiment was not completed because of the timescale of the thesis. Instead of mouse immunisation, a SPOT scan assay was used to prove the selected peptides were antigenic. In the next chapter, the mapping of the most antigenic regions of two 15-mer peptides DA15-1 and DA15-2 by a SPOT scan assay will be presented.

The successful selection of phage clones from a range of phage libraries using the low affinity mAb to DNP ensured that techniques had been employed correctly. What has not been reported is how during the above selection trials with a BVDV mAb were performed side by side with the DNP mAbs. This BVDV work will be reported in chapter 7 along with the use of an additional an 8-mer solid phase library and a SPOT scan assay for BVDV.

Chapter 6. The mapping of the most antigenic sites of two 15-mer peptides selected by a low affinity mAb to DNP using the SPOT scan assay

6.1. Introduction

Spot peptide synthesis is an easy and flexible technique for simultaneous, parallel chemical synthesis on cellulose membrane supports (SPOT membrane: pure cellulose chromatography paper) by using Fmoc protection chemistry (Frank, 1992; Frank and Overwin, 1996; Kramer and Schneider-Mergener, 1998; Kramer *et al.*, 1999). The reactivity of peptides on SPOT membrane with antibodies is then tested by a SPOT scan assay that is based on a solid phase ELISA (Hujer *et al.*, 2004; Martens *et al.*, 1995; Halimi *et al.*, 1996).

The most common application of the SPOT scan assay is the identification of linear B-cell epitopes on antigens that bind to antibodies. The entire amino acid sequence of an antigen is synthesised by displaying it as overlapping peptides that are usually 8-15 amino acids long (Reineke *et al.*, 1999; Hujer *et al.*, 2004). For instance, Martens *et al.*, (1995) used the SPOT scan assay to map linear epitopes of P (phosphoproteins) of Phocid distemper virus (PDV) 2558/Han88 strain and Canine distemper virus (CDV) by using five mAbs specific for P-protein. They identified three linear epitopes on P proteins; two of them were located with the COOH-terminal of the P proteins and third one was located on the NH_2 terminal part of the protein.

The SPOT scan assay also enables one to make amino acid substitutions on mimotopes that comprise all single possible substitution analogues of the wild-type sequence. This can lead to the identification of the aminoacid residues critical for antibody binding and to the reselection of better mimotopes as shown for RSV (Chargelegue *et al.*, 1998).

The identification of discontinuous epitopes compared to continuous epitopes is a much more complex and difficult task due to their nature and location within the three dimensional structure of the antigens (Reineke *et al.*, 1999). Korth *et al* in (1997) reported the first example for the identification of discontinuous epitopes by the spot scan assay. They identified a three-segmented binding site for the mAb 15B3 that specifically recognises the disease form of prion protein. Reineke *et al*., (1999) reported the second example of mapping a discontinuous epitope on interleukin-10 (IL-10) that composed of a two-segmented binding region. Connection of these two binding region and further optimisation by substitution analysis followed by a disulfide cyclisation scan resulted in a peptide (mimotope) mimicking IL-10 with a dissociation constant in the lower nanomolar range of high affinity.

In this study, the two 15-mer peptides selected by biopanning of a 15-mer phage library with the low affinity mAb DNP Pα47 (see chapter 5, section 5.3.4.1) and 8-mer overlapping peptides representing these peptides were synthesised on a SPOT's membrane. The probing these potential mimotope peptides with the selector mAb revealed their most antigenic 8-mer regions and critical amino acids contributing the antibody binding. This could also be an appropriate model study for combining combinatorial epitope library technology and the SPOT scan assay for selecting mimotopes of BVDV that was used earlier and later in the thesis (see chapter 3, section 3.3.9 and chapter 7, section 7.3.1.4).

6.2. Materials and Methods

6.2.1. Synthesis of peptides on SPOT's membrane

The presentation of SPOT peptides was performed by synthesising two complete 15-mer peptides DA15-1 LFDAVRPGPVGSVIT and DA15-2 DWDSACLDGNPRCLD and the 8-mer overlapping peptides (offset by one residue) derived from these peptides on a derivatised cellulose membrane (from SPOT No. 1 to 18) essentially as described by Frank, (1992) **(Figure 6.1).** A 15-mer (GFFPDHQLPDAFGAN) CP1 and and 8-mer (QLPDAFGA) CP2 peptides from HBV pre-S1 region comprising residues 22-36 were synthesised as unrelated control peptides (from SPOT No 19-20) (D'Mello *et al.*, 1997). In addition, an 6-mer peptide (CAHYID) (CP3) representing epitope of the extra cellular domain of human epidermal growth factor receptor (SPOT No 22) and the mAb (c-erb-1/EGF-R) specific for this domain were employed as assay positive controls (Parker *et al.*, 1984). These peptides were covalently bound at their COOH terminus to a SPOT's membrane while NH_2 terminus remains unbound. Approximately, 5-10 nmol per peptide was synthesised on the membrane by Fmoc-L-amino acid chemistry (SIGMA-Genosys-Custom SPOT's Technical Manual). The detailed description of peptide synthesis steps was given in General Material and Methods (chapter 2, section 2.11.1, 2.11.2). The SPOT peptides were renumbered in results for designing of presentation.

6.2.2. Testing the reactivity of SPOT peptides by SPOT scan assay

The reactivity of peptides with mAbs was tested by a solid-phase ELISA (Frank and Overvin, 1996). The reactivity of all peptides (No:1-22) was first tested with the sheep anti-mouse β-galactosidase conjugate to detect any non-specific binding (conjugate control assay). Following conjugate control assay, the membrane was regenerated and the reactivity of peptides with the mAbs was tested. Briefly, the mAbs diluted in 1:100 and incubated with the SPOT membrane overnight. After testing the peptides with each mAb, the membrane was regenerated for reusing.

The reactivity of peptides (No: 1-20) was tested with the mAb DNP Pα47 and assay control peptides (No: 21-22) with the mAb EGF-R at the same day side by side using same batch of reagents. The membrane was then regenerated again and the reactivity of peptides (No: 1-20) was tested with the mAb DNP Pα41. Following regenerating the membrane, it was dried and stored in -20°C. The detailed description of the SPOT scan assay development protocol is given in General Materials and Methods (chapter 2, section 2.11.3).

6.2.3. Assessment of the reactivity of peptides with mAbs

The strength of the reaction between peptides and mAbs were assessed by comparing visually the blue colour intensity following incubation with the conjugate and subsequent the development of the substrate. In order to produce more objective assessment, the colour intensity of reactive peptides was semi-quantified by using the Adobe PhotoShop program. This is based on quantifying the intensity of blue (cyan) colour with the program's colour picker and creating a histogram in which reactions are expressed as dye units (DU). The reactivity of a peptide is considered as positive if the DU of the peptide was twice above that of the mean value of peptides (from SPOT No 1 to 22) obtained with only conjugate control. The mean DU value of 22 peptides with only conjugate was 23 and two times above this value (46) was taken as cut off. Values more than 46 were considered as positive.

6.3. Results

6.3.1. Reactivity of the peptides with the mAbs by SPOT scan assay

Conjugate control assay

In order to ensure that any non-specific binding that might arise from the reactivity of the peptides with the secondary antibody; the assay was performed first in the absence of primary antibody. Only peptide No. 15 VRPGPVGS was reacted slightly with the secondary antibody (40 DU). No obvious non-specific reactions were observed with any other peptides **(Figure 6.1 and Table 6.1)**.

Assay control

The positive control peptide CP3, spot no. 22 CAHYID, showed a high reactivity with the control mAb EGF-R (76 DU) indicating a successful SPOT peptide synthesis and assay procedure **(Figure 6.1 and Table 6.1)**. This assay control experiment was performed at the same time with testing the reactivity of peptides with the mAb DNP Pα47.

Testing the reactivity of peptides with mAb DNP Pα47

Upon reacting the mAb DNP Pα47 with the peptide DA15-2, the reactivity was observed with the spot peptides No. 1 (DWDSACLDGNPRCLD), 7 (CLDGNPRC), 8 (LDGNPRCL) and 9 (DGNPRCLD) **(Figure 6.1 and Table 6.1)**. By aligning these reactive sequences (SPOT No.7-9), it was shown that the motifs or amino acids **DGNPR** are common in each peptide. However, the greatest reactivity was observed with the spot peptides No: 1 DWDSACLDGNPRCLD and 9 DGNPRCLD that were quantified as 77 DU and 79 DU, suggesting that the 8-mer peptide **DGNPRCLD** contains all the amino acids that are critical for optimal binding to the mAb DNP Pα47 **(Figure 6.1 and Table 6.1)**. The peptide DGNPRCLD was the most antigenic part of the DA15-2: DWDSACLDGNPRCLD and located at the carboxyl part of the 15-mer peptide.

