B cell epitopes in fish nodavirus

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by

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Declaration

I hereby declare that the work and results presented in this thesis was conducted by me at the Institute of Aquaculture, University of Stirling, Scotland. The work presented in this thesis has not been previously submitted for any other degree or qualification.

The literature consulted has been cited and where appropriated, collaborative assistance has been acknowledged.

Stirling, March 2005

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Abstract

Three epitope-mapping procedures were used to identify B-cell epitopes on Betanodaviruses: neutralisation escape mutant sequence analysis, phage display, and pepscan. Betanodaviruses have emerged as major pathogens of marine fish. These viruses are the aetiological agents of a disease referred to as viral nervous necrosis (VNN), which affects many species of fish that are economically valuable to the aquaculture industry. The identification of betanodavirus B-cell epitopes will facilitate the rational development of vaccines to counter VNN.

A panel of mouse monoclonal antibodies (MAbs) was produced using hybridoma methodology for use in each of the epitope mapping procedures. These antibodies were characterised in Western blotting, ELISA, and virus neutralisation tests. Rabbit polyclonal sera, and serum samples from nodavirus-infected fish were also used for pepscan analyses.

Attempts to produce betanodavirus neutralisation escape mutants, using plaque assay or limiting dilution based methods, were not successful.

Two phage libraries expressing random peptides of seven (Ph.D.7™) or twelve (Ph.D.12™) amino acids in length as fusions to the coat protein were used to identify the ligands recognised by MAbs directed against betanodavirus. Neither of these phage libraries yielded conclusive results. Phage clones containing tandem inserts were obtained after MAb selection from library Ph.D.7™. Extensive screening and nucleotide sequence analysis of MAb-selected clones from library Ph.D.12™) failed to yield a consensus sequence.

Pepscan analyses were performed using the recently developed suspension array technology (SAT). This was used to map the recognition sites of MAbs and serum samples onto a panel of overlapping synthetic peptides (12mers) that mimicked the betanodavirus coat protein. The results of pepscan analyses required careful interpretation due to the binding of antibodies and serum samples to multiple peptides. However, three regions of the nodavirus coat protein were identified as containing B-cell epitopes: amino acids 1-50, 141-162, and 181-212. These results are discussed in relation to previous studies of immune responses to betanodaviruses, and to the future development of betanodavirus vaccines and diagnostic reagents.

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Abbreviation list

aa	Amino Acids
Ab	Antibodies
ACNNV	Atlantic Cod Nervous Necrosis Virus
AHX	Amino hexanoic acid
AHNNV	Atlantic Halibut Nervous Necrosis Virus
BBV	Black Beetle Virus
BKD	Bacterial Kidney Disease
BFNNV	Barfin Flounder Nervous Necrosis Virus
BOV	Boolarra Virus
BNYVV	Beet Necrosis Yellow Vein Virus
BSA	Bovine Serum Albumin
CBA	Cytometric Bead Array
CBR	Critical-Binding Residues
CCVD	Channel Catfish Virus Disease
CDRS	Complementarity Determining Region
CNS	Central Nervous System
CPE	Cytopathic Effect
CsCl	Caesium Cloride
DMEM	Dulbecco's Modified Eagle Medium
DMEM+	Dulbecco's Modified Eagle Medium with sodium pyruvate, L-glutamine, Penicillin-Streptamycin and Fetal Bovine Serum (20%)
DIEV	*Dicentrarchus labrax* Encephalitis Virus
DINNV	*Dicentrarchus labrax* Nervous Necrosis Virus
D-PBS	Dulbecco's PBS without Ca and Mg
D-PBS+	Dulbecco's PBS (without Ca and Mg) supplemented with 1 % of BSA and 0.02 % of NaN_3
ELISA	Enzyme - Linked Immunosorbent Assay
ERM	Enteric Redmouth
FAT	Fluorescent Antibody Technique
FBS	Foetal Bovine Serum
FCA	Freund's Complete Adjuvant
FEV	Fish Encephalitis Viruses
FHV	Flock House Virus
FIV	Feline Immunodeficiency Virus
GF-1	grouper (*Epinephelus coioides*) cell line
GKHD	Grass Carp Haemorrhagic Disease
GGNNV	Greasy Grouper Nervous Necrosis Virus
GMO	Genetic Modified Organism
GNNV	Grouper Nervous Necrosis Virus
hrs	Hours
HAT	Hypoxanthine-Aminopterin-Thymidine
HBSS	Hanks' Balance Salt Solution
HGPRT	Hypoxanthine Guanine Phosphoribosyl Transferase
HIRRV	Hirame rhabdovirus
HIV	Human Immunodeficiency Virus
HT	Hypoxanthine-Thymidine
IHNV	Infectious Haematopoietic Necrosis Virus
IBDV	Infectious Bursal Disease Virus
ISAV	Infectious Salmon Anaemia Virus
IPNV	Infectious Pancreatic Necrosis Virus
IP	Intraperitoneal injection
IV	Intravenous injection
JFNNV	Japanese flounder Nervous Necrosis Virus
LcEV	*Lates calcarifer* Encephalitis Virus
L-15/FBS	Leibovitz's L-15 medium containing Glutamax-I supplemented with 10 % FBS
MAI	Micro-Array Immunoassay
MAbs	Monoclonal Antibodies

MBA	Multiplex Bead Assay
min(s)	Minute(s)
MFCT	Microparticle-based Flow Cytometric Technology
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
Mouse IgG-PE	Goat anti-mouse IgG antibody conjugated with phycoerythrin
MPBFC	Multiplexed Particle-based Flow Cytometry Assay
NCR	Non-Coding Region
NI	Neutralisation Index
NK	Natural killer cells
NNV	Nervous Necrosis Virus
NOV	Nodamura Virus
Nt	Nucleotide(s)
OIE	Office International des Epizooties
ORF	Open Reading Frame
PBMT	Particle-Based Micro-array Technology
PBS	Phosphate Buffered Saline
PBS-T-BSA	PBS with 0.05% Tween 20 and 1% BSA
PE	Phycoerythrin
PEG	Polyethylene Glycol
pfu	Plaque forming units
PNN	Piscine Neuropathy Nodavirus
Rabbit IgG-PE	Goat anti-rabbit IgG antibody conjugated with phycoerythrin
RdRp	RNA-dependent RNA polymerase
RF	Replicative Form
RI	Refractive Index
RGNNV	Red-Spotted Grouper Nervous Necrosis Virus
RPS	Relative Percent Survival
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
SAT	Suspension Array Technology
SBNN	Sea Bass Neuropathy Nodavirus
Sea bass IgM	Anti-European sea bass IgM monoclonal antibody
SC	Subcutaneous Injection
SDV	Sleeping Diseases Virus
SJNNV	Striped Jack Nervous Necrosis Virus
SPDV	Salmon Pancreatic Disease Virus
SNP	Single Nucleotide Polymorphism
SNN-1	Striped Snakehead (*Ophicephalus striatus*) cell line
SVE	Sea bass Viral Encephalitis
TPNNV	Tiger Puffer Nervous Necrosis Virus
TRO	Total Residual Oxidants
UV	Ultra-Violet
VER	Viral Encephalopathy and Retinopathy
VHSV	Viral Hemorrhagic Septicaemia Virus
VNN	Viral Nervous Necrosis
YGNNV	Yellow Grouper Nervous Necrosis Nodavirus

Chapter 1 - Introduction

1.1 - Betanodavirus

1.1.1 - History of the disease

Within the last 20 years a disease with distinct pathological features comprising vacuolation of the encephalon and retina has affected the marine fish-farming industry. The aetiologic agent of this disease has been identified as a virus belonging to the Nodaviridae family. Nodavirus infection is now recognised as a major problem for the marine aquaculture industry in many parts of the world. Nodavirus-associated disease can cause extensive mortalities (up to 100 % in larvae) in affected stocks.

Nodavirus disease in marine fish is neuropathogenic and for this reason it was first named SVE (Sea bass Viral Encephalitis) by Bellance and Gallet de Saint-Aurin (1988). Since then it has been referred to as Viral Nervous Necrosis (VNN) (Yoshikoshi and Inoue, 1990), Viral Encephalopathy and Retinopathy (VER) (Munday *et al.* 1992), and Fish Viral Encephalitis (FVE) (Comps *et al.* 1994). The disease is officially denominated Viral Encephalopathy and Retinopathy by the OIE (Office International des Èpizooties), based on the histopathological signs that accompany infection (Munday *et al.* 2002). Tanaka *et al.* (2004) suggested that the disease should be referred to as Viral Encephalitis and Retinitis based on histopathological studies.

The first report of fish nodaviruses was made in Martinique where the production of sea-bass (*Dicentrarchus labrax*) alevins was completely halted by this virus in 1985 (Bellance and Gallet de Saint-Aurin, 1988, Breuil *et al.* 1991). Since then further disease outbreaks with similar symptoms have been reported with global distribution. More than 38 species belonging to 21 families of ten different orders have been affected (Table 1.1).

Table 1.1 - Fish species in which nodavirus infection has been reported

Order	**Family**	***Species***	***Common name***	***Country***	***Reference***
Anguilliformes	Anguillidae	*Anguilla anguilla* L.	European eel	Taiwan	Lai *et al.* (2001b)
Acipenseriformes	Acipenseridae	*Acipenser gueldenstaedtii*	Russian sturgeon	Greece	Athanassopoulou *et al.* (2004)
Cyprinodontiformes	Poeciliidae	*Poecilia reticulata*	Guppy	Singapore	Hegde *et al.* (2003)
Gadiformes	Gadidae	*Gadus morhua* *Gadus macrocephalus* *Melanogrammus aeglefinus*	Atlantic cod Pacific cod Haddock	United Kingdom Canada Japan Canada	Starkey *et al.* (2001) Johnson *et al.* (2002) Arimoto *et al.* (1993) Gagné *et al.* (2004)
Mugiliformes	Mugilidae	*Mugil cephalus*	Black mullet	Israel	Ucko *et al.* (2004)
Perciformes	Carangidae	*Pseudocaranx dentex* *Seriola dumerili* *Trachinotus falcanus*	Striped jack Amberjack Yellow-wax pompano	Japan Taiwan	Mori *et al.* (1992) Arimoto *et al.* (1993) Nishizawa *et al.* (1997) Lai *et al.* (2001b)
	Centropomidae	*Lates calcarifer* Bloch	Barramundi	Australia Singapore Tahiti Israel	Glazebrook *et al.* (1990) Chew-Lim *et al.* (1998) Renault *et al.* (1991) Ucko *et al.* (2004)
	Lutjanidae	*Lutjarnus erythropterus*		Taiwan	Lai *et al.* (2001b)
	Moronidae	*Dicentrarchus labrax* L.	European sea bass	Martinique France Italy Israel	Bellance and Gallet de Saint-Aurin (1988) Breuil *et al.* (1991) Ucko *et al.* (2004)
	Rachycentridae	*Rachycentron canadum* L.	Cobia	Taiwan	Lai *et al.* (2001b)
	Sciaenidae	*Umbrina cirrosa* *Atractoscion nobilis* *Sciaenops ocellatus*	Shi drum White sea bass Red drum	Japan U.S.A Korea Israel	Comps *et al.* (1996) Nishizawa *et al.* (1997) Curtis *et al.* (2001) Oh *et al.* (2002) Ucko *et al.* (2004)

Table 1.1 (continued)- Fish species in which nodavirus infection has been reported

Order	**Family**	***Species***	***Common name***	***Country***	***Reference***
Perciformes	Serranidae	*Chromileptes altivelis*	Humpback grouper	Indonesia	Zafran *et al.* (2000)
		Epinephelus			
		akaara	Redspotted grouper	Japan	Mori *et al.* (1991)
					Chi *et al.* (1997)
		aeneus	White grouper	Israel	Ucko *et al.* (2004)
		awoara	Yellow grouper	Taiwan	Lai *et al.* (2001a)
		fuscoguttatus	Black-spotted grouper	Taiwan	Chi *et al.* (1997)
		lanceolatus	Dragon grouper	Taiwan	Lin *et al.* (2001)
		malabaricus	Brown-spotted grouper	Thailand	Danayadol and Direkbusarakom (1995)
		moara	Kelp grouper	Japan	Nakai *et al.* (1994)
		septemfasciatus	Sevenband grouper	Japan	Fukuda *et al.* (1996)
		tauvina	Greasy grouper	Singapore	Chua *et al.* (1995)
	Sparidae	*Pagrus major*	Red seabream	Japan	Nishizawa *et al.* (1997)
	Oplegnathidae	*Oplegnathus fascinatus*	Japonese parrotfish	Japan	Yoshikoshi and Inoue (1990)
Pleuronectiformes	Paralichthyidae	*Paralichthys olivaceus*	Japanese flounder	Japan	Nguyen *et al.* (1994)
	Pleuronectidae	*Hippoglossus hippoglossus*	Atlantic halibut	Norway	Grotmol *et al.* (1997)
				United Kingdom	Starkey *et al.* (2000)
		Pleuronectes americanus	Winter flounder	Canada	Barker *et al.* (2002)
		Verasper moseri	Barfin flounder	Japan	Nishizawa *et al.* (1997)
	Scophthalmidae	*Scophthalmus maximus*	Turbot	Denmark	Bloch *et al.* (1991)
	Soleidae	*Solea vulgaris*/*Solea solea*	Dover sole	United Kingdom	Starkey *et al.* (2001)
Scorpaeniformes	Platycephalidae	*Platycephalus indicus*	Bartail flathead	Japan	Song *et al.* (1997)
Siluriformes	Siluridae	*Parasilurus asotus*	Chinese catfish	Taiwan	Chi *et al.* (2003)
Tetraodontiformes	Triodontidae	*Takifugu rubripes*	Tiger puffer	Japan	Nakai *et al.* (1994)

Until very recently all fish nodavirus disease outbreaks were confined to marine species. The first indication that nodaviruses might infect freshwater fish was the occurrence of VER in barramundi, but this species is catadromous and part of the life cycle is in sea water. In 2000 the first occurrence of these viruses in a fresh water ornamental fish, guppy (*Poecilia reticulata*) was detected in Singapore (Hegde *et al.* 2003). In Greece two freshwater outbreaks of nodavirus disease have been described. One in sturgeon, (*Acipenser gueldestaedi*) and another in European sea bass (*Dicentrarchus labrax*) under production in freshwater (Athanassopoulou *et al.* 2003, Athanassopoulou *et al.* 2004). Mortalities over 70 % caused by VNN in European eel (*Anguilla anguilla*) were observed in fish-farms of Taiwan (Chi *et al.* 2003).

The confirmation of nodavirus as the aetiological agent of VER in fulfilment of Koch's postulates has been performed by experimental infection of several fish species including: *Dicentrarchus labrax* (Péducasse *et al.* 1999c, Skliris and Richards, 1999a), *Epinephelus akaara* (Mori *et al.* 1991), *Epinephelus malabaricus* (Boonyaratpalin *et al.* 1996), *Epinephelus septemfasciatus* (Tanaka *et al.* 1998), *Pseudocaranx dentex* (Arimoto *et al.* 1993, Nguyen *et al.* 1996), *Anarhicas minor* (Amundsen and Sommer, 1999, Johansen *et al.* 2003, Sommer *et al.* 2004) *Scophthalmus maximus* (Húsgarð *et al.* 1999) and *Hippoglossus hippoglossus* (Grotmol *et al.* 1999).

1.1.2 - Taxonomy

Based on virion size and genome characteristics Mori *et al.* (1992) classified the virus isolated from striped jack as a member of the Nodaviridae family. The virus was designated Striped Jack Nervous Necrosis Virus (SJNNV). Comps *et al.* (1994), characterised a virus isolated from outbreaks of VNN in *Dicentrarchus labrax* and *Lates calcarifer* and obtained results similar to those reported by Mori *et al.* (1992). Comps *et al.* (1994) also concluded that the aetiological agent of VNN in these species was a nodavirus, and named the causative

agents Fish Encephalitis Viruses (FEV) to distinguish them from the previously described insect nodaviruses.

Viruses with similar properties have been isolated from globally distributed outbreaks of VNN. In each instance, the virus particles isolated from diseased fish have exhibited physical and chemical properties characteristic of nodaviruses.

The Nodaviridae were first identified from insects (Garzon and Charpentier, 1992), then from fish. More recently a nodavirus has been identified in a crustacean (Arcier *et al.* 1999).

Recently the viruses isolated from cases of VNN have been classified as belonging to the genus *Betanodavirus* within the Nodaviridae family to distinguish them from the insect nodavirus, which are classified as belonging to the genus *Alphanodavirus* (Ball *et al.* 2000).

The lack of a permissive cell culture system for fish nodaviruses delayed the characterisation of the aetiological agent of VNN. The first studies described the agent of VNN as a "picorna-like virus" (Glazebrook *et al.* 1990, Breuil *et al.* 1991).

1.1.3 - Characterisation of the virus

Breuil *et al.* (1991) characterised virus particles isolated from European sea bass (*Dicentrarchus labrax*) larvae and juveniles as having a typical icosahedral shape and a diameter of 23 nm as determined by electron microscopy. After purification in CsCl gradients virus particles showed a diameter of 26 nm from side to side, 29 nm from point to point, and a buoyant density of 1.30 g per cm^3 (Breuil *et al.* 1991).

The nucleic acid and the structural proteins of striped jack nervous necrosis virus (SJNNV) were characterised by Mori *et al.* (1992). This study revealed that the virus associated with VNN consisted of non-enveloped particles about 25 nm in diameter, with two single-stranded positive-sense RNA molecules with molecular weights of 1.01×10^6 Da (RNA1) and 0.49×10^6 Da (RNA2), lacking a poly(A) sequence at the 3' terminus. The same

authors identified two major polypeptides, one of approximately 100 kDa encoded by RNA1, and another of 42 kDa encoded by RNA2, which was deduced to represent the coat protein gene. Barramundi NNV, European sea bass NNV (DlNNV) and grouper NNV have the same major RNA2 polypeptide (42 kDa) (Comps *et al.* 1994, Hegde *et al.* 2002).

The RNA1 genome segment encodes "protein A", which is the viral component of the RNA-dependent RNA polymerase (RdRp) (Nagai and Nishizawa, 1999). According to these authors, the nucleotide sequence of SJNNV RNA1 comprises 3081 bases and contains a single Open Reading Frame (ORF) encoding a protein of 983 aa (amino acids) of Mr 110 kDa. However, the RNA1 segment from a nodavirus isolated from Atlantic cod, contains 3100 nucleotides (nt), with one ORF between nt 79-3021 that encodes a 981 aa polypeptide (Sommerset and Nerland, 2004). In contrast, the greasy grouper NNV (GGNNV) RNA1 is 3103 nt in length with one ORF (nt 79-3027) encoding a protein of 982 aa (Tan *et al.* 2001).

Several studies of the RNA2 have indicated that this segment varies in length according to the virus strain analysed. The longest RNA2 was identified in GGNNV, 1433 nt, whereas SJNNV contained an RNA2 segment of 1410 nt, and that from SBNNV was only 1406 nt long. Both GGNNV and SBNNV RNA2s encode coat proteins of the same size (338 aa), while that from SJNNV is 2 amino acids longer. However all fish nodavirus coat proteins studied are of the same molecular weight (37 kDa) (Nishizawa *et al.* 1995a, Delsert *et al.* 1997a, Tan *et al.* 2001).

A nodavirus obtained from the freshwater fish species guppy (*Poecilia reticulata*) contained a shorter RNA2 (1367 base pairs) and the ORF coded for a protein of the same size as that found in sea water species – 338 amino acids (Hegde *et al.* 2003).

The longer sequence length described for both GGNNV RNAs by Tan *et al.* (2001) have been attributed to the use of a different sequencing methodology involving 5'RACE.

In alphanodavirus a subgenomic RNA3 is produced during RNA replication from the 3' terminus of RNA1. This transcript encodes one or two small proteins (B1 and B2) with unknown functions (Schneemann *et al.* 1998). A RNA3 transcript from a betanodavirus of approximately 400 base pairs was reported for the first time by Delsert *et al.* (1997b). Tan *et*

al. (2001) suggested that the RNA1 of GGNNV encoded two proteins designated B1 (111 aa) and B2 (75 aa). The B1 protein was translated in the same reading frame as the replicase protein A, whereas protein B2 was translated in the +1 reading frame and overlapped the C terminus of protein A. The amino acid sequence of Atlantic halibut RNA3 corresponded to nt 2730-3100 of the RNA1 Atlantic halibut sequence (Sommerset and Nerland, 2004). The amino acid sequence indicated that only the B2 protein was expressed during virus replication. In flock house virus (FHV) the B2 protein has been identified as a potent RNA silencing inhibitor (Li *et al.* 2002).

Protein α is the precursor of the capsid protein and is important for viral assembly (Guo *et al.* 2003). In alphanodaviruses, protein α is autocatalytically cleaved to form the mature coat protein subunits β and γ (Gallagher and Rueckert, 1988). With betanodaviruses this doublet formation does not occur as a result of autocatalysis, but instead results from the formation of an intramolecular disulfide bond between cystines 187 and 201 (Krondiris and Sideris, 2002).

Analysis of the RNA2 genome segment from Atlantic halibut indicates that the deduced amino acid sequence contains two stretches of arginine residues that are found in all nodaviruses and are assumed to participate in the binding of the RNA genome to the internal capsid wall (Grotmol *et al.* 2000). An aspartic acid residue is present at position 75 that is also common to all nodaviruses This residue is believed to represent part of a catalytic site that is involved in capsid protein cleavage (Grotmol *et al.* 2000).

The N-terminus of the coat protein is very rich in basic amino acids (nine arginines and six lysines), which might account for the observed irregularity in the mobility of this protein in SDS-PAGE (Sideris, 1997). The same pattern has been also observed in the coat protein of other insect nodaviruses and this region of the coat protein is thought to be involved in the coat protein-RNA interaction required for virus encapsidation (Schneemann *et al.* 1998).

Chi *et al.* (2001) demonstrated that the capsid proteins of GNNV is glycosylated based on their staining properties with periodic acid-silver.

1.1.4 - Clinical signs

1.1.4.1 - Signs

The clinical signs of VNN include abnormal colouration, anorexia, and altered swimming in association with extensive mortalities that can approach 100 % (Table 1.2). In most cases the fish affected are larvae or juveniles, but there are a number of reports of VNN in adult fish.

Table 1.2 - Clinical signs of nodavirus disease.

Species	***Age***	***Signs***	***Reference***
Lates calcarifer	15-18 dph	Lethargy, anorexia, pale-grey coloration, swimming in a darting or corkscrew fashion	Glazebrook *et al.* (1990)
Epinephelus akaara		Whirling swimmimg pattern	Munday and Nakai (1997)
Pseudocaranx dentex		Abnormal swimming behaviour, swim bladder hyperinflation	Munday and Nakai (1997)
Hippoglossus hippoglossus		Lethargy, belly-up at rest, abnormal swimming, pale colour	Grotmol *et al.* (1997)
Scophthalmus maximus	50-100 mm	Reduced feeding activity, dark colouration, lethargic, lying abdomen-up on the bottom, abnormal swimming such as rotating, spinning, horizontal looping	Bloch *et al.* (1991)
Oplegnathus fascinatus	6-25 mm TL	Loss of swimming activity and equilibrium, Spiral swimming, dark colour	Yoshikoshi and Inoue, (1990)
Paralichthys olivaceus	17-18 mm TL (35 dph)	Whirling, abnormal swimming behaviour	Nguyen *et al.* (1994)
Epinephelus akaara	7-20 mm	Listless swimming near the surface of the water, abrupt whirling, sinking to the bottom	Mori *et al.* (1991)
Epinephelus akaara	170-1850g	Floating upside down around the surface of water	Fukuda *et al.* (1996)
Epinephelus tauvina	Up to 40 mm	Loss of equilibrium, corkscrew swimming	Chua *et al.* (1995)
Cromileptes altivelis	10-52 dph	Twirling swimming, resting on the bottom, sluggish behaviour	Zafran *et al.* (2000)

TL – total length; dph - days post hatching

1.1.4.2 - Tissue distribution and histopathology

VNN is characterised by extensive necrosis of the central nervous system (CNS) with numerous virus particles present in the cytoplasm of affected nerve cells (Nishizawa *et al.* 1995b).

Glazebrook *et al.* (1990) observed extensive vacuolation and neuronal degeneration of the mid and hind brain and vacuolation of the retina in affected barramundi (*Lates calarifer* Bloch). Under electron microscopy, degeneration was evident in cells in the brain and retina. Virions were present within the cytoplasm of neurones, which showed margination of nuclear chromatin. In the retina, virions were either membrane bound by endoplasmic reticulum or free in the cytoplasm. Separation of the nuclear membrane was observed, and the inner cristae of mitochondria were disintegrated with only vestiges of the plasma membrane remaining.

Under light microscopic examination, larvae of *Oplegnathus fasciatus* showed conspicuous vacuolation and pyknosis associated with marked shrinkage and basophilia in affected cells of the spinal cord and brain (Yoshikoshi and Inoue, 1990). Numerous virus particles were evident in the cytoplasm of neurones, and within inclusion bodies and nerve fibres of affected neurones. These included oligodendrocytes which formed the myelin sheath and probably astrocytes. The authors were able to differentiate two kinds of degenerative change – pyknosis and cell lysis. In pyknotic cells the virus particles were densely packed in the cytoplasm, which contained a few degenerating mitochondria. These cells appeared to be highly necrotic. Cell lysis occurred much more frequently and was closely associated with vacuolation (Yoshikoshi and Inoue, 1990).

In larvae of *Epinephelus akaara* conspicuous vacuolation occurred in the nuclear layers of the retina and in various parts of the brain (Mori *et al.* 1991). When the same samples were observed by transmission electron microscopy, necrotic and lytic degeneration of neurons and other unidentified cells in the retina and brain, and numerous hexagonal virus particles in the heavily vacuolated cytoplasm and extracellular spaces were evident.

In the studies by Yoshikoshi and Inoue (1990) and Mori *et al.* (1991), lesions were not found in tissue samples taken from the gills, heart, alimentary canal, liver, exocrine pancreas, kidney, spleen, skin and skeletal muscles.

In nodavirus infected *Lates calcarifer* larvae Renault *et al.* (1991) described enlarged basophilic cells with a rounded shape, and inclusion bodies and cytoplasmic vacuolation in cells of the optic tectum, cerebellum, tegmentum, vagal lobes, medulla oblongata and spinal cord. The vacuoles were often very extensive and the resulting loss of neural substance gave a spongiform appearance to the tissue. A similar spongiform appearance was evident in the retina.

Nguyen *et al.* (1997) studied the tissue distribution of virus replication in striped jack using Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) and Fluorescent Antibody Technique (FAT) and detected virus in the liver, kidney, stomach, intestine and gonadal fluid of 13 year old broodstock fish.

1.1.5 - Route of infection

Renault *et al.* (1991) reported that the cerebellum, the optic tectum and the retina were the primary sites of lesions in nodavirus-infected barramundi. The spinal cord and spinal ganglia were the primary sites of lesions in Japanese parrotfish (*Oplegnathus fasciatus*) (Yoshikoshi and Inoue, 1990). In striped jack, necrosis and vacuolation of nerve cells were first observed in the spinal cord, particularly in the area situated near to the swim bladder, with lesions occurring later in the brain and then in the retina (Nguyen *et al.* 1996). The authors suggested that one of the initial multiplication sites of the virus was the area of the spinal cord situated immediately above the swim bladder. From there the virus was postulated to spread in an anterior direction to the base of the spinal cord and forward to the brain terminating in the retina.

In a study performed in Atlantic halibut by Grotmol *et al.* (1999) the initial major focus of lesions in the central nervous system was the caudal part of the brain stem together with the stratified epithelium of the anterior intestine. The route of infection to the CNS may have been axonal transport to the brain stem through cranial nerves including the vagus nerve.

Péducasse *et al.* (1999c) noted that after infecting *Dicentrarchus labrax* juveniles by subcutaneous injection, bath exposure, and caudal immersion, virus was first detected in the spinal cord, and then in the brain and in the retina. The infection appeared to progress from two major sites, the gills and skin and/or the lateral line to gain access to the central nervous system (Péducasse *et al.* 1999a).

Possible nodavirus infection of the host through the nasal cavity has been suggested by Mladineo (2003) and Tanaka *et al.* (2004). These authors reported that the virus penetrates the nasal epithelium, disseminating through the olfactory nerve and olfactory bulb, to the aboral brain tissue, medulla oblongata, spinal cord and finally to the retina.

Whereas there are histological differences according to the age of the host, there are no age related differences in tissue-distribution. Larvae exhibit heavy necrotization of the brain whereas juveniles and adults show higher concentrations of the virus in the brain but with lower necrotization of the tissue (Mladineo, 2003).

1.1.6 - Transmission of disease

Several outbreaks in fish larvae suggest that vertical transmission of VNN may occur. The detection of viral antigens in ovarian tissues, fertilised eggs and hatched larvae was achieved by Arimoto *et al.* (1992) and Breuil *et al.* (2002). The presence of Nodavirus in sperm and subsequent infection of eggs during fertilisation was demonstrated by Breuil *et al.* (2001). These authors infected sperm with Nodavirus and demonstrated infection of offspring.

Nguyen *et al.* (1997) suggested that the broodstock can carry the virus in the gonads and several other organs (liver, kidney, stomach and intestine) and during the stress of

spawning virus replication occurred in these organs. Virus can then be shed from the gonads and digestive tract and infect eggs, sperm or larvae (Arimoto *et al.* 1992, Mushiake *et al.* 1992). The carrier status of the broodstock was confirmed by the total absence of virus in nerve tissue (Nguyen *et al.* 1997).

The infection of healthy fish by cohabitation with infected fish was demonstrated by Arimoto *et al.* (1993) in striped jack larvae. The survival of purified nodavirus particles and the ability to use water as a transmission vehicle has been demonstrated by Breuil *et al.* (2002) in a challenge of European sea bass larvae.

Fish surviving natural infection can act as carriers. One year after infection, Atlantic halibut survivors were shown to carry betanodavirus (Johansen *et al.* 2004b).

Horizontal transmission of VNN is problematic with respect to asymptomatic species and the use of polyculture systems. Skliris and Richards (1999b) demonstrated the presence and the viability of nodavirus in tilapia (*Oreochromis mossambicus*), and the absence of symptoms of the disease. For many years gilthead sea bream (*Sparus aurata*) was believed to be an asymptomatic carrier of VNN, but the carrier state was eventually demonstrated by Castric *et al.* in 2001.

The transmission of VNN by contaminated equipment has been suggested with respect to the disease in sturgeon (Athanassopoulou *et al.* 2004).

All marine fish larvae need to be fed with live food and this can act as a reservoir for many pathogens. Skliris and Richards (1998) assessed the susceptibility of the brineshrimp *Artemia salina* and the rotifer *Brachionus plicatilis* to nodavirus infection. Their results indicated that these invertebrates may act as a carrier for nodaviruses. No evidence was found for viral replication in the invertebrate hosts.

1.1.7 - Pathogenicity

A number of factors have been found to influence the virulence of betanodavirus infections in marine fish. These include temperature, age and species of host. The genetic basis of virulence in betanodaviruses is currently poorly characterised, and this has impeded the development of effective vaccines.

Betanodavirus virulence is associated with variation of water temperature. VNN-associated mortalities tend to be more severe at elevated water temperatures (25-28°C). Mortality frequently decreases or ceases altogether at temperatures below 20-23°C (Mori *et al.* 1991, Le Breton *et al.* 1997). However, instances of VNN have occurred at low water temperatures (4-15°C) (Tanaka *et al.* 1998).

The implication of elevated water temperatures as a factor influencing the severity of VNN was suggested by Fukuda *et al.* (1996) and Skliris and Richards (1997), who also noted high mortalities and relatively early disease signs in European sea bass held at elevated water temperatures (Skliris and Richards, 1999a).

A relationship between water temperature and virulence in betanodaviruses was reported by Péducasse *et al.* (1999a) and Breuil *et al.* (2001). These authors found that a strain of DlNNV designated Sb2 was highly virulent at 25°C (Péducasse *et al.* 1999a) but less virulent at 14-15°C (Breuil *et al.* 2001).

Based on *in vitro* studies utilising the GF-1 cell line, Chi *et al.* (1999b) proposed that in winter, nodaviruses enter a state of persistent infection within host cells, and do not cause necrosis or mortality in fish. These authors suggest that lower water temperatures delay the occurrence of mortalities in groupers.

Betanodaviruses can replicate and cause disease with mortalities of 100 % in Atlantic halibut yolksac larvae at temperatures as low as 6°C (Grotmol *et al.* 1999). This high virulence may represent an adaptation to replication in cold-water fish, and may thus be a phenotypic feature of this virus strain compared to nodavirus pathogenic to warm-water species.

Significant differences in virulence between two nodavirus strains (striped jack and Atlantic halibut) in cross infections of their respective natural hosts were reported by Totland *et al.* (1999). However, the authors were unable to conclude whether the observed differences in mortality were an effect of water temperature or a result of the host specificity of the viruses studied. The inability of SJNNV to infect Atlantic halibut was also observed by Húsgarð *et al.* (2001).

The susceptibility of European sea bass to nodavirus infection was shown to be age dependent (Breuil *et al.* 2001). A betanodavirus strain (Sb1) was pathogenic for larvae, but less to juveniles or adults.

1.1.8 - Detection Methods

The detection of fish nodaviruses can be achieved by several methods, including histology, FAT, in-situ hybridization, virus isolation in cell culture, enzyme linked immunosorbent assay (ELISA), immunohistochemistry, and molecular procedures such as RT-PCR.

Histology using light and electron microscopy were the first techniques used for the identification of VNN (Yoshikoshi and Inoue, 1990). Recognition of the characteristic lesions of VNN in the brain and retina can easily be achieved by microscopy.

Nguyen *et al.* (1996) used a fluorescent antibody technique (FAT) to detect betanodaviruses. It was possible to detect virus antigen in the nervous system with this technique. Since then this FAT has been used routinely to identify and confirm the presence of betanodavirus in outbreaks of VNN.

The detection of betanodaviruses with *in situ* hybridisation using probes labelled radioactively or DIG-labelled in DIEV infection was reported by Comps *et al.* (1996).

The isolation of nodavirus in a cell line was reported for the first time by Frerichs *et al.* (1996), who used the SSN-1 cell line (derived from striped snakehead *Ophicephalus*

striatus) to isolate a nodavirus from a sample of *D. labrax* brain tissue. Nodavirus infected SSN-1 cells exhibited characteristic cytopathic effects. The GF-1 (derived from a grouper *Epinephelus coioides*) cell line has been used to successfully propagate GNNV (Chi *et al.* 1999a, Chi and Lin, 1999). More recently, the cell line GB (Grouper Brain, derived from yellow grouper *Epinephelus awoara*) has also been used for propagation of betanodaviruses (Lai *et al.* 2001b). In other cell lines such as that from barramundi (*Lates calcarifer*) cytopathic effects are not obtained during primary culture, and blind passage is required (Chua *et al.* 1995). Cell lines of this type are less useful for betanodavirus detection.

A major constraint of the above techniques is their requirement for lethal sampling of test fish. Consequently, they cannot be applied to the screening of broodstock fish. In these circumstances methods for detection of nodavirus antibody are advantageous, since they can be applied non destructively (Breuil and Romestad, 1999).

The detection of SJNNV antibodies in broodstock of striped jack using indirect ELISA was achieved for the first time by Mushiake *et al.* (1992). Further attempts to detect nodavirus antibodies have been carried out in European sea bass, striped jack, barramundi and barfin flounder (Mushiake *et al.* 1992, Mushiake *et al.* 1993, Breuil and Romestad, 1999, Watanabe *et al.* 2000, Huang *et al.* 2001). Antibodies against betanodavirus can be detected one year after infection in halibut (Johansen *et al.* 2004b).

Indirect ELISA was used for detection of nodavirus antigens in eggs, larvae and brood stock of striped jack (Arimoto *et al.* 1992), who used anti-nodavirus polyclonal antibodies produced in rabbits.

Monoclonal antibodies represent useful diagnostic tools for betanodaviruses. Nishizawa *et al.* (1995b) succeeded in generating monoclonal antibodies (MAbs) against SJNNV that recognised the coat protein (42 kDa), and had neutralising activity as assessed in *in vivo* neutralisation assays. These MAbs did not react with other nodavirus strains suggesting different neutralising epitopes between SJNNV and other nodaviruses. Another panel of MAbs produced against Yellow Grouper Nervous Necrosis Nodavirus (YGNNV) exhibited i*n vitro* neutralisation capacity and recognised the coat protein (42 kDa) (Lai *et al.*

2001a). MAbs against GNNV that were capable of neutralising several other strains of nodavirus isolated from grouper, tiger puffer, striped jack and barfin flounder were produced and characterised by Shieh and Chi (2005).

Molecular diagnostic procedures have been applied to the detection of betanodaviruses. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used to detect nodaviruses by Nishizawa *et al.* (1994), who tested combinations of five different primers (2 forward and 3 reverse). The most efficient amplification was achieved with primers designed to target a region designated T4 (592-1017 nt) (F2 - 5' CGTGTCAGTCATGTGTCGCT 3' and R3 3' AGAAGTGGGCACAACTGAGC 5'). This primer pair generates a 426 bp fragment and has a detection limit of 0.1 fg of virus.

Nested RT-PCR has proven to be more sensitive than single step RT-PCR for monitoring the presence of the nodavirus genome in asymptomatic carriers (Thiéry *et al.* 1999, Gomez *et al.* 2004).

Recently a real-time Nucleic Acid Sequence Based Amplification (NASBA) procedure has been described for detection of betanodaviruses. NASBA is an isothermal nucleic acid amplification method. This method was found to be more sensitive than conventional RT-PCR (Starkey *et al.* 2004).

1.1.9 - Phylogeny

1.1.9.1 - Fish nodavirus

The genus *Betanodavirus* has been divided into seven lineages: *Dicentrarchus labrax* encephalitis virus (DlEV); tiger puffer nervous necrosis virus (TPNNV), *Lates calcarifer* encephalitis virus (LcEV); Japanese flounder nervous necrosis virus (JFNNV); striped jack nervous necrosis virus (SJNNV)), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Ball *et al.* 2000).

The first phylogenetic analysis of fish nodaviruses was carried out with isolates from Japan, Italy and Australia (Nishizawa *et al.* 1997). The Japanese isolates diverged into four major clusters: TPNNV type (Tiger Puffer Nervous Necrosis Virus), SJNNV type (Striped Jack Nervous Necrosis Virus), BFNNV type (Barfin Flounder Nervous Necrosis Virus) and RGNNV type (Red-Spotted Grouper Nervous Necrosis Virus). The isolates from Italy and Australia were found to belong to the RGNNV type (see Figure 1.1).

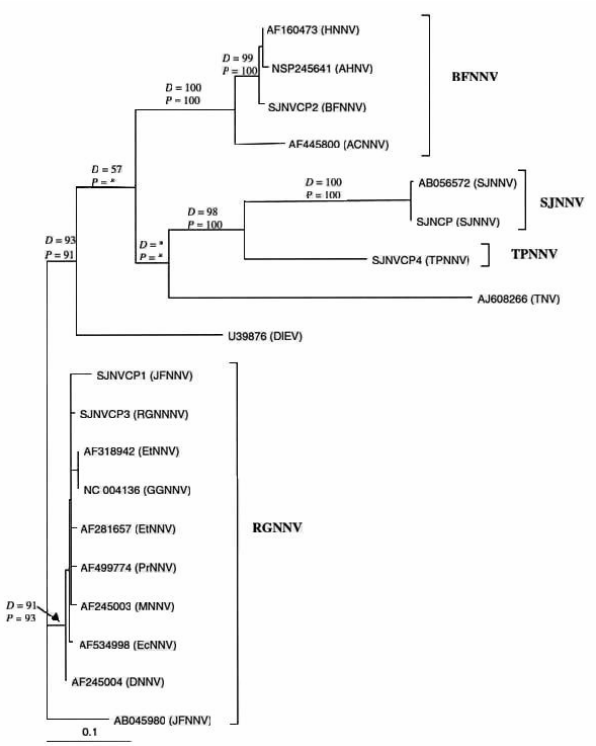


Figure 1.1 – Betanodavirus phylogenetic tree (Johansen *et al.* 2004a).

Phylogenetic analysis of nodaviruses from European sea bass from two different regions of France (Atlantic and Mediterranean areas) showed isolates from both geographic areas belonged to the RGNNV group, but the Mediterranean viruses were more closely related to the RGNNV type than the Atlantic viruses, which were more similar to the JFNNV group (Thiéry *et al.* 1999). The nucleotide sequence of the coat protein gene of the French Mediterranean SBNNV isolate was identical to nodaviruses isolated from Greece and Italy, suggesting that nodaviruses from the Mediterranean coast region are closely related (Thiéry *et al.* 1999).

Atlantic halibut nodavirus has been classified as belonging to the BFNNV clade, which also contains nodaviruses isolated from Pacific cod (*Gadus macrocephalus*) and barfin flounder (*Verasper moseri*) - both cold-water species (Grotmol *et al.* 2000). The T2 region of the Atlantic halibut nodavirus RNA2 has a nucleotide sequence practically identical (98.4 %) to the T2 region of the BFNNV and the T4 nucleotide sequence showed identities of 97.9 % with barfin flounder and 98.2 % with Pacific cod (Grotmol *et al.* 2000).

Betanodaviruses isolated from Atlantic halibut in Scotland show 98 % homology with viruses from Atlantic halibut in Norway (Starkey *et al.* 2000). This study also indicated that U.K. halibut nodavirus isolates belong to the BFNNV clade. Recently, nodaviruses from Atlantic cod and Dover sole farmed in the UK have also been found to cluster with the BFNNV nodaviruses (Starkey *et al.* 2001).

Skliris *et al.* (2001), studied the phylogenetic relationship between a collection of nodaviruses isolated from Europe, Asia and Japan. The results of this work show that 12 of the 13 isolates (all the isolates from European sea bass, brownspotted grouper, barramundi, rock porgy and shi drum) were found to belong to the RGNNV genotype while a single isolate from striped jack was found to belong to the SJNNV type.

A betanodavirus isolated from the guppy, a freshwater species, exhibited 98.2 % nucleotide identity with *Epinephelus tauvina* based on sequence analysis of the T4 region of RNA2, indicating that this virus belongs to the RGNNV clade (Hegde *et al.* 2003).

In 2004 two other genetic groups of betanodavirus were proposed, one for turbot and another for Atlantic North nodaviruses (from Atlantic cod, haddock and winter flounder). The designation of a new clade for turbot was based on relatively low sequence identity (77-78 %) between the turbot nodavirus and representatives of the other four phylogenetic groups (Johansen *et al.* 2004a). At the amino acid level, differences between the Atlantic North America viruses and Atlantic halibut NNV from Norway are not so pronounced (93 % identity), though Gagné *et al.* (2004) proposed that Atlantic Cod Nervous Necrosis Virus (ACNNV) represents a distinct phylogenetic group.