Upon reacting the mAb DNP Pα47 with the peptide DA15-1, the reactivity was observed with the spot peptides No. 10 (LFDAVRPGPVGSVIT), 11 (LFDAVRPG), 12 (FDAVRPGP) **(Figure 6.1 and Table 6.1)**. By aligning the reactive peptides, the

FDAVRPG sequence was common to each peptide. However, the greatest reactivity was observed with the 8-mer peptide No. 11 **LFDAVRPG** (87 DU) suggesting that the sequence contains all the critical amino acids for optimal binding to the mAb. Therefore, this 8-mer peptide is the most antigenic part of the DA15-1: LFDAVRPGPVGSVIT and located within the amino-terminal part of the 15-mer peptide. The spot peptide 19 (GFFPDHQLDPAFGAN) CP1 displaying 15-mer sequence from pre-S1 region of HBV showed a non-specific reactivity with the mAb DNP Pα47 but the spot peptide No.20 (QLDPAFGA) CP2 displaying an 8-mer sequences from the same peptide did not show any non-specific reactivity **(Figure 6.1 and Table 6.1)**.

Testing the reactivity of peptides with mAb DNP Pα41

No cross-reactivity was observed between the peptides and the high affinity DNP mAb DNP Pα41 **(Figure 6.1 and Table 6.1)**.

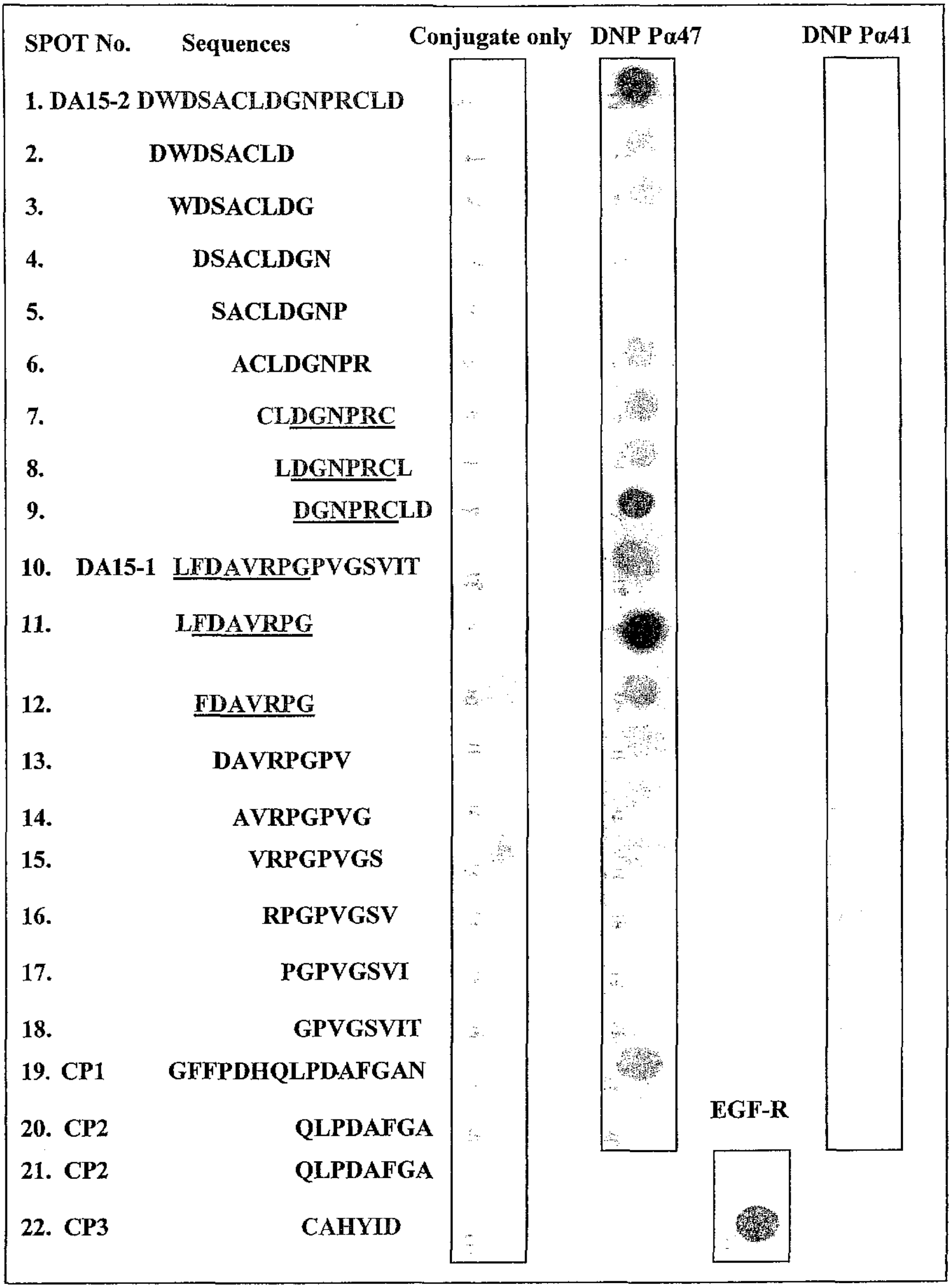


Figure 6.1. Reactivity of the peptides DA15-1 and DA15-2 with the mAbs by a SPOT scan assay

The peptides (DA15-1 and DA15-2) and 8-mer overlapping peptides derived from these peptides (1-18) and unrelated control peptides (19-22) were synthesised on SPOT membrane and their reactivity with the mAbs was tested by the SPOT scan assay. Antibody bound peptides on SPOT membrane gave blue colour reaction. Reactive peptide sequences were shown in red and the common amino acids within these reactive peptides were underlined. CP= control peptide

Table 6.1. Reactivity of Peptides DA15-1 and DA15-2with the mAbs by a SPOT scan assay

SPOT No.	Mimotope sequences	Reactivity as DYE UNIT (DU)			
		Conjugate only	DNP Pα47/ EGF-R		DNP Pα41
1.	DA15-2 DWDSACLDGNPRCLD	22	77		24
2.	DWDSACLD	24	38		25
3.	WDSACLDG	22	40		26
4.	DSACLDGN	22	22		20
5.	SACLDGNP	22	24		20
6.	ACLDGNPR	20	44		20
7.	CLDGNPRC	21	51		22
8.	LDGNPRCL	22	46		19
9.	DGNPRCLD	23	79		18
10.	DA15-1 LFDAVRPGPVGSVIT	24	54		20
11.	LFDAVRPG	23	87		21
12.	FDAVRPGP	28	49		18
13.	DAVRPGPV	21	35		19
14.	AVRPGPVG	21	29		19
15.	VRPGPVGS	40	33		18
16.	RPGPVGSV	18	24		18
17.	PGPVGSVI	18	22		17
18.	GPVGSVIT	21	26		18
19.	CP1 GFFPDHQLPDAFGAN	22	59		15
20.	CP2 QLPDAFGA	27	27		15
21.	CP1 QLPDAFGA	25	ND	24	ND
22.	CP3 CAHYID	26	ND	76	ND

The reactivity of a peptide is considered as positive if the DU value was at least two standard deviation (SD) above the mean DU value of 22 peptides reacted only with conjugate. The mean DU value of 22 peptides with conjugate control was ± 1.5 SD 23. The cut off for specific binding with mAbs was two times above the mean value (46 DU).
ND= Not determined
CP= Control peptide

6.4. Discussion

In this chapter, the SPOT scan assay was used to map the most antigenic 8-mer regions of two potential 15-mer DNP mimotopes of DA15-1 LFDAVRPGPVGSVIT and DA15-2 DWDSACLDGNPRCLD that were selected by the low affinity mAb DNP Pα47 (chapter 5). Both 15-mer peptides reacted specifically with the mAb DNP Pα47 when presented as solid phase peptides on the membrane in the SPOT scan assay **(Figure 6.1 and Table 6.1)**.

The lack of cross-reactivity between mimotopes and the high affinity mAb DNP Pα41 is suggestive for the unique feature of the mimotopes selected by the low affinity mAb **(Figure 6.1 and Table 6.1)**. This was not surprising since two 15-mer mimotopes DA15-1 and DA15-2 displayed on phage did not give a cross reactivity with the same mAb in ELISA either (see chapter 5, section 5.3.5).

The most antigenic 8-mer regions of the peptides DA15-1 and DA15-2 are corresponded to the amino terminal part of DA15-1 **LFDAVRPG**PVGSVIT and carboxyl terminal part of DA15-2 DWDSACL**DGNPRCLD** **(Table 6.1 and Figure 6.1)**. The reactivity obtained with these peptides as DU values was the highest as compared to the other 8-mer reactive peptides **(Table 6.1)**. This indicated that all amino acids within these regions are essential for optimal binding to the mAb DNP Pα47. In addition, this also confirms the fact that in epitopes the amino acid residues in contact with the binding sites (paratopes) of antibodies usually are consisted of 8-12 amino acids (van Regenmortel, 1998).

The location of the most antigenic regions of these potential mimotopes was also important for suggesting how the flexible peptide presentation on phage might mimic non-protein substances such as, the DNP. Most importantly, the location of the most antigenic 8-mer regions within the amino terminal part of the DA15-1 and carboxyl terminal part of the DA15-2 confirmed the flexibility of both ends of random peptides displayed on phage for adopting conformational epitopes.

The most important consequence of this study was to highlight the relevance of combining both combinatorial epitope library approach and the SPOT scan assay for selecting mimotopes of a non-protein substance, DNP, for the first time.

The other potential mimotope peptides selected by 6, 10 and 17-mer phage libraries should also be synthesised on SPOT's mebrane and their most antigenic regions should be searched in the SPOT scan assay. This would be a comparison of the amino acid composition of the most antigenic regions of peptides selected by different phage libraries with the low affinity mAb to DNP. A plan for this experiment was not completed due to the lack of the time. This experiment is suggested for future work (see chapter 8, section 8.3.5).

Earlier, the SPOT scan assay had been used to confirm binding specificity of potential mimotope peptides selected by using a 15-mer phage-display and an 8-mer solid phase library with BVDV neutralising antibodies (Wb166 and Wb214) (see chapter 3, section 3.3.9). Because of the inconclusive results, peptides selected with these mAbs were not considered as mimotopes.

In the next chapter, the SPOT scan assay was used to confirm the binding specificity of potential 8-mer mimotope peptides of BVDV E2 selected from an 8-mer solid phase library with another neutralising BVDV mAb157.

Chapter 7. The selection of BVDV mimotopes using an extended range of combinatorial libraries

7.1. Introduction

A study carried out by Deregt *et al.*, (1998) suggested that an E2 specific neutralising mAb157 was directed to a unique discontinuous BVDV-1 genotype specific epitope and neutralised a range of strains within BVDV-1 (see chapter 1, section 1.5). The characterisation of the epitope recognised by the mAb using combinatorial peptide libraries would facilitate to design a mimotope-based vaccine against BVDV-1, which would also be beneficial for future work with BVDV-2.

In this chapter, an extended range of two linear pIII-based (a 6 mer and a 15-mer) and two constrained pVIII-based (a 10-mer and a 17-mer) types of phage libraries and an 8-mer solid phase library were used to select mimotopes of BVDV. In parallel, the two mAbs to DNP (a low affinity DNP Pα47 and a high affinity DNP Pα41) were used to select mimotopes by using the same phage libraries side by side with BVDV mAb 157. The purpose of using DNP mAbs was to compare the effect of antibody affinity with regard to BVDV mAb157 and applicability of mAbs for mimotope selection using a simple DNP system as compared to BVDV epitopes.

Biopanning of phage libraries with the neutralising BVDV mAb157 and DNP mAbs gave enrichment of binding clones for only the low affinity mAb to DNP. The subsequent rounds of biopanning were carried out with the low affinity DNP mAb and results were presented in chapter 5. The use of an 8-mer solid phase library with BVDV mAb 157 selected potential mimotope peptides and their binding specificity was confirmed by a SPOT scan assay.

7.2. Materials and Methods

7.2.1. Monoclonal antibodies

The E2 specific neutralising BVDV mAb 157 (ascitic fluid) (Deregt *et al.*, 1998a) used in this study was described in Chapter 2 (section 2.1.1). The low affinity DNP Pα47 and the high affinity DNP Pα41 mAbs (Stanley *et al.*, 1983) were described in Chapter 5 (section 5.2.1). The mab c-erb-1/EGF-R to EGF-R described in chapter 3 (section 3.2.13) and the mAb U23 to fusion protein of Newcastle disease virus (Alexander *et al.*, 1997) were used as controls.

7.2.2. Combinatorial peptide libraries

The same phage libraries used in chapter 5 (section 5.2.2) were also used in this chapter. In addition, an 8-mer solid phase library was also used earlier in the thesis (see chapter 3, section 3.2.2 and 3.3.8).

7.2.3. Selection trials of BVDV E2 mimotopes using a range of phage-display and solid phase libraries

7.2.3.1. Biopanning of pIII-based linear and pVIII-based constained phage libraries with BVDV mAb157

Approximately $2X10^{10}$ TU/ml phage clones were incubated with beads, which had been coated with a 1:100 dilution of ascitic fluid containing BVDV mAb157. Three rounds of biopanning were carried out to select binding ligands to the mAb 157 as described in the General Materials and Methods (Chapter 2, section 2.5.1). Screening of the same phage libraries with DNP mAbs was carried out side by side with the BVDV mAb as described in chapter 5 (section 5.2.4).

7.2.3.2. Screening of an 8-mer solid phase library with the mAb 157 to select BVDV mimotopes

A portion of 8-mer solid phase library (0.05 g) was screened with the mAb 157 as described in General Materials and Methods (Chapter 2, Section 2.12 and 2.12.1).

7.2.4. Reactivity of peptides selected by screening an 8-mer solid phase library with the mAb157 by a SPOT scan assay

Synthesising peptides on SPOT's membrane

Peptides selected by screening an 8-mer solid phase library with the BVDV mAb 157 were synthesised on SPOT's derivatised cellulose membrane (see chapter 2, section 2.11.1 and 2.11.2). The same control peptides and control mAb used earlier (see chapter 3, section 3.2.13 and chapter 6, section 6.2.1) were used again namely, positive control peptide CP2 (CAHYID) from EGF-R and an irrelevant peptide CP1 (QLDPAFGA) from HBV (D'Mello *et al.*, 1997). The membrane was used on three occasions.

Conjugate control assay

The reactivity of all peptides (No: 1-7) was first tested with the sheep anti-mouse β-galactosidase conjugate. Following conjugate control assay, the SPOT membrane was regenerated for testing mAbs

The mAbs diluted in 1:100 and incubated with the SPOT membrane overnight. Antibody binding peptides were detected by addition of the conjugate followed by the subsequent development with the substrate (see chapter 2, section 2.11.3 for detail).

Testing the reactivity of peptides with the BVDV mAb 157 and control peptides with mAb EGF-R

The reactivity of peptides (No: 1-5) was tested with the mAb BVDV 157 and control peptides (No: 6-7) with the control mAb EGF-R (cerb-1) at the same day using the same batch of reagents.

Testing the reactivity of peptides with NDV mAb U23

After regenerating the membrane, peptides (No: 1-7) were tested with the mAb NDV U23.

Assesment of the reactivity of peptides

The colour intensity of reactive peptides was semi-quantified by using the Adobe PhotoShop program (see chapter 6, section 6.2.3 for detail). The reactivity of a peptide is considered as positive if the DU of the peptide was above twice the mean value of all peptides plus two SD obtained with only conjugate control. This vas found as 30 DU and taken as cut off. Values more than 30 DU considered as positive reactivity.

7.3. Results

7.3.1. Selection trials of BVDV mimotopes using a range of phage display and solid phase libraries

7.3.1.1. Biopanning of pIII-based linear and pVIII-based constained phage libraries with BVDV mAb157

Linear phage libraries (a 15-mer and a 6-mer)

A 15-mer phage library was biopanned with BVDV E2 mAb 157 and the DNP specific mAb DNP Pα47 in order to select binding ligands. Bound phage was recovered for both mAbs in the first round. However, enrichment of the bound phage clones was not observed with the BVDV mAb 157 during the second round of biopanning experiment and the yield was zero **(Table 7.1a)**. The ratio between yields observed between 2nd and 1st round of biopanning with the mAb DNP Pα47 was 200 suggesting an enrichment of bound phage clones to the mAb. The same library was used to select binding ligands of the high affinity mAb DNP Pα41 in a different experiment. However, it was not possible to select any bound phage clones (see chapter 5, section 5.3.2).

The same results were also observed with a 6-mer phage library. No phage clones were recovered and amplified with the BVDV mAb 157 and the mAb DNP Pα41 in the second round of biopanning **(Table 7.1b)**. Bound phage was recovered and successfully amplified for the low affinity mAb DNP Pα47 in the second round. The ratio between yields observed between 2nd and 1st round of biopanning with the mAb DNP Pα47 was 432 indicating an enrichment of bound phage clones. The final round of biopanning with the mAb DNP Pα47 was carried out successfully and the selected phage clones were tested for their mimotope potential for DNP (Chapter 5, Section 5.3.2).

It can be seen from the data presented above that the experimental protocol used with the mAb DNP Pα47 always gave a reproducible outcome comparing to the mAb157 and the high affinity mAb DNP Pα41.