Totland *et al.* (1999) claimed that genetic diversity among nodavirus strains reflects significant phenotypic differences, which may represent adaptation enabling infection of different host species and/or replication at different temperatures.

The capacity for different strains of nodavirus to affect the same species, when considered with the diversity of species that can be infected, suggests that the host range of nodaviruses may reflect at least in part host availability as opposed to the consequences of coevolution between pathogen and host (Dalla Valle *et al.* 2001). The same authors suggested that the observed relatedness between nodaviruses from Australian and Mediterranean waters reflects a parallel or convergent evolution rather than an exchange of viruses between these geographical areas.

1.1.9.2 - Fish and other nodavirus

The sequence identities between the RNA1 genome segments of SJNNV and the insect nodaviruses Black Beetle Virus (BBV) and Flock House Virus (FHV) were approximately 28.3 % at the nucleotide level and 27.6 % at amino acid level, although conserved motifs for the RNA-dependent RNA polymerase were located at almost identical positions within the deduced amino acid sequence of RNA 1 (Nagai and Nishizawa, 1999).

The sequence similarities between the coat protein gene of SJNNV and other known insect nodavirus (nodamura virus (NOV), black beetle virus, flock house virus and boolarra virus (BOV)) are 28.6 % or less at the nucleotide level, 10.6 % or less at amino acid level. There is no conserved region of RNA2 between the fish and insect viruses (Nishizawa *et al.* 1995a). This finding is supported by a sequencing study of the RNA2 segment of DlEV and other insect nodaviruses (Delsert *et al.* 1997a).

The coat proteins of DIEV (338 amino acids) and SJNNV (340 amino acids) are shorter than those of the insect nodaviruses (399 to 407 amino acids) (Delsert *et al.* 1997a).

Based on nucleotide sequencing studies of SJNNV RNA1 and RNA2, Nagai and Nishizawa (1999) and Nishizawa *et al.* (1999) suggested that fish nodaviruses represent a new genus designated *Piscinodavirus* within the family *Nodaviridae*.

In 2000 the Nodaviridae family was reclassified as having two genera: *Alphanodavirus* and *Betanodavirus* (Ball *et al.* 2000). The genus *Alphanodavirus* comprises insect nodaviruses and the genus *Betanodavirus* comprises fish nodaviruses.

In 1997 a nodavirus was detected in the crustacean (*Macrobrachium rosenbergii*) (Arcier *et al.* 1999) in Guadeloupe, West Indies. To date *Macrobrachium rosenbergii* nodavirus (*Mr*NV) has also been isolated in China and India (Hameed *et al.* 2004). Another unsually small virus named extra small virus (XSV) is invariably associated with MrNV infection in *M. rosenbergii* (Qian *et al.* 2003). MrNV has been characterised as a new type of nodavirus based on sequence analysis (Bonami *et al.* 2005) (Figure 1.2).

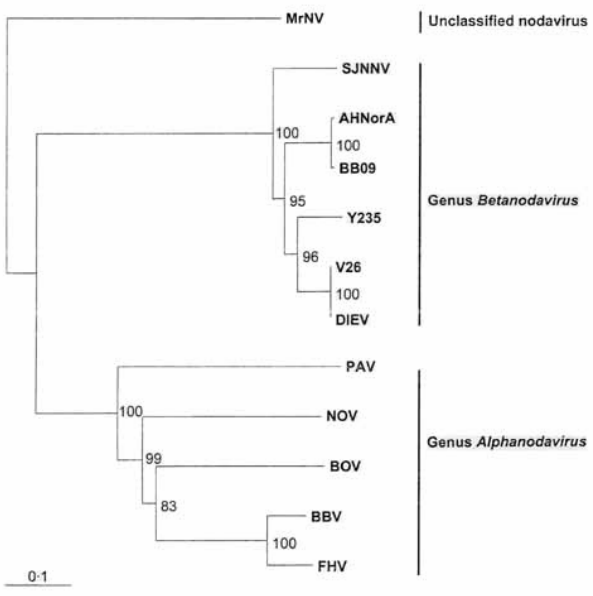


Figure 1.2 – Phylogenetic tree of the *Nodaviridae* family (Thiéry *et al.* 2004).

1.1.10 - Control Measures

1.1.10.1 - Disinfection

Attempts to control betanodaviruses in fish-farms are complicated by the relative stability of the nodavirus particle. These viruses are amongst the most stable of the fish viral pathogens (Frerichs *et al.* 1996).

The implementation of effective disinfection procedures is one method that can be used to control VNN. To this end, studies have been performed to determine the susceptibility of betanodaviruses to physical and chemical inactivation.

Striped jack NNV is completely inactivated at pH 12 so rubber shoes, plastic ware and nylon nets can be washed with an alkaline solution (pH 12) which can be neutralised with a hydrochloride solution (Arimoto *et al.* 1996). This betanodavirus strain is also inactivated by sodium hypochlorite, calcium hypochlorite, benzalkonium chloride and iodine. Heat treatment at 60°C for 30 min, ultra-violet light (UV) at an intensity of 410 μW per cm^2 for 4 mins and ozone at 0.1 μg ml^{-1} also efficiently inactivate SJNNV. However, SJNNV viability was not reduced as efficiently by formalin, ethanol, methanol, ether or chloroform.

The sensitivity of DlNNV to chlorine, iodine and peroxygen was noticeably different in the presence of organic matter (Frerichs *et al.* 2000). UV irradiation of 440 μW per cm^2 for 8 minutes reduced virus infectivity by 99.9%, and acid peroxygen also rapidly reduced the infectivity of DlNNV (Frerichs *et al.* 2000).

1.1.10.2 - Broodstock selection

Vertical transmission represents one of the major obstacles to the control of VNN. Striped jack spawners have been identified as an important reservoir of infection, allowing the virus to propagate in the ovaries and to be subsequently released with the eggs into the environment (Arimoto *et al.* 1992).

As a first approach towards the control of vertical transmission, Mushiake *et al.* (1993) have suggested that broodstock should be screened for the presence of virus and that improvements should be made to the spawning induction method to minimise stress. The authors screened spawners using ELISA to detect nodavirus antibodies.

However, VNN has occurred in offspring derived from spawners in which neither specific antibodies nor viral antigens were detected by ELISA (Mushiake *et al.* 1994). This illustrates the need for more sensitive screening methods for the detection of the virus. RT-PCR has been shown to be more effective and sensitive than ELISA in the selection of the striped jack spawners (Mushiake *et al.* 1994). Dalla Valle *et al.* (2000) suggested that broodstock can be screened by analysing the blood, but nested RT-PCR needs to be used

Watanabe *et al.* (1998) suggested that to prevent vertical transmission broodstock should be selected with ELISA titres less than 1:10 and subsequently reared in individual tanks. The eggs and sperm should be examined by RT-PCR in the spawning season, and only spawners that are negative by RT-PCR should be submitted to artificial fertilisation. Fertilised eggs should be disinfected with ozone and hatched larvae analysed by RT-PCR.

The selection of brood stock by detection of serum antibodies and antigen detection in ovarian biopsies was also described by Breuil *et al.* (2000).

Limiting the number of spawnings by broodstock fish to less than ten per season also reduces the incidence of vertical transmission, because nodavirus frequently occurs in larvae derived from eggs collected late in the spawning season (Mori *et al.* 1998).

In striped jack and barfin flounders disinfection of eggs with ozone (0.5 $\mu g\ l^{-1}$ of total residual oxidants (TROs) for 5 minutes) or with an oxidant (0.5 $\mu g\ ml^{-1}$ TRO for a duration of 30 sec generated by a corona discharge generator) has been shown to be effective (Mori *et al.* 1998, Watanabe *et al.* 1998).

The disinfection of the surface of Atlantic halibut eggs with 4 mg ozone per litre for 30 seconds also reduces mortality from VNN (Grotmol and Totland, 2000).

1.1.10.3 - Vaccines

Aquaculture is a fast growing industry. However, approximately 10 % of the total production within this industry is lost due to infectious diseases (Benmansour and de Kinkelin, 1997).

Measures for the treatment and control of infectious diseases in aquaculture are inadequate, particularly in the case of viral diseases. Furthermore, the use of chemicals and antibiotics is associated with adverse environmental effects and potential risks to human health. For this reason, disease prevention is considered to be of increasing importance in the aquaculture industry.

Vaccines can play a major role in disease prevention. They offer significant advantages over other methods of disease control as evidenced by the control of several important diseases like vibriosis, furunculosis and yersiniosis (Lillehaug, 1997). Vaccines have facilitated a reduction in the use of antibiotics in Norway, of which 30 000 kg were used in 1987 and only 5 000 kg in 1995 (Markestad and Grave, 1997).

Vaccination of fish can be performed by immersion, injection or by oral administration (Gudding *et al.* 1999). Immersion represents the only practical and economical technique for vaccinating fish fry, where efficacious protection is required at an early developmental stage. However, injection is preferred for mature fish since vaccination by this route offers better protection (Midtlyng, 1997).

A viral vaccine should fulfil certain requirements (Leong and Fryer, 1993). These include: inexpensive production costs; the ability to confer protection in intensive aquaculture systems; the protection of fish at susceptible developmental stages; protection against all circulating types of a viral pathogen; induction of long lasting immunity; safety; and ease of administration.

Since the early 1990s vaccination programs have been used in the aquaculture industry with promising results. Bacterial vaccines have proven to be easier to develop than

viral or parasitic vaccines. Some of the major bacterial diseases can now be controlled by vaccination programs.

Different types of vaccines have been developed for the fish farm industry (live, attenuated, recombinant protein). More recently, special attention has focussed on the development of DNA vaccines as a result of the success obtained with experimental VHSV and IHNV DNA vaccines in rainbow trout (97 % survival for VHSV and 77 % survival for IHNV) (Heppell *et al.* 1998, Corbeil *et al.* 2000, Lorenzen *et al.* 2000, LaPatra *et al.* 2001).

There are several bacterial vaccines commercially available or in development including: vibriosis (*Vibrio anguillarum*, *V.ordalii*, *V.salmonicida*, *V.vulnificus*); bacterial kidney disease (BKD) (*Renibacterium salmoninarum*); furunculosis (*Aeromonas salmonicida*); enteric redmouth disease (ERM) (*Yersinia ruckeri*); edwardsiellosis (*Edwardsiella ictaluri*); piscirickettiosis (*Piscirickettsia salmonis*); pasteurella (*Photobacterium damsela* subspecie *piscicida*); several *Flavobacterium* strains (Bernardet, 1997, Ellis, 1997, Kaattari and Piganelli, 1997, Romalde and Margariños, 1997, Smith *et al.* 1997, Stevenson, 1997, Toranzo *et al.* 1997, Thune *et al.* 1997, Marsden *et al.* 1998, Kanellos *et al.* 1999).

The development of efficacious viral vaccines has not lagged behind bacterial vaccine development. Some viral vaccines are currently available and others are in development including: infectious pancreatic necrosis (IPNV); spring viraemia of carp virus (SVC); grass carp haemorrhagic disease (GCHD); channel catfish virus disease (CCVD); several rhabdoviral diseases such as Hirame rhabdovirus (HIRRV), infectious hematopoietic necrosis (IHNV), viral hemorrhagic septicaemia virus (VHSV) (Heppell *et al.* 1995, Liao and Dobos, 1995, Lorenzo *et al.* 1995, Christie, 1997, Dixon, 1997, Winton, 1997, LaPatra *et al.* 2001, Roche and Gaudin, 2001, Mas *et al.* 2002, Perez *et al.* 2002, Ronen *et al.* 2003, Takano *et al.* 2004, Byon *et al.* 2005).

There are four basic types of vaccines - live attenuated, whole inactivated (killed), purified proteins produced from cloned genes and purified subunits (Leong *et al.* 1997).

Live attenuated vaccines are produced by attenuating viruses in serial passage in cell culture or by isolating naturally occurring variants (Winton, 1998). These vaccines generally offer excellent protection and are cost-effective, but concerns exist regarding residual virulence, loss of attenuation, or consequential effects on other aquatic species, and thus licensing live attenuated vaccines can be problematic (Winton, 1998). The motivation for developing synthetic vaccines was driven by the need to overcome the drawbacks associated with live attenuated vaccines (Arnon and van Regenmortel, 1992). Synthetic vaccines offer major advantages over the other type of vaccines because they are: inexpensive; easy to produce; easily modified when needed; and stable not requiring preservation or "cold-chain" supply. Multivalent vaccines can easily be produced by this means (Lorenzen, 1999, Heppell and Davis, 2000).

Fully synthetic vaccines have been demonstrated to induce protective B-cell and T-cell responses (Beck-Sickinger and Jung, 1993).

Synthetic vaccines can be divided into two main groups; a) non-expression based and b) expression based. DNA vaccines are expression based vaccines, with the protein of interest being expressed in immunised fish after an injection in the form of a DNA plasmid containing an expression construct. DNA vaccines offer the advantage of conservation of the native structure of the protein and can elicit humoral immune response (Heppell *et al.* 1998, Lorenzen *et al.* 2002). However, DNA vaccines have not gained public acceptance as they are perceived to be a product of genetic manipulation. Consequently, their use in the aquaculture industry may confer adverse economic effects.

Synthetic peptide based vaccines are an example of a non-expression based vaccine type. These are completely synthetic and thus relatively easy to licence. However there are also drawbacks with this type of vaccines. Firstly, they require the use of an adjuvant to increase their effectiveness, and secondly, knowledge of the antigenic structure of the target organism is required for their development (Winton, 1998). The use of synthetic peptides for immunisation of rainbow trout induced an antibody response against VHSV (Lorenzo *et al.* 1995).

Both DNA and peptide vaccine offer the advantage of safety with respect to reversion to virulence, which is a critical factor in relation to environmental safety in aquaculture (Gudding *et al.* 1999).

The potential advantages of synthetic vaccines has stimulated research aimed at an improved understanding of the molecular basis of the immunogenicity of proteins (Geysen *et al.* 1987b).

Usually protein antigens contain several unique molecular structures, each of which can elicit an immune response (Lydyard *et al.* 2000). Antibodies produced against an antigen are not directed against the whole molecule but against a specific region of the molecule (immunogenic region) (Lydyard *et al.* 2000).

The region of an antigen that is recognised by an antibody molecule is referred to as an *epitope* or an antigenic determinant (DeLisser, 1999, van Regenmortel, 2001). B-cell *epitope mapping* is the identification of the sites that are recognised by an antibody response to a defined antigen (Morris, 1996a).

Viral epitopes have been classified into four categories (van Regenmortel, 1989b, van Regenmortel, 1990a, Arnon and van Regenmortel, 1992, van Regenmortel, 1992): cryptotopes, neotopes, metatopes and neutralisation epitopes. Cryptotopes are epitopes that can only be recognised when native virus particles are fragmented or denatured. Neotopes are epitopes that are only present when the quaternary structure of virus proteins is intact. Neotopes commonly occur in viral capsids. Epitopes present in both dissociated and polymerised forms of the viral coat proteins are designated as metatopes. Neutralising epitopes represent those epitopes that are recognised by neutralising antibodies.

Neutralising epitopes are of great importance in the design of synthetic vaccines. Their identification can aid the rational design of vaccines by facilitating the induction of immune responses against relevant sites on pathogenic micro organisms (van Regenmortel, 1989b).

It is important to distinguish between antigenic and immunogenic epitopes. An antigenic epitope is the region of a protein molecule that an antibody can bind to, and an

immunogenic epitope is the region of a protein that can elicit an immune response (Geysen *et al.* 1984, Morris, 1996a). Immunogenicity is not solely a property of an antigen but is linked to features of the host immune system including B-cell and T-cell repertoires and the major histocompatibility complex (MHC) (Benjamin *et al.* 1984). Residues involved in the high-energy contacts between an antibody and its target epitope are called critical-binding residues (CBRs). Amino acid replacements at these sites usually reduce binding affinity greatly (Geysen *et al.* 1987b, Davies and Cohen, 1996). The CBRs located within a single contiguous polypeptide sequence are said to form a *linear epitope*, whereas CBRs comprised of residues from two or more distinct regions of a polypeptide that are brought together by protein folding form *conformational epitopes* (also referred to as *discontinuous epitopes*) (Barlow *et al.* 1986). It is now widely believed that the majority of the protein epitopes are discontinuous (van Regenmortel, 2001).

Structural studies (crystallography) reveal that 15-22 amino acids on the surface of a protein antigen make contact with a similar number of residues in an antibody binding site (Kuby, 1994). Contact involves ionic and hydrophobic interactions, and has between 75-120 hydrogen bonds (Kuby, 1994). The surface area of this large complementary interface is between 650-900 $Å^2$ (Kuby, 1994).

Functional studies of antibody binding peptides have led to the general consensus that most of the binding energy of the epitope-antibody interaction results from the binding of between three and eight amino acid residues (Geysen *et al.* 1987a, van Regenmortel, 1989b). Clearly, there is a difference in the number of residues implicated in binding identified by structural and functional studies. This is believed to be due to the presence of residues on the antigen-antibody interface whose main contribution to the binding complex is the formation of complementary surfaces that act as a scaffold. These residues are identified by structural studies, wheras only critical binding residues influence functional studies (Scott, 2001).

For the development of synthetic vaccines, efficient methods for epitope mapping are of considerable importance (Beck-Sickinger and Jung, 1993). Studies utilising monoclonal antibodies have been widely used to characterise viral epitopes (McCullough, 1986). The use

of monoclonal antibody based methods has allowed the delineation of antigenic structure at a level of precision not previously possible, because of their specificity for a single antigenic site (Benjamin *et al.* 1984).

Several approaches have been used to characterise epitopes using MAbs: ① competitive antibody binding; ② immunological screening of recombinant expression libraries of random cDNA fragments; ③ antibody binding to chemically synthesised overlapping peptides, or to fragments generated by proteolytic cleavage; ④ MAb binding to recombinant proteins using panels of deletion mutants or chimeric constructs composed of different species of the same molecule, or bacterially expressed fusion proteins or proteins generated by site-directed mutagenesis; ⑤ escape mutants (van Regenmortel, 1990a, Tzartos, 1996, DeLisser, 1999). Some of the methods used for localisation of viral epitopes are described in Table 1.3.

Table 1.3 – Methods used in localisation of viral epitopes (van Regenmortel, 1990a).

Epitope mapping method	***Type of epitope recognised***
X-ray crystallography of antigen-Mab complex	Discontinuous and linear epitope
Synthetic peptides as probes: free peptides peptides adsorbed to a solid-phase peptides conjugated to a carrier peptides attached to a support used for synthesis	Linear epitope
Use of fusion proteins and peptides: chimaeras prokaryotic expression vectors (e.g.phage display)	Linear epitope
Use of anti-peptide antibodies	Cross-reacting linear epitopes
Viral escape mutants with MAb	Neutralising epitopes, discontinuous epitopes
Topographic mapping by competitive MAb binding assay	Only relative position of epitope is defined

1.1.10.3.1 - Betanodavirus vaccines

Fish surviving betanodavirus infection have been found to produce neutralising antibodies, which is believed to explain their resistance to natural re-infection (Tanaka *et al.*

2001). This observation suggests that vaccination represents a logical and effective means of controlling VNN.

Intensive research efforts have been directed towards the development of a vaccine against betanodaviruses. The most successful approach to date has utilised recombinant nodavirus coat protein as a protective antigen. This type of vaccine successfully induced neutralising antibodies and reduced mortalities in experimentally challenged grouper (sevenband grouper, humpback grouper) (Tanaka *et al.* 2001, Yuasa *et al.* 2002), turbot and Atlantic halibut (Húsgarð *et al.* 2001, Sommerset *et al.* 2001). However, the survival rates indicate that the vaccine was not suitable for commercial use. For example Tanaka *et al.* (2001) obtained a relative percent survival (RPS) of 88 when fish were challenged with $10^{3.4}$ $TCID_{50}$/fish, but the RPS decreased to 35 when fish were challenged with $10^{4.4}$ $TCID_{50}$/fish. After vaccinating with recombinant protein and challenged the fish Yuasa *et al.* (2002) obtained a survival rate of 67.5 RPS. A similar RPS value (66) was obtained by Húsgarð *et al.* (2001) using as well a recombinant protein vaccine.

Initial work aimed at the development of DNA vaccines for use in fish suggest that this approach may be applicable to the control of VNN. Sommerset *et al.* (2005) developed an Atlantic halibut NNV (AHNNV) DNA vaccine. In protection trials, this vaccine was unable to induce an immune response and protect challenged fish. The analysis of immunised fish muscle demonstrated that transcription and translation of the construct had occured in vivo. However, a humoral immune response was not induced.

Rhabdovirus DNA vaccines can induce non-specific immune responses that confer protection against other viruses. Consequently, a DNA vaccine directed against the rhabdovirus VHSV has been investigated as a potential nodavirus vaccine. In challenge studies, this VHSV vaccine protected turbot against betanodavirus infection (Sommerset *et al.* 2003). The best results (100 RPS) were obtained when the fish were challenged 8 days post-vaccination. When the challenge was performed 35 days post-vaccination the RPS was 63. The encouraging results obtained after 8 days post-vaccination are a consequence of cross-protective anti-viral defence mechanisms that are not of long duration. The same non-specific

immune response was observed by Lorenzen *et al.* (2002) with rainbow trout vaccinated against VHSV or IHNV.

1.2 - Aim and objectives

In the past fifteen years, Betanodaviruses have emerged as major pathogens of marine fish, affecting many species of economic value to the aquaculture industry. Despite advances in our understanding of betanodaviruses, the development of effective vaccines to counter these viruses remains elusive. The betanodavirus vaccines that have been produced to date have not afforded a high degree of protection. The regions of the betanodavirus particle that serve as targets of protective immune responses are currently poorly characterised. This lack of knowledge represents a barrier to the rational development of betanodavirus vaccines. The identification of the targets of protective immune responses against fish nodaviruses could be used to inform future vaccine development programmes. It would be possible to direct immune responses towards protective epitopes through the use of vaccine constructs administered either alone, or in conjunction with adjuvants or other immunostimulants.

The goal of this thesis is to identify betanodavirus B-cell epitopes. The successful identification of these structures would represent a significant step towards the rational development of vaccines to counter VNN. The thesis is comprised of the following four specific objectives:

① To produce antibodies against betanodaviruses for use in epitope mapping studies. This work involves:

a) the production and characterisation of mouse monoclonal antibodies directed against betanodavirus using conventional hybridoma methodology. Monoclonal antibodies will be screened for antibody class/isotype and the ability to neutralise betanodavirus.

b) the production of rabbit polyclonal antibodies against betanodavirus.

c) the collection of serum samples from fish (European sea bass) naturally-infected with betanodavirus.

The antibodies obtained in this stage of the project will be used to identify betanodavirus epitopes using one or more of the epitope mapping procedures summarised

below in steps 2-4. In combination, these procedures permit the identification of both conformational and linear epitopes.

② To produce and characterise betanodavirus neutralisation escape mutants. Monoclonal antibodies will be used to select betanodaviruses resistant to *in-vitro* neutralisation. Nucleotide sequence analysis of the coat-protein gene of isolated escape mutants will permit identification of the sequence changes conferring neutralisation resistance.

③ To identify the recognition sites of betanodavirus monoclonal antibodies using a random peptide library displayed on the surface of a filamentous bacteriophage. Phage clones binding to monoclonal antibodies will be isolated using conventional panning procedures. Nucleotide sequence analysis of isolated phage clones will permit identification of the amino acid sequence recognised by monoclonal antibodies.

④ To identify the recognition sites of antibodies using the "Pepscan" procedure. A series of short overlapping synthetic peptides mimicking the betanodavirus coat protein will be used to map the recognition sites of antibodies directed against betanodavirus. The recently developed suspension array technology will be used to quantitate the interaction between antibodies and synthetic peptides. This procedure will be used to identify regions of the coat protein recognised by mouse monoclonal antibodies, rabbit polyclonal antibodies, and serum samples from fish naturally infected with nodavirus.

Chapter 2 - General methods

2.1 - Virology

2.1.1 - Cell culture

Betanodaviruses were cultured in an SSN-1 cell line. This cell line was produced from whole fry tissue of *Ophicephalus striatus* (striped snakehead) at the Institute of Aquaculture, and is infected with an endogenous type C retrovirus (Frerichs *et al.* 1991). The optimal growth temperature for SSN-1 is 28°C. No supplementation with CO_2 is required.

The SSN-1 cell line was maintained in Leibovitz's L-15 medium containing Glutamax-I (Gibco, Paisley, Scotland) supplemented with 10 % FBS (Foetal Bovine Serum, Gibco, Paisley, Scotland) (L-15/FBS).

SSN-1 cell monolayers were passaged every 4^{th} to 7^{th} day and were subcultured at a "split" ratio of between 1:3 and 1:4. This was chosen according to the degree of confluence of the monolayer and the anticipated cell growth period required for a given experiment

To passage SSN-1 cells, the growth medium was removed and cells were washed twice with Dulbecco's PBS without Ca and Mg (D-PBS, Gibco, Paisley, Scotland). Excess D-PBS was removed prior to the addition of Trypsin-EDTA (1x concentrated, Gibco, Paisley, Scotland). Cell monolayers were then incubated until their appearance became white and opaque. Trypsin-EDTA solution was then removed and cells detached by gently tapping the flask. One third of the final volume of fresh L-15/FBS was added, and cells were resuspended and aliquoted evenly into new cell culture flasks (Nunc, Fisher Scientific, Leicestershire U.K.) containing fresh medium.

The SSN-1 stock flasks were incubated at 28°C and split every 4-7 days.

Volumes of D-PBS, Trypsin-EDTA and L-15/FBS medium used for each size of growth vessel are shown in Table 2.1.

Table 2.1 - Volumes of Dulbecco's PBS, Trypsin-EDTA and L-15/FBS used for SSN-1 cell passage.

Flask (cm^2)	***Dulbecco's PBS (ml per wash)***	***Trypsin-EDTA (ml)***	***New flask (final volume in ml)***
25	4	1	5-6
75	10	2	18-20
175	15	4	40-50

2.1.2 - Virus culture

During this study four betanodavirus isolates were used. These viruses were grouped depending on the temperature that the isolate can be propagated, 25°C and 20°C, respectively. All the betanodavirus strains details are listed in Table 2.2.

Table 2.2 - Betanodavirus isolates used in this study.

Propagation	***Strain reference***	***Fish species***	***Country***
25°C	Mt/01/Sba	European sea bass (*Dicentrarchus labrax* L.)	Malta
	Jp/06/Sj	Striped jack (*Pseudocaranx dentex*)	Japan
20°C	GB/32/Cod	Atlantic cod (*Gadus morhua*)	United Kingdom
	GB/30/Hal	Halibut (*Hippoglossus hippoglossus*)	United Kingdom

2.1.2.1 - Inoculation of SSN-1 cell monolayers with betanodavirus

Betanodaviruses were propagated in either pre-formed SSN-1 monolayers or by simultaneous inoculation of SSN-1 cells. Both methods required highly confluent SSN-1 cell monolayers (typically seven days old).

For infection of pre-formed monolayers, SSN-1 cells were split and incubated until the monolayer reached the required degree of confluence. Temperature and period of incubation varied according to the betanodavirus isolate propagated. The growth medium was

then removed, and virus and sufficient L-15/FBS to cover the cell monolayer were added and permitted to adsorb. The volume of medium and virus used varied according to the size of the tissue culture flask used (see Table 2.3). After adsorption, L-15/FBS medium was added and cells were incubated at appropriate temperatures until an extensive cytopathic effect (CPE) was observed. Adsorption and incubation temperature and period used were according to the isolate under propagation. Viral supernatant was harvested and clarified by centrifugation for 15 mins at 1410 x *g* at 4°C (Eppendorf 5804R).

Virus preparations were stored at 4°C for periods up to 30 days. For longer periods the virus was stored at -20°C or -70°C.

For simultaneous inoculation SSN-1 cells were split and re-suspended in L-15/FBS as described in section 2.1.1. Virus was then added, and cells were incubated until an extensive CPE was evident. The amount of virus added was the same as used for infection of pre-formed monolayers (Table 2.3).

Table 2.3 – Volume of L15-FBS medium and virus used for infection of pre-formed monolayers.

Size of flask (cm^2)	***DMEM+ adsorption step (ml)***	***Virus (ml)***	***Final volume (ml)***
25	1	0.5	6
75	2	1.5	20
175	3	6.5	50

2.1.2.2 - Betanodavirus propagated at 25°C

Two different strains of betanodavirus, European sea bass and striped jack were used during this study. A growth temperature of 25°C was found to be optimal for culture of these isolates of betanodavirus. SSN-1 cells were maintained at 28°C for 6-7 days prior to infection. For simultaneous inoculation, cells were split at a ratio of 1:3 and re-suspended in L-15/FBS. Hanks' Balanced Salt Solution (HBSS, Gibco, Paisley, Scotland) was then added at a volume

necessary to decrease the FBS concentration to 5 % (v/v). Virus was added and mixed with the SSN-1 cells. Cells were incubated at 25°C until an extensive CPE was observed (typically 4 – 7 days).

For infection of pre-formed monolayers SSN-1 cells were split at a ratio of 1:3 and incubated at 28°C until monolayers attained 70 % of confluence (for growth of SJNNV) and 80 % (for growth of DlNNV). Virus was added and allowed to adsorb for 60 mins at 25°C. Sufficient L-15/FBS and HBSS were then added in order to achieve a final concentration of 5 % FBS. Cells were incubated at 25°C until an extensive CPE was observed (typically 4-7 days).

2.1.2.3 - Betanodavirus propagated at 20°C

During this study two isolates of betanodavirus that are propagated at lower temperatures were used – these were derived from Atlantic cod and Halibut. Optimal growth of these viruses was achieved at a growth temperature of 20°C and use of L-15/FBS. SSN-1 stock cells were kept at either 28°C or 22°C, depending of the availability of the incubators, the amount of cells needed for the experiments and the type of virus inoculation to be performed (pre-formed monolayer or simultaneous inoculation).

SSN-1 cells grown at 28°C were split at a ratio of 1:3 after 6-7 days growth and incubated overnight at 25°C. Cell monolayers exhibiting > 75 % confluence were then incubated at 22°C. Cells were used within 24 hours for infection experiments, or maintained at 22°C for up to 10 days for use as stock cells.

SSN-1 cells maintained at 22°C required a different treatment. These cells were incubated for 7-10 days at 22°C. After this period the cells were split at a ratio of 1:2 and either used immediately or incubated for up to 10 days as stock cells.

Cells used for infection of Atlantic cod and halibut betanodavirus by simultaneous inoculation were maintained at 22°C. These cells were split at a ratio of 1:2, infected with betanodavirus and incubated until extensive CPE was observed.

Infection of pre-formed SSN-1 monolayers was performed with the stock cells grown at 22°C or 28°C. Cells were infected when monolayers were > 85 % confluent. Virus was allowed to adsorb for 6 h at 20°C. L-15/FBS medium was then added and monolayers were incubated for 6-8 days until an extensive CPE was evident.

2.1.3 - Virus quantification

The concentration of the virus was determined by calculating the virus titre (number of infectious units per unit volume) by $TCID_{50}$.

The dilution of virus required to infect 50 % of inoculated cell cultures is defined as $TCID_{50}$, and relies on the presence and detection of cytocidal virus particles (Burleson *et al.* 1992).

Infectivity titrations were performed in flat bottom 96 well plates (Nunc, Fisher Scientific Leicestershire U.K.) by adding 90 µl of diluent to each well. HBSS was used as diluent for warm-water betanodavirus isolates and L-15/FBS for cold-water isolates. Virus (10 µl) or diluent (negative control) was added to the first well of each row. Virus samples were diluted ten-fold across the plate. Each individual dilution of virus was made with a new pipette tip to prevent carry-over. One 25 cm^2 flask of highly confluent SSN-1 cells was harvested for each 96 well plate with betanodaviruses propagated at 25°C. For betanodavirus strains propagated at 20°C isolates from two 75 cm^2 flasks of SSN-1 cells were used to prepare three 96 well plates. Cells (100 µl) were added to each well of titration plates. These were sealed with Nescofilm (Bando Chemical Ind. Ltd, Japan, Fisher Scientific Leicestershire U.K.) and incubated at the appropriate temperature for 7 days, when monolayers were visually

assessed for cytopathic effects. Virus titres were calculated by the method of Spearman-Karber (Hierholzer and Killington, 1996).

$$\text{Spearman-Karber formula: Mean log TCID}_{50} = X + \frac{1}{2} \times d - d \times \Sigma\left(\frac{r}{n}\right)$$

Where, X = log of the highest reciprocal dilution.
d = log of the dilution interval.
r = number of test subjects not infected at any dilution.
n = number of test subjects inoculated at any dilution.

2.1.4 - Virus purification

A Beckman L-80 ultracentrifuge was used for ultracentrifugation of betanodavirus preparations. Two sizes of centrifuge tubes (Ultra-Clear™ tubes, Beckman, High Wymcombe U.K.) were used, depending on the type of rotor used. For clarification an SW28 rotor was used together with 38.5 ml centrifuge tubes. The pelleting step and purification by caesium chloride (CsCl) gradient centrifugation were performed in an SW41Ti rotor together with 13.2 ml centrifuge tubes.

Centrifuge tubes were disinfected prior to use with 70 % ethanol (Fisher Scientific, Leicestershire U.K.).

2.1.4.1 - High speed clarification

The first stage of the purification procedure was a high-speed clarification step performed using a SW28 rotor. Disinfected and dry centrifuge tubes were placed on a scale and tared. Virus preparation (36 g) was added to each centrifuge tube, which was balanced prior to centrifugation. Tubes were centrifuged at 12 000 x *g* at 4°C for 35 mins. Virus

supernatants were collected in a clean flask. The virus supernatant (≈ 2 ml) near the bottom of the tube was discarded.

2.1.4.2 - Pelleting

Virus pelleting was performed using an SW41Ti rotor. High-speed clarified virus supernatant (12 g) was added to disinfected centrifuge tubes. The weight of tubes was adjusted to 12 g when necessary with HBSS. Tubes were cenrifuge at 100 000 x *g*, at 4°C for 95 mins. Supernatant was discarded, more high-speed clarified virus supernatant was added and spun. The procedure was repeated 3 or 4 times.

After the repeated pelleted centrifugations the tubes were inverted. After the majority of the supernatant had drained, 50 µl of TNE buffer (RNase free pH 7.4, Appendix 1) was added to the pellet and mixed well. Tubes were sealed with Nescofilm and incubated for 20 mins at 4°C. Supernatants were collected into a cryovial. A further 50 µl of TNE buffer was then added to tubes, which were then and incubated for 20 mins at room temperature. Supernatants were collected and stored at -20°C. For logistical reasons, tubes were on occasion harvested using an alternative method. TNE buffer (100 µl) was added to centrifuge tubes, which were then seased with Nescofilm and stored overnight at 4°C. Virus was then harvested as described above.

An average of 500 ml of virus supernatant was pelleted and resuspended in 1 ml of TNE buffer.

2.1.4.3 - Purification using caesium chloride

The use of isopycnic gradient centrifugation with a high density and low viscosity medium like caesium chloride for the purification of non-enveloped viruses is of great

advantage for obtaining a purer product and relatively high volumes of sample can be processed in each gradient (Easton *et al.* 2000).

2.1.4.3.1 - Caesium chloride gradient

A 50 % w/w stock of caesium chloride in TNE buffer (pH7.4, RNase free) was used to prepare CsCl gradients. This stock solution was autoclaved. All glassware was RNase-free treated with DEPC (Appendix 1).

A capillary tube was rinsed with RNase-free water. Solutions of 40 %, 35 %, 30 %, 25 % and 20 % w/v caesium chloride were prepared by diluting the stock solution in TNE buffer. The refractive index (RI) of each solution was measured using a refractometer (Bellingham & Stanley ABBE60).

Syringes (5 ml, Terumo Merseyside U.K.) were filled with different amounts of caesium chloride solutions: 1.5 ml of 20 %, 2.5 ml of 25 %, 2.5 ml of 30 %, 2.5 ml of 35 % and 2 ml of 40 %.

The capillary tube was placed adjacent to the bottom of the centrifuge tube and held in place with tape. A needle (25G) was attached to the other end of the tube (Figure 2.1 ①).

The caesium chloride solutions were added in sequence from the lowest to the highest concentration. Each solution was added very slowly to avoid the formation of bubbles. Care was taken not to disturb the CsCl solutions to ensure that the gradient formed efficiently.

With this method it was very difficult to avoid the formation of bubbles and the injection of air bubbles into the gradient. In order to avoid such problems an isopycnic gradient maker was used to make the caesium chloride gradient.

The gradient maker contains two chambers connected by a tap (Figure 2.1 ②). One of the chambers is joined to the centrifuge tube by an outlet tube. This chamber was filled with 7.25 ml of 20 % caesium chloride. The other chamber was filled with 6.75 ml of 40 % caesium chloride.

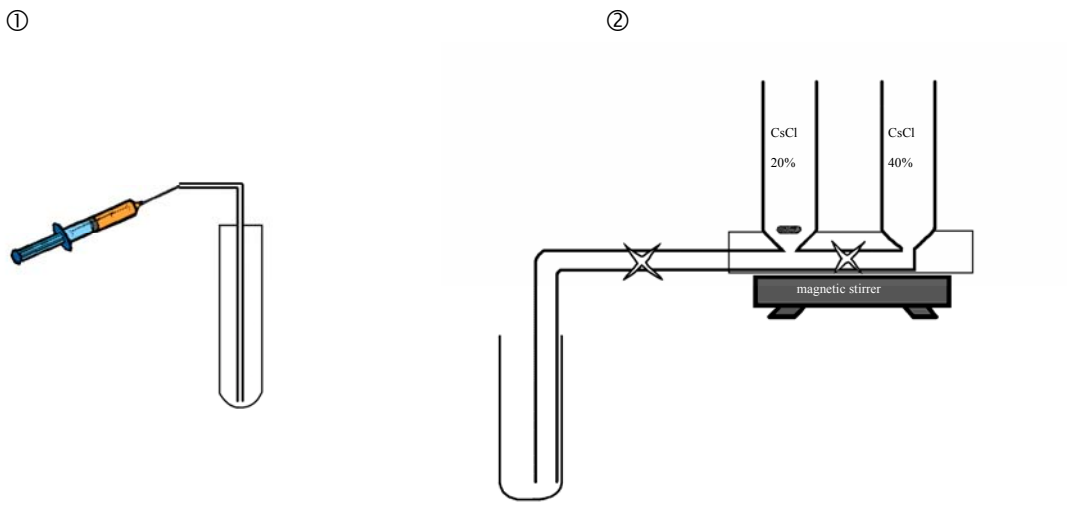


Figure 2.1- ① CsCl gradient by syringe method; ② CsCl gradient maker using a column maker.

The gradient maker was placed over a stirring plate and a magnetic flea was placed in the chamber with the outlet tube (Minor, 1999).

The tap of the outlet tube was opened and 1 ml of solution allowed to drain before the tap linking both chambers was opened. This allows the CsCl solution to mix. The gradient becomes progressively denser as the CsCl solutions mix and drain.

Betanodavirus in TNE buffer (1 ml) was added to gradients prepared by either of the methods described above.

Gradients were centrifuged in an SW41Ti rotor 150 000 x *g*, for 17 hours at 4°C.

2.1.4.3.2 - Harvesting purified virus

To harvest virus samples the centrifuge tube was held in the fraction collector (Fraction Recovery System 270-331580, Beckman). Because SSN-1 cells are endogenously infected with a type C retrovirus (Frerichs *et al.* 1991) two bands are visible in CsCl gradients of betanodaviruses grown in these cells. The upper band is composed of retrovirus particles and the lower band of betanodavirus particles. In runs where these bands were visible, the

centrifuge tube was pierced with a 25G needle immediately below the band corresponding to betanodavirus particles. A 5 ml syringe was used to collect between 750-1000 µl of sample.

When the bands were not visible fractions of the gradient were collected using a fraction collector. Prior to use, this collector was thoroughly rinsed with RNase-free water. Centrifuge tubes containing CsCl gradients were placed into the fraction collector holder and the top of the centrifuge tube was then closed to create a vacuum. The bottom of the tube was pierced, and the vacuum valve was used to control the release of 40 drop fractions which were stored at 4°C after collection. The RI of each fraction was measured using a refractometer. Betanodavirus particles were obtained from fractions with a RI between 1.365 and 1.355.

The presence of betanodavirus in harvested bands or fractions was confirmed by titration in SSN-1 cells as described in section 2.1.3. CsCl was removed from these samples by re-centrifugation through TNE buffer at 100 000 x *g* for 1 h and 35 mins at 4°C. The resulting pellet was re-suspended in TNE buffer and stored at -20°C.

2.2 - Monoclonal antibodies

2.2.1 - Culture of cell lines

Myeloma cells (SP2/0, Sigma, Dorset U.K.) and hybridomas were cultured in DMEM+ [(Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Dorset U.K.) supplemented with 5 ml sodium pyruvate (100 mM) (Sigma, Dorset U.K.), 5 ml L-glutamine (200 mM) (Sigma, Dorset U.K.), 2.5 ml streptomycin/penicillin (10000 units penicillin in 20 ml; 10 mg ml^{-1} streptomycin) (Sigma, Dorset U.K.), and 10 % FBS (Sigma, Dorset U.K.)] at 37° C with 5 % CO_2 supplementation.

The SP2 cells grew at a faster rate than the hybridoma cells and consequently needed to be split every 3-4 days. Hybridoma cells were passaged at weekly intervals.

2.2.2 - Storage in liquid nitrogen

Cells were cultured in 24 well plates (Nunc, Fisher Scientific, Leicestershire U.K.). The cells from two wells were pooled and centrifuged at 150 x *g* for 7 mins (Wifug 500E). The supernatant was discarded and the pellet re-suspended by gently flicking.

DMEM+ (1.5 ml) containing DMSO (10 % v/v, Sigma, Dorset U.K.) was added and mixed smoothly. The content of each tube was then aliquoted into two cryovials, which were immediately wrapped in bubble wrap and transferred to a -70°C freezer. The following day the cryovials were stored in liquid nitrogen.

2.2.2.1 - Thawing cells

To thaw cells 9 ml of DMEM with additives (5 ml sodium pyruvate (100 mM), 5 ml L-glutamine (200 mM), 2.5 ml streptomycin/penicillin (10000 units penicillin in 20 ml; 10 mg ml^{-1} streptomycin)), were placed into a 15 ml centrifuge tube (Sterilin, Scientific Laboratories Supplies, Lanarkshire, Scotland). To the bottom of the centrifuge tube 1 ml of FBS was added very carefully in order not to mix with the DMEM and 2 layers of solution could be observed.