Table 7.1 (a, b). Biopanning of pIII-based (a 15-mer and a 6-mer) linear phage libraries with BVDV mAb 157 and DNP mAbs

a) 15-mer library

		BVDV 157			DNP Pα47		
Round	**Incubation time**	**Input (I) TU**	**Output (A) TU**	**%Yield**	**Input (I) TU**	**Output (A) TU**	**% Yield**
1st	Overnight	4×10^8	1×10^3	0.00025	4×10^8	1.8×10^3	0.00045
2nd	4 hours	1×10^8	0	0 (NR)	1×10^8	9×10^4	0.09

b) 6-mer library

Monoclonal antibody	**Round**	**Incubation time**	**Input (I)TU**	**Output (A)TU**	**% Yield**
DNP Pα47	1st	Overnight	$8X10^8$	$1.5X10^3$	0.0001875
	2nd	4 hours	$3.7X10^8$	$3X10^4$	0.0081
BVDV 157	1st	Overnight	$8X10^8$	$9X10^2$	0.001125
	2nd	4 hours	$2X10^7$	0 (NR)	0 (NR)
DNP Pα41	1st	Overnight	$8X10^8$	$1.25X10^3$	0.0001563
	2nd	4 hours	$2X10^7$	0 (NR)	0 (NR)

Yields were obtained by biopanning of pIII-based a) 15-mer b) 6-mer phage libraries with the DNP mAbs (DNP Pα47 and DNP Pα41) and BVDV mAb 157. The percentage of yields for two rounds was determined by dividing the number of eluted phage (output) by the number of input phage times 100. Increases in % of yield with the low affinity DNP mAb DNP Pα47 between rounds 1st and 2nd were 200 for the 15-mer library and 432 for the 6-mer library.

Constrained phage libraries (a 17 mer and a 10-mer)

A 17-mer constrained phage library (X15CX) was used to select phage clones bound to BVDV mAb 157 and DNP mAbs (a low affinity mAb DNP Pα47 and a high affinity mAb DNP Pα41) respectively. Phage clones were recovered and amplified successfully for each mAbs in the first round and amplified eluates were subjected for the second round. However, it was not possible to recover and amplify any phage clones with the BVDV mAb 157 and the high affinity DNP. Phage clones were recovered and amplified using the low affinity mAb to DNP **(Table 7.2a)**. The difference of the yield between second and first round of biopanning with the DNP Pα47 was 1132, indicative of the successive amplification of the bound clones.

A 10-mer constrained phage library (XCX6CX) was also used to select binding ligands of the BVDV mAb157 and the mAb DNP Pα47. Because of insufficient amount of the DNP Pα41, only the low affinity DNP Pα47 mAb was used to select binding ligands. It was again not possible to recover and amplify any phage clones with the BVDV mAb 157 in the second round **(Table 7.2b)**. The ratio between yields observed between 2nd and 1st round of biopanning with the mAb DNP Pα47 was 25 indicating an enrichment of bound phage clones.

The final round of biopanning of 17-mer and 10-mer phage-display libraries was successfully carried out with the mAb DNP Pα47. The outcomes of this work were presented in chapter 5 (section 5.3.2).

Table 7.2 (a, b). Biopanning of pVIII-based (a 17-mer and a 10-mer) constrained phage-display library with BVDV mAb157 and DNP mAbs

a) 17-mer library

Monoclonal antibody	Incubation time	Input (I) TU	Output (A) TU	% Yield
BVDV 157	Overnight	5×10^8	3.3×10^2	0.000066
	4 hours	1×10^7	0	0 (NR)
DNP Pα41	Overnight	5×10^8	3×10^2	0.00006
	4 hours	4×10^7	0	0 (NR)
DNP Pα47	Overnight	5×10^8	7.95×10^3	0.00159
	4 hours	2.7×10^7	3.6×10^5	1.8

b) 10-mer library

		BVDV 157			DNP Pα47		
Rounds	**Incubation time**	**Input (I) TU**	**Output (A) TU**	**%Yield**	**Input (I) TU**	**Output (A) TU**	**%Yield**
1st	Overnight	9.6×10^8	6.5×10^2	0.000067	9.6×10^8	7.35×10^4	0.0076
2nd	4 hours	1.7×10^8	0	0 (NR)	$7X10^7$	1.35×10^5	0.192

Yields were obtained by biopanning of pVIII-based a) 17 mer b) 10-mer phage libraries with the DNP mAbs (DNP Pα47 and DNP Pα41) and BVDV mAb 157. Increases in % of yield with the low affinity mAb DNP Pα47 between rounds 1st and 2nd were 1132 for the 17-mer library and 25 for the 10-mer library.

7.3.1.2. Screening of an 8-mer solid phase library with the mAb 157 to select BVDV mimotopes

An 8-mer solid phase epitope library was screened with the mAb157 to select binding ligands. A countable number (~10) of reactive peptides with the mAb were selected at a dilution of 1:100. The intensity of brown colour development was taken as an indicator of the strength of the reactivity between peptides and the mAb 157 in the presence of the vast majority of remaining colourless non-reactive beads. The darkest four coloured beads were collected for sequencing **(Figure 7.1)**.

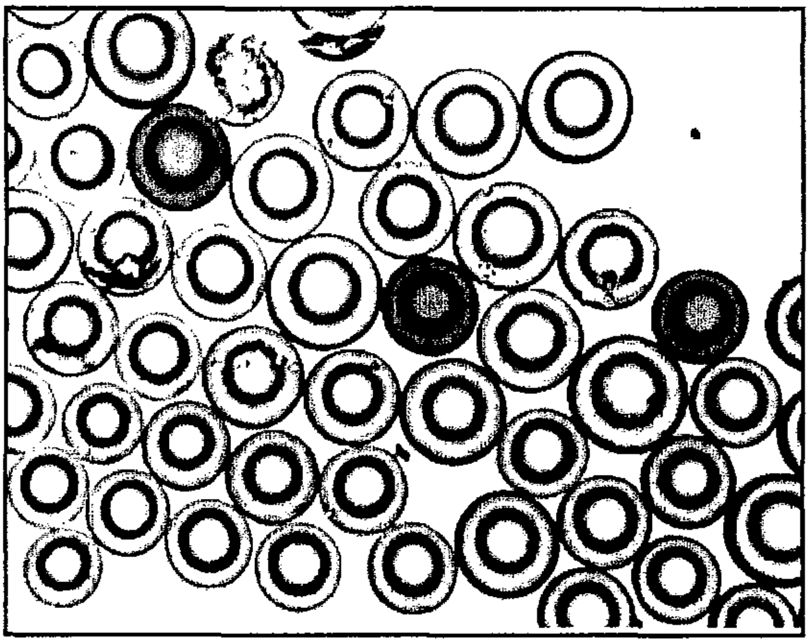


Figure 7.1. Photomicrograph of solid phase library screened with mAb157

The BVDV mAb 157 reactive beads are seen as stained dark brown beads among the nonreactive colourless beads after the addition of peroxidase-labelled rabbit-anti-mouse conjugate followed by staining with the substrate.

7.3.1.3. Sequences of the peptides and their comparison with the BVDV E2

Amino acid sequence analysis of the peptides selected by the E2 specific neutralising mAb 157 revealed the following sequences: LHEQYYYF, LYRFGEFD, IKDXX, and SXDKXX where X was indicative of unknown residues **(Table 7.3)**. This either might be due to of physical damage during storage of beads or incompletely deprotected residues resulting in low-level sequence (Dr. Joe Gray, Molecular Biology Unit (MBU), University of Newcastle upon Tyne, personal communication). These peptides are designated as 157A1, 157A2, 157A3 and 157A4. It can be seen from the sequences that they are rich in charged and aromatic amino acid residues. The amino acids L, Y, F, and E are common in 157 A1 and 157A2 sequences.

These sequences were compared with the primary amino acid sequence of the E2 of BVDV NADL (Collett *et al.*, 1988) that corresponds to 693-1193 amino acids by eye examination **(Table 7.3)**. This analysis identified several domains in which some amino acids of the peptides matched with parts within the E2 sequence. The peptide 157A2 LYRFGEFD matched with the domains (748 LQRCTRET 755, 848 YRRSKPFP 855 and 888 LYKGGSIE 895. First two amino acids L, H matched with the domain 761 LHTRALPT 769 and the 157A1 LHEQYYYF peptide sequence. The peptide 157A3 IKD matched with the domain 704 IAKDERIG 711. Three common amino acids were found between the 157A4 peptide SADK and the same domain. The summary of results is illustrated in Table 7.3.

The similarity of the two 8-mer peptides with the E2 of BVDV NADL was also compared with proasd program as described earlier in (chapter 3, section 3.3.3). The lowest ASD score (55.94) was observed with the peptide 157A1 LHEQYYYF and a region on E2 of BVDV LKGEYQYW corresponding to the amino acids 1012-1019 that is located within the carboxyl part of the E2.

The lowest ASD score (48.35) observed for 157A2 peptide sequence LYRFGEFD was located within the amino terminal part of the E2 that was corresponding to a region LYKGGSIE (888-895 amino acids).

Table 7.3. Comparison of amino acid sequences of peptides with the E2 of BVDV NADL

Peptide sequences	Sequence comparison with BVDV E2
157A1: L H E Q Y Y Y F	761 769 **L H** T R A L P T **L H** E Q Y Y Y F
157A2: L Y R F G E FD	748 755 **L** Q **R** C T R **E** T **L** Y **R** F G **E** F D 848 855 **Y R** R S K P **F** P L **Y R** F G E **F** D 888 895 **L Y** K G **G** S I **E** **L Y** R F **G E** F D
157A3: I K D	704 711 **I** A **K D** E R I G **I K D**
157A4: SADK	704 711 I **A K D** E R I G S **A D K**

Peptide sequences were compared with the primary amino acid sequence of the E2 region of BVDV NADL. Amino acids matching with the E2 region of the BVDV are shown in bold and underlined.

7.3.1.4. Reactivity of peptides with the mAb 157 by a SPOT scan assay

In order to confirm binding specificity of peptides (157A1, 157A2, 157A3, and 157A4) with the mAb157, their reactivity with the selector mAb was tested in a SPOT scan assay.

Conjugate control assay

In order to correct for any non-specific binding that might arise from the reactivity of the peptides with the secondary antibody (conjugate); peptides were first tested in the absence of primary antibody. Non-specific reactivity was not obtained any of the peptides **(Figure 7.2).**

Assay control

The positive control peptide CP2 CAHYID (No. 7) representing an epitope of EGF-R and the mAb EGR-R specific to this epitope showed a strong reactivity (70 DU) indicating a successful SPOT peptide synthesis and assay procedure. No non-specific reactivity was observed with an irrelevant control peptide CP1 (QLDPAFGA) representing an epitope of HBV (D'Mello *et al.*, 1997) **(Figure 7.2).**

Testing the reactivity of peptides with BVDV mAb 157

Upon reacting the mAb 157 (1:100 dilution) with the peptides, the reactivity was observed with SPOT peptides No. (1) (157A1 LHEQYYYF) (57 DU), (2) (157A2 LYRFGEFD) (36 DU) **(Figure 7.2).** The reactivity observed with the SPOT peptide 157A1 LHEQYYYF was nearly two-fold greater than the peptide 157A2 LYRFGEFD. No reactivity was observed with uncompleted peptides 157A3 IKD and 157A4 SADK. In addition, non-specific binding was not observed with the irrelevant control peptide CP1 QLDPAFG (No. 5)..

Testing the reactivity of peptides with mAb U23

An un-related neutralising mAb U23 specific for the F protein of the NDV (Alexander *et al.*, 1997) was used as irrelevant control mAb. No reactivity was observed with any of the peptides with the mAb U23 **(Figure 7.2).**

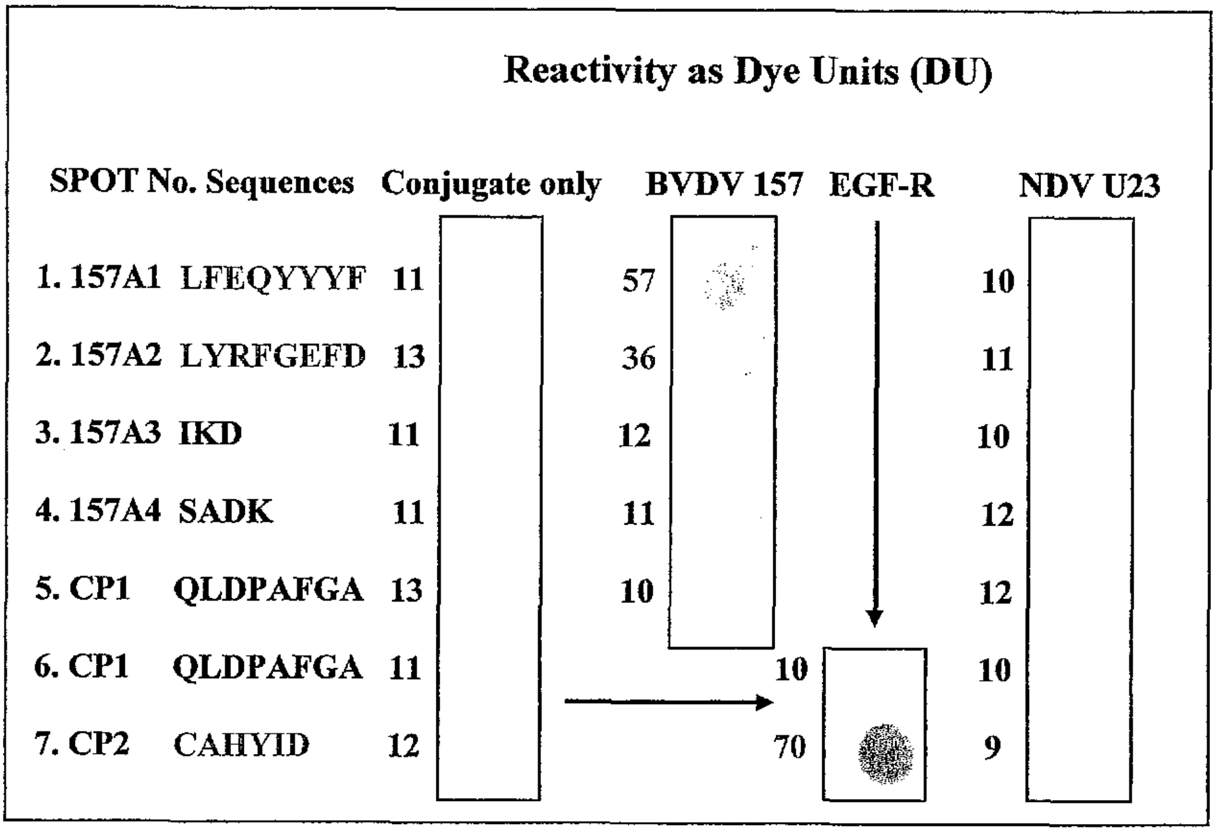


Figure 7.2. SPOT scan assay of peptides with mAbs (157, EGF-R and U23)

The reactivity of peptides was first tested with the sheep anti-mouse β-galactosidase conjugate and then with mAbs 157, U23 and c-erb-1/EGF-R by a SPOT scan assay. Reactive peptides were shown as blue SPOTs. After reaction of mAb with peptides the membrane was regenerated. The results were expressed as dye units (DU) representing colour intensity of reactive peptides. The reactivity of a peptide is considered as positive if the DU value was above twice the mean value plus 2 SD obtained in conjugate control assay. The mean DU value plus 2SD was 15 and twice above this value was 30 DU. Values more than 30 were taken as positive reactivity. The reactive peptides and their DU values were shown in red.

7.4. Discussion

This chapter describes the use of a range of phage and an 8-mer solid phase epitope libraries to select mimotopes mimicking the neutralising epitope on BVDV E2 recognised by the mAb157. In addition, the effect of antibody affinity and phage-display library application was tested by using a low and a high affinity DNP mAbs in parallel with the BVDV mAb 157.

7.4.1. Biopanning of phage libraries with BVDV mAb157 and DNP mAbs

It is believed that epitopes are usually six amino acid long and that all theoretically possible 64 million amino acid epitopes would be represented many times in a 6-mer epitope library (Parmley and Smith, 1988; Sparks *et al.*, 1996). However, by using a 6 and 15-mer pIII-based linear phage libraries, it was not possible to amplify any specifically bound phage clones to the BVDV mAb 157 and the high affinity DNP mAb in the second round. However, both libraries gave bound phage clones with the low affinity DNP mAb.

The random peptides displayed on these linear phage libraries are presumably very flexible. In one sense, this may be considered an advantage in that a single peptide sequence effectively represents a multitude of molecular shapes or conformations. However, this multiplicity of molecular conformation may also result in a weakening of affinity for any given ligate such as a mAb, which will only bind a very limited subset of the conformations available to a linear peptide. This will be critically important in the case of the discontinuous or conformation dependent epitopes that may not be mimicked by linear peptides (Smith and Petrenko, 1997; Irwing *et al.*, 2001).

In order to overcome to this problem, phage libraries where random peptides displayed as constrained forms as a result of terminal cysteine (cys) residue(s) flanking each of the random peptides have been constructed and used to identify ligands for a wide variety of ligates (Scott and Craig, 1994; Bonnycastle *et al.*, 1996). Bonnycastle *et al.*, (1996) suggested that the use of a range of libraries could enhance to identify binding ligands to any given ligate (see chapter 1, section 1.7.4.2). Therefore, a 17-mer and a 10-mer constrained type of phage libraries were used to select binding ligands to the mAb 157.

However, it was not possible to recover and amplify any bound phage clones to the mAb157 in the second round **(Table 7.2a, b).** In addition, biopanning of a 17-mer constrained type of phage library with the high affinity DNP mAb DNP Pα41 did not produce any bound phage clones either **(Table 7.2a).**

It was interesting to see that the first round of biopanning gave results with both BVDV mAb157 and the two DNP mAbs and phage libraries. However, in the second round it was not possible to recover and amplify phage clones selected by mAb 157 and the high affinity mAb DNP Pα41. The outcome with the low affinity mAb DNP Pα47 was consistently successful by using exactly same reagents and same conditions **(Table 7.2a, b).** From these results, it can be assumed that there are a number of limitations for the usage of phage libraries for identifying discontinuous epitopes and selecting ligands from high affinity mAbs. These limitations can be categorised according to biological limitations associated with phage libraries and the nature of epitopes recognised by the BVDV E2 mAbs (see chapter 3, section 3.4 for detail).