Cryovials were placed into a 37°C waterbath (Techne UB-8) until their contents thawed. Cells were carefully added to the top of the medium and centrifuged 150 x *g* for 7 mins. The medium was then discarded and the cell pellet resuspended by gently flicking the bottom of the tube. Myeloma cells were resuspended in 10 ml of medium and transferred to a 25 cm^3 flask (Nunc, Fisher Scientific Leicestershire U.K.). Hybridoma cells were resuspended in 1 ml of DMEM+ and transferred to a single well of a 24 well plate.

2.2.3 - Concentration of monoclonal antibodies

The concentration of MAbs was achieved by using 20 ml Vivaspin (VivaScience, Epsom U.K.) columns. These columns have a membrane with molecular weight cut off of 10 000. The MAbs samples were spun at 3 000 x *g* at 4°C.

2.2.4 - Purification of monoclonal antibodies

Monoclonal antibodies were purified with pre-packed Protein-G columns (Hi Trap Protein G HP, Amersham, Bucks U.K.). These columns were purchased pre-packed with

protein G Sepharose™. This protein is used for purification of IgG antibodies and all the MAbs purified are IgG.

Hybridoma supernatant (50 ml) was filtered twice through a 0.45 μm filter (Sartorius, Surrey U.K.) and then diluted 1:1 in binding buffer (Appendix 1). Buffers used were freshly prepared and filtered though a 0.45 μm filter (Appendix 1).

A liquid chromatography system (Econo System of Bio-Rad, composed of a pump, UV monitor, system controller and 2110 fraction collector) was used for purification of monoclonal antibodies. Prior to use by passing approximately 20 ml of distilled water through the tubing system the liquid chromatography system was cleaned. Binding buffer was run through the system prior to adding the purification columns.

The Hi Trap column was placed in the Econo System and washed with binding buffer (~10 ml). After washing, the MAb sample was added and run through the column. The sample was added carefully to prevent the formation of bubbles. The column was washed with binding buffer until all unbound material was removed as assessed by UV spectrophotometric analysis of the effluent.

Bound antibodies were eluted with Glycine-HCl (0.1 M, pH 2.7, Appendix 1) and collected using a fraction collector (Econo System - 2110 fraction collector, Bio-Rad). The low pH of the elution buffer promotes the disruption of the covalent bonds between the antibodies and the protein G Sepharose. Collection tubes were prepared by the addition of 130 μl of Tris-HCl (1 M, pH 9, Appendix 1). This buffer raises the pH of the antibody solution. Thirty drops of column eluante were collected per tube. The elution step was performed until no unbound material was present in the effluent as assessed by UV spectrophotometric analysis of the column eluante.

Columns were cleaned with 20 % v/v ethanol, and stored in the same solution at 4°C. All the procedures performed with the column were at a speed of 1 ml per minute, the maximum recommended for the column by the manufactures.

The liquid chromatography system was washed with distilled water (~50 ml) after use.

Spectrophotometric analysis (Cecil CE 2021) at a wavelength of 280 nm was performed to identify the fractions containing monoclonal antibodies.

2.3 - Determination of protein concentration

The protein concentration of purified betanodavirus samples was determined with either the Bio-Rad protein assay kit (Bio-Rad, Hertfordshire U.K.) or the BCA protein assay (Pierce, Northumberland U.K.). The protein concentration of purified monoclonal antibody samples was determined using the BCA protein assay.

2.3.1 - Bio-Rad protein assay kit

The Bio-Rad's protein assay is based on the Bradford method. The absorbance of acidic Coomassie Blue Brilliant G-250 shifts from 465 to 595 nm when bound to protein.

A 250 μg ml^{-1} stock solution of BSA (bovine serum albumine, Sigma, Dorset U.K.) in TNE buffer (pH 7.6) was used to prepare a set of standards with concentrations ranging from 2.5 and 25 μg ml^{-1}. Virus samples were diluted in the same buffer.

The dye reagent was filtered through Whatman (Fisher Scientific, Leicestershire U.K.) number 1 filter paper prior to use.

The standard or virus sample (800 μl) were mixed with 200 μl of dye reagent and vortexed gently. After 35 mins incubation at room temperature the absorbance of samples/standards was determined at a wavelength of 595 nm (Cecil CE 2041). A standard curve was constructed from the absorbance values of the BSA standards, and this was used to calculate the protein concentration of virus samples.

2.3.2 - BCA protein assay kit

The BCA protein assay reagent kit (Pierce) is based on the application of bicinchoninic acid (BCA) for colorimetric detection and quantification of total proteins.

The procedure could detect input concentrations of protein ranging from 20-2000 μg ml^{-1}. Samples and detection reagent were mixed (1:8 ratio) in 96 well plates. The detection reagent was prepared by the addition of 50 parts reagent A to 1 part of reagent B.

A series of protein standards was prepared by dilution of albumin standard (Pierce, Northumberland. U.K.) in Glycine-HCl (for analysis of Monoclonal antibodies) or TNE (for betanodavirus samples).

Twenty five μl of sample or standard were mixed with 200 μl of detection reagent and mixed gently with a pipette tip. The samples were microwaved for 30 seconds at 850 V and allowed to stand for 10 mins at room temperature. The absorbance of samples was then read in an ELISA plate reader (Dynex Technologies MRXII) at a wavelength of 540 nm. A standard curve was constructed from the absorbance values of the protein standards, and this was used to calculate the protein concentration of virus or monoclonal antibody samples.

2.4 - ELISA

ELISA plates (Immulon® 4 HBS flat bottom, Thermo Labsystems, Middlesex U.K.) were coated with 50 µl well^{-1} of 0.01 % poly-L-lysine (Sigma, Dorset U.K.) and left to adsorb for 60 mins at room temperature. The plates were washed 3 times with LSWB (Low Salt Washing Buffer, Appendix 1), 100 µl well^{-1} of antigen was added and incubated at 4°C overnight.

Virus supernatant, cell line supernatant and bacteria (suspension in PBS) were used as antigens for coating the ELISA plates.

The following morning, 50 µl well^{-1} of 0.05 % gluteraldehyde (Sigma, Dorset U.K.) (Appendix 1) was added and incubated for 20 mins at room temperature. Plates were washed 3 times with LSWB and 250 µl well^{-1} of blocking buffer (Appendix 1) was added and left to block for 2 hours at room temperature. The washing procedure was repeated.

Antibodies (mouse and rabbit sera, MAbs or polyclonal antibodies) were added (100 µl well^{-1}) and incubated for 90 mins at room temperature. Polyclonal antibody was used as a positive control and antibody buffer (Appendix 1) as a negative control. When needed, the antibodies were diluted in antibody buffer.

After this incubation the wells were washed 5 times with HSWB (High Salt Washing Buffer) and soaked for 5 mins in the last wash.

Either 100 µl well^{-1} of anti-mouse IgG conjugated with HRP (Diagnostics Scotland, Edinburgh Scotland) or anti-rabbit IgG conjugated with HRP (Sigma, Dorset U.K.) was added. The antibody conjugated with HRP was diluted 1:1000 v/v in conjugated buffer (Appendix 1) and incubated for 60 mins at room temperature. The washing procedure with HSWB was repeated.

Substrate (100 µl well^{-1}, Appendix 1) was added to each well and incubated for 10 mins at room temperature. After this period 50 µl well^{-1} of stop solution (Appendix 1) was added.

The ELISA assay was read at 450 nm in ELISA Reader (Dynex Technologies MRXII).

The average of the negative control was calculated. A sample was considered positive when its value was 3 times higher than the negative control average.

2.5 - Molecular biology

2.5.1 - Nucleotide Sequencing

2.5.1.1 - Cycle-Sequencing PCR

The DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Bucks U.K.) was used for nucleotide sequencing.

The polymerase chain reaction (PCR) procedure was performed according to the manufacturer's instruction. The premixed sequencing reagent was diluted 1:4 by addition of sequencing reagent buffer (Appendix 1).

The PCR reaction solution was prepared by mixing 1 µl of primer (5 pmol), 2 µl sequencing reagent premix, 6 µl sequencing reagent buffer and 11 µl sample. The DNA concentration was adjusted to between 0.1-0.2 pmol in nuclease-free water (Promega, Southamptan U.K.). The sequencing mix was briefly centrifuged (Heraus biofuge pico) prior to thermal cycling, which was performed in a Biometra Tgradient thermocycler.

Cycling parameters were 25 cycles of:

94°C, 10 seconds;
50°C, 20 seconds;
60°C, 1 minute.

Samples were stored at at 4°C or -20° C according to the availability of an automated nucleotide sequencer

2.5.1.2 - Precipitation of sequencing-PCR reaction products

Precipitation solution (52 µl, Appendix 1) was added to PCR reaction products which were vortexed briefly and incubated on ice for 5 mins. The tubes were then centrifuged at 13 793 × *g* (Denville Scientific Inc. Micro 240A) for 20 mins at room temperature and the supernatant discarded. The pellet was washed with 190 µl of 70 % ethanol, then samples were

centrifuged at 13 793 × *g* for 5 mins at room temperature. The supernatant was discarded and samples were dried prior to addition of 2 µl of Formamide Loading dye (supplied with DYEnamic ET Terminator Cycle Sequencing Kit, Amersham, Bucks U. K.).

Samples were stored at -20°C or analysed immediately.

2.5.1.3 - Sequencing

Sequencing reactions were analysed on an ABI Prism™ 377 sequencer using 0.2 mm acrylamide gels of 36 cm length.

Plates were washed with distilled water and allowed to air dry, then placed into the caster. A 0.2 mm thick spacers were placed between the 2 plates, aligned with the plates and clamped into the supporter.

The acrylamide sequencing gel was prepared (Appendix 1), a 50 ml syringe was filled with the gel mix and immediately injected between the plates. This procedure was performed very carefully to prevent the formation of bubbles. The caster comb was then positioned on top of the gel, which was allowed to polymerise for 2 hours at room temperature.

After this period the comb was removed and the upper surface of the gel was cleaned taking care to remove any gel debris. The area of the sequencing plates that are laser-scanned was cleaned with distilled water.

Samples were loaded either manually or by the Ficoll method as explained below.

Sequencing gels were electrophoresed at 3000 V, 50 mA, 150 W for 7 hours at 51°C. The power of the laser was 40 mW. ABI Prism® sequencing analysis software version 3.4.1 (Applied Biosystems) was used to analyse electropherograms. Nucleotide sequences were compiled with BioEdit Sequence Alignment Editor software (Hall, 1999). The samples were analysed visually in order to establish if the sequence data could be further analysed or if the sample needed to be repeated. A sample was considered for further analysis when the graph

produced automatically by the program revealed clear peaks for each nucleotide. When some background occurred the reading was analysed and sequenced again if necessary.

2.5.1.3.1 - Manual loading method

For manual loading a shark tooth comb (PE Biosystems, Cheshire U.K.) (48 or 62 wells) was inserted into the gel to a maximum depth of 1mm.

Sequencing gels were loaded into the sequencer and a plate check performed. The heating plate and the upper and lower buffer chambers were then clamped in position. The buffer chambers were filled with 1x TBE buffer (Appendix 1) and a pre-run was performed. When the gel reached a temperature of 48°C, the pre-run was stopped and the odd-numbered samples (0.8-1.3 µl) were loaded with Miniflex 0.2 mm tips (Sorenson™, Anachem, Bedfordshire U.K.). The gel was then run for 3-4 mins at 3 000 Volts and stopped before the even-numbered samples were loaded. When sample loading was complete, the gel was electrophoresed at 3 000 Volts for 7 hours.

2.5.1.3.2 - Ficoll loading method

Plates were cleaned, loaded into the sequencer, and a plate check performed as described above. The lower buffer chamber and heating plate were positioned, and the chamber filled with 1x TBE buffer. A pre-run was performed until the gel reached a temperature of 51°C. To the top of the gel plate 400 µl of Ficoll loading buffer (Appendix 1) was added using 1 ml syringe with a 25G needle taking care to avoid the formation of bubbles.

A loading tray was cooled down to 4°C and 0.8 µl of sample was added to the loading tray wells. Carefully a membrane comb (The Gel Company, Web Scientic Ltd, Cheshire

U.K.) was inserted into loading tray wells and allowed to absorb the samples for 10 seconds. The membrane was removed and immediately inserted into the sequencing gel. During this procedure the teeth of the comb slightly contacted the gel surface.

The upper buffer chamber was positioned and filled with 1x TBE buffer. The gel was electrophoresed at 3000 Volts for 90 seconds, the membrane comb was removed and the gel rinsed thoroughly with 5 ml of TBE to remove the Ficoll solution. Electrophoresis was then continued for 7 hours.

Chapter 3 - Production of monoclonal and polyclonal antibodies against betanodavirus

3.1 - Introduction

Immunity is defined as resistance to disease, especially infectious diseases. The collection of cells, tissues and organs involved in this resistance is the immune system, and the coordinated reaction of these cells and molecules to infectious microbes is the immune response (Abbas and Lichtman, 2001). The protective action of the immune system can range from simple biochemical and cellular defence mechanisms in invertebrates such as sponges and worms, to very complex networks of immune cells and molecules found in vertebrates like mammals and birds (Davey, 1989).

The host defence mechanism consists of innate immunity and adaptive immunity. The first line of immune defence is the former being mainly responsible for the first stage of expulsion of microbes and it may provoke inflammation (Lydyard *et al.* 2000). The principal components of innate immunity are: ① physical and chemical barriers, such as epithelial and antimicrobial substances produced at epithelial surfaces; ② phagocytic cells (neutrophils and macrophages) and natural killer (NK) cells; ③ blood proteins, including components of the complement system and other mediators of inflammation; ④ proteins called cytokines such as IL-1 and TNFα that regulate and coordinate many of the activities of the cells involved in innate immunity (Abbas *et al.* 2000, Roitt and Rabson, 2000). The main characteristics of this type of immune response are the fact that is rapid, has some specificity and no memory (Lydyard *et al.* 2000).

The second line of immune defence is adaptive immunity, comprising humoral and cell-mediated immunity. The former provides defence against extracellular microbes and is mediated by antibodies (Ab), which are produced by B-lymphocytes (Golub, 1981). The B-cells have a high diversity, with the potential to generate ~ 10^8-10^{10} different antigen binding

antibodies (Kuby, 1994). The B-cell population matures under the influence of bone marrow and/or gut-associated tissue in mammals (Lydyard *et al.* 2000). Antibodies do not have access to intracellular microbes thus cell-mediated responses involving T-lymphocytes come into play (Abbas and Lichtman, 2001). The T-cells mature under the influence of the thymus (Bier *et al.* 1986). All humoral and cell mediated immune responses to foreign antigens have a number of fundamental properties (specificity, diversity, memory, specialization, self-limitation and non-reactivity to self) that reflect the properties of the lymphocytes that mediate these responses (Table 3.1) (Abbas *et al.* 2000).

Table 3.1 – Properties of the adaptive immune system (Abbas *et al.* 2000).

Feature	***Function significance for immunity***
Specificity	Guarantees that distinct microbes elicit specific responses
Diversity	Allow the immune system to respond to a large variety of microbes
Memory	Leads to enhanced responses to repeated exposures to the same microbe
Specialization	Generates responses that are optimal for defence against different types of microbes
Self-limitation	Allows immune system to respond to newly encountered microbes
Non-reactivity to self	Prevents injury to the host during response to microbes

The adaptive immune response consists of sequential phases, each of them corresponding to a particular reaction of the lymphocytes and other components of the immune system (Figure 3.1).

When a pathogen infects a host for the first time a primary response will develop within 4-5 days (Lydyard *et al.* 2000). The response to this first exposure to an antigen is mediated by naïve lymphocytes and is called the recognition phase (Abbas and Lichtman, 2001). For B-cells this response results in IgM being secreted initially and often followed by IgG (Kuby, 1994). Interaction of B-cells with antigen and the appropriate costimulatory signals leads to B-cell activation (activation phase) (Welsh *et al.* 2004). The B-cells are able to bind to both continuous and discontinuous sequences of amino acids, whereas the T-cell

recognises linear processed peptide epitopes bound to the major histocompatibility complexes (MHC) on molecules (Atabani, 2001).

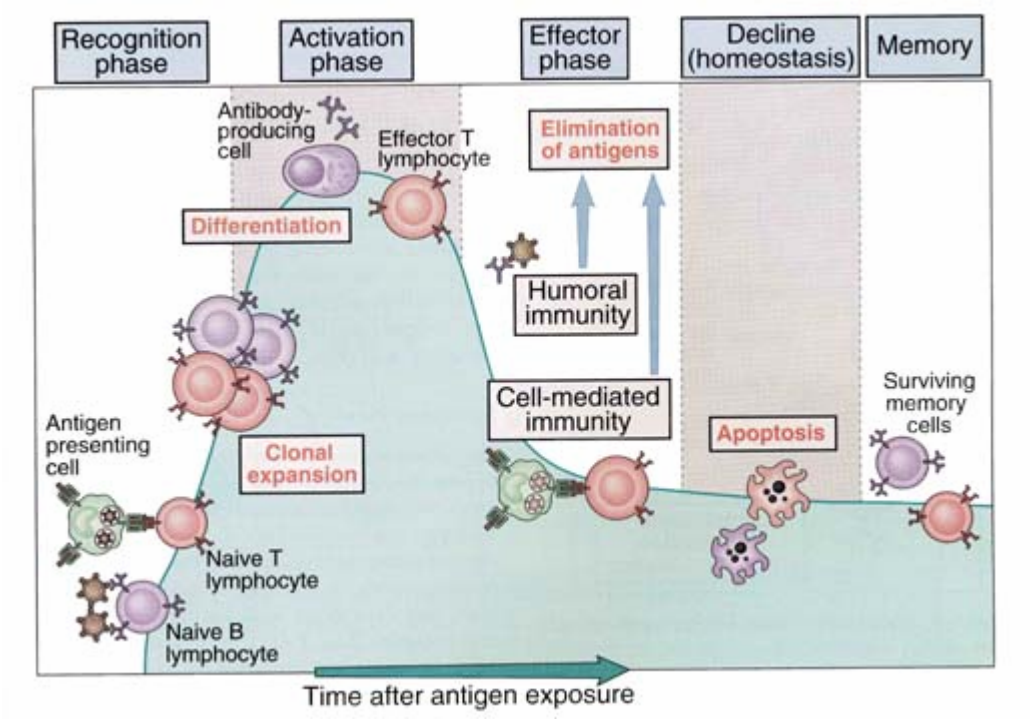


Figure 3.1 – Sequential phases of the adaptive immune response (Abbas and Lichtman, 2001).

The activated B-cells proliferate and differentiate either into memory cells or plasma cells also known as effectors (effector phase) (Davey, 1989). The plasma cells may produce IgM or may undergo isotype switching and secrete IgG or IgA (Welsh *et al.* 2004). When the antigen has been removed the antibody response will reach its peak; followed by a decrease in Ab concentration in the circulation (decline phase) as a result of the normal rate of catabolism of the antibody by the memory cells (memory phase) (Lydyard *et al.* 2000, Abbas and Lichtman, 2001). Subsequent encounters with the same antigen lead to immune responses that are usually faster, with higher affinity, reaching a greater magnitude and lasting longer (Kuby, 1994). The characteristics of this secondary responses are the result of activation of memory lymphocytes, which are long-lived cells that were induced during the primary immune

response (Abbas and Lichtman, 2001) (Figure 3.2). Within humoral immunity this secondary response usually corresponds to the IgG class (Lydyard *et al.* 2000).

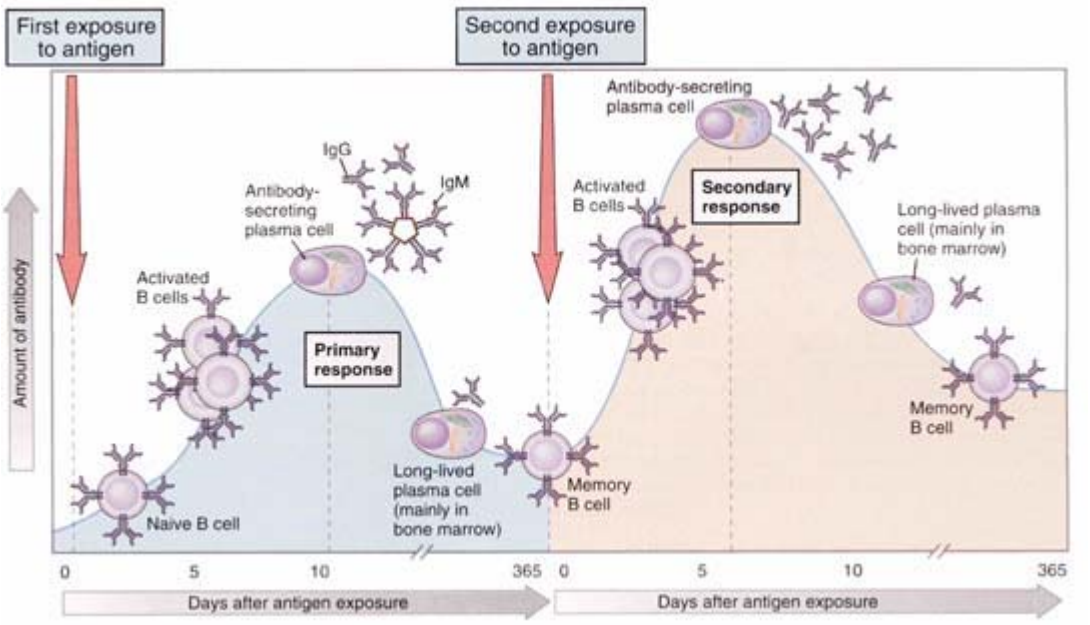


Figure 3.2 – Primary and secondary humoral immune response (Abbas *et al.* 2000).

Viruses may be extracellular before they enter host cells, or in the case of cytopathic viruses, when they are released from lysed infected cells (Abbas *et al.* 2000). Natural immunity to viral infections is associated with interferon type I (IFN) and NK cells. The interferons have a protective capacity before the virus infects a cell, by inducing resistance to viral multiplication (Roitt *et al.* 1985). Humoral immunity against viral infections is mediated by antibodies which block virus binding and entry into host cells (Abbas *et al.* 2000). Once a viral infection is established, cell mediated immune mechanisms are most important, with $CD8^+$ cytotoxic T-cells and $CD4^+$ T_H1 cells being the main components involved (Kuby, 1994). Both types of adaptive immunity interact with each other, with B-cells presenting peptide epitopes to $CD4^+$ T-cells, and T-cells producing cytokines and other immunomodulators that provide help for antibody production and induction of memory B-cells (Atabani, 2001).

Anti-viral antibodies are essential tools in the study of viruses and virus host interactions. B-cells can recognise a variety of viral proteins and glycoproteins, including components of the envelope and interior components of the nucleocapsid, which may be released from infected host-cells prior to complete viral assembly (Kuby, 1994).

Viruses are often antigenically complex and one of the major problems associated with the use of antiviral antisera has been the difficulty of producing antisera of unequivocally defined specificity for individual viral components (Yewdell and Gerhard, 1981). The problems associated with polyspecificity of antisera are largely avoided by the use of monoclonal antibodies (MAbs). A monoclonal antibody is an antibody with a unique specificity derived from a single B-cell clone (Nelson *et al.* 2000). The production of monoclonal antibodies was achieved for the first time 30 years a go by Köhler and Milstein (1975). In 1986 these scientists were awarded the Nobel Prize for their contribution for the production of monoclonal antibodies. This award reflects the importance that this technique has had in the investigation, diagnosis and even treatment of diseases.

Monoclonal antibodies against fish viruses have been developed against a variety of fish viruses including infectious haematopoietic necrosis virus (IHNV) (Schultz *et al.* 1985, Ristow and Arnzen, 1989), infectious pancreatic necrosis virus (IPNV) (Wolski *et al.* 1986), viral haemorrhagic septicaemia virus (VHSV) (Mourton *et al.* 1991), salmon pancreas disease virus (SPDV) (Todd *et al.* 2001), grouper iridovirus (Shi *et al.* 2003) and infectious salmon anaemia virus (ISAV) (Falk *et al.* 1998). The development of MAbs against nodavirus was already achieved by Nishizawa *et al.* (1995b) with the production of MAbs against striped jack betanodavirus strain (SJNNV). Lai *et al.* (2001a) also successfully obtained MAbs against yellow grouper betanodavirus strain (YGNNV).

The exquisite specificity of MAbs is the main reason why they are so often considered superior to polyclonal antibodies (Peppard, 2000). Monoclonal antibodies have replaced polyclonal antibodies in most large-scale serology. In the basic science laboratory, however where smaller amounts of antibodies are required, conventional polyclonal antisera may still be preferable for many purposes (Yelton and Scharff, 1981). It usually takes four to six

months to generate a stable hybridoma cell line, if all goes well, whereas producing small amounts of antiserum requires less time, energy and expense. If an antigen does not yield a good antiserum it is often difficult to generate monoclonal antibodies to it (Yelton and Scharff, 1981). Any given MAb may be very sensitive to physical conditions such as pH and temperature, which may change its reactivity and functional activity (Mosmann *et al.* 1980). Monoclonal antibodies have only a subset of the properties of a conventional antiserum, and therefore it is unlikely that antibody produced by just one hybridoma cell line can fulfil all requirements for all assays (Yelton and Scharff, 1981).

The recognition of multiple determinants limits antigenic analysis with polyspecific antisera and precludes characterization on individual antigenic sites of a protein (Yewdell and Gerhard, 1981). On the other hand, the use of monoclonal antibodies has allowed the delineation of antigenic structure at a level of precision not previously possible, because MAbs recognised only a single site and not an average of determinants (Benjamin *et al.* 1984).

Prior to the application of the MAbs to antigenic studies they need to be characterised, since the interpretation of antigenic analysis is dependent on the homogeneity and specificity of the hybridoma antibodies (Yewdell and Gerhard, 1981).

Monoclonal antibodies have become key components in clinical laboratory diagnostic tests since they enable the development of standardises immunoassay systems. The wide application in detecting and identifying serum analytes, cell markers and pathogenic agents has largely arisen through the exquisite specificity of these unique reagents (Nelson *et al.* 2000).

3.1.1 - Production of hybridoma cells

Monoclonal antibodies are obtained through the production of a specific hybridoma cell line to a pre-determined antigen.

Most MAbs have been produced using the BALB/C mouse because the fusion partners (myeloma cell lines) have been developed from plasmacytomas induced in this strain by the intraperitoneal injection (IP) of mineral oil (Dean and Shepherd, 2000).

When soluble proteins or carbohydrates are used for immunising mice they are usually mixed with an adjuvant. Adjuvants are substances which non-specifically enhance the immune response to antigens (Roitt *et al.* 1985). An adjuvant may function as an antigen-depot-forming substance, a delivery vehicle or inert carrier, an immunostimulator/ immunomodifier (able to stimulate cells of the immune system or modify immune cell activation), or a combination of these (Hanly *et al.* 1995). Certain adjuvants such as aluminium compounds, oil emulsions, liposomes, and synthetic polymers act through the effect of antigen localization ("depot" effect) (Jennings, 1995). This “depot” effect provides a protected reservoir of antigen for slow release to draining lymph nodes, helping promote formation of memory cells and prolonged Ab responses (Hanly *et al.* 1995), and inducing complex cell interactions between macrophages and lymphocytes (Jennings, 1995). Adjuvants can be been categorized according to: ① their origins, whether they are derived from mineral, bacterial, plant, synthetic, or whether they are host a product, such as Interleukin 1; ② their proposed mechanism of action. Freund’s complete adjuvant has been the most widely used adjuvant. This is a mineral oil containing mycobacteria that acts as a slow release agent preventing rapid dispersion of soluble immunogens and elicits a strong cellular infiltrate of neutrophils and macrophages at the site of injection (Dean and Shepherd, 2000).

As a result of the memory of the adaptive immune system of mice and the fact that the strongest immune response follows secondary encounters with the antigen, the immunisation of the mice is usually performed twice. The booster immunity is given with Freund’s incomplete adjuvant (i.e. mineral only). These immunisations should be performed several weeks apart when the serum titres have dropped after immunisation. Re-immunisation when the levels of antibodies are still high will lower the effective strength of the boost (Harlow and Lane, 1988).

The antibody response is also influenced by the anatomical site at which immunogens are administered (Liddell and Cryer, 1991). Intraperitoneal injection (IP) is the most common immunisation method for mice used due to two factors (Harlow and Lane, 1988). Firstly the capacity of the peritoneal cavity allows the use of larger volumes of the immunogens. Secondly IP immunisation does not deliver antigens directly into the blood system and therefore adjuvants can be used

Mature antibody-secreting cells fuse poorly with myeloma cells and it is the committed precursors that are required for hybridoma production. For this reason a third and final boost is given to the mouse 3-5 days before the fusion between the spleen cells and myeloma cells is performed (Dean and Shepherd, 2000). This boost is performed by intravenous injection (IV) without adjuvant and a rapid and strong response can be expected, as the antigen will be collected quickly in the spleen, liver and lungs (Harlow and Lane, 1988).

For MAb production the host spleen cells are mixed with a selected cell line and the mixed cells exposed to an agent that promotes the fusion between the cells (see Figure 3.3). The immortal cell partners for the antibody-producing cell are myeloma cells, cancerous cells derived from the immune system (Liddell and Cryer, 1991). The myeloma cell lines originally used secreted immunoglobulin molecules that were a mix of the spleen and the myeloma H and L chains (Yelton and Scharff, 1981). These problems were overcome by the use of myeloma cell lines, such as SP2/0, that do not produce L and H chains (Shulman *et al.* 1978). The maintenance and health of the myeloma fusion partner is of major importance for the success of the fusion, with the best results achieved with myeloma cells thawed from liquid nitrogen just a few days prior to the fusion procedure (Hunt *et al.* 2000).

The mechanism of fusion is complex, involving cell agglutination, membrane fusion, cell swelling and optimal environmental conditions (Knutton and Pasternak, 1979). The original agent used by Köhler and Milstein (1975) for enhancing the frequency of fusion between cells was inactivated Sendai virus. Nowadays a large number of chemicals can be

used to induce the fusion between cells, but the most commonly used chemical "fusogen" is polyethylene glycol (PEG) (Klebe and Mancuso, 1981).

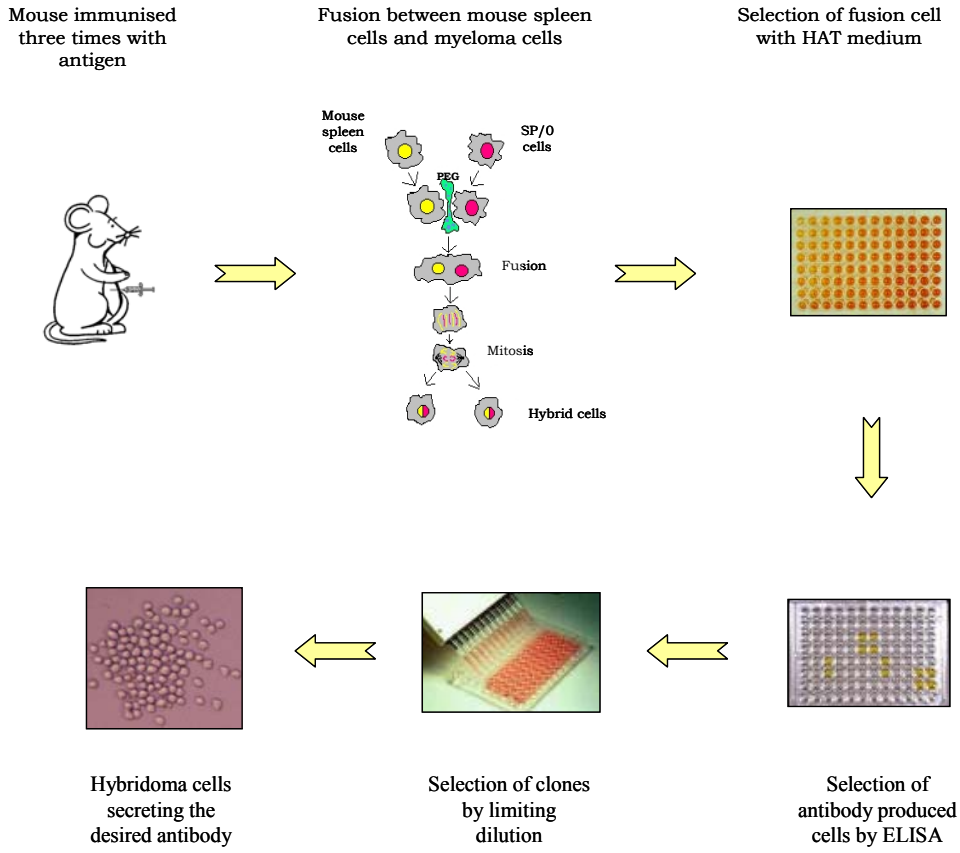


Figure 3.3 – Hybridoma cell line production.

Polyethylene glycol (PEG) fuses the plasma membranes of adjacent myeloma and antibody secreting cells, forming a single cell with two or more nuclei. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells (Harlow and Lane, 1988). In the presence of abnormal number of chromosomes, segregation does not always deliver identical sets of chromosomes to daughter cells, and chromosomes may be lost (Harlow and Lane, 1988, Nelson, 2001). If the chromosome that carries a functional gene (e.g. those responsible for rearranged immunoglobulin heavy or light chain genes) is lost then production of the antibody will stop, leading to unstable hybridoma cells

(Nelson, 2001). This unstable hybridoma cell line will be seen phenotypically by decreasing of antibody titres (Harlow and Lane, 1988).

The fusion protocol results in a mixture of parental cells, hybrids of each parent to itself, and most importantly, hybrids between one parent and the other (Yelton and Scharff, 1981). The selection of the hybrids between mouse spleen cells and myeloma cells is based on the fact that cells possess two pathways of nucleotide biosynthesis: ① the *de novo* pathway, that is the normal pathway and ② the salvage pathway which uses an enzyme called hypoxanthine guanine phosphoribosyl transferase (HGPRT) (Nelson, 2001). The hypoxanthine-aminopterin-thymidine (HAT) selection system is usually employed for the selection of the hybrids between the two parents. The myeloma parent has been engineered with a genetic defect for the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT-) (Dean and Shepherd, 2000). The HGPRT- cells cannot use exogenous hypoxanthine to synthesize purines (salvage pathway) just being able to use the *de novo* pathway for this synthesis (Yelton and Scharff, 1981). The *de novo* pathway is blocked by the aminopterin (Nelson, 2001). The myeloma cells die in the presence of hypoxanthine, aminopterin and thymidine because both pathways for the formation of the purine precursors of DNA are blocked (Liddell and Cryer, 1991). Spleen lymphocyte cells, although not killed by aminopterin, will not proliferate in culture, so after a few days the only rapidly dividing cells remaining are myeloma-spleen hybrids (Yelton and Scharff, 1981). Aminopterin inhibits many of the normal cell functions and should be removed from the feeding medium as soon as possible (Campbell, 1984). Usually the HAT is replaced with HT (hypoxanthine and thymidine) to allow the emerging cells to adapt to the main pathways of purine and pyrimidine biosynthesis (Campbell, 1984).

The hybrids need to be screened with a rapid, simple and sensitive assay because just a small percentage of the hybrids generated produce the desired antibody (Yelton and Scharff, 1981). Screening is normally carried out 10-14 days post-fusion by removing samples of the cell culture supernatant. This screening is vital for the final result, because the selection of the hybridoma colony to pick, expand and clone is based on it (Dean and Shepherd, 2000). Once a

positive hybrid is identified it should be cloned to avoid overgrowth by other hybrids or by non-producing variants (Yelton and Scharff, 1981). Cloning is an essential part of MAb production, ensuring that a single clonally expanded B-cell produces the antibody (Melamed and Sutherland, 1997). Cloning by limiting dilution is the most common method used to obtain single clone wells (Harlow and Lane, 1988). The use of feeder cells is vital during this procedure in order to achieve reasonable cloning efficiencies (Melamed and Sutherland, 1997). The isolated single cell clones are re-screened and re-cloned several times. After subclones are rescreened to identify those still producing the antibody of interest, just a few of them are grown to mass culture and frozen for future recovery and applications (Yelton and Scharff, 1981). Single-cell cloning ensures that the antibody producing cells are truly monoclonal (Harlow and Lane, 1988).

3.1.2 - Objectives

The aim of this study was to produce monoclonal antibodies against betanodavirus and to apply these in epitope mapping studies.

The MAbs obtained were characterised and their application as diagnostic tools was explored. Western Blot, immunohistochemistry and neutralisation assays were also optimised using the anti-betanodavirus MAbs obtained.

3.2 - Materials and Methods

3.2.1 - Antigen

The European sea bass (*Dicentrarcus labrax* L.) betanodavirus strain used was used for the production of polyclonal and monoclonal antibodies. The virus was cultured and purified as described in Chapter 2.

3.2.2 - Polyclonal antibodies

3.2.2.1 - Immunisation

One female New Zealand rabbit was immunised in order to obtain polyclonal antibodies against European sea bass nodavirus. The purified nodavirus sample was obtained as described in Chapter 2. The virus (50 μg ml^{-1}) was mixed 1:1 with Titremax® Gold (TitreMax, CyT Rx® corp. USA, Stratech Cambridgeshire U.K.). Titremax® Gold, used as an adjuvant and does not contain mineral oil, proteins, polysaccharides or other microbial products. These characteristics minimise or eliminate the undesirable side effects caused by most common adjuvant used, such as Freund's Complete Adjuvant (FCA). Titremax® is completely non-toxic to users and animals. It induces a moderate transitory inflammation, while FCA induces a severe inflammation. In addition it does not induce a hypersensitivity granulomatous response (http://www.titremax.com).

The virus was mixed with the adjuvant using two latex free syringes (1 ml, Henke Sass Wolf GMBH, Germany) linked by a Leur lock and the solution was mixed until a white, thick solution was obtained.

The rabbit was injected subcutaneously (SC) at 4 sites, with 0.1 ml of virus:adjuvant solution per site. This procedure was repeated 4 weeks later. The third and final immunisation

was given after another 4 weeks by intravenous injection (IV) with 600 µl of nodavirus (50 µg ml^{-1} of virus in TNE buffer).

The rabbit was sacrificed 12 days later and blood collected by cardiac puncture (5 ml syringe, Terumo, Merseyside U.K.).

3.2.2.2 - Collection of rabbit blood

In order to verify specific antibody production rabbit blood was collected. The blood was collected from the marginal ear vein. The fur on the ear was shaved off carefully with a scalpel blade, then the region was wiped with ethanol and the marginal ear vein was cut with a sterile scalpel. The drops of blood were collected into a 1.5 ml eppendorf tube.

The blood was allowed to clot overnight at 4°C. On the following day it was centrifuged for 5 mins at 15 800 x *g* (Thermo IEC microlite), the serum collected and stored at -20°C.

A pre-immunisation sample was collected on the same day as the first immunisation and another two samples were collected 10 days after the first and second immunisation. The rabbit serum was tested by ELISA (see Chapter 2).

3.2.3 - Monoclonal antibody production

3.2.3.1 - Immunisation

For the production of monoclonal antibodies against European sea bass betanodavirus 3 mice were immunised with 30 µg ml^{-1} of virus. The nodavirus was diluted in TNE buffer and mixed with adjuvant Titremax® Gold as described in section 3.2.2.1.

The mice were immunised IP with 0.2 ml of virus:adjuvant spread over 3 sites. The same procedure was performed for the first boost 4 weeks later. The final boost was given

without adjuvant IV (0.1 ml of virus in TNE buffer) after another 4 weeks. The mouse that presented the highest antibody titre by ELISA was chosen for the last boost.

3.2.3.2 - Collection of mouse blood

Mouse blood was collected in order to test the titre against the virus. This operation was performed once before the first immunisation and 10-14 days after the first IP and the first boost.

The mouse's tail was wiped with alcohol and the tail vein cut with a sterile scalpel. The blood drops were collected into a 1.5 ml eppendorf tube and allowed to clot overnight at 4°C. The following day the blood was centrifuged for 5 mins at 15 800 x *g* (Thermo IEC microlite), the upper serum layer collected and stored at -20°C.

On the day that the mouse was sacrificed the blood was collected by cardiac puncture with a 2 ml sterile syringe (Terumo, Merseyside U.K.).

Mice sera were screened by ELISA (as described in Chapter 2) using a 10-fold dilution series and the end-point titre of the sera antibodies was obtained.

3.2.3.3 - Fusion

3.2.3.3.1 - Myeloma cells

Myeloma cells (SP2/0, Sigma, U.S.A.) were thawed for the fusion and cultured in DMEM+ (Chapter 2).

On the fusion day, the cells were collected, DMEM with additives (sodium pyruvate, L-glutamine and streptamycin/penicillin) added up to 50 ml and then cells were centrifuged for 7 mins at 150 x *g* (Wifug 500E). The supernatant was discarded and the pellet was gently resuspended into 50 ml of DMEM with additives and placed in a CO_2 incubator at 37°C.

3.2.3.3.2 - Blood feeder medium

Blood was collected from a non-immunised mouse in order to be used as feeder cells. The mouse was sacrificed using CO_2 and blood collected by cardiac puncture using a 2 ml sterile syringe with sterile heparin (Sigma, Dorset U.K.) (10 i.u. in DMEM).

A solution of DMEM+ and 10 % of HAT (hypoxanthine-aminopterine-thymidine, Sigma, Dorset U.K.) was prepared and kept at 37°C until mixed with the mouse blood. The mouse blood was mixed with DMEM+/HAT in a ratio of 1:300 of blood:medium and kept in the incubator until further utilization.

3.2.3.3.3 - Mouse spleen cells

The immunised mouse was sacrificed using carbon dioxide. The abdomen was wiped with ethanol, aseptically opened and the spleen collected very carefully.

The spleen was placed into 20 ml of DMEM, fat trimmed off and it was then washed by passing through three petri dishes containing DMEM. The organ was then "blown" by cutting off both ends and injecting 5 ml of DMEM through the spleen using a syringe and a needle. All the DMEM used was previously warmed up to 37°C.

The cell suspension was placed into a centrifuge tube and DMEM with additives was added to make the volume up to 50 ml. The cells were then placed in the CO_2 incubator at 37°C.

3.2.3.3.4 - Fusion

The spleen cells and the SP2 cells were centrifuged at 82.3 x *g* for 7 mins, supernatant discarded and the pellet gently resuspended in 10 ml of warmed DMEM with additives.

An aliquot of each type of cell was made and counted using a haemocytometer.

All the spleen cells were mixed with myeloma cells to give a spleen:SP2 ratio of 4:1. The cell mix was centrifuged for 7 mins at 82.3 *g*. The pellet was aspirated as dry as possible and then resuspended by gently tapping.

To the pellet was added 1 ml of warmed 50 % PEG (Sigma, Dorset U.K.) over 1 min, allowed to stand for 1½ mins swirling occasionally. Then 1 ml of warm DMEM was added over ½ min, 3 ml warm DMEM over ½ min, 16 ml warm DMEM over 1 min and the cells were left to stand for 5 mins. After this period the cells were centrifuged for 7 mins at 82.3 *g*, the pellet resuspended by gently tapping and 200 ml of blood feeder medium, was added.

The cells were placed into sterile 96 well plates (Nunc, Fisher Scientific Leicestershire U.K) by adding 180 µl per well and incubated at 37°C with 5 % CO_2.

3.2.3.3.5 - Screening the clones

If the aim is generating as diverse an antibody panel as possible, a binary assay system should be used which is independent of antibody isotype and in which the binding of the antibody to the viral protein occurs in the absence of a competing interaction (Yewdell and Gerhard, 1981). Commonly used binary assays are the radioimmunoassay (RIA) and Enzyme-Linked-Immunosorbent-Assay (ELISA) (Yewdell and Gerhard, 1981). In the present study the ELISA was the selection method chosen.

The fused cells were allowed to stand for 10 days without being disturbed. After this period 100 µl of supernatant was collected and ELISA was performed to verify the production of antibodies (as described in Chapter 2). Positive antibody producing fusion cells were collected, placed into sterile 24 well plates (Nunc, Fisher Scientific Leicestershire U.K.) with 1 ml of DMEM+ supplemented with 10 % HT (hypoxanthine-thymidine, Sigma, Dorset U.K.) and incubated for 7 days at 37°C with 5 % CO_2.

After this period 100 µl of supernatant for each well was collected and ELISA was performed. The positive cells were selected, cloned by limiting dilution into 96 well plates and incubated for 7 days. The positive cells were also expanded to 2 wells of a 24 well plate and incubated for 7 days. After this period the cells were stored in liquid nitrogen (see Chapter 2). These cells are called Parental cells.

After a 7 day period of incubation the cloned cells were checked, wells with just one clone were selected and the supernatant screened by ELISA. The clones that were positive by ELISA were cloned again by limiting dilution in a 96 well plate and incubated for 7 days. This procedure was performed 3 times until the clone could be considered to be a hybridoma cell line producing antibodies.

The positive clones of all the stages were expanded into 2 wells of a 24 well plate, incubated for 7 days and stored in liquid nitrogen.

All the limiting dilution and expanding of the clones were performed with blood feeder medium.

3.2.3.3.6 - Expansion of hybridoma cell lines

Following the selection procedure the hybridoma cells were expanded to a 24 well plate using 1 ml of blood feeder medium. After one week the cells were split into 1.5 ml of DMEM+. This procedure was repeated until the cells were used to growing without the mouse blood cells. After this period of adaptation the cells were expanded into 25 cm^3 flask (Nunc, Fisher Scientific Leicestershire U.K.) in order to produce enough supernatant for further applications.

The expansion was performed by adding 5 ml of DMEM+ to 25 cm^3 flask. The content of 1 well of a 24 well plate was added per 25 cm^3 flask and incubated for 7-10 days. The supernatant was collected, following centrifugation at 515 *g* for 10 mins and stored at -20°C.

The cells were expanded into larger flasks, when larger volumes of antibody was required.

3.2.4 - Characterisation of the antibodies

The type of immunoglobulin and specificity of the monoclonal antibodies produced were determined.

For both monoclonal and polyclonal antibodies, the neutralisation ability, molecular weight of antigen that they recognised and performance in immunohistochemistry was determined.

3.2.4.1.1 - Immunotyping

The determination of the isotype serves not only to define the murine immunoglobulin class or subclass but also helps to identify the presence of a single isotype or a mixture isotype (Nelson *et al.* 2000).

Immunotyping was performed using a Sigma ImmunoType™ kit (Sigma, Dorset U.K.). The procedure followed the manufacturer's instructions. Forceps were used and all the incubations were performed at room temperature using a rocker shaker (Bibby Gyro-rocker).

Briefly, the strip was placed into an assay tube, 3 ml of MAb supernatant was added and incubated for 30 mins. After this period the supernatant was decanted, the strip washed by adding 3-4 ml of PBS-T-BSA (PBS (Sigma, Dorset U.K.) with 0.05 % (v/v) Tween 20 (Sigma, Dorset U.K.) and 2 % (w/v) BSA (Sigma, Dorset U.K.), and incubated for 5 mins. Meantime the biotinylated second antibody (anti-mouse conjugated with biotin) was prepared by adding 1 drop of biotinylated antibody to 2 ml of PBS-T-BSA (1:50 dilution). After the washing step, biotinylated antibody was added to the assay tube and incubated for 30 mins.

Once the incubation period had finished, the antibody was decanted and the strip was washed as previously described. The ExtrAvidin®-Peroxidase was added to the assay tube and incubated for 15 mins. The ExtrAvidin®-Peroxidase was prepared by adding 1 drop of extravidin to 2 ml of PBS-T-BSA. After the incubation period the ExtrAvidin®-Peroxidase was decanted and the strip was washed for 5 mins with PBS-T-BSA and 5 mins with PBS. The buffer was discarded and 2 ml of substrate solution was added. The substrate solution was prepared by mixing thoroughly 4 ml of water, 2 drops of substrate buffer (2.5 M Acetate buffer pH 5.0), 1 drop of substrate chromogen (3-amino-9-ethyl-carbazole in N,N-dimethyl formamide; AEC-DMF) and 1 drop of substrate (2 % v/v hydrogen peroxide). The reagents were added in this order. The strip was incubated until a clear, red insoluble signal was obtained for the positive control. Incubation was performed for a maximum of 10 mins. The strips were preserved by immersing them in distilled water for a few mins, drying in a paper towel and storing in a plastic sleeve protected from the light.

3.2.4.2 - Specificity

The specificity of the MAbs was determined by screening them in ELISA (Chapter 2) against other strains of betanodavirus and different pathogens that may be present in fish or aquatic environment (Table 3.2).

For the viruses tested the ELISA assays were performed with virus supernatant. The bacteria tested were resuspended in PBS.

The antigenic specificity of the MAbs was analysed with other strains of betanodavirus belonging to different genotype clades. From BFNNV clade, Atlantic cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*) strains were used. Striped jack (*Pseudocaranx dentex*) strain was used for representing the SJNNV clade.

Table 3.2 – Strains of betanodavirus, cell lines and other fish viruses and bacteria used for test MAbs specificity.

Antigen	
Virus	Betanodavirus strains: European sea bass (*Dicentrarchus labrax*) Atlantic cod (*Gadus morhua*) Halibut (*Hippoglossus hippoglossus*) Striped jack (*Pseudocaranx dentex*) Infectious Pancreatic Necrosis Virus - IPNV (serotype) Salmon Pancreatic Disease Virus - SPPD Infectious Salmon Anaemia Virus - ISAV Sleeping Disease Virus -SDV
Cell lines	CHSE - 214 SSN-1 SHK-1
Bacteria	*Vibrio vulnificus* (biotype 2) *Vibrio harveyii* (CECT 5978) *Vibrio anguillarum* (type II) *Vibrio parahaemolyticus* *Vibrio splendidus* *Vibrio alginolyticus* *Vibrio mimicus* *Photobacterium damselae* subspecies *piscicida* *Mycobacterium fortuitum* (1294) *Mycobacterium chelonae* *Piscirickettsia salmonis* LF-89 (ATCC VR 1361)

The viral supernatants (SPDV, ISAV and SDV) and cell line supernatants (SHK-1 and CHSE – 214) were kindly supplied by Dr. Kimberly Thompson (Aquatic Vaccine Unit, University of Stirling). The SPDV, ISAV and SDV isolated were originally obtained from Dr. David Smail (FRS Marine Lab, Aberdeen).

The bacteria used for testing MAbs specificity belong to the Institute of Aquaculture (University of Stirling) bacteriology's collection and were kindly supplied by several PhD students at the Institute of Aquaculture (Stirling) namely Fuad Matori (*Vibrio* species and *Photobacterium damsela* subspecies *piscicida*), Sandra Laffon (*Mycobacterium* species) and Úna McCarthy (*Piscirickettsia salmonis* and CHSE – 214).

3.2.4.3 - Neutralisation

The neutralisation test is used to verify if an antibody has the ability to prevent the infection of cells by viruses (Cann, 1999). Neutralisation activity was performed using β neutralisation. This was performed in sterile flat bottom 96 well microtitre plates (Nunc, Fisher Scientific Leicestershire U.K.) by adding 40 µl of 5 fold serial dilutions of antibodies and 40 µl of 100 $TCID_{50}$ ml^{-1} of betanodavirus. The plates were incubated for 90 mins. After this period 100 µl of SSN-1 cells was added to each well and incubated for 7 days at 25° C for European sea bass betanodavirus strain, and at 22°C for cod betanodavirus strain. After this period the cells were scored for cytopathic effect and antibodies titre was estimated by 50 % end-point Spearman-Karber method (LD50) (Chapter 2).

The neutralisation index (NI) was calculated according to Rovozzo and Burke (1973).

$$NI = \log_{10}[(LD_{50} \text{ of virus with diluent}) - (LD_{50} \text{ of virus with Mabs})]$$

With: $NI < 1$ considered not significant;
$1 > NI < 1.6$ considered questionable;
$NI \geq 1.7$ considered significant.

3.2.4.4 - Western Blot

Western blot was performed to determined the molecular weight of the antigen that the monoclonal antibodies recognised.

3.2.4.4.1 - SDS-PAGE gel

The SDS-PAGE was optimised to betanodavirus using the protocol established by Weins *et al.* (1990).

The plates for running the SDS-PAGE were washed with tap water, rinsed with distilled water and dried with paper tissue. A spacer was placed between the plates and slid into the plate's holder. The set was made even and the holder was screwed from the top to the bottom. A small amount of Vaseline® was spread on the bottom of the holder/plates in order to promote the adhesion of the plates to the caster (Dual gel caster, Might™ Small SE245, Hoefer Pharmacia Biotech Inc.).

The plates where then placed in the caster and the separating gel (12 % acrylamide, Appendix 1) was added. A layer of butanol-2 (Fisher Scientific, Leicestershire U.K.) was added to the top of the gel in order to prevent gel oxidation. The gel polymerised for 60 mins at room temperature. After polymerisation of the gel, the butanol was washed out with plentiful amounts of distilled water. The comb was placed, the stacking gel (4 % acrylamide, Appendix 1) loaded and allowed to polymerise for 45 mins. After this period the gel plates were placed into the electrophoresis chamber (Hoefer Pharmacia Biotech Inc. SE250) and reservoir buffer was added (Appendix 1).

Betanodavirus was prepared as described in Chapter 2. Several concentrations of purified virus were utilised (Table 3.2.) in order to verify the optimal concentration of betanodavirus for running the SDS-Gel. The virus sample diluted in TNE buffer was mixed with SDS sample buffer (Appendix 1), boiled for 4 mins and centrifuged for 5 mins at 15 800 x *g* (Thermo IEC microlite). The ability of antibodies to bind to unboiled samples was also determined, as in Table 3.3.

Table 3.3 – Concentration of Betanodavirus samples analysed by SDS-PAGE, with or without boiling.

	***Betanodavirus* ($\mu g\ l^{-1}$)**							
	1	***2.5***	***5***	***15***	***25***	***30***	***50***	***100***
Boiled	+	+	+	+	+	+	+	+
Not boiled	-	-	-	-	-	+	-	-

Two different molecular weight markers were used, Protein Marker Broad Range (BioLabs Inc., New England) for SDS-PAGE gel, and Full Range Rainbow (Amersham) for Western-blot.

The samples and molecular weights were loaded with loading tips and gel was electrophoresed for 90 mins at 120 V (Amersham Pharmacia Biotech EPS 1001).

The gel was stained with Coomassie Brilliant Blue (Appendix 1) in order to observe the molecular weight of the betanodavirus proteins and to confirm the success of the gel.

3.2.4.4.2 - Western blot

Optimisation of Western blot to use the MABs against betanodavirus was performed using the protocol established by Weins *et al.* (1990).

Unstained SDS-PAGE gel, filter paper (Whatman n.1), nitrocellulose membrane (Hybond™-ECL™, Amersham) and sponge pads were equilibrated in transblot buffer (Appendix 1) for 20 mins.

The blot holder was mounted from the cathode to the anode in the following order – sponge pad, three sheets of filter paper, nitrocellulose membrane, SDS gel, three sheets of filter paper and sponge pad. The procedure was carefully carried out to prevent the formation of bubbles.

The blot holder was placed into the blotting chamber (Fisher FEB10) and filled with transblot buffer. The nitrocellulose membrane was then blotted at 60 V for 70 mins (Amersham Pharmacia Biotech EPS 1001).

The lanes with molecular markers were cut, rinsed in distilled water, dried and stored in the dark. The non-specific binding sites on the membrane containing sample lanes were blocked over-night at 4°C with 1 % (w/v) BSA in TBS (Appendix 1).

The following morning the membrane was washed 3 times with TTBS (Appendix 1) for 5 mins each and incubated with hybridoma supernatant. Two incubation periods, 1 h at room temperature and over-night at 4°C, and several concentrations of hybridoma cells (1:100; 1:50, 1:25, 1:10 (v/v in TNE buffer), neat and 2 × concentrated) and polyclonal antibodies (1:100; 1:50; 1:30) were tested.

After this incubation the washing procedure with TTBS was repeated and the membrane was incubated for 1 h at room temperature with anti-mouse IgG HRP conjugated (1:100 in PBS) (Diagnostics Scotland, Edinburgh, Scotland). The membrane was washed 3 times with TTBS for 5 mins each and finally washed 1 min in TBS. The membrane was developed with chromogen/ substrate solution (4CN Membrane Peroxidase Substrate System (2-C), KPL, Wembley U.K.). The reaction was allowed to proceed for a period no longer then 30 mins and stopped with distilled water.

3.2.4.5 - Immunohistochemistry

The immunohistochemistry protocol used was developed according to Adams and Marin de Mateo (1994).

Tissue blocks were trimmed to facilitate access to the embedded tissues, and soaked in distilled water for 60 mins. Sections of 5 μm thickness were cut using a microtome (Jung Biocut 2035). Ribbons of sections were floated on 40°C distilled water and then slipped onto labelled glass slides. These were allowed to dry at 40°C, then transferred to a drying oven and further dried at 60°C overnight.

The sections were de-waxed in xylene (2 × xylene, 5 mins each), re-hydrated through ethanol 100 % (5 mins), ethanol 70 % (3 mins) and distilled water (5 mins).

The tissues were encircled with a PAP pen (Liquid blocker Super pap pen, Agar Scientific, Essex U.K.) and the endogenous peroxidase was blocked by incubation with 3 % (v/v) hydrogen peroxide (Sigma, Dorset U.K.).

After this period the slides were washed 3 times with buffer (TBS or PBS, see Table 3.4) and the non-specific binding sites were blocked with 10 % (v/v) goat serum (Diagnostics Scotland, Edinburgh, Scotland). Excess liquid was tapped off and anti-betanodavirus MAb was added and slides incubated for a variety of times (Table 3.4).

Table 3.4 – Variables tested for optimisation of the immunohistochemistry protocol for the detection of Betanodavirus.

Protocol step	***Variable***		***Incubation time (mins)***
	Reagent	***Diluted in***	
Washing buffer	TBS PBS		
Antigen Retrieval	TBS Formic Acid Citric Acid		850 W - 1'45 s; 80 W - 6' 850 W - 1'45 s; 80 W - 2' 30s 850 W - 2' 30 s; 80 W - 2' 30s 850 W - 2' 30 s; 80 W - 3' 850 W - 3'; 80 W - 3' 850 W - 3'30 s; 80 W - 3' 850 W - 6'; 80 W - 6'
Endogenous peroxidase	H_2O_2	Methanol PBS	20; 30 30
Non-specific binding	Goat serum	1:10 in TBS 1:10 in PBS	10; 20 20
MAbs	Supernatant ♦ Supernatant ✳ Supernatant 3x concentrated ✯ Supernatant 10x concentrated ✯ Freeze drye ✯	 1:10 in PBS	60; 120 60 180 60; 180 120
Secondary antibody	Anti-mouse HRP Anti-mouse Biotin Anti-mouse biotin (Vector ABC)	1:75 in TBS 1:50 in TBS 1:100 in PBS 1:1000 in PBS PBS and serum	60; 75 60 60; 30 30 30
Streptavidin-biotin amplification	Extravidin HRP Streptavidin HRP Streptavidin (Vector ABC)	1:50 in TBS 1:100 in PBS 1:200 in PBS 1:2000 in PBS PBS	60 60 20 20 30
Chromogen	DAB True Blue Vector VIP peroxidase substrate	TBS	20; 24 10 10
Counterstain	Mayer's Haematoxilin Contrast red Methyl Green		3 10; 2 3 mins at 60°C

♦ - MAbs 4C3, 3B10 and 4A12; ✳ cocktail of 1:1:1 of MAbs 4C3, 3B10 and 4A12;
✯ - MAb 4A12. All incubations performed at room temperature.

The slides were then washed 3 times with buffer (TBS or PBS), and incubated with secondary antibody. This antibody was anti-mouse or anti-rabbit antibody conjugated with HorseRadish Peroxidase (HRP) or biotin. These antibodies were diluted from a range between 1:50 to 1:100 (v/v) in TBS or PBS, as described in Table 3.4.

After washing 3 times with buffer (TBS or PBS) the slides were incubated with the chromogen or with the streptavidin-biotin complex. Following incubation with the chromogen and conterstaining the slides were de-hydrated (3 mins in ethanol 70 %; 5 mins in ethanol 100 %; 2 × xylene, 5 mins each) and coverslipped with Pertex (Cellpath Ltd., Hemel Hempstead U.K.).

The Vectorstain® ABC kit (Vector, Peterborough U. K.) was also tested for optimisation of the streptavidin-biotin amplification step. The chromogen used with this kit was Vector® VIP Peroxidase Substrate Kit (Vector, Peterborough U. K.) and the counterstain was performed with Methyl Green (Vector, Peterborough U. K.).

The Atlantic cod tissue was kindly provided by Dr. David Gromam (University of Prince Edward Island, Canada). Doctor Bovo (Istituto Zooprofilattico Sperimentale, Venice Italy) and Professor Hugh Ferguson (IoA, Stirling Scotland) kindly provided the tissue samples from European sea bass and grouper.

3.2.4.5.1 - Antigen Retrieval

After de-waxing and re-hydrating the slides were placed in a Copeland jar with buffer (Appendix 1) covered with microwaveable cling film and placed into a microwave oven (Sanyo). The slides were microwaved at 850 W, then at 80 W (Table 3.3). After these boiling periods the slides were allowed to cool down in the buffer for 5 mins. The slides were incubated another 5 mins in TBS (pH 7.6, Appendix 1) at room temperature and immunohistochemistry was performed as described in section 3.2.4.5.

3.3 - Results

3.3.1 - Monoclonal antibody production

In order to obtain monoclonal antibodies two fusions needed to be performed. On the first fusion the clones obtained were not considered to be useful for further applications because the antibody titre was too low.

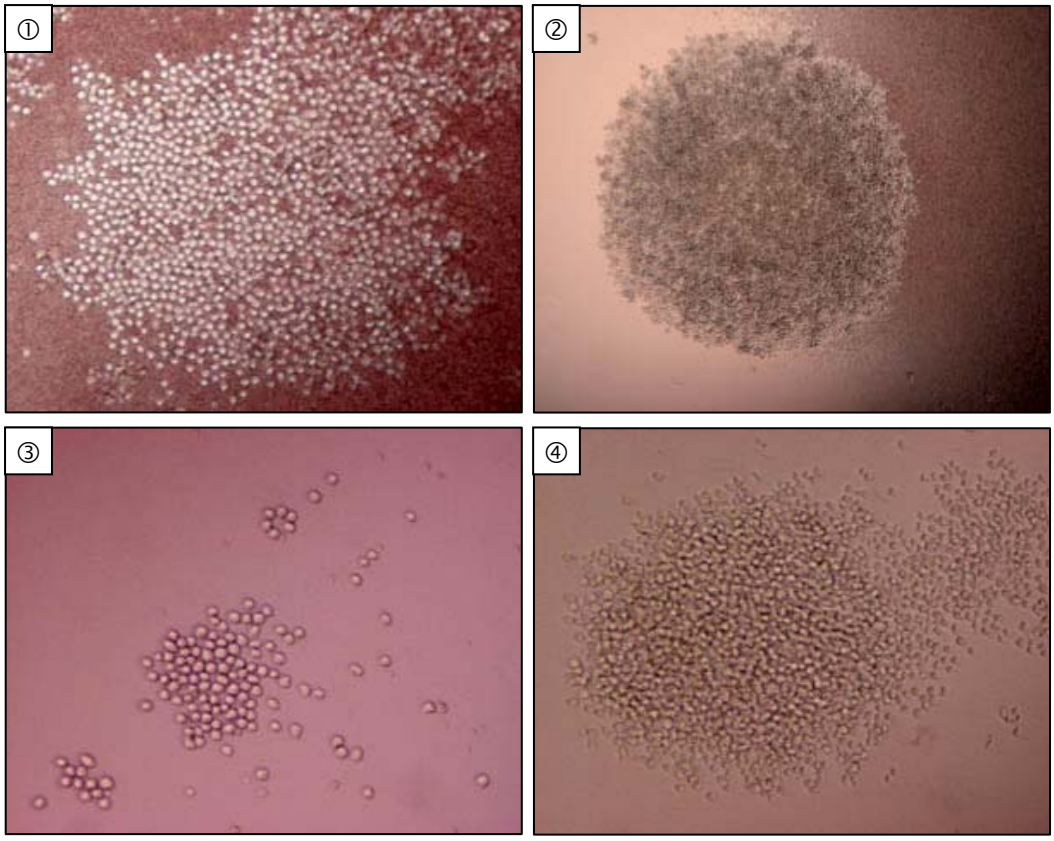


Figure 3.4 – ① Cloned cells surrounded by feeder cells (magnification 200x); ② single clone adjacent to feeder cells (magnification 40x); hybridoma cells producing monoclonal antibodies (③ magnification 400x and ④ magnification 100x).

Some examples of clone cells surrounded by the feeder cells or hybridoma cells obtained are shown in Figure 3.4.

With the second fusion 21.3 % of the clones showed a high positive response by ELISA against betanodavirus supernatant. These clones were amplified and screened once

more. After this amplification 10 % showed a positive response against the virus. These positive clones were classified as parental cells, cloned by limited dilution and screened three times. After this selection 3 clones (21 %) still give a positive result by ELISA and were considered to be hybridoma cells producing monoclonal antibodies (3B10, 4A12 and 4C3).

The parental cells stored in liquid nitrogen were thawed and the cloning procedure was repeated once more. This process allowed a further two hybridoma cell lines – 1E3 and 5G10, to be obtained.

3.3.2 - Immunotyping

All five MAbs were shown to belong to the same immunoglobin class IgG but have different subclasses. MAbs 4C3, 4A12 and 1E3 were IgG2a, and MAbs 3B10 and 5G10 were IgG2b, as demonstrated in Figure 3.5.

Figure 3.5 – Immonotyping strips used for determining the type of immunoglobin of the MAbs. ① 4C3, 4A12 and 1E3; ② 3B10 and 5G10.

3.3.3 - Cross-reactivity

The reaction of the anti-betanodavirus MAbs against other betanodavirus strains and a variety of fish pathogens was determined by ELISA (Table 3.5).).

As well as reacting against betanodavirus from European sea bass MAbs 4A12, 1E3 and 5G10 also reacted with betanodavirus isolated from Atlantic cod and halibut strains. The MAbs 3B10 and 4C3 only showed reactivity against the strain of betanodavirus that was used to immunised the mice, i.e. the European sea bass strain. None of the MAbs reacted against the striped jack betanodavirus strain.

None of the MAbs showed cross-reactivity against other viruses (IPNV, WISAV, SPPD, sleeping disease), or against the cell lines CHSE-214 and SKH-1 used to grow these viruses or to the cell line used to grow betanodavirus, SSN-1.

Table 3.5 – Cross-reactivity of the anti-betanodavirus MAbs with several strains of betanodavirus and other fish viruses and bacterioses.

Antigen	***MAbs***				
	3B10	***4A12***	***4C3***	***1E3***	***5G10***
Virus					
IPNV (serotype)	-	-	-	-	-
SPPD	-	-	-	-	-
WISAV	-	-	-	-	-
SDV	-	-	-	-	-
Betanodavirus strains					
European sea bass (*Dicentrarchus labrax*)	+	+	+	+	+
Atlantic cod (*Gadus morhua*)	-	+	-	+	+
Halibut (*Hippoglossus hippoglossus*)	-	+	-	+	+
Striped jack (*Pseudocaranx dentex*)	-	-	-	-	-
Cell lines					
CHSE - 214	-	-	-	-	-
SSN-1	-	-	-	-	-
SHK-1	-	-	-	-	-
Bacteria					
Vibrio vulnificus (biotype 2)	-	-	-	-	-
Vibrio harveyii (CECT 5978)	-	-	-	-	-
Vibrio anguillarum (type II)	-	-	-	-	-
Vibrio parahaemolyticus	-	-	-	-	-
Vibrio splendidus	-	-	-	-	-
Vibrio alginolyticus	-	-	-	-	-
Vibrio mimicus	-	-	-	-	-
Photobacterium damselae subspecies *piscicida*	-	-	-	-	-
Mycobacterium fortuitum (1294)	-	-	-	-	-
Mycobacterium chelonae	-	-	-	-	-
Piscirickettsia salmonis LF-89	-	-	-	-	-

Bacterial pathogens including *Piscirickettsia salmonis*, *Photobacterium damselae* subspecies *piscicida* and several strains of *Vibrio* and *Mycobacterium* were also tested with negative results.

3.3.4 - Neutralisation

The supernatant of five clone hybridoma cells and one polyclonal antibody were tested using an *in vivo* neutralisation test (Table 3.6). The Mabs 3B10 and 4C3 neutralised the European sea bass nodavirus strain.

Table 3.6 – Neutralisation index of polyclonal antibodies and MAbs 3B10, 4A12, 4C3, 1E3 and 5G10.

Neutralisation Index (NI)		
	European sea bass	***Atlantic cod***
Mabs		
3B10	3.16	-
4A12	0.36	1.26
4C3	3.44	-
1E3	1.68	0.20
5G10	2.74	1.80
Polyclonal antibody	4.56	3.74

MAb 4A12 had no neutralisation capacity against the European sea bass strain but gave a questionable NI with cod nodavirus strain (NI = 1.26). MAb 1E3 showed the opposite behaviour. This MAb had no neutralisation capacity against the betanodavirus cod strain (NI < 1), but with the European sea bass strain the NI value (1.68) can be considerered significant (NI ≥ 1.7). Both nodavirus strains were neutralised by MAb 5G10.

The polyclonal antibodies produced against the European sea bass strain revealed neutralisation ability against betanodavirus strain from European sea bass (NI = 4.56) and against the Atlantic cod strain (NI = 3.74).

3.3.5 - Western-Blot

Western Blot analysis showed that all the MAbs obtained have the ability to bind to a virus protein of approximately 42 kDa.

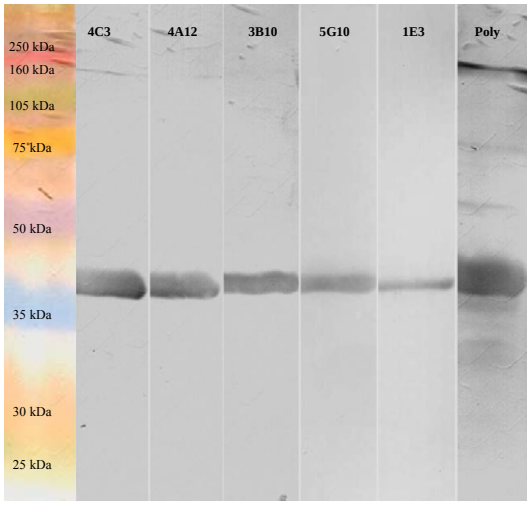


Figure 3.6 – Western Blot of all the MAbs and polyclonal antibodies. The virus sample was boiled.

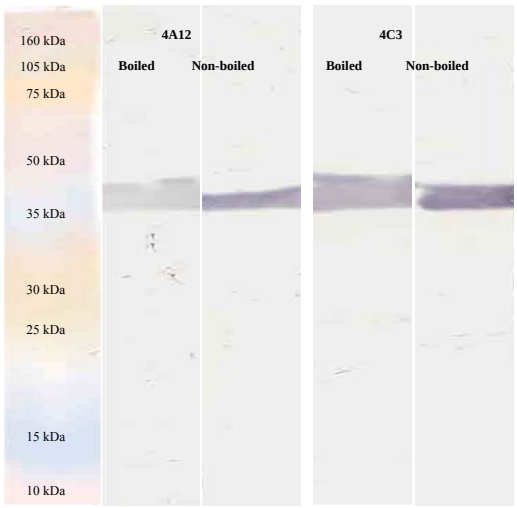


Figure 3.7 – Western Blot with boiled and non-boiled virus samples.

The polyclonal antibody recognised virus proteins of several different molecular weights at 160 kDa; 75 kDa; 55 kDa and the 42 kDa recognised by the MAbs.

MAbs 4A12 and 4C3 bound to boiled and non-boiled betanodavirus samples. These MAbs appear to recognise virus protein with different intensities, showing heavier staining to non-boiled virus samples (see Figure 3.6 and 3.7).

3.3.6 - Immunohistochemistry

The application of the MAbs in immunohistochemistry was possible only when streptavidin-biotin amplification or when antigen retrieval was performed. Monoclonal antibody 4A12 was the only one capable of recognising the virus by immunohistochemistry.

For both methods the optimised protocol is described in Table 3.7. Both methods show similar results but biotin-streptavidin amplification protocol is less labour and time demanding.

Using immunohistochemistry it was possible to verify that the virus is widely spread in the infected tissue, and it is not possible to detect localised areas of virus at the stage where these tissue samples were collected (Figure 3.8 ④, ⑥ and Figure 3.9 ② to ⑥).

When non-infected tissue sample was analysed MAb 4A12 revealed no cross-reactivity with non-infected tissue (Figure 3.8 ②). The use of PBS instead of MAb revealed that none of the reagents used react with fish tissue (Figure 3.8 ①, ③ and ⑤; Figure 3.9 ①).

Immunohistochemistry using MAb 4A12 appeared to be very sensitive for detecting virus in European sea bass diagnosed with VNN. These samples were diagnosed with VNN but did not show clear histological symptoms (Figure 3.8 ④ and ⑥). However, with immunohistochemistry they revealed the presence of the virus and allowed the confirmation of the diagnosis. Fish tissue samples with the characteristic vacuoles in the retina layers and brain could be observed in Atlantic cod (Figure 3.9 ③ to ⑥).

Table 3.7 – Immunohistochemistry optimised protocols.

Protocol	**Protocol step**	***Variable***		***Incubation time (mins)***
		Reagent	***Diluted in***	
	Washing buffer	TBS		
Antigen Retrieval	Antigen Retrieval	Formic Acid		850 W - 3'30 s 80 W - 3'
	Endogenous peroxidase	H_2O_2	Methanol	30
	Non-specific binding	Goat serum	1:10 in PBS	20
	MAb	Supernatant 10x concentrated		180
	Secondary antibody	Anti-mouse HRP	1:100 in PBS	60
	Chromogen	DAB	TBS	24
	Counterstain	Mayer's Haematoxilin		3
Biotin-streptavidin amplification	Endogenous peroxidase	H_2O_2	Methanol	30
	Non-specific binding	Goat serum	1:10 in TBS	20
	MAb	ADL	1:10 in PBS	120
	Secondary antibody	Anti-mouse biotin (Vector ABC)	PBS and serum	30
	Streptavidin-biotin amplification	Streptavidin (Vector ABC)	PBS	30
	Chromogen	Vector VIP peroxidase substrate	PBS	10
	Counterstain	Methyl Green		3 mins at 60°C

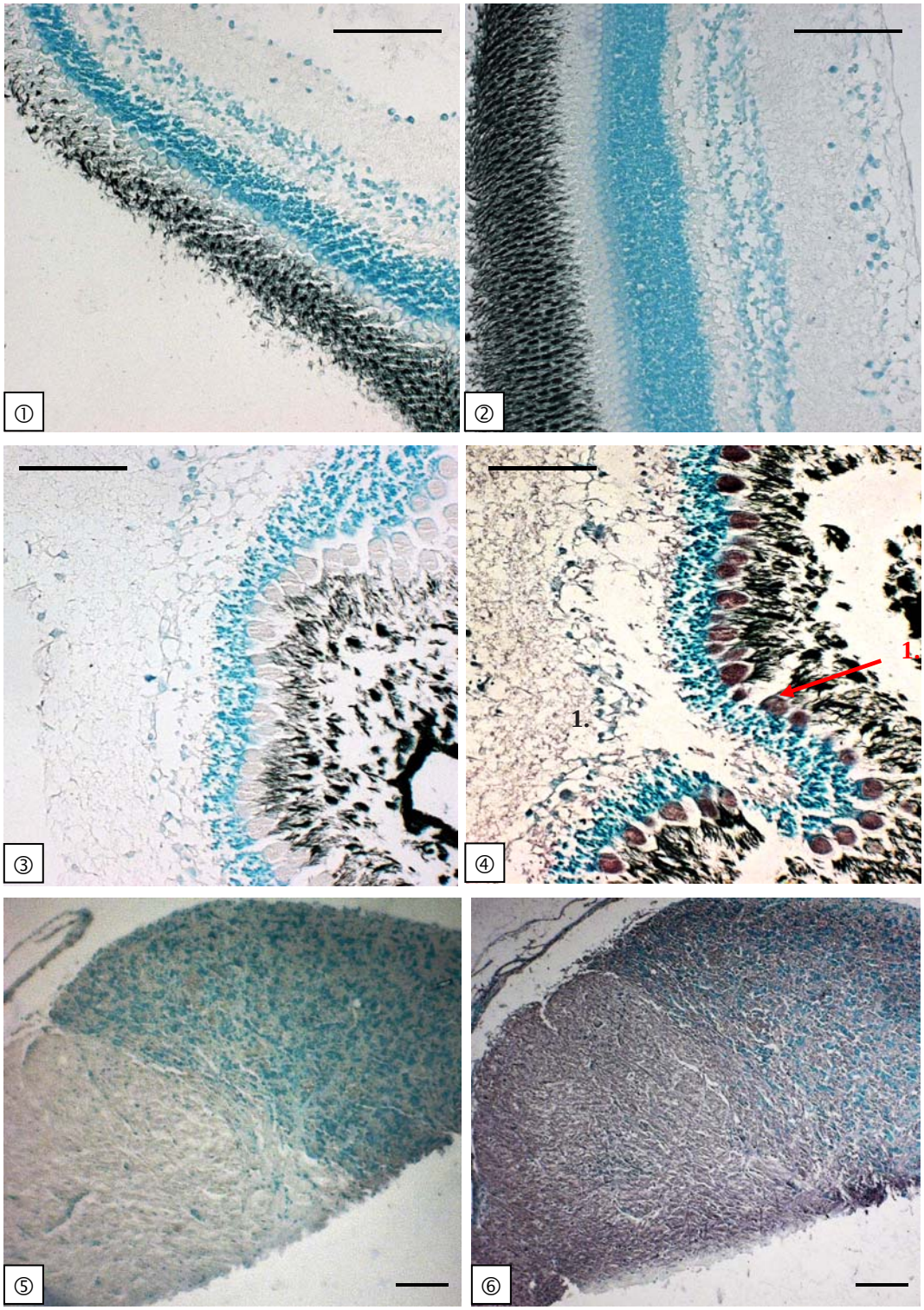


Figure 3.8 – Immunohistochemistry of paraffin section of normal and infected fish tissue. Streptavidin-biotin amplification was performed with Vectorstain ABC kit. The presence of virus is revealed by the colour purple. Eye of non-infected Atlantic cod incubated with PBS ① and MAb ② shows no immunostaining. Immunostaining can be observed in the photo-receptors (PR) and in the ganglionic layer (GL) of European sea bass infected with VNN incubated with MAb ④. The same sample incubated with PBS ③ shows no immunostained. Brain of European sea bass infected with VNN was incubated with PBS ⑤ and MAb ⑥ and shows the widely spread disposition of the virus in the brain tissue. Bar = 100 μm.

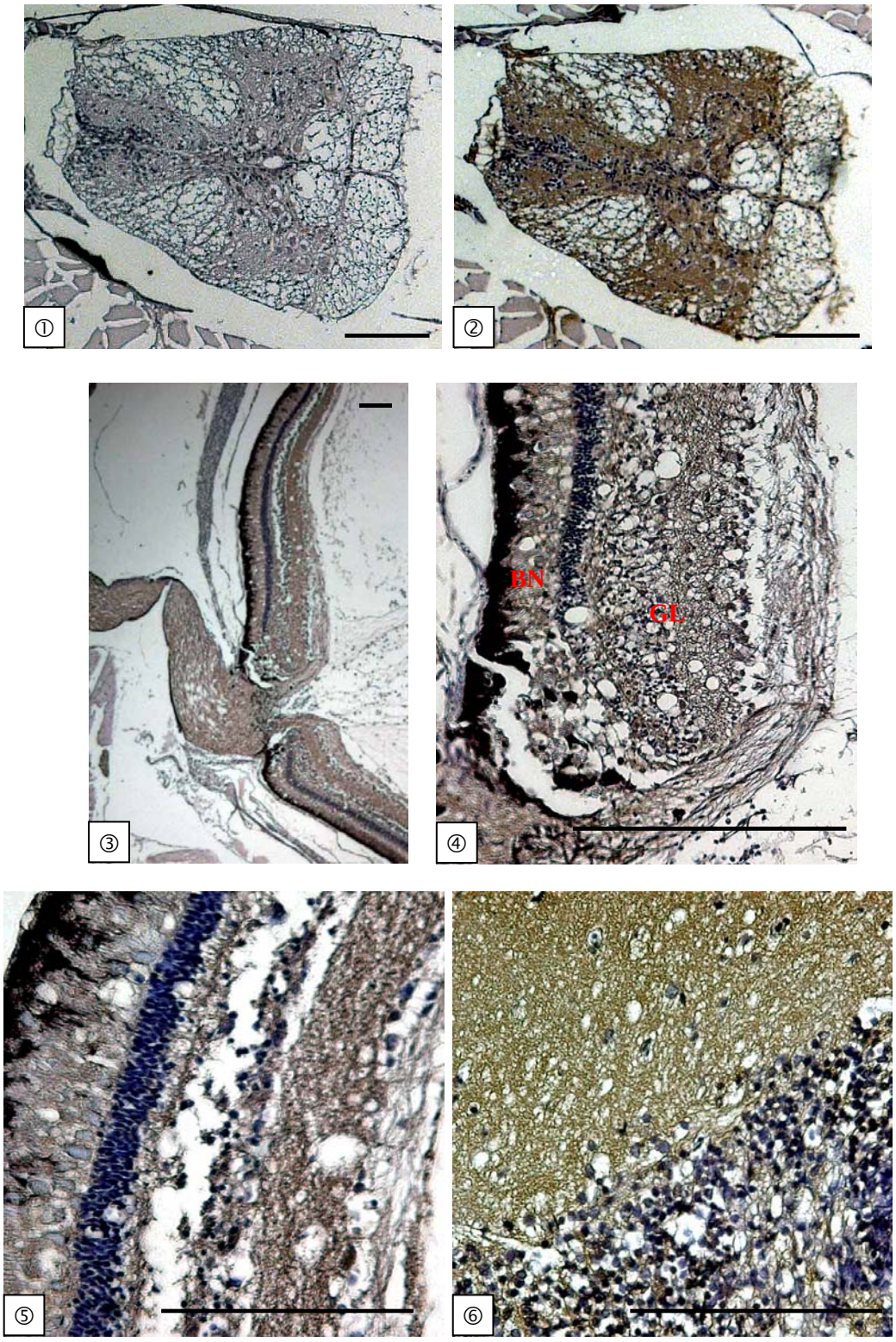


Figure 3.9 – Immunohistochemistry of paraffin section of infected Atlantic cod using antigen retrieval step. The presence of virus is revealed by a brown colour. Spinal cord was incubated with ① PBS and ② MAb. Immunostaining and vacuoles can be observed in the major parts of the spinal cord ②, eye ③, ④, ⑤ (bipolar nuclear layer (BN) and ganglionic layer (GL)) and brain ⑥. Bar = 100 µm.

3.4 - Discussion

The production of MAbs for use as diagnostic tools is widespread and has been carried outfor 30 years. Monoclonal antibodies are attractive reagents since, once generated, they provide a perpetual source of a well-defined antibody (Yelton and Scharff, 1981). The discriminatory power of monoclonal antibodies makes them ideal reagents for investigation of antigenic relationships between viral proteins (Yewdell and Gerhard, 1981).

The ultimate aim in the production of Mabs that recognise European sea bass nodavirus strain was to apply them in antigenic analysis. It is known that the relevance of this type of analysis with MAbs may depend largely on the availability of a diverse panel of antibodies (Yewdell and Gerhard, 1981). In order to try to obtain the most diverse and best panel possible two fusions were performed. The clones obtained with the first fusion revealed very low titre values. Usually this type of result is a consequence of the chromosomal instability associated with the fusion process of the spleen cells and the myeloma cells. The MAbs obtained with the first fusion were therefore discarded and a second fusion was performed. With this second fusion it was possible to obtain and characterise five hybridoma cell lines. The produced MAbs all belonged to the IgG class. As the affinity to the antigen of IgG is higher than that of the IgM antibodies (Nakajima and Sorimachi, 1995), this suggests that the MAbs obtained can be used in serological and diagnostic assays.

Monoclonal antibody may not be specific, as it is possible that they will react not only with the antigen of interest but also with other antigens with similar structural features (Yelton and Scharff, 1981). This cross-reaction demonstrates shared determinants that are indicative of structural or chemical similarities between the antigen and the other “substance” (Yelton and Scharff, 1981, Nelson *et al.* 2000). Thus, an ELISA was used to determine the cross-reactivity of the five MAbs against other betanodavirus strains, viruses and bacterial organisms that can be found in fish and the aquatic environment. The MAbs 4A12, 1E3 and 5G10 recognised Atlantic cod and halibut strains as well as European sea bass betanodavirus

strain. However MAbs 4C3 and 3B10 just recognised the strain used for immunising the mice – European sea bass strain. European sea bass is grouped into the RGNNV clade (Red-Spotted Grouper Nervous Necrosis Virus) (Skliris *et al.* 2001), Atlantic cod and halibut into the BFNNV clade (Barfin Flounder Nervous Necrosis Virus) (Starkey *et al.* 2000, Starkey *et al.* 2001) and the striped jack into the SJNNV clade (Striped Jack Nervous Necrosis Virus) (Nishizawa *et al.* 1997). Benjamin *et al.* (1984), suggested that by applying the multi-determinant-regulatory model nearly all evolutionary substitutions would directly affect immunologic cross-reactivity because most amino acid substitutions are immunologically detected. The lack of response of some of the MAbs against the different nodavirus strains may reveal that the MAbs recognise a less conserved region of the coat protein, and this region is not shared by RGNNV, BFNNV and SJNNV clades. Nishizawa *et al.* (1995b) suggested that there is a difference in the epitopes between SJNNV and other nodavirus strains based on the lack of binding of MAbs to these strains. Monoclonal antibodies 4A12, 1E3 and 5G10 appear to recognise a region on the coat protein shared by RGNNV and the BFNNV clusters, but 3B10 and 4C3 recognised a region that is not shared between these two clades and is specific to the RGNNV cluster. The results suggests the presence of five MAbs binding to at least two different epitopes, one where MAbs only recognise the European sea bass strain (MAbs 4C3 and 3B10) and another where MAbs also recognise Atlantic cod and halibut strains (MAbs 4A12, 5G10 and 1E3).