The neutralising BVDV mAb 157 was suggested as recognising a discontinuous epitope located at the amino terminal part of the E2 of BVDV (Deregt *et al.*, 1998a). In some cases, peptides representing parts of the discontinuous epitopes containing critical binding residues may react with mAbs (van Regenmortel, 1996; 2001). However, in the case of BVDV E2 neutralising mAbs tested in this study, it was not possible to select binding ligands to them by phage libraries. In addition, antibody affinity may also be a limiting factor for phage library selection process since high affinity binding between an antigen and antibody requires a very specific interaction (Janeway *et al.,* 2005). Especially, the consistent positive outcome obtained with the low affinity DNP mAb with each phage library and no results with the high affinity DNP mAb strengthen this possibility.

Another critical factor is that E2 is a glycosylated viral protein (Brock, 1995) and the glycosylation process could probably be critical factor for its antigenic and immunogenic properties. For instance, the recognition of a broadly neutralising mAb 2G12 against an epitope on gp120 surface glycoprotein of HIV-1 depended on glycosylation (Trkola *et al.*, 1996). Thus, a linear peptide would not reproduce the epitope.

7.4.2. Screening of a solid phase library with BVDV mAb

An 8-mer solid phase epitope library was therefore considered to be an alternative approach for identifying binding ligands to the BVDV E2 mAb 157. The presentation of random peptides in high density on the beads in solid phase library and the simplicity of the selection procedure make it an attractive alternative way for identifying ligands to ligates (Lam *et al.*, 1996). Screening an 8-mer solid phase library with the mAb 157 gave four binding peptides (157A1, 157A2, 157A3 and 157A4) **(Table 7.3)**. However, the comparison of sequences of these peptides with the BVDV E2 by eye examination and proasd program did now show a high similarity between the peptides and BVDV E2. This was not surprising since peptides representing discontinuous epitopes do not have to show similarity to the primary amino acid of the antigenic protein (Steward *et al.*, Chargelegue *et al.*, 1998).

7.4.3. SPOT scan assay to test the reactivity of peptides

Because of the similarity of presentation and accessibility of peptides on SPOT's cellulose membrane, the SPOT scan assay was decided to be an appropriate method to confirm the binding specificity between peptides (potential mimotopes) and the mAb 157. The reactivity of peptides 157A1 LHEQYYYF (57 DU) and 157A2 LYRFGEFD (36 DU) with the mAb 157 was indeed confirmed in the SPOT scan assay **(Figure 7.2).** The reactivity obtained with the peptide 157A1 was nearly two-fold greater than that of the peptide 157A2, indicative of the former is better fit and more complementary for the antigen binding site of the mAb 157. It can be seen from the sequences that they are rich of charged and aromatic amino acid residues. The amino acids L, Y, F, and E are common in 157A1 and 157A2 sequences. This could be further indicative for the importance of these amino acids for forming an epitope structure (Getzoff *et al.*, 1987; Appel *et al.*, 1990).

The promising results obtained with the use of an 8-mer solid phase library with the mAb157 are likely related to several factors. Firstly, the use of the solid phase library is much simpler than phage library application and this could avoid biological limitations of phage library selection procedure. Secondly, the presentation of peptides on solid phase with higher density could make peptides appropriately accessible to paratope region of the mAb157. Another, probably the most important factor could be the presence of binding ligands for the mAb 157 in the portion of the solid phase library. This highlights the usefulness of using a range of phage display and solid phase epitope libraries for selecting binding peptides.

7.4.4. Future studies

The next stage should be synthesising the reactive peptides (157A1 and 157A2) preferably as MAP form and testing them as antigenic and immunogenic mimics of the epitope recognised by the mAb157. These experiments were suggested for the future work on mimotope vaccines against BVDV and would parallel past work on MV and RSV (Steward *et al.*, 1995; Chargelegue *et al.*, 1998).

Chapter 8. General Discussion

The original aim of this study was to identify neutralising epitopes on BVDV E2 for the development of a mimotope-based vaccine against BVDV. In order to achieve this, combinatorial peptide libraries were used with neutralising BVDV mAbs to select vaccinal mimotopes. It was soon realised that the type of epitopes and antibody affinity could be problematic for successful mimotope selection. To test the effect of antibody affinity on mimotope selection, the DNP hapten specific mAbs with known affinity and another neutralising BVDV mAb were used with an extended range of libraries.

In this general discussion chapter, the outcomes of this study will be discussed in following order:

8.1. The influence of epitopes and antibody affinity on the selection of mimotopes of BVDV and DNP using combinatorial peptide libraries

8.1.1. The mimotope selection of DNP compared to BVDV by using a range of phage libraries

8.1.2. The effect of antibody affinity for mimotope selection of BVDV and DNP

8.1.3. The difficulty of making mimotopes from discontinuous epitopes of BVDV and other viruses

8.2. The reasons for the success of the 8-mer solid phase library and the SPOT scan assay for selecting potential BVDV mimotopes

8.3. Future studies involving mimotopes of BVDV and DNP

8.3.1. MAP peptides as antigenic and immunogenic mimics of BVDV

8.3.2. Additional phage libraries for biopanning of mAbs to BVDV

8.3.3. Gene-fragment libraries for mapping epitopes with mimotope potential of BVDV

8.3.4. The use of additional DNP mAbs for selecting mimotopes

8.3.5. Testing the immunogenicity of DNP mimotopes

8.3.6. SPOT scan assay to improve DNP mimotopes

8.4. Final statements of the thesis

8.1. The influence of epitopes and antibody affinity on selection of mimotopes of BVDV and DNP using combinatorial peptide libraries

8.1.1. The mimotope selection of DNP compared to BVDV by using a range of phage libraries

In the first part of this study, four peptides were selected by biopanning of pIII-based linear a 15-mer phage library with the neutralising BVDV E2 mAb Wb166. However, the selected peptides could not be shown to be mimotopes by standard assays (see chapter 3, section 3.3.6 and 3.3.7). In addition, an 8-mer solid phase library was screened with BVDV E2 mAbs Wb166 and Wb214 to select mimotopes of BVDV. The immunisation trial of the first peptide (Th-Wb214-P1) selected by screening an 8-mer solid phase library with the mAb Wb214 failed to induce anti-BVDV antibodies in mice (see chapter 3, section 3.3.7.2, 3.3.7.3). Since the specific reactivity of all selected peptides was not confirmed by a SPOT scan assay (chapter 3, section 3.3.9), no more peptides were used for immunisation.

The failure to select BVDV mimotopes was first considered to due to the discontinuous nature of epitopes recognised by neutralising BVDV mAbs. The testing relative affinity of neutralising BVDV mAbs revealed that the mAb Wb166 had a relative affinity higher than other neutralising mAbs (see chapter 4, section 4.3.4). Accordingly, it was also considered that antibody affinity could also be a limiting factor for mimotope selection. Therefore, it was decided to refine the applicability of phage library technology by using a low and a high affinity DNP hapten mAbs. In addition, a new neutralising mAb157 directed to a discontinuous epitope on BVDV E2 (Deregt *et al.*, 1998a) was also used in parallel with DNP mAbs by using an extended range of linear and constrained phage libraries (see chapter 7).

The trials of selecting binding ligands to BVDV E2 mAb 157 were not successful by using pIII-based linear (a 15-mer and a 6-mer) phage libraries (see chapter 7, section 7.3.1.1). The use of low affinity DNP mAb led to the selection of binding ligands of the DNP when using the same libraries. However, the selected ligands did not give a cross reactivity with the high affinity DNP mAb. Furthermore, the high affinity DNP mAb failed to select binding ligands from the same libraries (see chapter 5, section 5.3.2).

The presentation of random peptides on phage is the critical factor for the successful selection of binding ligands to ligates. Presumably, if the presentation of peptides on phage does not resemble the natural three-dimensional structure of the native antigen, it may not be possible to select peptide mimics (mimotopes) of the discontinuous epitopes of natural ligand (eg. a viral antigen) (Rodi and Makowski, 1999; Smith and Petrenko, 1997; Irwing *et al.*, 2001).

Bonnycastle *et al.*, (1996) studied the factors enhancing a successive application of phage libraries for identifying binding ligands to any given ligate using a large range of phage libraries with a range of mAbs and polyclonal antibodies recognising peptides, folded proteins, carbohydrates and linear and discontinuous epitopes. Their study highlighted the critical importance of using a large range of phage libraries including linear and constrained types for selecting tight binding ligands of ligates.

Following the advice of Professor Jamie Scott who was the corresponding author on the above paper (Bonncastle *et al.*, 1996), it was decided to try constrained phage libraries in order to enhance the chance for selecting mimotopes of BVDV. Phage libraries in which random peptides are displayed as constrained forms by having fixed cysteine (cys) residues within the random amino acid sequences have been used for identifying binding ligands for variety of ligates (Bonnycastle *et al.*, 1996). The neutralising BVDV E2 mAb 157 was then tested with two constrained phage libraries. However, it was not possible to select binding ligands of the mAb157. In parallel work with the DNP mAbs, the binding ligands were selected with a low affinity DNP mAb from the constrained libraries (see chapter 7, section 7.3.1.1). It was also not possible to select binding ligands recognised by a high affinity DNP mAb.