Photobacterium damselae subspecies *piscicida*, *Piscirickettsia salmonis* and several species of *Vibrio* and *Mycobacterium* were tested for MAbs specificity. There was no cross-reaction between these organisms and the MAbs. The same result was observed when four different fish viruses (IPNV, SPDV, WISAV and sleeping disease) were screened.

An enormous advantage of hybridoma technology is the ability to generate specific antibodies even with impure antigens, due to the selection performed during the screening assays (Secher and Burke, 1980). The immunisation of the mice was performed with purified betanodavirus, but the selection of MAbs was performed with virus supernatant from tissue culture. There is always the possibility that the MAbs may recognise SSN-1 cell components

present in the supernatant. This could occur due to the presence of cell particles in the purified virus suspension used for the immunisation of the mice. In fact, the MAbs did not cross-react with SSN-1 supernatant. The lack of cross-reactivity between the MAbs and the cell lines (CHSE-214 and SHK-1) used to grow the other viruses tested was also confirmed.

Antibodies specific for viral antigens are often crucial in containing the spread of a virus during acute infection and in protecting against re-infection (Kuby, 1994). Antibodies can reduce virus growth by preventing virus adsorption or penetration into host cells (Dimmock, 1984, Abbas *et al.* 2000). Work with polio virus suggested that different MAbs can neutralise virus by different mechanisms or a single MAb can neutralise by a number of alternative mechanisms (McCullough, 1986). The neutralisation index (NI) suggests the presence of MAbs that bind to different epitopes. The group comprising the MAbs that cross-reacted with other betanodavirus strains can be sub-divided. The lack of neutralisation ability of MAb 4A12 to the European sea bass strain indicated that this MAb recognised a different epitope region to MAbs 5G10 and 1E3. Even these two MAbs do not appear to share the same epitope as the lack of neutralisation against Atlantic cod presented by MAb 1E3 indicates.

Western blot analysis revealed that all the MAbs recognised a protein with an approximate molecular weight of 42 kDa, the size of the betanodavirus coat protein (Mori *et al.* 1992). The MAbs developed by Nishizawa *et al.* (1995b) against striped jack nodavirus and by Lai *et al.* (2001a) for Yellow Grouper Nervous Necrosis Nodavirus (YGNNV) also recognised this protein. The generation of MAbs that recognise this protein is not surprising as the coat protein is the outer structural component of the virion particle.

Monoclonal antibodies recognised the proteins in their three-dimensional configuration on the viral surface and often recognise epitopes that depend upon the folded three-dimensional shape of the polypeptide chain (Dulbecco, 1988a). The detection of the coat protein is very important for future antigenic studies because it indicates the type of epitope that the MAbs recognise. Two of them (MAbs 4C3 and 4A12) revealed the ability to recognise the coat protein in both boiled and non-boiled samples suggesting that the epitope of these MAbs bind may not be dependent on protein folding.

Immunohistochemistry with the anti-nodavirus MAbs was only successful with MAb 4A12 and the process required either an amplification or antigen retrieval step. Both antigen retrieval and signal amplification with streptavidin-biotin revealed similar results and a general binding of the MAb to the cytoplasm of infected tissue cells was observed. Such a result is not surprising as the betanodavirus particles are present in the cytoplasm of infected cells (Nishizawa *et al.* 1995b), where virus assembly occurs (Chi *et al.* 1997). Such results, a generalised staining of the brain and eye were also observed by Lai *et al.* (2001a) and Shieh and Chi (2005) using anti-grouper nodavirus MAbs. When tested MAb 4A12 revealed no cross-reativity with normal nerve cell, eye or even with other organs tissue. These results indicate that MAb 4A12 can be useful for diagnosis and confirmational diagnosis of VER.

Polyclonal antisera contain many different antibodies that will perform optimally in different assays (Yelton and Scharff, 1981). Therefore polyclonal antibodies can be use in a vast number of immunoassays. The exquisite specificity and homogenous structure of MAbs is the main reason why MAbs are so often considered to be superior to polyclonal antibodies. In the context of immunoassays this is not always an advantage (Peppard, 2000) because they may not performe well in as many immunoassays as polyclonal antibodies. These differences between MAbs and polyclonal antibodies were confirmed by the polyclonal antisera showing the highest neutralisation index to the betanodavirus strains and the binding to several proteins by Western Blot.

A crucial aspect of the characterisation of MAbs is the study of how the MAbs react in different assay systems. This is especially pertinent for the use of MAbs as diagnostic reagents because some antibodies perform well is some assays but not in others. This phenomenon relates to how an antibody recognises its target epitope in the context of the assay system used with the target epitope being masked, denatured or rendered inaccessible by the immobilisation procedure adopted within a given technique (Nelson *et al.* 2000). The most crucial advantage of MAb is that once a useful hybridoma cell line has been generated it can be used by different laboratories eliminating a source of variation and providing a standard reagent (Pollock *et al.* 1984).

3.4.1 - Final reflection

The production of hybridoma cell lines is a well-established technique. However, it is not a straightforward technique and often more than one fusion needs to be performed and this can be costly and time consuming. In order to obtain hybridoma cell lines producing antibodies against European sea bass betanodavirus two fusions were performed and five MAbs were generated.

The results obtained suggest that the five hybridoma cell lines recognised different regions of the coat protein. This fact is extremely useful for achieving the major aim of the thesis i.e. producing MAbs against betanodavirus for application in antigenic mapping studies. Due to the characteristics shown by the MAbs several epitope mapping techniques could be used, e.g. escape mutants and pepscan.

Cross-reactivity tests revealed that three of the MAbs (4A12, 5G10 and 1E3) recognised Atlantic cod and halibut betanodavirus strains. None of the MAbs produced cross-reacted with other fish viruses, cell line culture supernatant or bacteria species common to fish and aquatic environment. This indicates the specificity of the MAbs to betanodavirus and the possibility that they may be utilised as diagnostic tools.

The monoclonal antibodies produced can be applied to several techniques that require the use of antibodies against betanodavirus. For example the isolation of betanodavirus in cell culture can be confirmed by performing a neutralisation assay with the MAbs produced. The high signal obtained when infected tissue was staining using immunohistochemistry shows that the MAb 4A12 can be used for diagnosis and confirmation of VER in fish tissue samples.

Further studies should be performed in order to confirm the ability of the MAbs to bind to other betanodavirus strains e.g. barramundi, turbot, Dover sole, groupers, guppy and sturgeon and to establish a wider range of possible application of these MAbs.

Chapter 4 - Epitope mapping with escape mutants

4.1 - Introduction

During infection with bacteria or viruses, the host immune system produces an antibody response directed against the pathogen. These antibodies protect the organism by interaction between the paratope (antibody binding site) and the epitope (antigen binding site) (Klasse and Sattentau, 2002).

The development of techniques for the production of monoclonal antibodies allowed the perpetual supply of antibodies directed against a single epitope (Yelton and Scharff, 1981). Consequently, monoclonal antibodies stimulated the study of the antigen-antibody interaction and the characterisation of epitopes within viruses and other antigenic structures.

Several techniques have been employed for epitope mapping, including random peptide phage display (Scott and Smith, 1990) and pepscan using overlapping panels of synthetic peptides (Geysen *et al.* 1984). However, these techniques are largely limited by their inability to represent conformational epitopes.

Monoclonal antibodies directed against viral antigens have been available for over 20 years. Some of these antibodies exhibit viral neutralising activity (Strauss *et al.* 1991). The biological processes involved in virus neutralisation by antibodies are not completely understood. However, it has been suggested that antibodies may neutralise viruses by preventing the binding of viral particles to cellular receptors or by blocking viral penetration into the cytoplasm (Kuby, 1994).

When viruses are incubated with neutralising antibodies, rare mutants can be recovered that possess the ability to replicate despite the presence of the antibody neutralising activity (Gerhard and Webster, 1978, Laver *et al.* 1979). These virus mutants are referred to as *neutralisation escape mutants*. Nucleotide sequencing of these viruses can then be used to identify which changes in the virus protein structure confers neutralisation resistance (Mateu

et al. 1990, Strauss *et al.* 1991, Balasuriya *et al.* 1995). This procedure is particularly suitable for the analysis of RNA viruses since these exhibit relatively high genetic variability due to error-prone replication machinery (Gitlin *et al.* 2002, Boden *et al.* 2003).

The principle underlying this technique is that virus escape mutants are able to replicate in the presence of a neutralising antibody because they possess a mutation that confers a change in an amino acid residue situated at the antibody binding site. Thus the antibody is unable to bind and neutralise the escape mutant (Morris, 1996b). It has been demonstrated that a single amino acid substitution is sufficient to shift the antigenicity of a virus as occurs for example in the influenza virus HA1 polypeptide (Berton *et al.* 1984). The interaction between an antibody and an antigen can be abolished by the loss of a single hydrogen bond, provoking a reduction in the binding constant of nearly three orders of magnitude (Colman *et al.* 1987).

The generation of mutants is a routine procedure exploited widely in structure-function analyses and for the development of engineered viruses as vaccines or drugs (Yoshiyama *et al.* 1994, Shotton *et al.* 1995, Zhao *et al.* 2004, Schubert *et al.* 2005)

The most widely used method for isolating and selecting escape mutants is based on the viral plaque reduction assay. However, for some viruses such as influenza virus the production of escape mutants has been achieved in eggs (Berton *et al.* 1984, Saito *et al.* 1994).

In the plaque reduction assay the growth of the virus occurs in the presence of a neutralising antibody, so any plaques that occur are formed by the replication of a viral escape mutant. Plaques are formed by cytopathic viruses (Rovozzo and Burke, 1973) and these can in many cases be produced by the addition of an overlay such as agarose to an infected monolayer of cells (Dulbecco and Vogt, 1954).

In the majority of studies the plaque process is preceded by several passages of the virus in cell culture in the presence of the neutralising antibody (Weiland *et al.* 1999). However, selection of escape mutants may take place in the absence of an overlay (Borrego *et al.* 2002). This methodology is of use for the analysis of viruses that are refractory to plaque formation.

Escape mutants have been used for epitope mapping studies of the intracytoplasmic tail of Human Immunodeficiency Virus (HIV) type 1 gp41 (Kalia *et al.* 2005), xenotropic murine leukaemia virus (Li *et al.* 1999); equine arteritis virus (Balasuriya *et al.* 1995); hepatitis A virus (Ping and Lemon, 1992); influenza virus (Gerhard *et al.* 1981); measles virus, mumps virus, rubella virus (Tischer and Gerike, 2000) and poliovirus (Minor *et al.* 1983).

4.1.1 - Plaque Formation

Zones of cell lysis or CPE occurring in virus infected cell monolayers are referred to as plaques (Hierholzer and Killington, 1996). Each plaque originates from a single virus particle (Rovozzo and Burke, 1973).

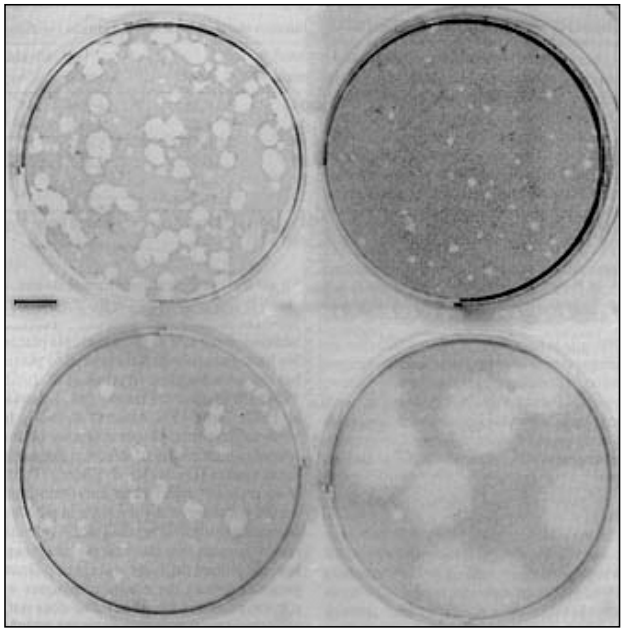


Figure 4.1 - Plaques produced by measles virus. Overlay agarose stained with neutral red (bar = 0.5 cm) (Borges *et al.* 1996). It is possible to observed the different plaque sizes produce by different measles strains.

In order to obtain plaques the virus is added to the cell culture monolayer and allowed to adsorb (Burleson *et al.* 1992) (see Figure 4.1). An overlay, typically agarose or carboxyl methylcellulose is then added and the infected monolayers are incubated. The overlay prevents diffusion of viral particles and permits better resolution of plaques by preventing the spread of virus to other areas of the cell monolayer (Bachmann *et al.* 1999, Hierholzer and Killington, 1996).

After incubation a stain is (e.g. neutral red or tetrazolium) is used to visualise plaques (Burleson *et al.* 1992). Virus isolated from individual plaques can be isolated by "picking".

4.1.2 - Objectives

The aim of this chapter was to isolate and characterise betanodavirus escape mutants. This would permit identification of the site(s) recognised by neutralising antibodies that play a major role in protection and recovery from infection. Neutralising monoclonal antibodies were used for escape mutant isolation. The production and characterisation of the MAbs are described in Chapter 3. Determination of the nucleotide sequence of the coat protein gene of isolated escape mutants would enable identification of amino acid changes in the coat protein associated with resistance to neutralisation. Two approaches were used to produce betanodavirus escape mutants: a) plaque isolation and b) isolation by limiting dilution. For producing betanodavirus plaques, several different overlays were tested. A limiting dilution procedure developed by Borrego *et al.* (2002) was also used.

4.2 - Material and Methods

4.2.1 - Plaque isolation

The procedure for obtaining betanodavirus plaques was performed with a nodavirus isolated from European sea bass designated Mt/01/Sba. Virus growth was performed in 6 well plates (Nunc, Fisher Scientific Leicestershire U.K.).

Agar number 1 (Oxoid, Hampshire U.K.); agarose type VII, low melting point (Sigma, Dorset U.K.); agar gum (Sigma, Dorset U.K.); carboxyl-methylcellulose medium viscosity (Sigma, Dorset U.K.) and carboxyl-methylcellulose high viscosity (Sigma, Dorset U.K.) were used as overlay.

All the overlays were tested at different concentrations, 0.5 %, 1 % and 1.5% (w/v) in a solution 1:1 of distilled water and L-15/FBS. The gelling compounds were dissolved in distilled water, autoclaved and held above gelling temperature until Lebovitz L-15 medium containing 10 % FBS (L-15/FBS) was added. The L-15/FBS was added immediately before the overlay was used.

Betanodavirus plaques were produced in pre-formed SSN-1 monolayers and also by simultaneous inoculation of SSN-1 monolayers.

Culture of SSN-1 stock cells is described in Chapter 2. Seven day old cultures of SSN-1 cells (25 cm^3 flask) were harvested and used to inoculate 6 well plates which were then incubated at 25°C. Cell monolayers were allowed to develop until 80 % confluent. Growth medium was then removed and monolayers washed three times with HBSS (supplemented with 2 % FBS). Betanodavirus strain Mt/01/Sba (500 µl) was added to each well and the plates were sealed with Nesco film. Virus was allowed to adsorb to the SSN-1 cells for one hour at 25°C, with gentle agitation (Mini Rocking platform, Biometra™). Monolayers were infected with between 10^2 to 10^6 $TCID_{50}$ of virus. Negative controls were inoculated with L-15 supplemented with 5 % of FBS.

After removal of non-adsorbed virus, cells were washed with HBSS (supplemented with 2 % FBS). The overlay (2 ml) was added and allowed to solidify. Plates were sealed with Nesco film and incubated at 25 °C.

Plates were incubated for 2, 3, 4 and 5 days. After this period 500 µl of neutral red (0.1 % in HBSS/FBS 2 %) was added and monolayers were examined microscopically for the formation of plaques.

For the simultaneous inoculation 2 ml of SSN-1 cells were mixed with 500 µl of the virus and allowed to seed for 24 hrs. Monolayers were then processed as described above for pre-formed monolayer.

4.2.2 - Isolation by limiting dilution

Two strains of betanodavirus were used for escape mutant isolation by limiting dilution, MT/01/Sba isolated from European sea bass and GB/32/cod, isolated from cod. Three MAbs were used 4C3, 4A12 and 3B10 (see Chapter 3 for details). The neutralisation ability of each of the MAb against each virus strain was determined as described in Chapter 3. MAbs 4C3 and 3B10 were used for MT/01/sba, and 4A12 was used for GB/32/cod.

Virus neutralisation assay was performed as described by Borrego *et al.* (2002).

HBSS (45 µl well^{-1}) was added to each well of a 96 well plate. Betanodavirus (5 µl) was added to the wells in the first column wells and a 10-fold series of dilutions was then made. Equal volumes of MAbs (1:20 and 1:100 v/v in HBSS) were added to virus-containing wells. The virus and MAbs were allowed to interact for 90 mins at 25°C or 20°C with gentle agitation (Mini Rocking platform, Biometra™). After this period 90 µl of SSN-1 cells were added to wells, then plates were sealed with Nesco film and incubated for 4 to 6 days at 25°C (European sea bass strain) or 20°C (Atlantic cod strain). After this period monolayers were

examined for CPE. Virus was harvested from cells showing CPE that had received the greatest input dilution of virus.

The harvested virus (100 µl well^{-1}) was added to 5 wells of a 24 well plate and incubated with 100 µl of MAb (1:20 v/v in HBSS) for 90 mins at 25°C or 20°C with gentle agitation. After this period 1 ml of SSN-1 cells was added to each well, and plates sealed with Nesco film and incubated for 4-6 days. After this period the supernatant was collected and the procedure repeated two further times. After the 3rd passage in the presence of MAb the virus was grown twice in the absence of MAb in order to exclude the MAb from the sample.

Positive and negative controls were incorporated into the limiting dilution procedure. These controls were processed in the same way as for samples. HBSS was added instead of MAb for a positive control, and HBSS (supplemented with 5 % FBS) was added instead of virus for a negative control.

Borrego *et al.* (2002) screened the escape mutants obtained by ELISA. This step was performed in order to gain reassurance that the escape mutants were not recognised by the MAbs. This screening was performed prior to sequencing the escape mutant's cDNA. An ELISA was performed as described in Chapter 2. An ELISA plate was coated with mutant virus supernatant from tissue culture of the virus. The MAbs used for the selection of the escape mutants were used as first antibody in the ELISA.

Once escape mutants have been identified the coat protein should be sequenced and the results compared with the coat protein of the non-escape mutants virus.

4.3 - Results

The aim of this study was to isolate betanodavirus neutralisation escape mutants. Two approaches were used; plaque isolation and isolation by limiting dilution.

4.3.1 - Plaque isolation

Plaque assays were performed using five different types of overlay: agar n^{o} 1; agar gum, agarose type VII, of high viscosity carboxyl-methylcellulose and medium viscosity carboxy-methylcellulose. None of these techniques permitted the isolation of betanodavirus escape mutants, when either infection of pre-formed monolayers or simultaneous inoculation was used as the route of infection.

Results varied considerably between experiments. On occasions, virus appeared to grow efficiently, whereas at other times, little growth was evident in infected monolayers. Agar type VII yielded plaques most consistently, but these were very small and tended to be surrounded by many other plaques. Cytopathic effects tended to be diffuse and present throughout infected monolayers rather than confined to discrete plaques, as shown in Figure 4.2.

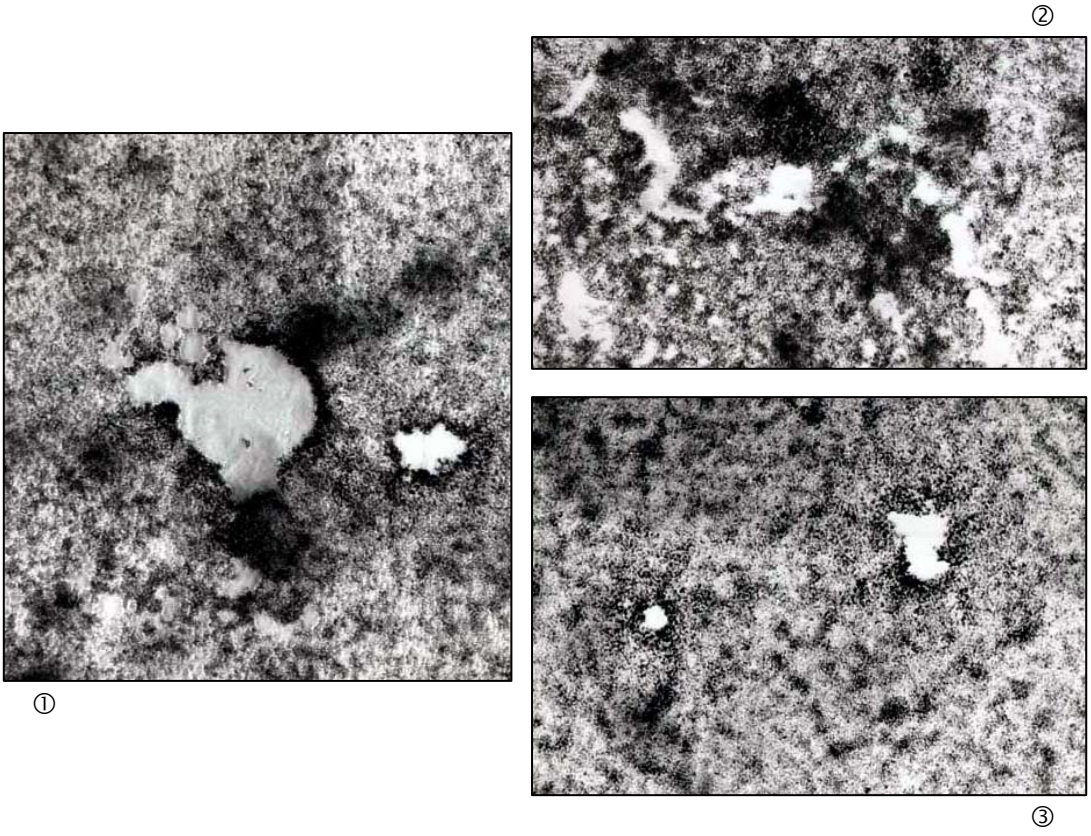


Figure 4.2 - SSN-1 monolayer showing cytopathic effects induced by betanodavirus MT/01/Sba. Monolayers were overlayed with Agar type VI and stained with 0.1% neutral red (① and ② magnification 100x, ③ magnification 40x)

4.3.2 - Isolation by limiting dilution

Limiting dilution was also assessed as a method for betanodavirus escape mutant isolation. In Figure 4.3. and 4.4 can be observed the SSN-1 monolayer in the presence or absence of the virus.

Escape mutants obtained were screened by ELISA prior to the nucleotide sequence analysis. The ELISA results revealed that the neutralising escape mutants were recognised by the MAbs. The nucleotide sequence would be performed in the mutants revealing a negative result by ELISA. The lack of such negative results led to none of the escape mutants obtained being sequenced.

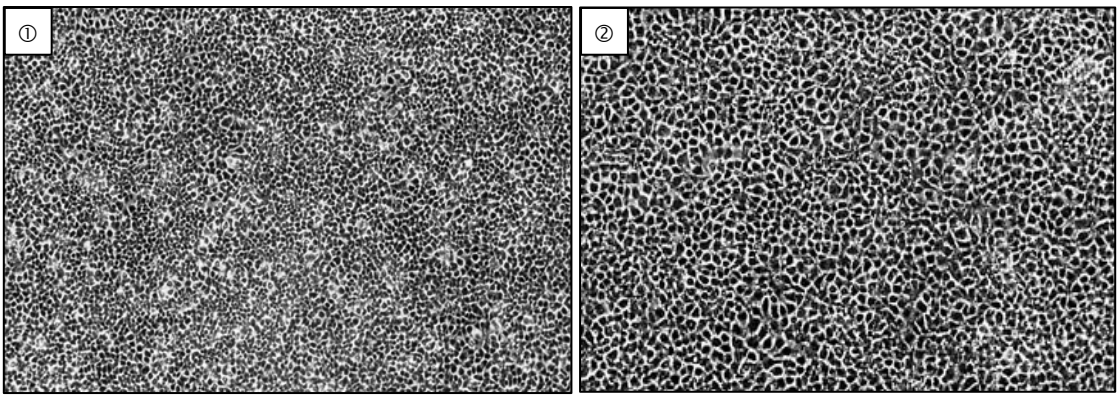


Figure 4.3 – SSN-1 monolayer (① magnification 40 x and ② magnification 100 x).

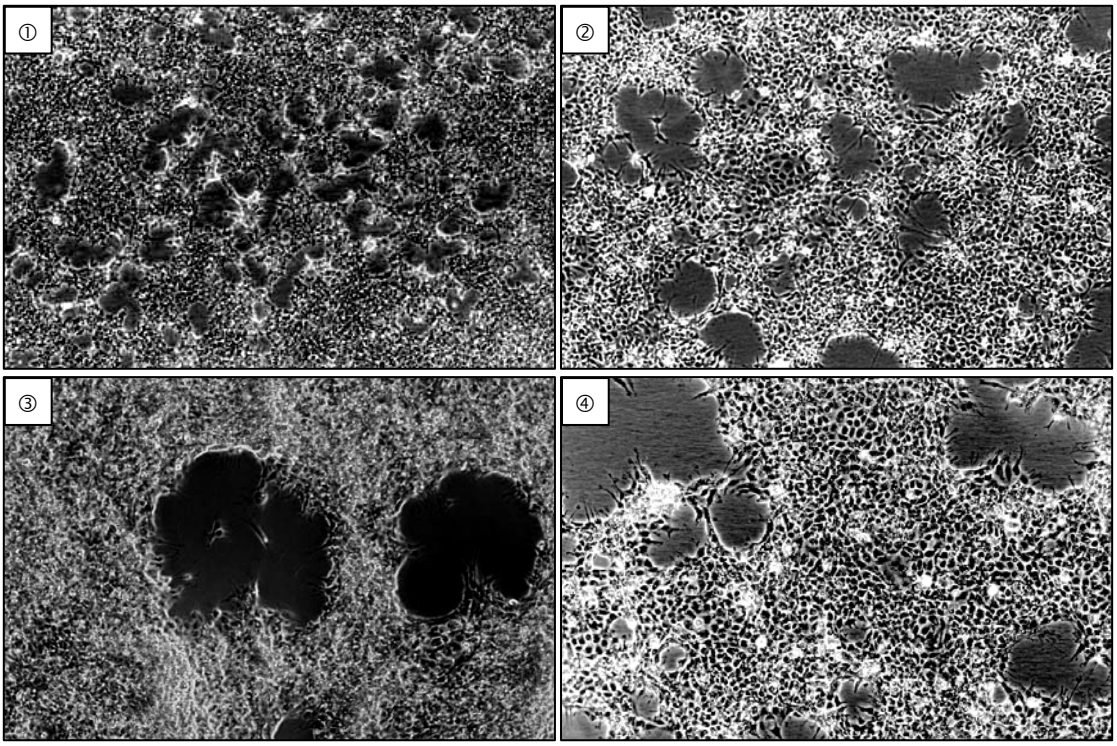


Figure 4.4 – Production of escape mutants by limiting dilution. European sea bass NNV infecting SNN-1 cells in the presence of 4C3 MAb (① magnification 40 x; ② magnification 100 x; ③ magnification 200 x). Atlantic cod NNV infecting SNN-1 cells in the presence of MAb 4A12 (④ magnification 200x).

4.4 - Discussion

Sequence analysis of viral neutralisation escape mutants is a powerful tool for analysing the molecular interaction between the humoral immune response and viral pathogens. This methodology has been applied successfully to several viruses including Hantaan virus (Wang *et al.* 1993), influenza A virus (Laver *et al.* 1979), rabies virus (Raux *et al.* 1995) and HIV type 1 (Shotton *et al.* 1995). The results of epitope mapping studies can be used to aid the rational design of vaccines and immunodiagnostic reagents. The method is particularly suited to the study of RNA viruses, which exhibit high mutation rates (Morris, 1996b, Das *et al.* 2004).

Attempts to isolate betanodavirus neutralisation escape mutants were performed using the two classical virological procedures of plaque purification and limiting dilution. Escape mutants were selected with a panel of neutralising MAbs produced in an earlier stage of the project (Chapter 3).

The plaque assay is ideally suited to escape mutant isolation because it allows for the easy isolation and propagation of viral clones resistant to the effects of neutralisation. This method had been used with success for the isolation of escape mutants to the rainbow trout virus VHSV (Béarzotti *et al.* 1995). The use of plaque assay required optimisation for betanodaviruses since plaque assays have not previously been described for this virus family. There is not a single method that can be used to plaque all known virus groups (Rovozzo and Burke, 1973).

Virus plaques are formed after infection of a cell monolayer. Virus is allowed to adsorb prior to the addition of a solid or semi-solid overlay to restrict diffusion of replicating virus (Dulbecco, 1988b, Cann, 2001). In the present study 5 different types of overlay were used: agar n^{o}1, agar gum, agar type VII and carboxyl methylcellulose (high and medium viscosity). None of these methods permitted the formation and isolation of betanodavirus plaques. This result is in contrast to numerous other studies in which virus plaques have been successfully produced using the same experimental procedures. For example, plaque

formation has been described for coxsackievirus, influenza virus, measles virus, mumps virus, and rubella virus using carboxy-methylcellulose as an overlay (Bachmann *et al.* 1999, Pipkin *et al.* 1999, Triantafilou *et al.* 1999, Tischer and Gerike, 2000). Furthermore, Wolf and Quimby (1973) have produced plaques of a number of fish viruses (bluegill myxovirus, channel catfish virus, eel virus, Egtved virus, IPNV, lymphocystis virus and spring viraemia of carp). Agar has been widely used as an overlay since the 1950s (Dulbecco and Vogt, 1954, Moss and Gravell, 1969, Borges *et al.* 1996, Scotti and Dearing, 1996, Li *et al.* 1999, LaBarre and Lowy, 2001).

In the present study, the betanodavirus strains studied formed microscopic plaques, however it was not technically feasible to collect the virus from plaques of this size. When longer incubation periods were used in attempts to produce larger plaques, the CPE spread throughout infected monolayers.

Several factors can influence the growth and plaquing characteristics of viruses. These include the sensitivity of the cells used for virus growth, health status of the cells, adsorption time, type of overlay, incubation period (Burleson *et al.* 1992, Hierholzer and Killington, 1996). All of these points were taken in to consideration in attempts to obtain betanodavirus plaques.

The SSN-1 cells (Frerichs *et al.* 1996) are the most commonly used cell line for isolation and propagation of Betanodaviruses. Other cell lines have been developed (Lai *et al.* 2001b) but it was not possible to obtain these. Whilst the use of other cell lines may ultimately permit the successful production of betanodavirus plaques, many of these are not available due to patent restrictions surrounding betanodavirus vaccine manufacture.

The adsorption period could also be of critical importance in the formation of virus plaques. During the present study, a 90 mins adsorption step was used. This length of time should be sufficient for virus attachment and penetration to occur. Previous studies have shown that 60 mins is sufficient for adsorption of viruses to cells, and no gain is obtained by increasing the adsorption time (Fendrick and Hallick, 1983, Bushar and Sagripanti, 1990).

Some studies have reported better results when infected cells were fixed with formalin and then stained with crystal violet (Bushar and Sagripanti, 1990, Gaertner *et al.* 1993). However, these methods could not be utilised in the present study because they are destructive procedures that do not permit the recovery of viable virus for nucleotide sequencing analysis.

Successful selection of escape mutants to IHNV, a fish rhabdovirus, was performed by Huang *et al.* (1996), when the escape mutants were selected by limiting dilution previous to plaque-purification. The use of limiting dilution is especially suitable for obtaining escape mutants of viruses that do not form plaques, but are capable of inducing cytopathic effects (Barclay and Almond, 2000). Since plaque based procedures did not yield betanodavirus escape mutants, attempts were made to isolate escape mutants using a limiting dilution procedure developed by Borrego *et al.* (2002). With this procedure selection was achieved with two dilutions of MAb (1:100 and 1:20 v/v). These were followed by further incubations in the presence of MAb at a dilution of 1:20. The dilutions of neutralising MAb were found to be suitable for obtaining escape mutants by limiting dilution (Minor *et al.* 1983, Ping and Lemon, 1992).

The isolation of mutant virus by limiting dilution is possible because rare variants in a virus sample (typically $1{:}10^6$) are not neutralised and these represent antigenic variants of the parental virus (Yewdell *et al.* 1979). Betanodavirus is an RNA virus and the strains used replicated to a titre greater than 10^7 which is appropriate for use in limiting dilution based procedures for escape mutant isolation. RNA viruses are particularly suited for escape mutant selection because there are no error correction mechanisms operating in RNA replication, and thus RNA viruses tend to exhibit high mutation frequencies that favours the creation of escape mutants (Dimmock and Primrose, 1987).

Neutralisation escape mutants are predicted to contain a single nucleotide sequence change corresponding to a single amino acid change compared to the parental virus (Parry *et al.* 1990). Several methods can be used to confirm resistance to the neutralising MAb used for selection such as ELISA and neutralisation test (Zhou *et al.* 1994, Béarzotti *et al.* 1995, Hörling and Lundkvist, 1997). In the present study the protocol developed by Borrego *et al.*

(2002) was followed so the escape mutants were screened by ELISA and found to be still recognised by the selecting antibody. Nucleotide sequencing of escape mutants was not performed because Borrego *et al.* (2002) considered the lack of recognition by ELISA as a pre-requisite for nucleotide sequencing. This may have lead to a failure to recognise the presence of mutations that had occurred in phage clones but did not prevent the binding of the MAbs. Nucleotide sequencing could be utilised to determine whether escape mutants were produced.

A possibility for the inability to produce escape mutants may be due to an insufficient number of passages in the presence of neutralising MAbs. It is possible that three passages of betanodavirus were insufficient and a longer selection is necessary.

4.4.1 - Final considerations

The aim of this thesis section was not achieved. It was not possible to develop a method for the production of betanodavirus neutralisation escape mutants by plaque-selection.

The use of different cell lines may have enabled this objective to be achieved. Virus isolated by limiting dilution was recognised by the selecting MAb in ELISA tests. According to Borrego *et al.* (2002) this recognition indicates that the production of escape mutants was unsuccessful. It is possible that several mutations are needed to alter the antigenicity of the betanodavirus or a higher number of passages may be required to obtain escape mutants.

Chapter 5 - Epitope mapping by phage display

5.1 - Introduction

Antibodies represent a major component of the array of immune defence mechanisms directed against pathogens such as bacteria and viruses. Humoral immune responses are of great importance in the protective effects induced by vaccines (Irving *et al.* 2001). Antibody molecules interact with their target antigen through specific receptor regions generated through somatic recombination of V, D, and J gene segments during B-lymphocyte maturation. Hypervariable regions of V_L and V_H domains are brought together in three dimensional space to form an antigen binding surface (Abbas and Lichtman, 2001). The specific region of an antibody molecule that interacts with the antigen is referred to as the *paratope*, and the corresponding region of the target antigen recognised by this region of an antibody molecule is referred to as an *epitope* (Wang and Yu, 2004).

Epitopes can be classified into two types. Those comprising a short sequential stretch of amino acids are referred to as *linear* epitopes. In contrast, epitopes that are formed by the juxtaposition of two or more distinct regions of a protein molecule are referred to as *discontinuous* or *conformational* epitopes (van Regenmortel, 1989a). Within this classification scheme, epitopes whose affinity is affected by unfolding of the protein are said to be constrained; almost all discontinuous and many linear epitopes are constrained (Geysen *et al.* 1987b).

Characterisation of the primary amino acid sequence or structural conformation of epitopes has many applications in biological sciences including the deciphering of protein-protein interactions, immunoassay development, elucidation of pathogen neutralising sites, vaccine development, investigation into the pathogenesis of autoimmune diseases and analysis of protein topology in intact cells or organelles (Morris, 1996a).

Several techniques have been developed for epitope mapping. One of the most commonly used techniques during the last 20 years has been phage display. This technique was first described in 1985 when George Smith demonstrated that it was possible to create a fusion between one of the phage fl minor coat protein genes with short stretches of DNA encoding foreign proteins. Smith showed that the sequence insert was translated and expressed as a fusion protein on the surface of the bacteriophage. Consequently, it was possible to create an epitope library by inserting tens of millions of short random DNA sequences into a phage population (Scott and Smith, 1990).

The phage display methodology provides a direct physical link between phenotype and genotype (Hoogenboom and Chames, 2000). This is achieved because the fusion peptide is part of the capsid enclosing the phage genomic DNA (Cull *et al.* 1992). Thus phages expressing a single insert can be purified from the phage library by affinity binding to an antibody or other protein (Scott and Smith, 1990). The use of the simple and cost efficient process of bacteriophage replication is applied to the propagation of individual clones (Rodi and Makowsi, 1999). The DNA insert of the phage is identified by DNA sequence analysis (Burton, 1995). One of the key advantages of this technique is that it can be performed without the previous knowledge of the protein structure (Williams *et al.* 2001).

A number of different phage display methods have been described (Kay and Hoess, 1996): ① phage display of natural peptides, used for mapping epitopes of monoclonal and polyclonal antibodies and generating immunogens; ② phage display of random peptides, used for mapping epitopes of antibodies, identifying peptide ligands and mapping substrate sites for proteases and kinases; ③ phage display of proteins and protein domains, utilised in the study of directed evolution of proteins, isolation of high-affinity antibodies and cDNA expression screening.

For the study of antigen-antibody interactions two types of phage display libraries have been used, random peptide libraries (RPL) and gene fragment libraries. Random peptide libraries are ideal for identifying peptide ligands of an antibody without prior knowledge of the recognition site (Irving *et al.* 2001). Both linear and discontinuous epitopes can be

mapped by phage display, but mapping of discontinuous epitopes represents a much more difficult task than the mapping of linear epitopes (Yip and Ward, 1999). Numerous attempts at epitope mapping discontinuous epitopes with phage display have been unsuccessful (Williams *et al.* 2001).

Random peptide phage display has been used in several epitope mapping studies and analyses of protein-protein interaction, including: the VP2 protein of the coronavirus infectious bursal disease virus (IBDV) (Cui *et al.* 2003); feline immunodeficiency virus (FIV) coat protein (D'Mello *et al.* 1999); hepatitis B virus (Germaschewski and Murray, 1996, Pál *et al.* 2003), hepatitis C viruses (Barban *et al.* 2000, Hadlock *et al.* 2000, Bugli *et al.* 2001); apical membrane antigen-1 (AMA1) of *Plasmodium falciparum* (malaria) (Coley *et al.* 2001); multi-drug resistance associated protein MRP1 (one of the most important members of the ATO-binding cassette (ABC) protein family) (Poloni *et al.* 1995, Flego *et al.* 2003); crotoxin, a lethal venom of rattlesnake *Crotalus durissus terrificus* (Demangel *et al.* 2000); hormones (Li *et al.* 1995); interaction of the phosphorylation site of kinase CK2 with other peptides and proteins (Cardellini *et al.* 2004) and screening MAbs using antigen-displaying phage (Lijnen *et al.* 1997).

5.1.1 - Bacteriophage

Filamentous phages (Figure 5.1) constitute a large family of bacterial viruses that infect a variety of Gram-negative bacteria, and use pilin as a receptor (Russel *et al.* 1997).

The best characterised phages are M13, fd and fl. The genomes of these phages are highly conserved, exhibiting 98 % sequence identity (Hill and Petersen, 1982). Because of this conservation and the dependence on the F plasmid for infection M13, fd and fl are collectively referred to as the Ff phage (Webster, 1996). This group of bacteriophages uses the tip of the F conjugative pilus as a receptor and thus are specific for *Escherichia coli* containing the F plasmid (F+) (Wang and Yu, 2004). In contrast to other groups of

bacteriophages that complete their life cycle by lysing the bacterial cell, filamentous phages do not kill their host. New phage particles are secreted without breaking the integrity of the cell envelope (Pereboev and Morris, 1996).

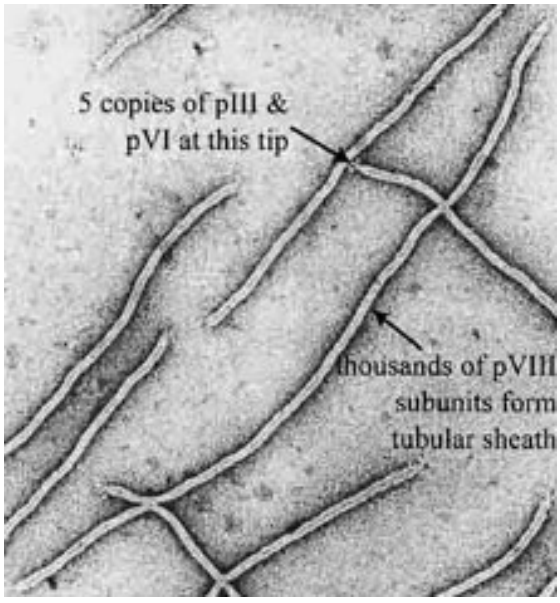


Figure 5.1 – Filamentous phage by electron microscope (Petrenko and Vodyanoy, 2003).

The filamentous phages have a single stranded, covalently closed DNA genome, which is encased in a long cylinder (approximately 7 nm wide and 900-2000 nm in length) (Wilson and Finlay, 1998). The phage genome encodes 10 proteins, five of which are virion structural proteins, three are required for phage DNA synthesis, and two serve assembly functions (Webster, 1996).

Five copies of each of the minor coat proteins pIII and pVI are located at one end of the phage particle. The minor coat proteins pVII e pIX are located at the other end, and 2700 copies of the major coat protein pVII are spread along the filamentous tube (Russel *et al.* 1997, Smith and Petrenko, 1997).

Infection of *E.coli* by the Ff phage is initiated by the specific interaction of the phage with the tip of the F pilus (Webster, 2001). The N-terminus of the pIII protein is responsible for this interaction (Pereboev and Morris, 1996).