8.1.2. The effect of antibody affinity for mimotope selection of BVDV and DNP

It is known that a mAb specific for a particular epitope always retains the capability of recognising related structures on antigens that are considered as potentially cross-reactive. However, a high affinity DNP mAb did not give a cross reactivity with the mimotopes identified by using a low affinity DNP mAb. More importantly, it suggests the preference and selectivity of high affinity antibodies for their specific ligands even in the case of a simple DNP hapten example. If this is the real case for antibodies directed to a simple defined chemical compounds such as haptens, it is more likely to happen with antibodies directed against viral epitopes. From these findings, it can be suggested that high antibody affinity might be a disadvantage for the selection of binding ligands as exemplified by the DNP system for two possible reasons. Firstly, the ligands for high affinity antibodies are rare and absent in the libraries. Secondly, phage clones displaying ligands for high affinity mAbs probably bind tightly and they are not recovered by acid elution step by biopanning. In order to overcome the second possibility, an 8-mer solid phase library was also used in this study.

The neutralising BVDV mAbs used in this study had high neutralising titre (chapter 4, section 4.3.1) and they are directed to the discontinuous epitopes on BVDV E2 (Paton *et al.*, 1992). The estimated relative affinity of the Wb166 that had highest neutralising titre was higher than other neutralising mAbs (chapter 4, section 4.3.4). The discontinuous nature of the epitopes they recognise and their high neutralising titre probably make more complicated for selecting binding ligands for these BVDV mAbs. This latter possibility can perhaps be tested by using non-neutralising or low affinity BVDV mAbs with phage libraries. As shown by Steward *et al.*, (1995), it was possible to select mimotopes inducing neutralising antibody by using non-neutralising mAb directed to an epitope on F protein of MV. Such non-neutralising E2 specific BVDV mAbs could similarly be used in future work.

8.1.3. The difficulty of making mimotopes from discontinuous epitopes of BVDV and other viruses

Most of the mAbs recognising discontinuous epitopes tested in the study of Bonnycastle *et al.*, (1996) either isolated weakly binding ligands or did not isolate binding ligands at all. One of the mAb used for screening was a human mAb12 that recognised the $CD4^+$ receptor for the HIV-1 envelope protein gp120 and neutralised a wide spectrum of HIV-1 primary isolates *in vitro* (Parren *et al.*, 1999). Two weakly binding ligands were selected from a 12 and a 15-mer constrained type of phage libraries sharing a five-residue consensus within the gp120 protein. Later on, Zwick *et al.*, (2001) used this consensus sequence to make two sublibraries by fixing the sequence and randomising the residues around it. The tightest binding peptide (ligand) was then selected by biopanning of a sublibrary with the mAb b12. Furthermore, the screening of an antigen fragment library derived from the gp120 gene with the mAb b12 was not successful, providing further evidence that the epitope recognised by this mAb b12 was discontinuous and complex. This example clearly demonstrates the difficulties and one of the most important limitations of phage library approach for selecting mimotopes of discontinuous epitopes, and this might be the reason for the failure to successfully identify E2 specific mimotopes using neutralising BVDV mAbs in this study.

Another relevant study was carried out by Yu *et al.*, (2000). Yu and Scott screened three *in vitro* neutralising and *in vivo* protecting mAbs (7-10A, 5B19, and 5B170) recognising linear and discontinuous epitopes of S glycoprotein of a neurotropic murine hepatitis virus (MHV-A59) using 12 different conformationally constrained and linear phage libraries. The mAb 7-10A was directed to a discontinuous epitope on the S1 subunit of MHV-A59 whereas two other mAbs, 5B19 and 5B170, recognised linear epitopes within an immunodominant region on the S2 subunit of the virus. They found that all three mAbs selected binding peptides from constrained libraries but not from the linear phage libraries. Three constrained libraries gave binding peptides with mAbs 5B170 and 7-10A and only one library gave binding peptide with the mAb 5B19. Peptides selected by mAbs 5B19 and 5B170 showed consensus sequences that shared homology with S2 subunit of the S glycoprotein since they were directed against linear epitopes. The critical binding residues of these mAbs were similar since peptides selected by both mAbs were cross-reactive: peptides selected by the mAb5B170 were recognised strongly by the mAb5B19 and vice versa. Although these two mAbs were cross reactive and recognised linear epitopes, not all 12 phage libraries selected binding peptides to them.

Immunisation studies with a number of the tightest binding ligands revealed that mice immunised with a phage clone whose displayed peptide mimicked the linear epitope was protected from MHV-A59 induced lethal encephalitis However, binding peptides identified with the mAb 7-10A did not induce protective immune responses in mice, suggesting peptides identified by this mAb failed to mimic the complete discontinuous epitope.

It can be suggested from Yu and Scott's study (Yu *et al.*, 200), the use of a large number of phage libraries (e.g 12) is very critical to select binding ligands (mimotopes) from mAbs even directed to linear epitopes. In addition, the difficulty of appropriate mimicry of selected mimotopes with discontinuous epitopes was exemplified by the failure of inducing protective antibody against MHV-A59. In our study, it was not possible to use a large range of all available phage libraries and test our neutralising E2 specific BVDV mAbs.

It is very likely that all epitopes are discontinuous to some extent, that short peptides probably cross-react with a native antigen mimic via the part of the discontinuous epitope that is often regarded as a linear epitope (Barlow *et al.*, 1986). For this to occur, the critical binding residues (CBRs) must be located on a single part of the protein so that they can make extensive contact with antibody combining site. The locations of most linear epitopes identified by Craig *et al.*, (1998) corresponded to regions of high mobility and accessibility. Such regions tend to have high protrusion indices that allow antibody to make a critical, central contact with a continuous segment of the protein (Barlow *et al.*, 1986; Thronton *et al.*, 1986). Antibodies that bind to such regions, as well as to cross-reactive peptides, must have unique binding sites that will accommodate the epitopes on both antigens (Craig *et al.*, 1998). Thus, it can be assumed that the successful identification of binding ligands of mAbs recognising discontinuous epitopes from phage libraries may depend on their ability to bind short peptides derived from the primary amino acid sequence of the antigen as shown by Heiskanen *et al.*, (1999) using puumala hantavirus neutralising mAb. This is one of the critical aspects for BVDV neutralising mAbs that should be tested in future studies.

The majority of anti-protein antibodies that are recognised by peptides bind to linear epitopes on the protein antigens. Peptides mimicking with such epitopes have been identified using phage libraries (D'Mello *et al.*, 1997; Craig *et al.*, 1998; Yip and Ward, 1999). The binding of peptides to antibodies takes place in a groove or a cleft, and the binding site is buried deep within the Fab interface (Wilson *et al.*, 1994). However, antibody binding to the discontinuous epitopes on folded proteins usually occurs over a large, shallow surface (Davies *et al.*, 1988; Braden and Poljak, 1995). Importantly, the CBRs of discontinuous epitopes seem to be larger and spread over two or more segments of the protein as compared to linear epitopes and peptide mimics of discontinuous epitopes are likely be present in smaller numbers in the phage libraries (Irwing *et al.*, 2001). It is likely that many antibodies recognising discontinuous epitopes frequently will not often bind peptides or bind peptides weakly (Bonnycastle *et al.*, 1996). This could be due to their large, shallow binding sites that may not often allow a peptide to become buried (Wilson *et al.*, 1994). These features might be possible reasons to explain the difficulty of selecting binding peptide ligands with mAbs recognising discontinuous epitopes and their weak binding tendency for selecting mAbs (Bonnycastle *et al.*, 1996; Craig *et al.*, 1998; Zwick *et al.*, 2001).

The neutralising BVDV mAbs used in this study are suggested to recognise discontinuous epitopes (Paton *et al.,* 1992; Deregt *et al.*, 1998a). Therefore, because of above discussed reasons, they may not bind to short peptides displayed on phage and may be poor candidates for mimotope selection.

8.2. The reasons for the success of the 8-mer solid phase library and the SPOT scan assay for selecting potential BVDV mimotopes

An 8-mer solid phase library selected two potential mimotope peptides 157A1 and 157A2 of BVDV. They were bound by the selector mAb157 in a SPOT scan assay (chapter 7, section 7.3.1.2 and 7.3.1.4).

Because of the similarity of peptide presentation on both resin beads in a solid phase library and on a cellulose membrane in a SPOT scan assay, the confirmation of the reactivity of two 8-mer peptides (157A1 and 157A2) with the mAb157 in SPOT scan assay was suggestive for the selection of specifically binding ligands to the mAb157. It is very likely that the high density of the peptides presented as multiple copies on both systems was critical for successful mimotope selection from the mAb157. This result was also in accordance with the study carried out by Chargelegue *et al* (1998). They proved that a SPOT scan assay was the most appropriate way to confirm the binding of peptides selected by screening an 8-mer solid phase library with the mAb19 directed to a conformational epitope on F protein of RSV. They also improved the binding of the RSV specific mimotopes to the selector mAb in the SPOT scan assay by making amino acid substitution analysis. The improved mimotopes in MAP system induced protective neutralising antibodies to RSV in mice.