After infection, the phage positive (+) strand DNA (ssDNA) enters the cytoplasm and the complementary strand (-) is synthesised by bacterial enzymes, creating a double-stranded replicative form (RF) (Wilson and Finlay, 1998). When the phage specific single-stranded DNA binding protein, pV, reaches a critical concentration, it binds to the newly synthesised DNA strands to form a pV-DNA complex (Webster, 2001). This complex prevents the (+) DNA strand from being used by host replication enzymes, facilitating virus encapsidation (Russel, 1995). Eventually, further increases in pV concentration halt RF synthesis, and the viral assembly process starts at the bacterial membrane (Webster, 1996). During assembly, pV molecules are displaced from the pV-DNA complex, and the capsid proteins assemble around the DNA as it is extruded through the envelope (Webster, 2001). The extrusion process is very well tolerated by the host bacteria, and they continue to multiply for several generations while extruding phage into the culture medium (Pereboev and Morris, 1996). The concentration of RF DNA and of progeny phage produced are very high (10^{11} to 10^{12} phage particles ml^{-1}). Because of these high levels of replication it is relatively easy to isolate large quantities of both double and single-stranded phage DNA (Bainbridge, 2000).

5.1.2 - Phage display system

The life cycle of filamentous bacteriophages can be exploited using molecular biological techniques to generate large and diverse libraries of phage-displayed peptides, which can be used to isolate and characterise bioactive ligands such as antibodies (Smith and Petrenko, 1997, Zwick *et al.* 1998, Sidhu, 2001).

Although it is theoretically possible to create phage libraries in any bacterial virus, the filamentous bacteriophages are particularly suited for this purpose in that they replicate to

high levels and have a small, easily manipulated genome (Hoess, 1993). Many of the vectors used in genetic manipulation studies are phages that infect *Escherichia coli* (Smith and Petrenko, 1997). Bacteriophage M13 has been the most frequently used biological vehicle for phage display, not just because it was the first and the best characterised library display, but also because it has proven to be amenable to successful screening (Rodi and Makowsi, 1999).

In phage display, peptides or proteins are fused to one of the capsid proteins and are accessible to molecular interactions (Smith, 1985). Peptides have been expressed as fusions to a number of M13 capsid proteins, including pIII (Scott and Smith, 1990; Cwirla *et al.* 1990; Devlin *et al.* 1990) and the major capsid protein pVIII (Felici *et al.* 1991) (Figure 5.2).

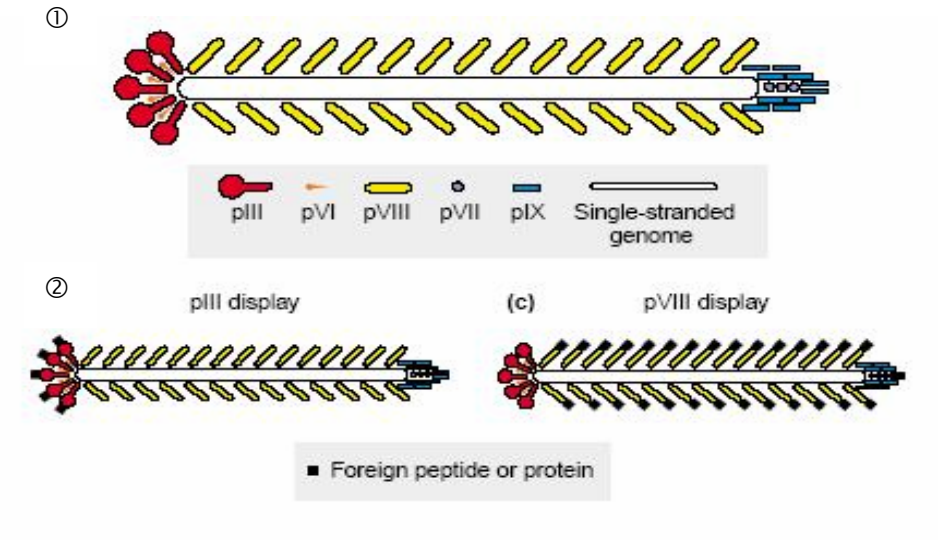


Figure 5.2 – ① Localisation of phage encoded proteins in the filamentous phage Ff; ② localisation of the fusion proteins on the filamentous phage surface (Irving *et al.* 2001).

The major coat protein, pVIII, may represent the best candidate for protein fusion because it is present at a high copy number (2700 copies) compared to the minor coat protein pIII of which only five copies are expressed on the phage surface (Kay *et al.* 1998). However, most studies have employed pIII, since this protein can accommodate longer inserts than pVIII, which can only accept a maximum of six amino acids (Greenwood *et al.* 1991).

Longer inserts in pVIII interfere with the phage assembly process (Smith and Petrenko, 1997). Consequently, phagemid vectors are required to implement long inserts in this phage protein (Williams *et al.* 2001).

The key concept of phage display is that the binding activity of a ligand displayed by the phage (the phenotype) is physically linked to the genetic information encoding that ligand, (the genotype) (Wang and Yu, 2004). It is this linkage between phenotype and genotype that enables the selection and characterisation of a specific peptide ligand (Sparks *et al.* 1996).

Vast numbers of unique peptide sequences ($\geq 10^8$) can be generated and screened simultaneously through a process called biopanning (Dottavio, 1996). During this process, the target, e.g. a MAb, is immobilised onto a surface and interact with a phage display library (Gershoni *et al.* 1997) (Figure 5.3). Phages expressing inserts identical or similar to the epitope recognised by the target MAb will be recognised and bound (Pereboev and Morris, 1996, Gershoni *et al.* 1997). Enrichment of bound phages is achieved by amplification in *Escherichia coli* between each round of the panning procedure (Pereboev and Morris, 1996). Clones derived from a single bound phage can thus be propagated, facilitating DNA isolation. Conventional nucleotide sequence analysis can then be used to determine the amino acid sequence of the peptide insert displayed on the phage surface (Dottavio, 1996, Sidhu, 2001). The propagation of individual clones also enables the application of further immunological techniques such as phage ELISA (Williams *et al.* 2001).

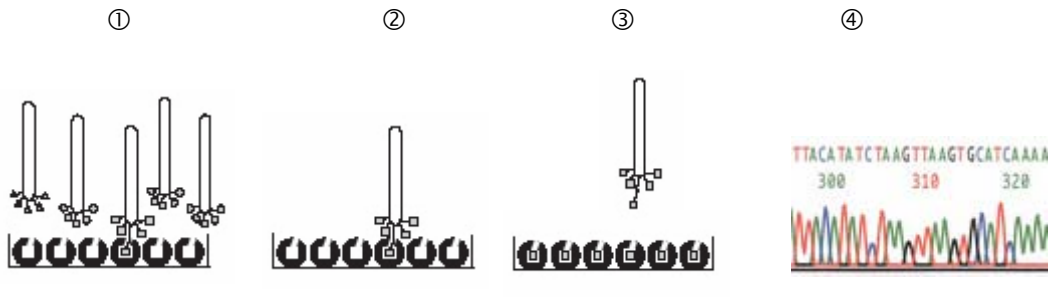


Figure 5.3 - Biopanning of phage libraries: ① Screening of phage library with the target; ② Washing to remove non-specific phage; ③ Elution of specific phage binders; ④ DNA sequencing of individual phage clones.

The success of this technique is dependent on the phage-display library quality. A given phage component of a library can only be selected if the insert DNA sequence is efficiently expressed and displayed on the phage surface (Sidhu, 2001).

5.1.2.1 - Random peptide phage libraries

The first generation of phage display random peptide libraries was described in 1990 (Scott and Smith, 1990, Cwirla *et al.* 1990, Devlin *et al.* 1990). The fusion of random DNA sequences to the coat protein gene III permitted the construction of a large library that contained a vast number of different peptide sequences. Although each phage clone displayed a single peptide, the library as a whole may represent billions of various peptides altogether (Gershoni *et al.* 1997).

When constructing a random peptide library the peptides are encoded by synthetic degenerate oligonucleotides and cloned into the pIII coat protein gene (Adey *et al.* 1996). If a stop codon (TAG, TAG and TGA) is present in the synthetic insert in a recombinant protein-insert DNA molecule, translation of the capsid protein is terminated, precluding assembly of progeny phage (Burrit *et al.* 1996). This possibility is reduced by the use of the degenerate codon NNK or NNS, where N can be any of the four nucleotides (G, T, C and A), K is for G or T, and S is for G or C (Beck-Sickinger and Jung, 1993). Each degenerate codon can represent all of the 32 codons that encode all 20 amino acids plus one amber stop codon (TAG) (Scott, 2001).

One of the principal objectives of a random peptide library is the maximisation of library complexity. The total number of variants, referred to as complexity of the library, is simply calculated as 20^n, where n is the number of peptide residues (Burrit *et al.* 1996, Gershoni *et al.* 1997). The complexity of the library is dependent on the number of inserted peptides. Not even a relatively large size library can contain all possible amino acid sequences longer than six residues, thus not all peptides can be represented in any random peptide library (Fack *et al.* 1997).

5.1.2.2 - Ph.D.™ libraries

Phage display is a selection technique in which a library of variants are expressed on the outside of phage particles, coupled to the genetic material encoding each variant (Wilson and Finlay, 1998, Rodi and Makowsi, 1999).

The Ph.D™ system is a commercially available random peptide library developed at the New England BioLabs. This phage library uses the phage vector M13KE for displaying random peptides as N-terminal fusions to the minor coat protein pIII (Noren and Noren, 2001). M13KE is a conventional M13mp19 derivative that can be simply and rapidly propagated using standard techniques, without the requirement for antibiotic selection or helper phage super-infection (Devlin *et al.* 1990). The vector is based on wild-type M13, but contains a *lacZ* gene cloned into the plus-strand origin and the restriction sites *KpnI* and *EagI* in the gene III coding sequence to allow the construction of the libraries (Scott and Barbas III, 2001).

The Ph.D.™ system comprises three different libraries, two not constrained (insert length 7 and 12 mer) and one constrained (insert length 7 mer flanked by a pair of cysteine residues). On this library a complexity of ~10^9 clones is sufficient to encode most of the 1.28 x10^9 possible 7-mers, but only 1 millionth of the 4 x10^{15} possible 12-mers (Noren and Noren, 2001).

With just five copies of pIII per virion this type of library is more suited for the identification of high-affinity ligands (Noren and Noren, 2001). The use of a library with shorter peptide inserts is appropriate for targets that bind to short, contiguous stretches of amino acids e.g. an antibody with a linear epitope. The longer peptide library (12-mer) is useful for targets where ligand binding is spread out over a longer randomised "window" than that contained in a library of shorter (≤ 7-mer) peptides (Noren and Noren, 2001).

The Ph.D™ system has been widely applied in biomedical research. Examples are included in Table 5.1.

Table 5.1 – Examples of studies performed with Ph.D™ system

Ph.D ™system application studies		***Author***
Epitope mapping with:		
Mabs	Human IgG1 Syndecan-1 (human plasma cell) Receptor binding domain of human α2-macroglobulin (α2-Mn) Human p53 protein *Neisseria meningitidis* outer membrane lipooligosaccharide (*Nm*LOS) Rabies virus (RABV) Scorpion toxins Cn2 and noxiustoxin (NTX)	Ehrlich and Bailon (1998) Dore *et al.* (1998) Birkenmeier *et al.* (1997) Blaydes *et al.* (2001) Charalambous and Feavers (2000) Mansfield *et al.* (2004) Gazarian *et al.* (2003 Hernández *et al.* (2002 Gazarian *et al.* (2000a)
Rabbit polyclonal antibodies	Anti-gliadin *Mycoplasma hyopneumoniae* IgG N-terminal of *Taenia solium* paramyosin	Yang and Shiuan (2003) Gazarian *et al.* (2000b) Osman *et al.* (1998)
Human polyclonal antibodies	Diabetes type 1; Primary biliary cirrhosis (PBC) Autoimmune thrombocytopenic purpura (AITP) Gliadin	Davies *et al.* (1999) Gevorkian *et al.* (1998) Osman *et al.* (2000)
Characterisation of immune factors		
	C1q (subunit of C1 the initiator of the classical complement pathway) Tumor Necrosis Factor α (TNFα) Zinc sites	Zhang *et al.* (2003) Messmer and Thaler (2000) Matsubara *et al.* (2003)
Development of drugs		
	gp120 of Human Immunodeficiency Virus type 1 (HIV) Biotinylated derivate of paclitaxel (high effective anti-neoplastic agent) Prolyl-tRNA synthetase (ProRS)] and protein kinase C alpha (PKCα)	Ferrer and Harrison (1999) Ashraf *et al.* (2003) Tao *et al.* (2000) Rodi and Makowsi (1999)
Enzyme inhibitors		
	Class ligase (tyrosyl tRNA synthetase (*haemophilus influenza*) and proline RS (*E. Coli*)) Class oxidoreductase (alcohol dehydrogenase (*Saccharomyces cerevisiae*)) Class hydrolase (carboxypeptidase B (*Sus scrofa* – pig) and β-glucosidase (*Agrobacterium faecaelis*)) Class transferase (hexokinase (*Saccharomyces cerevisiae*) Glycogen phosphorylase a (*Oryctolagus cuniculus* – rabbit)	Hyde-DeRuyscher *et al.* (2000)
Peptides directly to the antigen		
	Spores of: *Bacillus anthracis, B. cereus, B. subtilis* and *B. globigii* *Eimeria acervulina* and *E. tenella*	Turnbough Jr. (2003) Silva *et al.* (2002)

5.1.3 - Objectives

The objective of the work described in this chapter was to identify the epitope/s on the betanodavirus coat protein recognised by a panel of MAbs using phage display technology.

Two non-constrained phage libraries presenting inserts of either 7 or 12 random peptides fused to the coat protein pIII of M13 phage were used in this study.

5.2 - Materials and Methods

Phage display libraries Ph.D.-7™ and Ph.D.-12™ were obtained from New England Biolabs (Herts U.K.). The manufacturer's instructions were followed for all the phage manipulation procedures. Media and buffers are described in Appendix 1.

The Ph.D.-7™ and Ph.D.-12™ phage display libraries are non-constrained combinatorial libraries of random peptides with inserts encoding 7 or 12 amino acid residues respectively. These are fused to the minor coat protein (pIII) of phage M13. The insert sequence is positioned such that expressed peptides are located at the N-terminus of the pIII protein. A short spacer (Gly-Gly-Gly-Ser) is present between the insert and the pIII coding sequence. The 7-mer phage display library (Ph.D.-7™) has a complexity of ≈ 2.8 x 10^9 transformants. Approximately 70 copies of each insert sequence is contained in the 10 µl of phage supplied. The 12-mer phage display library (Ph.D.-12™) has a complexity of ≈ 2.7 x 10^{10} transformants, and the 10 µl of phage supplied contains ≈ 55 copies of each sequence.

Escherichia coli (ER2738) was used for phage-amplification. This *E. coli* strain is F´ lacI^q Δ(lacZ)M15 proA$^+$B$^+$ zzf::Tn10(Tet^R)/fhuA2 supE thi Δ(lac-proAB) Δ(hsdMS-mcrB)5 (r_k^- m_k^- McrBC$^-$). The *E. coli* host strain was supplied in TBS supplemented with 50 % glycerol and was stored at -70°C.

Streptavidin and biotin were used for a positive control.

All the procedures were executed with sterile filter tips.

The MAbs used for the assay were produced as described in Chapter 3. Purification and protein concentration of MAb preparations was also performed (Chapter 2).

For phage display studies, MAbs were diluted to a concentration of 100 µg ml^{-1} in coating solution (Appendix 1). The Ph.D.-7™ library was used to characterise MAbs 3B10, 4A12 and 4C3. The Ph.D.-12™ library was used to study MAbs 3B10, 4A12, 4C3, 1E3, and 5G10.

5.2.1 - *Escherichia coli* host

Although *Escherichia coli* strain ER2738 is defined as $recA^+$, spontaneous *in vivo* recombination events were never observed.

Escherichia coli used for M13 propagation was grown on LB-Tet media to select for the F-factor, since M13 is a male-specific coliphage.

When *E. coli* of F+ Δ-tet background is used to create an M13-based library, only phage-infected cells exhibit tetracycline resistance, so the ability of the cells to grow in the presence of tetracycline is a selective marker (Pereboev and Morris, 1996). The mini-transposon, which confers tetracycline resistance, was inserted in the F-factor of *E. coli* ER2738 strain. This resistance allows the selection of cells harboring the F-factor by plating and propagating on tetracycline-containing media.

The *E. coli* (ER2738) were grown on LB-Tet plates (Appendix 1), incubated at 37°C overnight. Plates were sealed with Nescofilm (Bando Chemical Ind. Ltd Japan, Fisher Scientific Leicestershire U.K.). Plates were stored at 4°C wrapped with aluminium foil for a maximum of 1 month.

The *E. coli* (ER2738) used in phage display studies was cultured overnight prior to use.

5.2.2 - Biopanning

The biopanning procedure comprises several steps, which are described below in the order of execution.

5.2.2.1 - Biopanning - 1st day

Purified MAbs were diluted in antibody buffer (100 μg ml^{-1}, Appendix 1) and 150 μl added to each well of a 96 well plate (Nunc, Fisher Scientific, Leicestershire U.K.). The plate was incubated overnight at 4°C, with gentle agitation (Mini Rocking platform, Biometra™) in a humidified container. The MAbs were tested in duplicate. Streptavidin (1.5 mg ml^{-1}) (prepared as described in Appendix 1) was added to plates as a positive control.

Escherichia coli was plated and incubated overnight at 37°C.

5.2.2.2 - Biopanning - 2nd day

The coating solution was removed and wells drained by firmly slapping plates face down onto a clean paper towel. Each well was filled with blocking buffer (Appendix 1) and incubated for 60 mins at 4°C with gentle agitation. Wells containing streptavidin (+ control) were blocked with blocking solution supplemented with 0.1 μg ml^{-1} streptavidin, to complex to any biotin in BSA.

Blocking solution was discarded and wells thoroughly drained. The wells were then washed 6 x with TBST (0.1 %) (Appendix 1). Washing was performed quickly to prevent plates drying. For Ph.D.-7™ the TBST was added with a pipette, while for Ph.D.-12™, TBST was added with a beaker. The latter method was adopted with Ph.D.-12™ due to the unsatisfactory results obtained with Ph.D.-7™, which potentially could have arisen due to the type of washing procedure used.

The library, as supplied, was diluted 1:100 in TBST (0.1 %) and 100 μl added per well. Plates were incubated 60 mins at room temperature with gentle agitation.

Non-binding phage was discarded and plates washed 10 x with TBST (0.1 %) as described above.

Bound phages were eluted with 100 µl of elution buffer (Appendix 1) by incubation for 8 mins at room temperature with gentle agitation. Elutes were pipetted into a microfuge tube and neutralised with 15 µl neutralising elution buffer (Appendix 1).

Streptavidin containing wells (+ control) were eluted with 100 µl of biotin (0.1 mM in TBS, Appendix 1). Incubation was performed for 30 mins at room temperature with gentle agitation.

An aliquot of 1 µl was used to quantitate eluted phage (see below). The remainder of the eluted phage was amplified in *E. coli*.

LB medium was inoculated with *E. coli* in a 250 ml Erlenmeyer flask. *E. coli* were incubated at 37°C with vigorous shaking until mid-log phase was attained. After sample elution *E. coli* was aliquoted (20 ml per sample) into culture flasks and eluted phage samples were added. Phages were amplified for 4.5 hrs at 37°C with vigorous shaking.

Cultures were transferred to 50 ml centrifuge tubes and centrifuge for 20 mins at 13 500 x *g* at 4°C (Sanyo Mistral 3000i). The upper 80 % of the supernatant transferred to a fresh centrifuge tube to which 1:6 volumes of PEG/NaCl (Sigma, Dorset U.K.) (Appendix 1) was added. Phages were allowed to precipitate at 4°C overnight.

Phage titration

Five ml of LB broth (Appendix 1) was inoculated with a single colony *E. coli* and incubated at 37°C with shaking until mid-log phase was attained. The mid-log was based on a O.D. value of 0.5, on spectrophotometric analysis using a wave length of 600 nm.

Agarose Top (Appendix 1) was melted in a microwave oven (Philips) and dispensed (3 ml) into sterile bijoux, one for each dilution of phage tested. Bijoux were kept at 45°C until used.

For logistical and financial reasons one plate per dilution was used for phage titration.

LB/IPTG/Xgal plates (Appendix 1) (one per dilution), were pre-warmed at 37°C.

A 10-fold serial dilution of phage was prepared in LB (Appendix 1). For amplified phage a dilution of 10^8-10^{10} was used. For un-amplified phage the dilution series ranged from 10^1 and 10^3.

When the *E.* coli culture had reached mid-log phase, 200 µl of culture was dispensed into 1.5 ml microfuge tubes. Ten µl of the appropriate phage dilution was added to tubes, which were vortexed briefly, then incubated at room temperature for 4 mins.

Microfuge tubes containing phage-infected *E. coli* were transferred to bijoux containing Agarose Top, vortexed briefly, and then poured onto a pre-warmed LB/IPTG/Xgal plate. The Agarose Top was spread evenly by tilting the plate.

The plates were sealed with Nescofilm and incubated overnight at 37°C.

The plates with ~100 plaques were counted and the phage titre expressed as plaque forming units (pfu) was calculated using the formula:

$$\text{Phage titre (pfu) per 10 µl} = \frac{n \times d}{s}$$

Where: n is the number of clones.
d is the dilution factor.
s is the volume of phage added.

5.2.2.3 - Biopanning - 3rd day

Phage samples precipitated in PEG were centrifuged for 30 mins 3500 x *g* at 4°C. The supernatant was decanted, re-centrifuged briefly and the residual supernatant removed with a pipette. The pellets were resuspended in 1 ml TBS and transferred into 1.5 ml microfuge tubes. After centrifugation for 5 mins at 12 000 x *g* at room temperature (Sanyo Micro centaur) to pellet residual cells, supernatants were transferred to fresh microfuge tubes

and 1:6 volumes of PEG/NaCl was added. Phages were re-precipitated on ice for 60 mins. Tubes were centrifuge at 9500 *g* for 10 mins at room temperature, the supernatant was then discarded, prior to re-centrifugation and removal of residual supernatant. The pellet was resuspended in 200 µl storage buffer (Appendix 1), centrifuged at 9500 *g* for 1 min, and the supernatant transferred to a fresh tube prior to storage at 4°C. This is the first amplified eluate.

The quantity of phage in the amplified product was titrated as described above.

A 96 well plate was coated with MAbs and Streptavidin in order to performed the second round of panning.

5.2.2.4 - Biopanning - 4th day

Coating buffer removal, blocking of non-specific sites, incubation, elution, titration and amplification were performed as described above for day 2. Washing steps were performed using TBST (0.5 %).

An input volume corresponding to 2 x 10^{11} phage-pfu from the first round amplified elute was added to the wells of a 96 well plate for the incubation step. The sample chosen was that exhibiting the highest titre. Phage samples were diluted with TBST (0.1 %).

5.2.2.5 - Biopanning - 5th day

The procedure on the fifth day was identical to that described above for day 3. The final product is the second amplified elute.

A third 96 well plate was coated with MAbs and Streptavidin for the third round of panning.

5.2.2.6 - Biopanning - 6th day

On the 6th day the procedure was identical to that described above for day 4. Plate wells were incubated with 2 x 10^{11} pfu of phage from the second round amplified elute.

Amplification of phages eluted in the third panning step is not require. These phages can be used for nucleotide sequencing of inserts in the pIII minor coat protein gene.

Eluted phage samples were titred and the resulting plates were used in subsequent nucleotide sequencing analysis. Consequently, the plates were incubated longer (16 –18 hrs at 36°C).

5.2.2.7 - Blocking experiment

Taking into consideration the results obtained after three rounds of biopanning using both Ph.D.-7™ and Ph.D.-12™, it was possible that the blocking procedure was not functioning efficiently. Consequently, an experiment was performed to test this hypothesis.

Blocking solution was added to one 96 well plate and incubated at 4°C for 60 mins with gentle agitation. Plates were drained and washed 6 x with TBST (0.5 % Tween 20) as described above.

The original library (PhD 12™) was diluted 1:100 v/v in TBST (0.1% Tween 20) and incubated for 60 mins at room temperature with gentle agitation.

The non-binding phage was discarded and the plate was washed 10 x with TBST (0.5 %).

Elution buffer (100 µl) was added to each well and incubated for 8 mins at room temperature with gentle agitation. The elute was transferred into a microfuge tube and neutralised with 15 µl neutralising elution buffer.

The resulting elute was titred as described above.

5.2.2.8 - 4th Biopanning

A 4th panning step was performed for assays using Ph.D.-12™. All of the steps in this procedure were identical to those described above, with the exception of the elution step. The elution step was performed with TBS supplemented with 20 μg ml^{-1} and 100 μg ml^{-1} of the target molecule (MAb used to coat the well) and the usual elution buffer (Zhang *et al.* 2003).

The elution procedure was performed in 3 steps. The first elution step was performed by incubating the wells with 100 μl of TBS (20 μg ml^{-1} target) for 15 mins. The eluate was collected and transferred to a microfuge tube. For the second elution step, 100 μl TBS (100 μg l^{-1} target) was added and incubated for 1 h. The eluate product was then collected and the 3rd elution step performed with elution buffer incubated for 8 mins followed by addition of the neutralising elution buffer.

The eluted products were titred and the clones harvested for nucleotide sequence analysis of inserts.

5.2.3 - Nucleotide sequence analysis of phage

5.2.3.1 - Plaque amplification

For characterisation of the clones only plates showing less then 100 colonies were used. Where this was not possible, eluted phage samples were further diluted to yield plates with suitable numbers of 100 plaques.

LB medium was inoculated with *E. coli* and incubated until a value of mid-log phase was attained. Bacteria were aliquoted (1 ml) into culture flasks.

Using a sterile pipette tip, blue plaques were stabbed and inoculated into a culture flask containing *E. coli*. Flasks were incubated at 37°C with vigorous shaking for 4.5–5 hrs.

With Ph.D.-7™ 10 plaques were selected and amplified for characterisation of the insert. For Ph.D.-12™ 20 plaques were used. An example of the plaques is depicted in Figure 5.4.

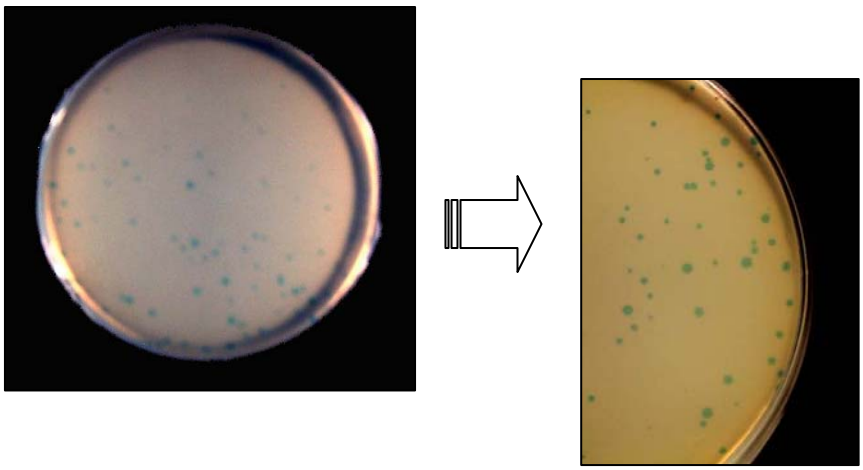


Figure 5.4 - Selection of the phage with Xgal-IPTG. Phage clones grow blue. These clones were selected for amplification and DNA extraction. Contamination with wild M13 would result in the growth of white plaque phage

After incubation, the cultures were transferred to 1.5 ml microfuge tubes, and centrifuged at 9500 x *g* for 30 seconds. The supernatant was transferred to a fresh tube and re-centrifuged. The upper 80 % of the supernatant was then transferred to a fresh tube. This product represents the amplified phage stock and was stored at 4°C for maximum of 8 weeks. For long-term storage, the phage samples were diluted 1:1 with sterile glycerol and stored at -20°C.

Phage clones selected and amplified as described above were found to contain insufficient DNA for nucleotide sequencing.

Consequently, an extra amplification step was introduced to produce greater amounts of DNA. This was performed by adding 100 µl of phage to 10 ml of LB containing mid-log phase *E. coli*. After 4.5 hrs of incubation the product was transferred to a 15 ml centrifuge tubes and centrifuged for 10 mins at 3500 x *g*, 4°C.

The amplification products were always plated on LB/IPTG/Xgal plates to confirm the presence of phage clones.

5.2.3.2 - Rapid purification of amplified phage clones

Purification of amplified phage clones was performed by transferring 500 µl of phage to a 1.5 ml microfuge tube. PEG/NaCl (200 µl) was added and mixed by inverting the tubes, which were allowed to stand at room temperature for 10 mins. The tubes were centrifuged for 10 mins at 12 000 x *g*. The supernatant was discarded and the tubes re-centrifuged briefly. Any remaining supernatant was carefully pipetted away.

In initial sequencing work, the quantity of DNA obtained from phage preparations was found to be insufficient. Consequently, greater amounts of purified phage were used. For each clone two extraction reactions were performed. In each reaction 1000 µl of amplified phage sample was mixed with 400 µl of PEG/NaCl. The procedure was performed as described above.

The buffer used to re-suspend pellets depended on the DNA extraction method used. For the iodine DNA extraction method, the pellet was re-suspended with iodine buffer (Appendix 1) and with TE (appendix 1) for the phenol-chloroform DNA extraction method.

5.2.3.3 - Phage DNA extraction

5.2.3.3.1 - Iodine DNA extraction method

The amplified phage pellet was re-suspended in 100 µl iodide buffer. Ethanol (250 µl, Fisher Scientific, Leicestershire U.K.) was added and the mix incubated for 10 mins at room temperature. Samples were centrifuged for 10 mins at 12 000 x *g* and the supernatant

discarded. The pellet was washed in 500 µl of 70 % ethanol, and then allowed to air dry prior to re-suspension in 30 µl of TE buffer.

Extracted DNA was found to be of low quality using this method, so the phenol-chloroform extraction method was used as an alternative.

5.2.3.3.2 - Phenol-Chloroform DNA extraction method

This method was kindly provided by Dr. David Landry of New England Biolabs, who suggested the use of Phenol buffered with TrisHCl (pH 8) as an alternative method to iodine DNA extraction.

Phage pellets were re-suspended in 70 µl TE buffer. The same volume of Phenol (Sigma, Dorset U.K.) or Aquafenol (Qbiogene, Cambridge U.K.) was added and samples were then vortexed. The samples were centrifuged for 2 minutes at 15 800 x *g* (Thermo IEC microlite). The upper layer was collected, transferred to a clean tube and 70 µl of chloroform (Sigma, Dorset U.K.) was added. The tubes were vortexed and centrifuged for 2 minutes at 15 800 x *g*. The upper layer was collected and transferred to a clean tube and 3 volumes of ethanol (92 %) was added. After incubation on ice for 3 mins the mix was centrifuged at 15 800 x *g* for 15 minutes. The supernatants were discarded and pellets washed with 500 µl of ethanol (70 %) by centrifugation as described above. The supernatants were discarded and the pellets were dried, and then re-suspended in 30 µl nuclease-free water (Promega, Southampton U.K.).

5.2.3.4 - Nucleotide sequencing

For nucleotide sequencing of the inserts fused to a minor coat protein gene (pIII) of M13 phage, the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Bucks U.K.) was used.

All the procedures for nucleotide sequence have been described in Chapter 2.

The sequencing primer (5' CGT TAC GCT AAC TAT GAG GGC 3') was supplied with the phage display kit. The amount supplied was found to be insufficient, so this primer was subsequently obtained from MWG (Milton Keynes, U.K.).

Amino acid sequences were deduced from the nucleotide sequence of inserts (Figure 5.5). This was processed using BioEdit software (Chapter 2).

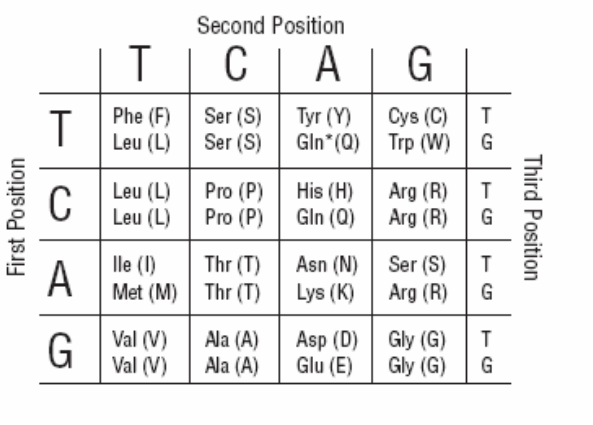


Figure 5.5 – Reduced genetic code (Phage display peptide library kit, NE BioLabs).

The following steps were performed to ensure the fidelity of nucleotide sequence analysis of phage inserts:

① The reverse complement sequence of the insert was determined;

② The sequence read accuracy of the *Kpn* I (upstream of the insert) and the *Eag* I (downstream of the insert) containing regions was confirmed;

③ The third position of each codon in the randomised region was confirmed to be G or T;

④ TAG codons were considered to represent glutamine (Q).

⑤ The phage clones obtained with streptavidin should exhibit amino acid sequence S/T/N_L_L/I/V_A/N_HPQ as the insert sequence. Being the major consensus amino acids the HPQ sequence.

5.2.3.5 - Detection of phage clones by ELISA

Phages corresponding to each amino acid sequence variant obtained were analysed by ELISA for verifying recognition by the selecting MAbs.

The ELISA protocol was performed as described in Chapter 2, using the amplified phage samples (200 μl) to coat the 96 well ELISA plates. Phage samples were incubated overnight at 4°C. Monoclonal antibodies (neat supernatant) was added as the primary antibody.

5.3 - Results

After three - four biopanning rounds, the nucleotide sequence of the inserts in the M13 pIII gene was determined. This was used to deduce the amino acid sequence of the peptide displayed on the phage surface. The results obtained with the phage systems Ph.D.-7™ and Ph.D.-12™ are presented below.

5.3.1 - Ph.D.-7™

The Ph.D.-7™ system was used to characterise epitopes recognised by MAbs 4C3, 4A12 and 3B10. Streptavidin was used as a positive control. Phage clones were sequenced after the third biopanning round.

5.3.1.1 - MAb 4C3

The deduced amino acid sequence data obtained with Ph.D.-7™ when panning was performed with MAb 4C3 are presented in Table 5.2.

No consensus sequence was evident based on the analysis of nine sequenced inserts. Attempts were made to sequence phage clone 4 but based on the criteria of validity described in 5.2.3.4, the sequence data was rejected. Clone 2 was found to contain a double insert (2a and 2b).

Some of the clones contained the same amino acids in a given position, as can be observed with clone 2b and 5 (xxWSxxx). Clone 5 and 6 shared 3 amino acids, but their position within the insert was different, (x)HWSxx.

Table 5.2 – Amino acid sequence of the Ph.D.-7™ inserts obtained after panning with MAbs 4C3.

4C3			
Clone	***Insert sequence***	***Clone***	***Insert sequence***
1	HLRWHHT		
2a	HSPSVLS	2b	AKWSSRH
3	QFSHYFN		
4	-		
5	NHWSLNG		
6	HWSHARH		
7	ALNYTNS		
8	HMRFIHY		
9	GHIMINR		
10	WPHKHFY		

5.3.1.2 - MAb 4A12

Table 5.3 present the deduced amino acid sequence data obtained with Ph.D.7™ when panning was performed with MAb 4A12.

No consensus sequence was obtained from the clones analysed. Clones 3 and 8, revealed the same amino acid in the same position (xxxHHHx).

Table 5.3 – Amino acid sequence of the Ph.D.-7™ inserts obtained after panning with MAbs 4A12.

4A12			
Clone	***Insert sequence***	***Clone***	***Insert sequence***
1a	HRLHSYM	1b	LPTNLHW
2	HTSSKLV		
3	WTPHHHF		
4	MHRPHWH		
5	SPLHAWW		
6	LLPSYIY		
7a	HYQSSVT	7b	GPKIWHI
8	HLRHHHY		
9a	WQFHLPH	9b	PRQYPRA
10	-		

Clones 1, 7 and 9 contained double inserts. Clones 1a and 7a contained the same two amino acids in the same position: HxxxSxx. Clones 1b and 7b also shared two amino acids: xPxxxHx.

It was not possible to analyse sequence data for clone 10.

5.3.1.3 - MAb 3B10

The deduced amino acid sequence data obtained with Ph.D.7™ when panning was performed with MAb 3B10 are presented in Table 5.4

No consensus was obtained. Clones 9b and 10 showed the greatest similarity with the sequence RxxLRxL.

Table 5.4 – Amino acid sequence of the Ph.D.-7™ inserts obtained after panning with MAbs 3B10.

3B10			
Clone	***Insert sequence***	***Clone***	***Insert sequence***
1a	GPKIWHT	1b	HYQSSVT
2	RPKRSPI		
3	NAMLQLR		
4a	TVKYHHH	4b	VSNMNTV
5a	ITPENST	5b	HPRIHFW
6	MSSAEAR		
7	MNLGALP		
8	APPSNLP		
9a	HSNHLHN	9b	RNVLRCL
10	RLTLRSL		

Almost half of the clones (1, 4, 5 and 9) contained a double insert. Some of these clones contained common amino acids, e.g. xSNxxxx (clone 4b and 9a).

5.3.1.4 - Streptavidin

Streptavidin was used as a positive control. A consensus sequence was obtained in the clones that contained a double insert, which was present in 40 % of clones (Table 5.5).

Table 5.5 – Amino acid sequence of the Ph.D.-7™ inserts obtained after panning with Streptavidin.

Streptavidin			
Clone	***Insert sequence***	***Clone***	***Insert sequence***
1a	DPAPRPR	1b	NHAHSTP
2	HLHIRFP		
3	HKRPRNN		
4a	DPAPRPR	4b	NHAHSTP
5	RPKRSPI		
6a	DPAPRPR	6b	NHAHSTP
7	-		
8a	DPAPRPR	8b	NHAHSTP
9	AQRQPEH		
10	INHVHRL		

5.3.2 - Ph.D.-12™.

The Ph.D.-12™ system was used to characterise epitopes recognised by MAbs 4C3, 4A12 and 3B10, 1E3 and 5G10. Streptavidin was used as a positive control. Phage clones were sequenced after the third and fourth biopanning round.

5.3.2.1 - MAb 4C3

No consensus sequence was obtained with MAb 4C3 after three rounds of panning (Table 5.6). Several clones contained the same amino acids in the same position, e.g. clones 15 and 16 shared WHxxxLxPxxxx amino acids; clones 1 and 10 shared residues

xxxHHxxxxSxx and clones 5 and 6 shared residues xxxHxxxxxSRx. Clone 6 had some residues in common with clone 18, HLxxxYxxxxxx.

The 4th panning revealed inter and intra-elution consensus sequences. Insert sequence HHRHNYAVEAPF occurred in 4 of 52 clones sequenced (two clones from the 1st elution, one clone of the 2nd and one clone of the 3rd elution) (Table 5.7). The sequence WHKHSYNSMPVY was present in two 1st elution clones and one 3rd elution clone. Clone 17 from the 1st elution and clone 7 of the 2nd elution contained the same amino acid sequence, GWKSHHNHERVF. Clone 4 of 1st panning and clone 13 of the 2nd panning contained the insert sequence HHKHGINQISP. This sequence, HHHKHGINQISP, was almost identical to clone 11 of the 1st panning sequence, differing by just a single residue (I or D) at the 10th position. Clones 5 and 7 of the 1st elution shared the same amino acid sequence with the exception of the final amino acid (L or P).

Table 5.6 – Amino acid sequence of Ph.D.-12™ clones obtained after panning with MAb 4C3.

MAbs 4C3	***Amino acid sequence***			
	3rd panning	***4th panning***		
		1st elution	***2nd elution***	***3rd elution***
1	HRSHHMHLPSPW	KPYHSWHQWQTS	HPRPHSHLEMPR	-
2	Double insert	HHRHNYAVEAPF	GHKHWQHNHSTH	WHKHSYNSMPVY
3	ALYKHSHHVWRL	HHRHNYAVEAPF	HTKVPWWGAFIT	IPHHYQFLKHRH
4	Double insert	HHHKHGINQISP	FHKHSYNYAHMH	WHKNTNWPWRTL
5	WPYHKHAFPSRP	WHKPWYSQPWPL	HSQWNTMQAIAT	WHRTYQPPLEPR
6	HLNHAYWQHSRA	VHWKNPTVFSYY	LPWHWHTSQRSL	HSRHHYNVHLNA
7	LHKPRPWHEFNR	WHKPWYSQPWPP	GWKSHHNHERVF	HHRHNYAVEAPF
8	HWKHFNGTRLLD	WHKHSYNSMPVY	HPLSKMHYRIHM	RFVIFILVIGLL
9	Double insert	-	-	IIVIIRIFLGGI
10	WPHHHHTRLSTV	APWWYHQWKAEQ	HSYHHTQRLLTR	FHKPSWHAWSGR
11	HHRYFNSNYLAW	HHHKHGINQDSP	YPWHKSHLREVT	HHWHQNNRQALV
12	FHKHPHSGRWYP	-	HHRHNYAVEAPF	FPRNHHQWLPHR
13	GQISNLPPLFRT	HFKHQHSYARPP	HHHKHGINQISP	YPHHHNSRYFPM
14	HLSKINRHFDHY	GLRHHHTIPNVS	HYKHHHTPILLN	Double insert
15	WHANKLPPRYFY	-	-	SHKHYNNYAHMH
16	WHKYPLFPPMTA	WHKHSYNSMPVY	HPSTHHRGASHI	FHKHSPRSPIFI
17	IIVIRLCRLLLM	GWKSHHNHERVF	HHWHSRSQLSWF	WHKPRLHTFDFA
18	HLRMNYPLHTYH	FHKHSPISPIFI	DHGLWRYYLYYQ	FHKHSPRSPIFI
19	HHRHTFVPLTPN	WNPHNHYRWFPH	-	HHRHIHTALWQN
20	MHRDYYPRYVPW	WNPHNHYRWFPH	HPFHQRHWLQLP	HHRAVPTFTWYS

Note – consensus clones are marked in colour.

Table 5.7 – Occurrence of insert selected with MAb 4C3.

MAb 4C3	***Occurrence***	***Panning***	
		3rd	***4th***
HHRHNYAVEAPF	4/52		1st elution (2/17) 2nd elution (1/17) 3rd elution (1/18)
WHKHSYNSMPVY	3/52		1st elution (2/17) 3rd elution (1/18)
HHHKHGINQISP	2/52		1st elution (1/17) 3rd elution (1/18)
GWKSHHNHERVF	3/52		1st elution (2/17) 2nd elution (1/17)
WHKPWYSQPWPL	1/52		1st elution (2/17)
WHKPWYSQPWPP	1/52		
WNPHNHYRWFPH	2/52		1st elution (2/17)
FHKHSPRSPIFI	2/52		3rd elution (2/18)

Note – the remaining insert sequences just occurred once.