The present promising results obtained with the mAb157 confirmed and extended the study of Bonnycastle *et al.*, (1996) where they suggested the use of a large range of phage libraries with a given antibody for successful selection of binding ligands. However, their study did not include a solid phase library. The presentation of peptides on solid phase seems to be more appropriate way for the recognition of the mAb157.

Moreover, in this study, the use of a solid phase library might have solved the biological limitations associated with phage libraries earlier because the selection procedure is more straightforward and possibility antibody bound peptides do not get lost during the amplification steps as in the case of biopanning. Importantly, peptide ligands for the mAb157 were likely present in the solid phase library.

It could also be possible to improve binding of the potential mimotopes 157A1 and particularly 157A2 by using a SPOT scan assay by doing amino acid substitutions. Mimotopes could then be synthesised in MAP system and their antigenic and immunogenic mimicry for inducing neutralising antibody response to the BVDV would be tested in mice as done for RSV by Chargelegue *et al.*, (1998).

In earlier part of this study, the same 8-mer solid phase library was also screened with other high affinity neutralising BVDV mAbs (Wb166 and Wb214) to select binding ligands. Although peptides were selected with both mAbs, their reactivity was not confirmed in a SPOT scan assay (chapter 3, section 3.3.9).

8.3. Future studies involving mimotopes of BVDV and DNP

8.3.1. MAP peptides as antigenic and immunogenic mimics of BVDV

The peptides (157A1 and 157A2) selected by screening an 8-mer solid phase library with the neutralising BVDV mAb 157 could be synthesised using MAP system and tested for antigenic and immunogenic mimicry of the neutralising epitope recognised by the selector mAb. Since the reactivity of the peptides was confirmed by the SPOT scan assay, it would be useful to synthesise peptides by using MAP system as well as synthesising linear peptides for comparing their antigenicity and immunogenicity (Chargelegue *et al.*, 1998). The MAP peptides would first be tested for antigenic mimicry by inhibition assay as used earlier in the thesis for peptides selected by a 15-mer phage library (chapter 3, section 3.3.6). The MAP peptides would secondly be used to immunise mice to test for antibodies to BVDV as earlier in the thesis (chapter 3, section 3.3.10.2 and 3.3.10.3).

8.3.2 Additional phage libraries for biopanning mAbs to BVDV

The E2 specific neutralising BVDV mAbs used in this study could also be tested by using a large range of additional phage libraries as recommended by Bonnycastle *et al.*, (1996) and Yu *et al.*, (2000). This would probably increase the chance of selecting optimal binding ligands for these mAbs. In addition, non-neutralising E2 specific BVDV mAbs (Paton *et al.*, 1992; Deregt *et al.*, 1998a) should also be tested for the selection of binding ligands. In our study, it has been suggested that antibody affinity could affect the identification of binding ligands. The non-neutralising E2 specific BVDV mAbs may have binding sites that could accommodate peptides and thereby select mimotopes better than neutralising antibodies. Thus, non-neutralising BVDV mAbs should also be tested with these additional libraries.

8.3.3. Gene-fragment libraries for mapping epitopes with mimotope potential of BVDV

The gene-fragment libraries offer a good alternative to combinatorial random peptide libraries for epitope mapping studies. In contrast to phage display random peptide libraries, they display linear peptides 30 to 100 amino acids long derived from the target antigen in their native structural context (Bentley *et al.*, 2000). The long peptide fragments are considered to adopt a structural conformation better than short peptides (Fack *et al.*, 1997; Holzem *et al.*, 2001).

A gene fragment library would make from E2 and screened with a range of neutralising polyclonal and monoclonal antibodies and even with non-neutralising antibodies. This might give one or more reactive fragments that could be further analysed for the epitope of interest. This could be done synthesising reactive fragments as overlapping peptides by using SPOT scan. They can then be further tested with the antibodies to determine the localization of the epitopes. This would also show if BVDV mAbs bind short peptides or not.

8.3.4. The use of additional DNP mAbs for selecting mimotopes

In the previous study, only one low and one high affinity DNP mAbs were used to select mimotopes. To determine whether the antibody affinity is a real limiting factor for mimotope selection, the DNP mimotope study should be extended by using at least three mAbs of low and high affinity in a larger study.

8.3.5. Testing the immunogenicity of DNP mimotopes

The mimotope DA17-1 that was selected by using a 17-mer constrained phage library inhibited the binding of the selector mAb DNP Pα47 to the DNP_{237} casein-conjugate in an inhibition ELISA stronger than other DNP mimotopes, thus, it was considered the best antigenic mimic of the DNP (chapter 5, section 5.3.7.2). The next question would be seeing if the mimotope could induce antibody response cross-reactive with the DNP hapten. If so, it will prove the possibility of identifying mimotopes mimicking the both antigenicity and immunogenicity of the DNP hapten for the first time. Particularly, it would be very interesting to see that the best antigenic mimotope DA17-1 would also be best immunogenic mimotope. In addition, it would also be interesting to see the priming with the DNP mimotope(s) could induce memory response that could be boosted following secondary injection with DNP (e.g DNP_{237} casein). This is very critical aspect for the development of mimotope-based vaccines against viral pathogens and thus the use of DNP mimotopes would be a good model study.

8.3.6. SPOT scan assay to improve DNP mimotopes

It would also be interesting to determine if it is possible to make mimotopes selected by the low affinity DNP mAb that are capable of recognising high affinity DNP mAb. This would be possible to make appropriate amino acid substitution analysis by using SPOT scan technology (Chargelegue *et al.*, 1998). This could also show the preferred peptide structures recognised by either high affinity or low affinity DNP mAbs. In addition, the SPOT scan assay should be used to map the most antigenic regions of all potential DNP mimotopes selected by each phage library (e.g 6, 17, 10-mer). This would show the amino acid composition of the most reactive regions of the mimotopes.

8.4. Final statements of the thesis

This study included two new discoveries:

1) DNP mimotopes could be made using phage libraries

2) Two potential mimotopes of BVDV was identified by a solid phase library

This study concluded that a large range of epitope libraries is necessary to identify discontinuous epitopes of BVDV, particularly when using neutralising mAbs.

Chapter 9. References

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Chapter 10. Appendices

10.1. Buffers and Bacteriological Media

10.1.1. Buffers

20X phosphate buffered Saline

800g	NaCl
20g	KCl
144g	Na_2HPO_4
24g	KH_2PO_4

Make up to 5 lt in SDW, and adjust pH 7.4, before use adjust the final concentration to 1x.

Tris-Borate EDTA buffer (10x TBE)

800 mM	Tris base (BDH)
890 mM	Orthoboric acid (BDH)
2mM	EDTA (BDH)

Before use dilute to 1x in deionised water

Bioarbonate buffer

0.1 M $NaHCO_3$
0.02% NaN_3

Make 100 ml, filter sterilize and store in the fridge

Blocking solution

0.1 M $NaHCO_3$
5 mg/ml dialyzed BSA
0.1 mg/ml streptavidin
0.02% NaN_3

Make 150 ml, filter-sterilize and store in the fridge.

Elution buffer

0.1 N HCl, pH adjusted to 2.2 with glycine
1 mg/ml BSA

0.1 mg/ml phenol red is optional.

The glycine.HCl buffer is filter-sterilized, and stored at room temperature.

PEG/NaCl (16.7%/3.3 M stock)

100 g PEG 8000 (Union Carbide; used to be called PEG 6000)
116.9 g NaCl
475 ml water

Stir until solutes dissolve (may be necessary to heat to 65° briefly to dissolve the last crystals of PEG). Make total volume 600 ml, autoclave and store at + 4°C.

TBS (1 ×)

50 mM Tris.HCl pH 7.5
150 mM NaCl

Autoclave and store at room temperature.

10.1.2. Bacteriological media

LB broth

In 1 lt of SDW dissolve

10g	Bactotryptone (Difco)
5g	Yeast extract (Difco)
10g	NaCl

in 1000 ml of SDW and adjust pH 7.5 with NaOH. Autoclave and store at room temperature.

LB agar

6g	Bactoagar (Difco)
400 ml	LB broth

Autoclave and keep at room temperature

LB top agar

0.75g	Bacto-agar (Difco)
100ml	LB broth

Autoclave and keep at room temperature

Terrific broth

In 900 ml SDW dissolve:

12 g	bacto-tryptone
24 g	yeast extract
4 ml (5.04 g)	glycerol

Autoclave 90-ml portions in 125-ml polypropylene bottles. 10 ml of separately autoclaved potassium phosphate buffer (0.17 M KH_2PO_4, 0.72 M K_2HPO_4).