No insert consensus sequences were obtained from phages derived from the second elution.

For the 3rd elution just two clones (16 and 18) exhibited a consensus sequence (FHKHSPRSPIFI). Clone 18 from the 3rd panning had a similar sequence that differed by a single amino acid, with an I instead of an R residue at position 7. Clone 14 from the 3rd panning contained a double insert.

Both pannings yielded samples that were impossible to sequence.

5.3.2.2 - MAb 4A12

No consensus sequence was observed in clones from the 3rd panning after selection with MAb 4A12 (Table 5.8). Some clones contained amino acid residues in the same position. For example clones 13 and 14 contained the sequence xxxxxHHRSxxx and clones 17 and 20 contained xxxxxHxxPWRS. Clones 7, 9 and 15 were impossible to sequence.

A consensus sequence was observed for seven clones from the 1st elution of the 4th panning (HHHKHGINQISP/R) (Table 5.8). This sequence occurred at a frequency of 34/60 clones from the 2nd and 3rd elutions (Table 5.9). With this sequence it was impossible to distinguish between a P or an R at the last amino acid position of the insert.

Table 5.8 – Amino acid sequence of Ph.D.-12™ clones obtained after panning with MAb 4A12.

MAbs 4A12	***Amino acid sequence***			
	3rd panning	***4th panning***		
		1st elution	***2nd elution***	***3rd elution***
1	WNMPHISHRHWR	HVKSHYHSLPHS	HHHKHGINQISP/R	HHHKHGINQISP/R
2	HIRHNHYTTSPF	HSKHRHLLWQAI	HHHKHGINQISR	HHHKHGINQISP/R
3	HHVHTPRWKLPL	SPPLYHKHHRHY	HHHKHGINQISP	HHHKHGINQISR
4	GKYHHHTPAHPQ	HHHKHGINQISP/R	HHHKHGINQISP	HHHKHGINQISP/R
5	VPFTKGYPPFDT	HHWHSGTHMSHV	HHHKHGINQISR	HHHKHGINQISP/R
6	FHRHHISPTWSP	SIWHSHHRYSWL	HHHKHGINQISP	HHHKHGINQISP/R
7	-	HHNHIRTYVWSA	HHHKHGINQISP/R	HHHKHGINQISP
8	PFHHKSTVAKNR	HNKHQHPPFMFG	HHHKHGINQISP/R	HHHKHGINQISP/R
9	-	VHRHHYWAPWSQ	HHHKHGINQISP/R	HHHKHGINQISR
10	LPHHHRYWDYPY	HSKVFHGLHSLK	HHHKHGINQISP/R	HHHKHGINQISP
11	TWSWHNSHIHMR	HHHKHGINQISP/R	HHHKHGINQISP/R	HHHKHGINQISP/R
12	YKSHHTRVAPST	HHHKHGINQISP	HHHKHGINQISP/R	HHHKHGINQISP
13	PKSTVHHRSAAA	HHHKHGINQISR	HHHKHGINQISP/R	HHHKHGINQISR
14	ISLMHHHRSTVP	HHHKHGINQISP/R	HHHKHGINQISP/R	HHHKHGINQISP/R
15	-	HHHKHGINQISP/R	HHHKHGINQISP/R	HHHKHGINQISP/R
16	MQMILSPVKHHL	HHHKHGINQISP	HHHKHGINQISP/R	HHHKHGINQISP/R
17	HKSCTHVPPWRS	HHHKHGINQISP/R	HHHKHGINQISP/R	HHHKHGINQISP/R
18	LLQIVSATRHHH	HHHKHGINQISP/R	HHHKHGINQISP/R	HHHKHGINQISP/R
19	Double insert	HHHKHGINQISP/R	HHHKHGINQISR	HHHKHGINQISP
20	GIVLLHHRPWRS	HHHKHGINQISP/R	HHHKHGINQISP/R	HHHKHGINQISP

Note – consensus clones are marked in colour.

Table 5.9 - Occurrence of insert selected with MAb 4A12.

MAb 4A12	***Occurrence***	***Panning***	
		3rd	***4th***
HHHKHGINQISP/R	34/60		1st elution (8/20) 2nd elution (14/20) 3rd elution (12/20)
HHHKHGINQISP	10/60		1st elution (2/20) 2nd elution (3/20) 3rd elution (5/20)
HHHKHGINQISR	7/60		1st elution (1/20) 2nd elution (3/20) 3rd elution (3/20)

Note – the remaining insert sequences just occurred once

5.3.2.3 - MAb 3B10

The insert sequences obtained after the 3rd and 4th pannings with MAb 3B10 are presented in Table 5.10. After the 3rd panning, clones 14 and 18 contained an insert consensus sequence (NHWSWYWSAQLN). Some of the clones had the same amino acids in the same sequence position. As an example clone 10 and 17 shared the first four amino acids of the insert sequence (FHKHxxxxxxxx).

Table 5.10 – Amino acid sequence of Ph.D.-12™ clones obtained after panning with MAb 3B10.

MAbs 3B10	*Amino acid sequence*			
	3rd panning	*4th panning*		
		1st elution	*2nd elution*	*3rd elution*
1	HHNHIRTYVWSA	HHHKHDINQISP	HHHKHGINQISP	SARIHTHFPSHT
2	STFKFHKHHLPL	HHHKHGINQISP/R	HHHKHGINQISP	Double insert
3	HHWHIRLPSSES	HHHKHGINQISP/R	HHHKHGINQISP	-
4	FPHHPKIHVPWR	HHHKHDINQISP	HHHKHGINQISP	WHKAPRPTYLSY
5	HWWDYNMRYHKS	HHHKHGINQISP	HHHKHGINQQSP	-
6	AKSPHWYHSHQR	HHHKHGINQISP	HHHKHGINQQSP	IHRTPHHHYYLY
7	LSTNHHTHPNPR	HHHKHGINQISP/R	HASRHPHPPWHP	Double insert
8	HMKHPRPLWPYW	HHHKHGINQISP/R	HHHKHGINQISP	HHHKHGINQISP
9	WSWHIHRPPILP	HHHKHDINQISP	-	WHKAPRPTYLSY
10	FHKHSPRSPIFI	HHHKHGINQISP/R	HHHKHGINQISP	HSIRLYQEPQIH
11	WHGSLKQNLWWY	HHHKHGINQISP	WHRIPEKIFVWQ	HTRTVHYHSLVP
12	HMPHKFHTHTRL	HHHKHGINQISP	IHRTPHHHYYLY	WHKAPRPTYLSY
13	SHWYQRTYWLST	HHHKHGINQISP	IHRTPHHHYYLY	IHRTPHHHYYLY
14	NHWSWYWSAQLN	HHHKHGINQISP/R	SHWHWRMLPPFS	WHKAPRPTYLSY
15	WHRHSPPTLKFQ	HHHKHGINQISP	IHRTHPSVWWHL	HHHKHGINQISP
16	-	HHHKHGINQISP	WPSYFTRHNQNM	HHTSAHGINPSV
17	FHKHHKAPALVR	HHHKHGINQISP	SARIHTHFPSHT	LHKAPRPTYLSY
18	NHWSWYWSAQLN	HHHKHGINQISP	HHRLHAHHWAIN	-
19	HGTAHGWKAYWY	HHHKHGINQISP	-	SARIHTHFPSHT
20	-	HHHKHGINQISP/R	-	IHRTHPSVWWHL

Note – consensus clones are marked in colour.

A consensus sequence (HHHKHGINQISP) was obtained from the 1st elution of the 4th panning in half of the clones analysed. This sequence was also obtained with six clones of 2nd elution and two clones of 3rd elution. In total this insert sequence occurred in 17 of the 53 clones analysed (Table 5.11).

The remaining insert clones sequences of 1st elution were very similar, exhibiting a single amino acid difference. Clones 1, 4 and 9 contained a D instead of a G at the 6th position of the insert. With the remaining clones derived from the 1st elution, it was not possible to distinguish between a P or a R at the last amino acid position of the insert.

With the 2nd elution two clones (5 and 6) contained a similar insert sequence (HHHKHGINQISP), which varied by a single amino acid – Q instead of an I in the 10th position.

The insert IHRTPHHHYYLY occurred in four clones, representing 4 of the 53 clones analysed.

Table 5.11 - Occurrence of insert selected with MAb 3B10.

MAb 3B10	***Occurrence***	***Panning***	
		3rd	***4th***
HHHKHDINQISP	3/53		1st elution (3/20)
HHHKHGINQISP	17/53		1st elution (10/20) 2nd elution (5/17) 3rd elution (2/16)
HHHKHGINQISP/R	8/53		1st elution (7/20) 2nd elution (1/17)
HHHKHGINQQSP	2/53		2nd elution (2/17)
IHRTPHHHYYLY	2/53		2nd elution (2/17)
SARIHTHFPSHT	3/53		2nd elution (1/17) 3rd elution (2/16)
WHKAPRPTYLSY	4/53		3rd elution (4/16)
IHRTPHHHYYLY	2/53		3rd elution (2/16)

The amino acid sequence WHKAPRPTYLSY occurred in four clones of the 3rd elution. SARIHTHFPSHT and HHHKHGINQISP were each present in two clones. Clones 2 and 7 contained a double insert.

5.3.2.4 - MAb 5G10

The insert sequences derived from phage panning with MAb 5G10 are presented in Table 5.12. A consensus sequence (VPHWHTEYLRWL) occurred in six clones from the 3rd

panning round (6:16 clones - Table 5.13). Clone 5 of the 3rd elution contained the same amino acid sequence. Clones 9, 15 and 18 from the 3rd panning were impossible to sequence and clone 14 contained a double insert.

In the 4th panning, clones 16 and 18 from the 1st elution contained a consensus sequence (SHRTTKTQSLTQ). The sequence HGSHQHRWQHSV occurred in 2/58 samples, (clone 7 of 1st elution and clone 7 of 2nd elution) (Table 5.13).

Table 5.12 – Amino acid sequence of Ph.D.-12™ clones obtained after panning with MAb 5G10.

MAbs 5G10	***Amino acid sequence***			
	3rd panning	***4th panning***		
		1st elution	***2nd elution***	***3rd elution***
1	GHWKLFPYWARS	SIWHSHHRYSWL	WHKIPQKAPLNP	LPHHHRWPIPRV
2	VPHWHTEYLRWL	YHKNYRSLPYFM	APRWHHHIILIG	LPHHHRWPIPRV
3	VPHWHTEYLRWL	LCAGQRHETSLL	GPHTYHSKHRLF	HHKLPTRITHYW
4	HMNKHSAHLVHL	HPKWHSFPPQLL	SFRHHHPTYQHL	LPHHHRWPIPRV
5	APWHLHNPIYRL	HTTYKSHHFFRT	HTWHLKYPTHRT	VPHWHTEYLRWL
6	VPHWHTEYLRWL	SWPSRHYHHLLP	HHNHIRTYVWSA	HHKHQIQPMLNL
7	VPHWHTEYLRWL	HGSHQHRWQHSV	HGSHQHRWQHSV	HFRKFHAERHLR
8	YHTEYMLWLGST	HVKHTHSYGIHY	Double insert	TPHAHPLKTGLS
9	-	HTNHSHWPLVRN	SHWHSKLRYFPP	HFRIHDNTHSLR
10	VPHWHTEYLRWL	FHKHSPRSPIFI	HHKHRALEPFLL	YPHHHHSWRLHT
11	HSRIHNHTDRNI	SPYHFHHRYTPT	SWPALSRHNDHT	TLFKHHPHSPRT
12	HTSLNYRPWLTI	HDRHKVHTPYYS	WHKHSYNSMPVY	LPHHHRWPIPRV
13	HSTHHLSKHILA	GNKNTNWPWRTL	HSFHNHLSRARL	HHRHFYSPWMSN
14	Double insert	FHKPYFKAPHMF	WHKIPQKAPLNP	FPKHSFHNHHAP
15	-	HHRHFSSPWMSN	YERMCHLDNYSD	FPSKWHGHWTAF
16	VPHWHTEYLRWL	SHRTTKTQSLTQ	VHKTHSHVNWRF	-
17	W/GHRHTPPPYPVI	WHKTSWQSWPGS	FPSKWHGHWTAF	APGHRHHYHQSF
18	-	SHRTTKTQSLTQ	HSLHSLYRLHSH	LPQWHHWHAPSR
19	HHRHFVQPPPTF	HHRHIPGWQLHT	HHTHQRPFYLAS	HIRFPYSHFFHA
20	KGIHWHHWNYGV	HVRHFYNTPPSY	LPSPKHHSREPH	GVNHWWHHGHLK

Note – consensus clones are marked in colour.

An insert consensus sequence was also present in clone 15 of the 1st panning and clone 13 of the 3rd panning (HHRHFSSPWMSN).

The 2nd elution contained a consensus sequence (WHKIPQKAPLNP) in 2 clones (1 and 14). A consensus sequence (FPSKWHGHWTAF) was present in clone 17 of this elution and clone 15 of the 3rd elution. Clone 8 contained a double insert.

In the 3rd elution a consensus insert sequence was obtained between clone 1, 2 and 4 (LPHHHRWPIPRV).

Table 5.13 – Occurrence of insert selected with MAb 5G10.

MAb 5G10	***Occurrence***	***Panning***	
		3rd	***4th***
VPHWHTEYLRWL	6/16 1/58	6/16	3rd elution (1/19)
HGSHQHRWQHSV	2/58		1st elution (1/20) 2nd elution (1/20)
HHRHFSSPWMSN	1/58		1st elution (1/20)
HHRHFYSPWMSN	1/58		3rd elution (1/20)
SHRTTKTQSLTQ	2/58		1st elution (2/20)
WHKIPQKAPLNP	2/58		2nd elution (2/19)
FPSKWHGHWTAF	2/58		2nd elution (1/19) 3rd elution (1/19)
LPHHHRWPIPRV	3/58		3rd elution (3/19)

5.3.2.5 - MAb 1E3

An insert consensus sequence (FHKHSPRSPIFI) was identified in phage clones derived from the 3rd and 4th panning with MAb 1E3 (Table 5.14). This sequence was present in 4/18 clones from the 3rd panning and 27/56 clones from the 4th panning (eleven clones of the 1st elution and eight clones of 2nd elution and 3rd elution) (Table 5.15). The sequence LHKHPHSHYNLE was also present in clones from each elution from the 3rd and 4th panning rounds. This insert sequence was present in four clones from the 3rd panning. The same sequence occurred in several clones from the fourth panning: clone 12 from the 1st elution, clones 1 and 19 of the 2nd elution and clones 4, 6, 7 and 19 of the 3rd elution.

Clones 13 and 15 from the 3rd panning contained the sequence FHRPHVHAHPPY.

As described above, the insert sequence FHKHSPRSPIFI occurred in phage clones (27/56) derived from all of the 4th panning elutions.

A similar sequence was present in two other 1st elution clones (2 and 4), which differed by a single amino acid residue (D instead of a P at the 6th position). The insert

sequence HHHKHGINQISP was also present in clones derived from each of the elutions from the 4th panning round. Clone 3 of the 1st elution, clones 7, 8 and 15 of the 2nd elution and clone 17 of the 3rd elution contained this insert amino acid sequence.

Table 5.14 – Amino acid sequence of Ph.D.-12™ clones obtained after panning with MAb 1E3.

Mabs 1E3	***Amino acid sequence***			
	3rd panning	***4th panning***		
		1st elution	***2nd elution***	***3rd elution***
1	LHKHPHSHYNLE	FHKHSPRSPIFI	LHKHPHSHYNLE	FHKHSPRSPIFI
2	FHKHSPRSPIFI	FHKHSDRSPIFI	-	-
3	-	HHHKHGINQISP	FHKHSPRSPIFI	FHKHSPRSPIFI
4	FHKHSPRSPIFI	FHKHSDRSPIFI	FHKHSPRSPIFI	LHKHPHSHYNLE
5	FHKHSPRSPIFI	FHKHSPRSPIFI	FHKHSPRSPIFI	-
6	FHKHSPRSPIFI	FHKHSPRSPIFI	HSAHSHRWSGHP	LHKHPHSHYNLE
7	HRTHNHTNWPPH	FHKHSPRSPIFI	HHHKHGINQISP	LHKHPHSHYNLE
8	IHRTPHHHYYLY	FHKHSPRSPIFI	HHHKHGINQISP	FHKHSPRSPIFI
9	ASQHHNHKWTLR	RRPHQRLHSTSH	FHKHSPRSPIFI	GKPHREPVLTLR
10	LHKHPHSHYNLE	FHKHSPRSPIFI	FHKHSPRSPIFI	FHKHSPRSPIFI
11	AHKHLSFWLRDG	FHKHSPRSPIFI	FHKHSPRSPIFI	FHKHSPRSPIFI
12	LHKHPHSHYNLE	LHKHPHSHYNLE	FHKHSPRSPIFI	HSAHSHRWSGHP
13	FHRPHVHAHPPY	FHKHSPRSPIFI	FHKHNYKSPPII	FHKHSPRSPIFI
14	SHRHIHNHLLSR	FHKHSPRSPIFI	AHKAHMHTHSRP	FHKHSPRSPIFI
15	FHRPHVHAHPPY	FHKHSPRSPIFI	HHHKHGINQISP	GKPHREPVLTLR
16	-	FHKHSPRSPIFI	VLKPTYQSFKLH	HHKHTSPRTVLT
17	SPPLYHKHHRHY	HMNKHSAHLVHL	FHKHSPRSPIFI	HHHKHGINQISP
18	YYPLHLHRHGLH	HRLHVPHQVTHM	AHKAHMHTHSRP	HSAHSHRWSGHP
19	HHKYSSTLYSSP	WHKHIPSPRASS	LHKHPHSHYNLE	LHKHPHSHYNLE
20	LHKHPHSHYNLE	**double insert**	GKPHREPVLTLR	FHKHSPRSPIFI

Note – consensus clones are marked in colour.

The 2nd elution showed a consensus sequence (AHKAHMHTHSRP) occurring in phage clones 14 and 18 from the 2nd elution. Two other clones from this elution shared insert sequences with clones derived from the 3rd elution (GKPHREPVLTLR and HSAHSHRWSGHP, respectively).

The insert sequence GKPHREPVLTLR amino acid sequence was present in clone 20 of the 2nd elution and clones 9 and 15 of the 3rd elution. Clone 6 of 2nd elution and clones 12 and 18 of the 3rd elution shared the amino acid insert sequence HSAHSHRWSGHP.

Table 5.15 - Occurrence of insert selected with MAb 5G10.

MAb 1E3	***Occurrence***	***Panning***	
		3rd	***4th***
LHKHPHSHYNLE	4/18 7/56	4/18	1st elution (1/19) 2nd elution (2/19) 3rd elution (4/18)
FHKHSPRSPIFI	4/18 27/56	4/18	1st elution (11/19) 2nd elution (8/19) 3rd elution (8/18)
FHRPHVHAHPPY	2/18	2/18	
FHKHSDRSPIFI	2/56		1st elution (2/19)
HHHKHGINQISP	5/56		1st elution (1/19) 2nd elution (3/19) 3rd elution (1/18)
HSAHSHRWSGHP	2/56		2nd elution (1/19) 3rd elution (1/18)
AHKAHMHTHSRP	2/56		2nd elution (2/19)
GKPHREPVLTLR	3/56		2nd elution (1/19) 3rd elution (2/18)

5.3.2.6 - Streptavidin

Steptavidin was used as a positive control. After biopanning a consensus sequence containing as a minimum the amino acid residues HPQ should be obtained.

Three different consensus sequences were present in phage clones derived from the 3rd panning using streptavidin (Table 5.16). The amino acid sequence IGHHTLYINHPQ occurred in 3 out of 18 clones sequenced (clones 7, 16 and 17) (Table 5.17).

The two other consensus sequences were obtained from clones 3 and 8 (WHKPHARPALDL), and clones 1 and 12 (AHKAHMHTHSRP). Clones 10, 15 and 20 shared four amino acid residues (WHKxxRxxxxxx). Clones 10 and 15 also shared a P residue at the 5th position and an L residue at the 10th position.

All of the phage clones analysed (n = 19), obtained from the 4th panning contained the same amino acid insert sequence – HHHKHGINQISP.

Table 5.16 – Amino acid sequence of the Ph.D.-12™ inserts obtained after panning with Streptavidin.

Streptavidin	***Amino acid sequence***	
	3rd panning	***4th panning***
1	HIRLGHLVSPHP	-
2	FHRHSDLSWFTI	HHHKHGINQISP
3	WHKPHARPALDL	HHHKHGINQISP
4	-	HHHKHGINQISP
5	WHNSWRSYSSSF	HHHKHGINQISP
6	HHKHFERVPRPP	HHHKHGINQISP
7	IGHHTLYINHPQ	HHHKHGINQISP
8	WHKPHARPALDL	HHNKHGINQISP
9	AHKAHMHTHSRP	HHHKHGINQISP
10	WHKHPRYYPLPP	HHHKHGINQISP
11	LPSYWHFSHYMR	HHHKHGINQISP
12	AHKAHMHTHSRP	HHHKHGINQISP
13	YPHQHTHPPKKT	HHHKHGINQISP
14	-	HHHKHGINQISP
15	WHKAPRPTYLSY	HHHKHGINQISP
16	IGHHTLYINHPQ	HHHKHGINQISP
17	IGHHTLYINHPQ	HHHKHGINQISP
18	LQTQYRPHQNHL	HHHKHGINQISP
19	AHKAHMHTHSRP	HHHKHGINQISP
20	WHKPVRRWSTLI	HHHKHGINQISP

Note – consensus clones are marked in colour.

Table 5.17 - Insert consensus sequences in phage clones selected with streptavidin.

Streptavidin	***Occurrence***	***Panning***	
		3rd	***4th***
WHKPHARPALDL	2/18	2/18	
IGHHTLYINHPQ	3/18	3/18	
AHKAHMHTHSRP	3/18	3/18	
HHHKHGINQISP	19/19		19/19

5.3.3 - Blocking experiment

The inability to obtain consensus sequences after the third panning suggested that it may be due to the blocking step not working correctly. In order to confirm or reject this possibility a blocking experiment was performed.

The titre of the phage recovered after incubation and elution exhibited the same range of values for both kits used. The amount of phage Ph.D-7™ and Ph.D-12™ added to the wells was 2×10^9 pfu μl^{-1} and 4×10^8, respectively. With Ph.D-7™ was possible to recover 2×10^2 pfu μl^{-1}, and with Ph.D-12™ 2.65×10^2 pfu μl^{-1} was recovered.

5.3.4 - ELISA

Elisa was performed in order to confirm the reactivity of the clone phage with the MAb used in the biopanning selection assays. The reactivity of the MAbs was only tested against phages recovered from the 4th panning round. Samples exhibiting an absorbance value $< 3 \times$ negative control absorbance value were considered negative and rejected. The rejected samples are not presented in table 5.18. The ELISA results indicate that all of the phage clones selected after panning with MAbs 4A12, 3B10 and 1E3 were not recognised by these monoclonal antibodies.

The majority of the phage clones isolated, 37 out of 52, were identified by MAb 4C3 in ELISA. Only one of the clones selected by panning with MAb 5G10 was recognised by ELISA.

Table 5.18 – Insert sequences of phage clones identified by MAbs 4C3 and 5G10 in ELISA.

MAb	*Insert - amino acid sequence*	*Occurrence*		*Insert - amino acid sequence*	*Occurrence*
4C3	HHRHNYAVEAPF	4/52		HPLSKMHYRIHM	1/52
	WHKHSYNSMPVY	3/52		HYKHHHTPILLN	1/52
	GWKSHHNHERVF	2/52		HHWHSRSQLSWF	1/52
	WHKPWYSQPWPL	1/52		IPHHYQFLKHRH	1/52
	WHKPWYSQPWPP	1/52		HHRAVPTFTWYS	1/52
	WNPHNHYRWFPH	2/52		WHKNTNWPWRTL	1/52
	FHKHSPRSPIFI	2/52		WHRTYQPPLEPR	1/52
	VHWKNPTVFSYY	1/52		HSRHHYNVHLNA	1/52
	APWWYHQWKAEQ	1/52		RFVIFILVIGLL	1/52
	HFKHQHSYARPP	1/52		HHWHQNNRQALV	1/52
	GLRHHHTIPNVS	1/52		FPRNHHQWLPHR	1/52
	WHKHSYNSMPVY	1/52		YPHHHNSRYFPM	1/52
	FHKHSPISPIFI	1/52		SHKHYNNYAHMH	1/52
	GHKHWQHNHSTH	1/52		WHKPRLHTFDFA	1/52
	FHKHSYNYAHMH	1/52		HHRHIHTALWQN	1/52
	LPWHWHTSQRSL	1/52		HSQWNTMQAIAT	1/52
5G10	YHTEYMLWLGST	1/16			

5.4 - Discussion

The discussion of the epitope mapping by phage display results comprises two sections. In the first section, the validity of the experimental protocol is analysed. The second section is concerned with the significance of the phage display data with respect to betanodavirus epitopes.

Section 1 - Validity of the phage display assays

The development of filamentous bacteriophage vectors has facilitated the construction of phage-displayed random peptide libraries that can mimic hundreds of millions of unique peptide sequences (Burrit *et al.* 1996). The small size and stability of the phage particle allows phage libraries of high titre (10^{14} particles ml^{-1}) to be produced and stored at 4°C for long periods (Smith and Scott, 1993).

The basic strategy of this technique is affinity selection of individual clones from a random peptide phage library. This is achieved through biopanning with a selected analyte (e.g. a MAb) immobilised on the surface of 96 well plates or on beads. After several biopanning steps the DNA of individual clones can be sequenced, permitting the deduced amino acid sequence to be determined. This can then be compared to the amino acid sequence of the protein under investigation. A consensus between the sequences of individual phage clones selected by a given analyte points to the interacting site(s) of the target protein.

According to Jefferies (1998) the main advantages of phage display are: ① biopanning efficacy, that permits the selection of high affinity phage present at extremely low levels in a phage library; ② amplification of the selected phage in bacteria allowing the enrichment of rare binding phage; ③ the physical link between genotype and phenotype that enables a fast and simple method for elucidation the binding sequence.

There are some studies that point to a small bias in random peptide libraries. Some of them are not explicable, but one is described as "a bias in the oligonucleotide sequence" (Bonnycastle *et al.* 1996). Generally phage display libraries show higher levels of glycine (G) and lysine (K) and lower levels of proline (P) and cysteine (C) than expected (Wilson and Finlay, 1998). However, provided that the affinity between displayed peptides and MAbs is high (as is the case for antibody-antigen interactions), the results obtained in the present study are unlikely to be influenced by this possible source of bias.

When a phage display experiment is designed, it is important to bear in mind that a given phage library is composed of different populations: non-binding phage (the dominant population); non-specific-binding phage, that binds for example to plastic or BSA (known as plate-binders); phage that specifically binds the screened molecule weakly and phage that specifically binds the screened molecule tightly (Menendez *et al.* 2001).

Several steps may be taken to enhance the elimination of undesired components of the phage population. High stringency washing can greatly reduce the background absorption of non-specifically binding phage (Lowman, 1997). However, a very high stringency in the first panning may lead to an unwanted decrease in the library complexity. A strategy using a combination of conditions favouring multivalent and monovalent interactions may thus be advantageous. The first rounds of panning are performed with conditions that promote multivalent interactions by using a lower stringency washing buffer. Subsequent panning rounds are performed on the basis of monovalent interactions that enrich for the higher affinity peptides using high stringency washing buffer (Cwirla *et al.* 1990, Dyson and Murray, 1995). In the present study, this strategy was used by performing the first panning washing steps with a buffer containing a relatively low amount of the detergent Tween 20 (0.1 %) and increasing the Tween 20 content to 0.5 % in the washing steps of the remaining panning rounds.

The plate-binder phages are usually removed by pre-adsorption of the libraries in microwells containing all the components of the screening assay, except the screening (target) molecule (Menendez *et al.* 2001). Alternatively, the phage library can be diluted in the

blocking agent. In this study, BSA was used as a blocking agent. Pre-incubation or dilution of the phage library in the presence of BSA was not performed during this experiment. Such an additional step was not considered to influence results, because the majority of previously published studies (14 of 19) performed with the Ph.D.™ system successfully yielded results without pre-incubation with BSA.

It has been observed that with libraries of different peptide length the yield of phage clones on panning with a given antibody may vary significantly and some instances the yield may be very low (Wilson and Finlay, 1998). In order to exclude this potential problem two different peptide length libraries were used in the present study, Ph.D-7™ and Ph.D-12™, which contain inserts of 7 and 12 residues respectively. The use of two different phage libraries was based on the major advantages and disadvantages of each phage display system. Longer inserts are more likely to assume a folded structure that may be required for binding to a selecting antibody. However, phage libraries constructed with peptides longer than six or seven residues will not fully represent all the possible peptide combinations (Miceli *et al.* 1994). Thus, the use of these two libraries (Ph.D-7™ and Ph.D-12™) will increase the overall chances of identifying the ligand of interest.

Section 2 - Analysis of the phage display assays data

Detailed analyse of the data obtained with the Ph.D-7™ and Ph.D-12™ phage libraries are discussed separately. The first part of this section will focus on the data obtained with Ph.D-7™. The second part will focus on discussion of the results obtained with Ph.D-12™.

Ph.D.-7™.

The phage library Ph.D-7™ has been successfully applied to the screening of antibodies to many different targets including: syndecan-1 core protein (Dore *et al.* 1998),

protein kinase C alfa (PKCα, (Ashraf *et al.* 2003), and protozoan sporozoite (*Eimeria acervulina* and *E. tenella*) (Silva *et al.* 2002). When screening MAbs to human α2-macroglobulin, Birkenmeier *et al.* (1997) obtained a consensus sequence SxxAxxL in each of twenty-five clones sequenced. However, in some studies performed using the Ph.D-7™ system the consensus sequence present amongst selected clones was less clear. For instance in the study performed by Yang and Shiuan (2003) only 21 of 70 clones sequenced formed a consensus sequence. All clones sequenced by Messmer and Thaler (2000) shared the motif PF, which was present in different positions of the insert sequence.

No consensus sequence was unequivocally present in phage clones selected by panning with the three MAbs analysed during this study. For this reason the insert sequences present in selected phage clones were analysed for the occurrence of smaller amino-acid motifs. In phage clones selected by MAb 4C3, several pairs of clones shared the same motifs, HxR, RH, HxxxN and HWS. Another clone also exhibited a WS motif (Table 5.19). When the phage clones insert sequences selected by MAb 4C3 were aligned, W, S, H residues were found to occur in five clones. This suggests, but does not definitively prove that WSH may represent a motif contained in the binding region of MAb 4C3.

In the phage clones selected by MAb 4A12, three inserts contained a PH motif and several clones exhibited WH, SY, SS, HHH and LP motifs (Table 5.19). An alignment of the deduced amino acid sequence of inserts from these clones suggests that a WH motif may be present in the recognition site of MAb 4A12.

When the same analysis was applied to MAb 3B10 two clones shared the RxxLRxL motif (Table 5.19). Another two clones exhibited some similarity with this motif with LxxL and LR. The sequence SN was present in three clones and two-residue motifs (SS, LP, MN) were present in several clones. It is possible that the motif LRxL is a component of the betanodavirus coat protein epitope recognised by MAb 3B10. W and P residues may also be involved.

Table 5.19 - Common motifs in phage clones selected by MAbs 4C3, 4A12 and 3B10 with Ph.D-7™.

4C3	***insert sequence***	***4A12***	***insert sequence***	***3B10***	***insert sequence***
1	HLRWHHT	1a	HRLHSYM	1a	GPKIWHT
2a	HSPSVLS	1b	LPTNLHW	1b	HYQSSVT
2b	AKWSSRH	2	HT-SSKLV	2	RPKRSPI
3	QFSHYFN	3	WTPHHHF	3	NAMLQLR
5	NHWSLNG	4	MHRPHWH	4a	TVKYHHH
6	HWSHARH	5	SPLHAWW	4b	VSNMNTV
7	ALNYTNS	6	LLPSYIY	5a	ITPENST
8	HMRFIHY	7a	HYQSSVT	5b	HPRIHFW
9	GHIMINR	7b	GPKIWHI	6	MSSAEAR
10	WPHKHFY	8	HLRHHHY	7	MNLGALP
		9a	WQFHLPH	8	APPSNLP
		9b	PRQYPRA	9a	HSNHLHN
				9b	RNVLRCL
				10	RLTLRSL

Note – the residues highlighted occurred more than once at that particular position. Italic residues are shared by 2 clone inserts; underlined residues are shared by ≥ 3 clone inserts; blue bold underlines the same motif shared by ≥ 2 clone inserts; red bold underlines residues of the proposed motif.

One of the drawbacks of epitope mapping by phage display arises from the analysis of an insufficient number of phage clones. This may lead to a failure to detect important insert sequences (Scott, 2001). During the present study, the number of clones sequenced was that recommended by the manufacturer. Other studies suggest that greater numbers of phage clones should be sequenced. Screening of thirty-two clones selected with MAbs to *Neisseria meningitidis* outer membrane lipooligosaccharide (*Nm*LOS) revealed only three clones containing a consensus sequence. A second consensus sequence was contained in two further clones (Charalambous and Feavers, 2000). Thus, it is possible that the absence of consensus sequences in the present study is a reflection of the relatively small number of clones sequenced. However, greater numbers of clones were not studied because of the high percentage of double inserts discovered in the clones analysed.

The presence of double inserts in Ph.D.-7™ phage clones selected by MAbs has not been described previously. A small percentage (< 1 %) of clones in each library can pick up more than one insert during ligation to the coat protein gene (Ph.D.™ Manual, New England

BioLabs). The high occurrence of phage clones containing double inserts may be a consequence of using a phage display system expressing a relatively short insert. An epitope has 15-22 contact residues with an antibody molecule (Laver *et al.* 1990). The region of the antigen in contact with an antibody is estimated to be between 650-900 $Å^2$ in area (Laver *et al.* 1990). Important contact residues may be further apart than can be mimicked by a short peptide. Phages with tandem inserts are potentially capable of binding to larger ligands.

Screening performed with the streptavidin positive control failed to yield either a consensus sequence or any phage clones with inserts containing the streptavidin consensus motif (HPQ) described by Devlin *et al.* (1990). The most plausible explanation for these observations is failure to remove non-specifically bound phages during the washing steps.

Ph.D.-12™

The phage library Ph.D.-12™ has been used to characterise ligand interactions in diverse areas of biological science, including: Human and rabbit sera recognition of gliadin (Osman *et al.* 1998, Osman *et al.* 2000); MAbs to Human Immunodeficiency Virus type 1 gp120 (HIV-1) (Ferrer and Harrison, 1999); rabies virus glycoprotein (Mansfield *et al.* 2004).

Nucleotide sequence analysis of twenty phage clones from the third panning with MAbs 4C3 and 4A12 indicated that no consensus sequence was present in any of these clones. Two clones selected with MAb 3B10 contained the same insert sequence. Five clones selected by MAb 5G10 also contained the same insert sequence. Clones selected with MAb 1E3, contained three consensus sequence groups, two of these were present in four clones, and the third in two clones.

The absence of a clear consensus sequence in any Ph.D.12™ clone could indicate the requirement for further rounds of biopanning, or else point to inefficient blocking or the use of non-specific elution buffer.

A similar failure to detect a consensus sequence in clones derived from three panning rounds was described by Gevorkian *et al.* (1998) who were only able to generate clones exhibiting insert consensus sequences after a total of five pannings. An extra panning round (four in total) with Ph.D.-12™ was performed with success by several researchers (Ehrlich and Bailon, 1998, Gazarian *et al.* 2000b, Turnbough Jr., 2003, Mansfield *et al.* 2004).

A "blocking" experiment designed to estimate phage recovery rates indicated that only 0.00001 % and 0.0000663 % were recovered to Ph.D-7™ and Ph.D-12™, respectively. This suggests that non-specific binding of phage particles has not biased the results of the present study. However, these recovery values represent $\approx 2 \times 10^2$ pfu μl^{-1} that may be sufficient to introducing error by amplification and sequencing of non-consensus insert sequence. The non-specific binding to plastic has been reported by Adey *et al.* (1995) and Gebhardt *et al.* (1996) and could potentially explain the lack of consensus motifs detected during this study.

The majority of studies employing Ph.D.™ phage libraries have utilised a non-specific elution buffer (Glycine-HCl). However the results obtained with the streptavidin positive control, eluted with biotin, indicated that the use of a specific elution buffer might be advantageous. Whilst specific elution is not technically possible, methods for competitive elution have been described. Zhang *et al.* (2003) used a competitive elution followed by non-specific elution in their work with successful results.

These observations (greater selection efficiency of increased panning rounds, phage recovery, and efficacy of multi-step elution) considered with the presence of consensus sequences within the MAb-selected phage inserts, and an HPQ motif in three positive control clones, indicate that the biopanning selection procedure was successful, and that better results may have been obtained with a fourth panning round. Consequently, a series of experiments was performed using a fourth panning combined with two competitive elutions and one non-specific elution.

The fourth panning revealed an increase in the number of insert consensus sequences in phage clones selected by all of the MAbs studied. A visible enrichment of the library was

obtained with the insert sequence FHKHSPRSPIFI in clones selected with MAb 1E3. This sequence revealed an occurrence frequency of 12.5 % in the third panning, and 52 % in the fourth panning. A consensus sequence (HHHKHGINQISP/R) occurred in 85 % of clones selected with MAb 4A12. This sequence also occurred in 57 % of clones selected with MAb 3B10, 6 % of clones selected with MAb 4C3 and 9 % of 1E3-selected clones. The high occurrence of one insert sequence in four of the five MAbs analysed suggests the presence of a bias towards this sequence (HHHKHGINQISP/R). Gazarian *et al.* (2003) considered clones with inserts occurring at a frequency of 70 % to result from bias and as such should be rejected.

In the present study, bias may result from overpanning of the phage library, such that only a limited number of clones predominate in the population to the exclusion of others which have similar or better affinities (Scott, 2001), even though the characteristics of the predominant clone bears no relevance to the selecting antibody (Burrit *et al.* 1996). The positive streptavidin control experiment strongly suggests that panning bias had influenced results. The HPQ control motif was not detected in any of the clones and all of the clones studied matched a single sequence (HHHKHGINQISP/R).

There is an inherent bias of growth competition within a biological system, which may explain the observed results. Each round of affinity selection is followed by competitive growth of the MAb-selected phages. During the phage growth of the screening cycles there is the chance that specifically-binding phages will be over-grown by poor or none-specific binders which have better growth properties (Rodi and Makowsi, 1999). This overgrowth can be favoured by the use of liquid medium for amplification of the adherent clones (Burrit *et al.* 1996).

An ELISA experiment was performed to confirm that panning bias in the Ph.D-12™ 4th panning had influenced the results of the present study, and to verify that the phage-clones were recognised by the MAbs used for selection. This is a recognised final screening procedure to identify reactive clones (Yip and Ward, 1999). The majority (37 of 52) of clones selected with MAb 4C3 and one clone selected by MAb 5G10 were identified in ELISA tests.

The sequence, HHHKHGINQISP/R, suggested to result from bias in the 4th panning was not recognised by any of the MAbs by ELISA. These data strongly suggest that HHHKHGINQISP/R sequence may be a consequence of a phage overgrowth. No binding was observed between any of the phage clones selected with MAbs 4A12 and 3B10. Similar findings were reported by Lane and Stephen (1993) in epitope mapping studies of the p53 protein.

The analysis of the clones identified by ELISA revealed that no consensus sequence is unequivocally present. Following the same approach as for Ph.D-7™, ELISA selected phage clones were analysed for the occurrence of smaller amino acid motifs.

As can be observed in Table 5.20 it is impossible to suggest just one or few smaller amino acid motifs. However, the amino acid residues known for their involvement in active sites, serine (S), aspartic acid (D), histidine (H), tryptophan (W), tyrosine (Y) and arginine (R) (Villar and Kauvar, 1994, Ma *et al.* 2003, Mansfield *et al.* 2004), are present in the majority of the reactive clones.

In the present study, the alignment of the deduced amino acid sequences of phage inserts with the betanodavirus coat protein did not produce meaningful results. Unlike earlier studies where alignments were restricted to a target of 20 amino acid residues in length (Gazarian *et al.* 2000b), the betanodavirus coat protein is comprised of 338 amino acid residues, significantly reducing the probability of identifying the sites mimicked by short consensus motifs.

Table 5.20 – Common motifs in clones selected by MAb 4C3 identified with Ph.D-12™.

MAbs	***Amino acid sequence***			
	3rd panning	***4th panning***		
		1st elution	***2nd elution***	***3rd elution***
4C3				
HL H-S H--------S	**HL**SKINRHFDHY **HL**RMN**Y**PLHTYH **HL**NHA**Y**WQH**S**RA **H**R**S**HHM**HL**P**S**PW			
HK----Q--P HK-----(-)P HK--(-)FP HKHSYN HKH—N HKH HK-S	WPY**HKH**A**FP**SR**P** W**HK**YPL**FPP**MTA F**HKH**PHSGRWYP	HH**HKH**GI**NQ**IS**P** W**HK**PWYS**Q**PW**P**P W**HKHSYN**SMPVY	F**HKHSYN**YAHMH G**HKH**WQHNHSTH	W**HKHSYN**SM**P**VY S**HKH**YN**N**YAHMH F**HK**P**S**WHAWSGR
HHRH HHR HHR---T--W	**HHRH**TFVPLTPN	**HHRH**NYAVEAPF	**HHRH**NYAVEAPF HPST**HHR**GASHI	**HHRH**NYAVEAPF **HHRH**IH**T**AL**W**QN **HHR**AVP**T**FT**W**YS
HHH	WP**HHH**HTRLSTV	**HHH**KHGINQISP	**HHH**KHGINQISP HYK**HHH**TPILLN	YP**HHH**NSRYFPM
WHK—Y WHKP WHK WHK-----P WHK--------A WH---L-P WH---(-)R WH------P HHWH WH HW WQ W---QP HQW WHP WP-H-H---S W-H---TRL	**WHK**YP**L**F**PP**MT**A** **WH**ANK**L**P**P**RYFY LHKPRP**WH**EFNR HLNHAY**WQ**HSRA **WP**Y**H**K**H**AFP**S**RP **WP**H**H**H**HTRLS**TV H**W**K**H**FNG**TRL**LD	**WHKP**W**Y**SQ**P**WPL **WHKP**W**Y**SQ**P**WPP **WHK**HS**Y**NSMPVY KPYHS**WH**QWQTS V**HW**KNPTVFSYY AP**W**WY**HQW**KAEQ	YP**WHK**SHL**R**EVT LPW**HWH**TS**Q**RSL **HHWH**SRSQLSWF GHK**HWQ**HNHSTH HSQ**W**NTM**Q**AIAT	**WHK**HSYNSMPVY **WHKP**RLHTFDF**A** FHKPS**WH**AWSG**R** **HHWHQ**NN**R**QALV **WHK**NTNW**P**WRTL **WH**RTYQP**P**LEPR HHRHIHTAL**WQ**N FPRNH**HQW**LPHR
PRY	WHANKLP**PRY**FY MHRDYY**PRY**VPW			
PLF	GQISNLP**PLF**RT WHKY**PLF**PPMTA			
FHKHS FHK-S F-KH			**FHKHS**YNYAHMH	**FHKHS**PRSPIFI **FHK**P**S**WHAWSGR IPHHYQ**F**L**KH**RH
VI------L	II**VI**RLCRLL**L**M			RF**VI**FILVIG**L**L
E--F		HHRHNYAV**E**AP**F** WKSHHNH**E**RV**F**	GWKSHHNH**E**RV**F** HHRHNYAV**E**AP**F**	HHRHNYAV**E**AP**F**
SHH	HR**SHH**MHLPSPW ALYKH**SHH**VWRL			
RH H-RH-Y-V	HLSKIN**RH**FDHY	HH**RH**NYAVEAPF GL**RH**HHTIPNVS	HH**RH**NYAVEAPF	H**S****RH**H**Y**N**V**HLNA **H**H**RH**N**Y**A**V**EAPF
5G10				
YH—Y Y--WL HS--H WH--N PY	VP**HWH**TEYLRWL G**HW**KLF**PY**WARS **YH**TE**Y**ML**WL**GST **HS**RI**H**NHTDRNI **HS**TH**H**LSKHILA HTSLN**Y**RP**WL**TI KGIH**WH**HW**N**YGV AP**WH**LH**N**PIYRL	**YH**KN**Y**RSL**PY**FM		

Note – the residues depicted in bold type underline same motif are shared by ≥ 2 clone inserts.

The failure to identify unequivocally a consensus sequence has been associated with plate-binder clones represent a probable explanation for the results. Insert sequences containing tryptophan (e.g. WxxWxxxW) and tyrosine are usually associated with this phenomenon (Adey *et al.* 1995, Gebhardt *et al.* 1996). Six clones of seventy-one sequenced contained two or more W or Y residues.

Christian *et al.* (1992) were unable to map epitopes recognised by MAbs to HIV-1 gp120. The authors associated the failure with an inability of their phage display protocol to discriminate between rare high-affinity insert sequences and more common low affinity sequences.

5.4.1 - Final reflection

Although the phage display methodology has been applied to epitope mapping studies for over 15 years, to our knowledge this is the first time that the technique has been used to identify epitopes on a fish virus.

Two phage libraries that expressed different sizes of random peptides fused to the pIII coat protein were used. After several affinity selection steps, biopanning, and sequence analysis of MAb selected clones, a consensus insert amino acid sequence should be obtained. However, the recognition of a consensus sequence is dependent upon the interpretation of short amino acid sequences and their relationship to the target antigen. The relationship between phage insert and the target antigen can "range from obvious to surprisingly obscure" (Burrit *et al.* 1996).

In the present study, no consensus sequence was identified in inserts from phages selected with MAbs raised against the betanodavirus coat protein. Phage clones selected with the Ph.D.-7™ system contained double inserts. An epitope comprises 14-22 residues (Kuby, 1994) and sometimes a peptide comprised of seven amino acid residues in length is not large enough to bind sufficient critical residues on an antibody molecule.

The use of the Ph.D.-12™ system, which expresses longer peptides (12 amino acids) also failed to yield a consensus sequence in MAb-selected phage clones after three rounds of panning. For this reason, a fourth round of panning was performed. However, this additional step created a bias in the MAb-selected procedure that was confirmed by the non-reactivity of peptides with the selecting MAbs in ELISA tests. The lack of consensus can also be associated with the presence of plate-binder phages that contaminate the elution step.

In future work, these problems should be taken into consideration and may potentially be solved with a pre-incubation of the phage library with possible sources of non-specific binding, such as for example, the plastic plate, BSA and buffers.

Chapter 6 - Epitope mapping with synthetic peptides

6.1 - Introduction

6.1.1 - Synthetic peptides

An important component of immune responses to many diseases is the ability of the host's antibodies to recognise foreign antigens, such as surface proteins or toxins (Carter, 1994).

The region of an antigen that is recognised by an antibody is designated as an antigenic site or B-cell epitope. In the case of viruses, the antigenic sites frequently correspond to parts of the capsid or envelope protein. The region of the antibody molecule that recognises an epitope is known as the combining site or paratope (van Regenmortel, 1992). Antibodies to protein epitopes are usually highly specific and able to distinguish between proteins differing by only a single amino acid in the target epitope (Alexander *et al.* 1983).

There are two main approaches to identifying the regions of a protein that are recognised by antibodies. These are based on either a) generation of successively smaller fragments of the native protein, or b) use of chemically synthesised synthetic peptides. Knowledge of the amino sequence of a given protein is a requirement of the latter approach.

Over the last two decades, the simultaneous synthesis of large numbers of peptides, either as discrete members of sets or as mixtures, has become an important tool in both molecular immunology and the drug discovery process (Ede, 2002). A revolutionary advance in this technology was described by the development of parallel synthesis of large sets of peptides by Geysen *et al.* in 1984. This methodology represented a major advance in epitope mapping of protein antigens because of its ability to characterise the interaction between antibodies and large numbers of overlapping synthetic peptides required to identify epitopes of protein molecules (Geysen *et al.* 1987a).

The use of synthetic peptides to identify epitopes recognised by monoclonal antibodies as described by Geysen *et al.* (1984) has been widely used in epitope mapping studies, e.g influenza A virus (Hatta *et al.* 2000), foot and mouth virus (Geysen *et al.* 1984), beet necrosis yellow vein virus (BNYVV) (Commandeur *et al.* 1994). For fish viruses the synthetic peptides have been applied to epitope mapping of Viral Haemorrhagic Septicaemia Virus (VHSV) (Estepa and Coll, 1996, Fernandéz-Alonso *et al.* 1998).

The principle of the pepscan technique is based on the fact that antibodies produced against a native protein are, by definition, directed to the immunogenic epitopes. Thus by representing an entire protein in the form of short overlapping synthetic peptides, antibody recognition sites can be mapped to regions defined by the length of the peptides used and sometimes even less, when the overlap of adjacent peptides is taken into consideration (Gershoni *et al.* 1997). Peptides are frequently coupled to a solid surface or a carrier molecule in order to allow detection of binding interactions between the peptide and the monoclonal antibody. This coupling procedure can significantly affect the presentation of the peptide, even to the extent of masking potentially reactive amino acid side chains (Geysen *et al.* 1987a).

The physical state of the peptides used in epitope mapping studies depends on the immune recognition molecule studied. For mapping B-cell epitopes either bound or cleaved peptides can be used. For T-cell epitope mapping cleaved peptides must be used (Sumar, 2001).

The synthesis of peptides has been a challenge to organic chemists since the turn of the 20th century. Classical methods of peptide synthesis are poorly suited for high throughput, simultaneous synthesis of peptides (Atherton and Sheppard, 1989). In 1964 Merrifield developed a completely new approach for peptide synthesis utilising solid phase synthesis (Merrifield, 1964). In this procedure the nascent peptide molecule is constructed whilst bound to a stable, solid particle throughout all of the synthesis steps, and is separated from soluble reagents and solvents by simple filtration and washing (Atherton and Sheppard, 1989).

The PEPSCAN procedure is a variation of the solid-phase peptide synthesis method developed by Geysen et al. (1984) and uses synthesis of peptides on the tip of polyacrylated grafted polyethylene rods arranged in an 8x12 microtitre plate array. The modular format that complements a 96 well plate permits the simultaneous handling of hundreds to thousands of individual peptides (Maeji *et al.* 1995). The original application of this technique enabled the identification and characterisation of epitopes within protein antigens to a resolution of one amino acid (Geysen *et al.* 1984). Although the PEPSCAN procedure has been shown to be particularly effective for mapping linear/continuous epitopes, it is also possible to detect discontinuous epitopes (Gao and Esnouf, 1996).

A further variation of the solid phase peptide synthesis was developed by Frank and Döring (1988), who used cellulose paper discs for the solid phase. With this technique individual amino acid coupling reactions in a multiple parallel synthesis are performed simultaneously on distinct areas of a continuous pure cellulose chromatography paper sheet (Frank, 1992, Frank, 2002). This technique has been named SPOT-synthesis.

The overlapping peptide scan utilising SPOT synthesis is a popular tool for characterisation of molecular interactions (Reineke *et al.* 2001). The best-known applications of this technology are the analysis of epitope mapping and receptor-ligand interaction sites (Laune *et al.* 2002). Continuous and discontinuous epitopes can be detected. The identification of discontinuous epitopes is based on the detection of low affinity interactions (Reineke *et al.* 1998, Reineke *et al.* 1999). These low affinity interactions can be detected by measuring the dissociation constant (Kramer *et al.* 1997, Reineke *et al.* 2002).

6.1.2 - Flow cytometry

Flow cytometry enables discrete measurements of optical signals from a single particle such as cells or beads (Edwards *et al.* 2004). These signals can be read by electrical impedance, light scattering and fluorescence (Shapiro, 2003).

Flow cytometers are divided in to two main types, analysers and sorters. The sorters have the ability to collect data on cells (analyse cells) and sort cells with particular properties (Chapman, 2000). The Becton Dickinson FACScan is an example of the sorters currently available. xMAP-based products from Luminex are of the analyser type.

For flow cytometric analysis, mono-dispersed cell suspensions must be produced (Mandy *et al.* 2001). These are achieved by the use of a laminar sheath-flow technique to confine cells to the center of a flow stream, reducing clogging due to clumps (Horan and Wheeless Jr., 1977). Single particles or cells are scanned as they flow in a liquid medium past an excitation light source (Radcliff and Jaroszeski, 1998).

With the flow cytometry technique, each cell is exposed to excitation light only for the brief period during which it passes through the illuminating beam, usually a few microseconds, and the flow velocity is typically constant for all the cells examined (Shapiro, 2003). The use of these uniform conditions of measurement make it possible to attain high precision, by obtaining nearly equal measurement values for cells containing equal amounts of fluorescent material (Shapiro, 2003).

Typically fluorochromes are fluorescent probes conjugated to monoclonal antibodies. These fluorescent probes react with the cells or particles of interest before analysis. Therefore the amount of fluorescence emitted as a particle passes the light source is proportional to the amount of fluorescent probe bound to the cell (Radcliff and Jaroszeski, 1998).

Cytometers can be used for sensitive chemical analyses involving the binding of suitable labelled ligands to solid substrates or to particles such as polystyrene beads (Shapiro, 2003). During development, the terminology describing microsphere-based assays saw many variations including: multiplex bead assay (MBA), particle-based micro-array technology

(PBMT), microparticle-based flow cytometric technology (MFCT), multiplexed particle-based flow cytometry assay (MPBFC), micro-array immunoassay (MAI), cytometric bead array (CBA) and suspension array technology (SAT) (Mandy *et al.* 2001, Nolan and Mandy, 2001).

For many years, the concept of performing immunological assays on microspheres has been both theorized and applied. Recently the technology been developed to the extent that both reagents and software are now commercially available (McHugh, 1994, McDade and Spain, 1997).

In 1977, Horan and Wheeless Jr. described for the first time the use of flow cytometry combined with microspheres for the determination of analytes in serum and other fluids. Initially two different sizes of beads were used. Rapid advances in instrumentation permitted the use of four different bead sizes (McHugh *et al.* 1988, Scillian *et al.* 1989). The ability to discriminate between different sized microspheres facilitated the simultaneous detection of several analytes. However the inability to distinguish aggregates of smaller microspheres from larger microspheres severely limited the extent of multiplexing that could be achieved (Fulton *et al.* 1997). This limitation jeopardised the opportunities that multiplexed binding assays offered for exploration of complex binding interactions in a single reaction volume (Iannone *et al.* 2001). Eventually, codifying the microspheres provided an elegant solution to this methodological problem.

The most commonly used encoding method in bead-based assays is optical encoding with fluorescent dyes (Yingyongnarongkul *et al.* 2003). To prepare microsphere sets, two hydrophobic fluorophores are used, one emitting in the red region of the light spectrum, and one emitting in the orange region (McDade and Spain, 1997). These two fluorophores are combined within microspheres to a unique ratio (Smith *et al.* 1998). By adjusting these intrinsic optical properties, it became possible to prepare arrays of microspheres in which individual microsphere subsets possess a unique *spectral address*, and thus can be used to perform multiplexed analyses (Nolan and Sklar, 2002).

These techniques require microspheres coated with a unique capture reagent (McHugh, 1994). There are several types of microspheres coated with different linkers for non-covalent coupling (Kellar and Iannone, 2002). However, when the orientation of the protein on the bead surface is critical, as is the case of antibodies, covalent coupling should be used (McHugh and Stites, 1991). Carboxyl (COOH), amine/hydrazide and maleimide groups are used for covalent coupling (Kellar and Iannone, 2002).

Microspheres of several sizes and composed of different polymers are currently available commercially.

Assays that combine flow cytometry and microspheres consist of four basic steps: ① coating of the beads with the antibody/antigen or capture reagent; ② addition of the analyte to the microspheres; ③ addition of a fluorescent reporter molecule that binds to the analyte and ④ fluorescent quantitation. These four steps are depicted in Figure 6.1.

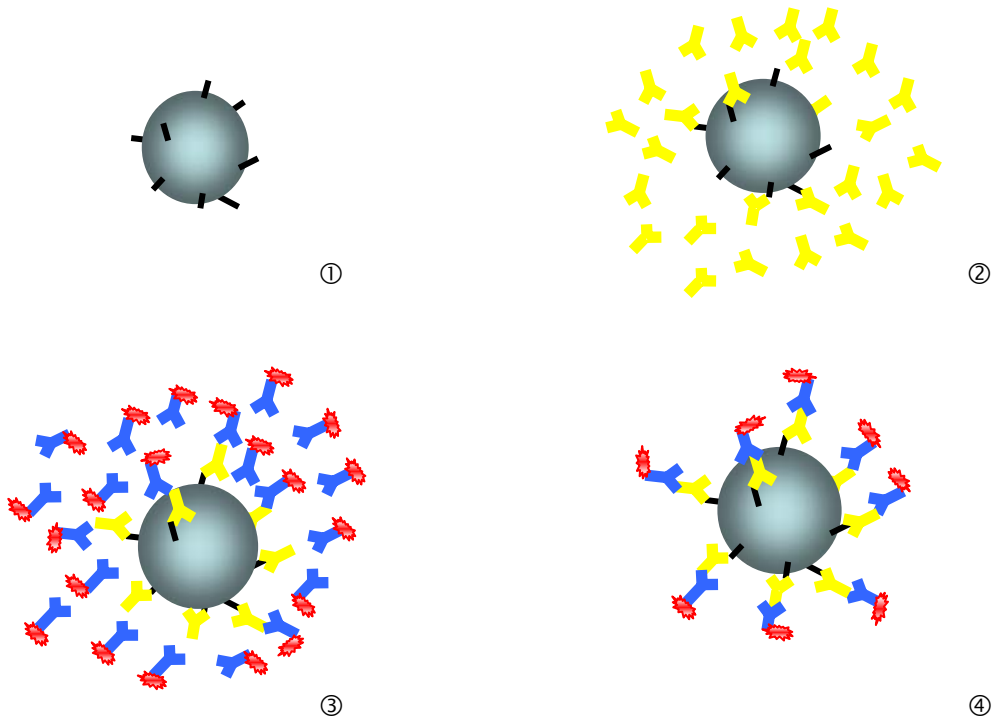


Figure 6.1 – Flow cytometry using SAT. ① beads coated with antibody/antigen; ② sample containing analyte added; ③ molecular reporter added; ④ fluorescent quantitation.

The combination of microspheres, flow cytometry technology and digital signal processing has facilitated the development of a fast and precise assay platform (McDade and Spain, 1997). This new generation of fluorescent microsphere-based immunological assays reduces the time and cost of traditional microtitre plate-based assays, by relying on the rapid association kinetics of antibodies and antigens in solution, as well as the use of a smaller surface area. Consequently, smaller quantities of reactants are required than in conventional immunoassays (Kellar *et al.* 2001).

Since the 1960s, flow cytometry has been applied to several fields of research. Until recently, the combination of this technique with labelled microspheres has predominantly been employed in immunological research (Table 6.1).

Table 6.1– Application of the microspheres and flow cytometry technology.

	Reference
Immunology studies	
Quantification of cytokines	
Tumour necrosis factor-alpha (TNF-α) Intereukin: IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-18 Gamma interferon (IFN-γ) Granulocyte-macrophage colony-stimulating factor (GM-CFS)	Prabhakar *et al.* (2002 de Jager *et al.* (2003) Kellar and Douglass (2003) Hulse *et al.* (2004)
Quantification of antibodies	
Human papilloma virus Toxins of anthrax, tetanus and diphtheria *Haemophilus influenzae* type b	Opalka *et al.* (2003) Biagini *et al.* (2004b) Pickering *et al.* (2002a)
Detection of antibodies in autoimmune diseases	
Systemic rheumatic disorders Systemic lupus erythematosus	Rouquette *et al.* (2003 Mahler *et al.* (2004)
Characterisation of antibodies	
HIV-1 infected individuals	Opalka *et al.* (2004)
Detection of antibodies	
Flavivirus Pneumococcal polysaccharides (PnPs) Genotype-specific to respiratory syncytial virus	Wong *et al.* (2004) Pickering *et al.* (2002b) Jones *et al.* (2002)
Expression	
CD40 in diabetic people	Vosters *et al.* (2004)
Other studies	
Detection and/or quantification	
Phosphorylation levels in cell signalling proteins: phospho-JNK phospho-p38 MAPK phospho-IκB-α phospho-ERK2 phospho-Akt phospho-ATF-2 phospho-MEK2phospho-STAT3 Herbicides in human urine (glyphosate, atrazine and metolachlor) Common foodborne illnesses (*E. coli*, *Listeria monocytogenes, Salmonella* and *Campylobacter jejuni*) Staphylococcal protein toxins (ETA and ETB)	Gao *et al.* (2003) Biagini *et al.* (2004b) Dunbar *et al.* (2003) Joubert *et al.* (2003)
Molecular techniques	
Single nucleotide polymorphism (SNP) Forensic analyses with human identity testing (SNP of African American vs Caucasian) Bacterial identification using 16S rDNA Identification and quantification of DNA in environmental samples Detection and quantification of viral nucleic acids	Chen *et al.* (2000) Vallone and Butler (2004) Ye *et al.* (2001) Spiro *et al.* (2000) Smith *et al.* (1998)
Hybridoma technology	
Characterisation of monoclonal antibodies	Jia *et al.* (2004)

6.1.2.1 - Suspension array technology with xMAP

For suspension array technology (SAT) the interaction between analyte and target is detected on the surface of solid fluorospheres (Mandy *et al.* 2001).

The Luminex-100 instrument has been designed by the Luminex Corporation (Austin, Texas) for fluorosphere-based flow cytometry (Nolan and Mandy, 2001). This technique is referred to as xMAP technology.

The Luminex-100 uses traditional flow cytometry hardware and spectrally discrete polystyrene beads, or microspheres, to measure multiple analytes simultaneously (Seideman and Peritt, 2002).

By varying the ratio of the two fluorophores (red and orange), up to 100 different bead sets can be distinguished and each bead set can be coupled to a different biological probe (Figure 6.2 ①) (Dasso *et al.* 2002).

5.5 μm, cross linked polystyrene microspheres with surface carboxylate groups were found to provide the best combination of thermal stability, uniformity (coefficient of variation 1% for diameter) and reagent density required for a wide range of possible assays (McDade and Spain, 1997).

The Luminex-100 has a dual laser system. The 532-nm, 13-mW yttrium aluminium garnet (YAG) laser (green laser) is used because it improves the excitation efficiency of R-phycoerythrin (PE, 578-nm emission) as compared to a 488-nm laser (Kellar and Iannone, 2002), and is dedicated to the quantitative analysis (Mandy *et al.* 2001). The 635-nm 10-mW red diode laser excites the two classification fluorochromes embedded within the microspheres (Kellar and Iannone, 2002) that are used to define a spectral address (Mandy *et al.* 2001) (Figure 6.2 ②).

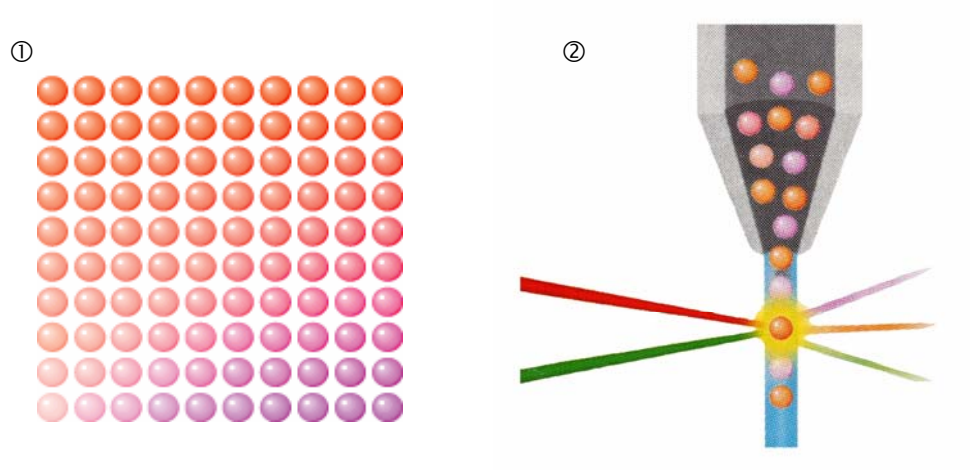


Figure 6.2 - ① Matrix of beads available by Bio-Rad; ② Dual lasers identifying the beads.

Data is acquired on the Luminex-100 by a PC equipped with a special digital signal processing board and control software that runs in the Windows environment. The software uses the orange and red data to separate the pool of microspheres into individual bead sets, and presents the average amount of green to each bead set (Vignali, 2000). The concentration of the analytes can be determined by extrapolation to a standard curve. The data can be exported to a spreadsheet program such as Microsoft-Excel for further analysis.

The most commonly used reporter molecules are antibodies either conjugated with a fluorochrome or biotin, which can be used to bind a streptavidin-fluorochrome conjugate (Sklar *et al.* 2002). R-phycoerythrin (R-PE) is considered the most suitable fluorochrome for quantitative fluorescence measurement in clinical flow cytometry (Haugland, 1994, Gratama *et al.* 1998), because its extinction coefficient and quantum yield are high (Mandy *et al.* 2001). Low background fluorescence is achieved through the use of homogenous multi-parameter measurements coupled with quantitative fluorescence detection capacity at low emission levels (Nolan and Mandy, 2001). The sensitivity of the instrument is high, and it can detect less than 500 PE molecules bound to beads (Shapiro, 2003).

6.1.3 - Objectives

The objective of this chapter was to identify B-cell epitopes on the betanodavirus coat protein using a new approach. This was achieved through the use of xMAP technology and the synthesis of a panel of overlapping synthetic peptides that mimicked the betanodavirus coat protein. This procedure was used by Opalka *et al.* (2004) for epitope mapping in Human Immunodeficiency Virus (HIV). Sets of overlapping peptides that mimic the betanodavirus coat protein were synthesised and coupled to polystyrene beads. The peptide-bead sets were incubated with antibodies and the reaction read in a Bio-Plex™ system array, to determine which region(s) of the betanodavirus coat protein are recognised. This technique was used to characterise the binding sites of murine monoclonal antibodies, rabbit polyclonal antibodies, and sera from fish (European sea bass *Dicentrarchus labrax*) naturally-infected with nodavirus. The production of monoclonal and polyclonal antibodies use in this study is described in Chapter 3.

6.2 - Materials and Methods

6.2.1 - Synthetic peptides

The Dragon Fish Nervous Necrosis Virus (DFNNV) coat protein (Genbank protein access number AAG22496) was used as template for the production of the synthetic peptides. Synthetic peptides were 12 amino acid residues in length (12 mers) and overlapped by two residues.

The cross-reactivity of peptides may be influenced by the immunoassay format, simply due to the fact that different conformations can be adopted by peptides in the various experimental conditions of different immunoassays (van Regenmortel, 1989b). An N-terminal AHX linker (amino hexanoic acid) was introduced in the peptide sequence in order to prevent conformation problems related with direct binding of the peptide sequence to the beads.

Two additional peptides were synthesised for use as negative controls. These peptides corresponded to the amino acid 30-41 and 305-316 but were synthesised in reverse sequence order. The sequences of peptides used in this study are presented in Table 6.2. The negative control peptides are marked in bold.

COOH beads were chosen for coupling to synthetic peptides. These beads are most useful for binding antibodies or small molecules, which can lose function when passively adsorbed to the microsphere surface (McHugh, 1994). The amine group of the peptides was coupled to the carboxyl group of the COOH beads (Bio-Rad, Hertfordshire U.K.). The coupling reaction was performed using the Bio-Plex Amine Coupling kit (Bio-Rad, Hertfordshire U.K.) according to the manufacturer's instructions.

The overlapping peptides were produced by Pepscan Systems (Lelystad, The Netherlands) and were supplied coupled to beads. After coupling beads were stored in the dark at 4°C.

Bead concentration was determined with a hemocytometer.

Table 6.2 – Sequences of synthetic peptides used for epitope mapping. Sequences were derived from the DFNNV coat protein.

Peptide (residue)	*Amino acid sequence*	*Bead reference*		*Peptide (residue)*	*Amino acid sequence*	*Bead reference*
1 (1-12)	MVRKGEKKLAKP	171-506046		19 (181-192)	GRLILLCVGNNT	171-506042
2 (11-22)	KPPTTKAANPQP	171-506046		20 (191-202)	NTDVVNVSVLCR	171-506042
3 (21-32)	QPRRRANNRRRS	171-506046		21 (201-212)	CRWSVRLSVPSL	171-506042
4 (31-42)	RSNRTDAPVSKA	171-506046		22 (211-222)	SLETPEETTAPI	171-506042
5 (41-52)	KASTVTGFGRGT	171-506046		23 (212-232)	PIMTQGSLYNDS	171-506042
6 (51-62)	GTNDVHLSGMSR	171-506046		24 (231-242)	DSLSTNDFKSIL	171-506042
7 (61-72)	SRISQAVLPAGT	171-506046		25 (241-252)	ILLGSTPLDIAP	171-506042
8 (71-82)	GTGTDGYVVVDA	171-506046		26 (251-262)	APDGAVFQLDRP	171-506042
9 (81-92)	DATIVPDLLPRL	171-506046		27 (261-272)	RPLSIDYSLGTG	171-506042
10 (91-102)	RLGHAARIFQRY	171-506028		28 (271-282)	TGDVDRAVYWHL	171-506024
11 (101-112)	RYAVETLEFEIQ	171-506028		29 (281-292)	HLKKFAGNAGTP	171-506024
12 (111-122)	IQPMCPANTGGG	171-506028		30 (291-302)	TPAGWFRWGIWD	171-506024
13 (121-132)	GGYVAGFLPDPT	171-506028		31 (301-312)	WDNFNKTFTDGV	171-506024
14 (131-142)	PTDNDHTFDALQ	171-506028		32 (311-322)	GVAYYSDEQPRQ	171-506024
15 (141-152)	LQATRGAVVAKW	171-506028		33 (321-332)	RQILLPVGTVCT	171-506024
16 (151-162)	KWWESRTVRPQY	171-506028		34 (327-338)	VGTVCTRVDSEN	171-506024
17 (161-172)	QYTRTLLWTSSG	171-506028		**35 (41-30)**	**KSVPADTRNSRR**	**171-506024**
18 (171-182)	SGKEQRLTSPGR	171-506028		**36 (316-305)**	**SYYAVGDTFTKN**	**171-506024**

The assay was multiplexed to 4 different peptides per well, because peptides were coupled with beads of four different spectral addresses. Peptides were grouped into 9 groups for analysis as indicated in Table 6.3.

Table 6.3 – Synthetic peptide groupings used for multiplex analysis in the Bio-Plex™ system.

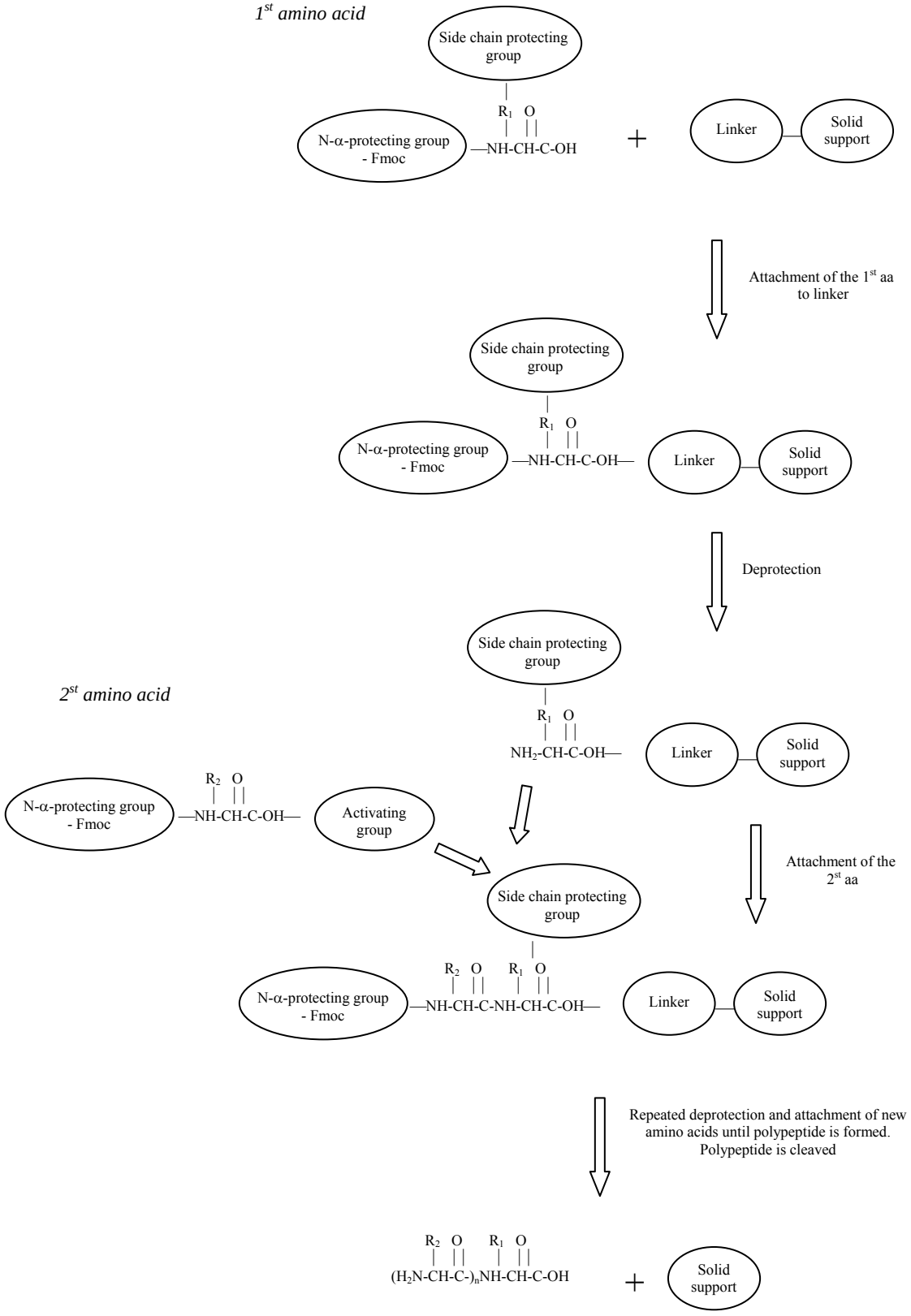
Group	*Peptide*	*Bead*		*Group*	*Peptide*	*Bead*
PepS 1	1 10 19 28	171-506046 171-506028 171-506042 171-506024		PepS 6	6 15 24 33	171-506046 171-506028 171-506042 171-506024
PepS 2	2 11 20 29	171-506046 171-506028 171-506042 171-506024		PepS 7	7 16 25 34	171-506046 171-506028 171-506042 171-506024
PepS 3	3 12 21 30	171-506046 171-506028 171-506042 171-506024		PepS 8	8 17 26 35	171-506046 171-506028 171-506042 171-506024
PepS 4	4 13 22 31	171-506046 171-506028 171-506042 171-506024		PepS 9	9 18 27 36	171-506046 171-506028 171-506042 171-506024
PepS 5	5 14 23 32	171-506046 171-506028 171-506042 171-506024				

6.2.1.1 - Peptide synthesis

The overlapping peptides were produced by Pepscan Systems (Lelystad, Netherlands) using solid phase synthesis and Fmoc chemistry.

To ensure the correct synthesis of a peptide it is necessary for inappropriate binding of the amino group of one amino acid and the carboxyl group of the other amino acid be prevented (Atherton and Sheppard, 1989). This is achievable by linking a protective group to the amino acids. The protective group used depends on the type of synthesis chosen. However, it must be easily removed during or after the polypeptide synthesis (Atherton and Sheppard, 1989). Fmoc (flurenylmethyloxycarbonyl) was used as for the α-N amino protecting groups during the synthesis of the peptides produced by Pepscan Systems. Usually this protective group is chosen because it requires mild conditions for removal and for the cleavage from the solid support (Sumar, 2001) (Figure 6.3).

Figure 6.3 – Diagram of solid phase peptide synthesis using the Fmoc technique (Adapted from Sumar (2001).



6.2.2 - xMAP technology

The xMAP technology (Luminex™) used in this study was the Luminex-100 marketed by Bio-Rad (Hertfordshire U.K.). Bio-Rad have named this equipment Bio-Plex™.

The Bio-Plex™ suspension array system integrates the xMAP technology with instrument controls and data analysis software, system validation and calibration tools together with all supplementary materials required to perform xMAP-based assays in a 96-well plate format (Willis *et al.* 2003).

The Bio-Plex™ system is comprised of an array reader, an XY platform, an HTF system (Figure 6.4) and a computer (PC). The Bio-Plex™ array reader is a compact flow analysis unit integrating a dual laser detection system, optics, fluidics and advanced digital signal processing.

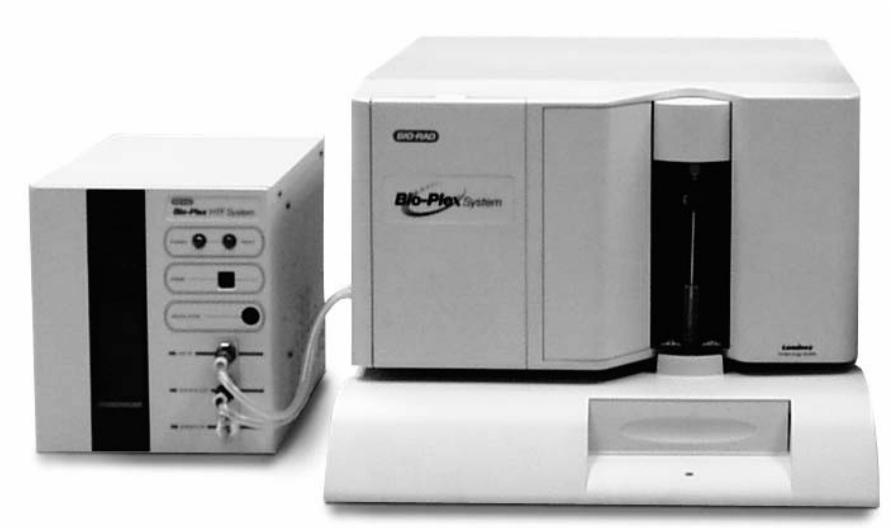


Figure 6.4 - Bio-Plex™ system: HTF system, array reader and XY platform.

The XY platform allows the automated processing of samples from a 96 well plate. The HTF (High Throughput Fluidics) system is designed to automate the introduction of sheath fluid into the Bio-Plex™ array reader.

The HTF system automatically draws sheath fluid from a non-pressurized bulk container to constantly maintain a reservoir of pressurized sheath fluid.

Minimum computer hardware and software requirements are IBM PB, Pentium III processor. Microsoft Windows 2000 operating system, Excel and Access software are essential. A high-speed digital signal processor and Bio-Plex Manager™ 3.0 software record the fluorescent signals simultaneously for each bead, and translate the signals into data for each bead-based assay. The data can then be exported to Microsoft Excel for further analysis.

The Bio-Plex™ has a MCV plate specially designed for needle adjustments and for performing the validation, calibration and washing
(Figure 6.5).

Figure 6.5 - MCV plate.

6.2.3 - Antibody samples

In this study, mouse MAbs, rabbit polyclonal antibodies and fish polyclonal sera were used.

The production of mouse MAbs was described in Chapter 3. Five monoclonal antibodies were used. These were designated 1E3, 3B10, 4A12, 4C3 and 5G10. The purification and estimation of MAb concentration using the BCA protein kit (Pierce, Northumberland U.K.) were performed as described in Chapter 2.

Polyclonal antibodies were produced in Rabbit immunised with European sea bass betanodavirus as described in Chapter 3.

The fish serum samples were kindly supplied by Professor Alexandra Adams (Aquatic Vaccine Unit, Stirling University, U.K.). The samples were collected from European sea bass (*Dicentrarchus labrax* L.) naturally infected with betanodavirus. The samples were tested for betanodavirus antibodies by ELISA. Nine fish serum samples were used. Seven of these were positive and two negative. The positive samples chosen represented those samples with the highest nodavirus antibody titres as presented in Table 6.4.

Table 6.4 – Betanodavirus antibody titres in samples of European sea bass serum as determined by ELISA.

Sample	1	3	4	7	10	15	17	2	9
Titre	1/16	1/32	1/32	1/32	1/32	1/64	1/32	-	-

As a negative control one betanodavirus-negative European sea bass serum sample was kindly supplied by Dr. W. Roy of the Machrihanish fish farm. European sea bass were sourced from a hatchery in Wales. No outbreaks of betanodavirus infection have been recorded at this site.

6.2.4 - Protocol for epitope mapping with SAT

The experimental xMAP protocol used in this thesis was based on previously published work (Kellar *et al.* 2001, Dasso *et al.* 2002, Prabhakar *et al.* 2002, Seideman and Peritt, 2002, Joubert *et al.* 2003). Some of the experimental steps were optimised as described in Table 6.5.

Table 6.5 – Overview of the protocol used for epitope mapping with SAT.

				Amount	***Volume per well***	***Incubation***
Preliminary Steps	**Blocking analyte**	Block interactions between synthetic peptides and buffer	Analyte (bead/peptide) in DPBS+	2000 beads per well of each peptide		30 minutes RT
	Pre-wetting plate	Block filter plate Wetting the filter membrane	DPBS+		200 µl	2 x 30 minutes RT
Protocol Steps	**Analyte**	Analyte added to the well plate	Analyte in DPBS+	2000 beads per well of each peptide	50 µl	
	Sample incubation	Binding bead-peptide to antibodies	Antibodies (polyclonal, MAbs and fish antibodies) in DPBS+	Required optimisation	50 µl	Required optimisation
	Wash		DPBS+		3 x 100 µl	
	Primary antibody*	Binding the primary antibody to fish serum	Anti-sea bass IgM	Required optimisation	DPBS+	3.5 hours RT
	Wash		DPBS+		3 x 100 µl	
	Reporter molecule	Binding the antibody conjugated with fluorochrome to the bead-peptide-abs	Anti-mouse IgG PE Anti-rabbit IgG PE	Required optimisation	50 µl	30 minutes RT
	Wash		DPBS+		3 x 100 µl	
	Pre-reading	Re-suspend the beads for reading	DPBS+		100 µl	
	Reading	50 µl of the sample were read by Bio-Plex™ and minimum of 100 beads per analyte are analysed				

RT – room temperature; * required only for fish serum samples

All antibody dilution, blocking and washing steps were performed using Dulbecco's PBS (Gibco, Paisley U.K.) containing 1 % BSA (w/v) (Sigma, Dorset U.K.) and 0.02 % (w/v) sodium azide (Sigma, Dorset U.K) (DPBS+).

Assays were performed in sterile MultiScreen HTS™ (Millipore, Watford U.K.) 96 well filter plates. These plates have a 1.2 μm hydrophil, low protein binding Durapore® membrane.

The fluorochrome phycoerythrin (PE) conjugated directly to antibodies was used as a reporter molecule. Goat anti-mouse IgG conjugated with PE (mouse IgG-PE) and goat anti-rabbit IgG conjugated with PE (rabbit IgG-PE) were obtained from Molecular Probes (Paisley Scotland).

All washing steps were performed with a vacuum manifold (Bio-Rad, Hertfordshire, U.K.) connected to a vacuum pump. The vacuum pressure was kept at –10 mm Hg.

The protocol used for screening the polyclonal and MAbs was identical. For fish sera an additional step was incorporated due to a requirement for the inclusion of a primary antibody (anti-fish IgM monoclonal antibody). The antibody used was mouse anti-European sea bass IgM monoclonal antibody (sea bass IgM) (Aquatic Diagnostics Ld, Stirling University, Stirlinghire Scotland). The IgG MAb was reconstituted in 1 ml of DPBS+.

The beads and phycoerythrin (PE) are photosensitive, and exposure to light for a period of two hours has been demonstrated to adversely influence assays due to photo-bleaching (Carson and Vignali, 1999). Special precautions were taken to avoid photobleaching. Beads and reporter molecules were protected from light at all steps of the experimental protocol. Laboratory illumination was minimised and directed away from benches used for experimental work. Light exposure was reduced to minimum levels. The beads, reporter molecules and the assay plates were wrapped in aluminium foil at all times.

6.2.5 - Antibody Screening Assay

Before screening the full set of synthetic peptides with antibody samples three steps of the protocol were optimised.

6.2.5.1 - Sample Incubation Time

The incubation time for allowing an optimal rate of antibody-peptide was the first step to be optimised, by incubating MAbs with PepS1 group (Table 6.6).

Table 6.6 – Optimisation of sample incubation time for epitope mapping with SAT.

Step	***Antibody***	***Reporter***	***Sample Incubation***	
			Time	**Temperature**
Sample Incubation	MAbs 1E3, 3B10, 4A12, 4C3, 5G10 20 μg ml^{-1}	Mouse IgG PE 7.5 μg ml^{-1}	60 minutes	Room temperature
			Overnight	4°C

The concentration of MAbs and reporter molecule used are shown in Table 6.6. All protocol steps were performed as described in Table 6.5.

6.2.5.2 - Antibody concentration

Different concentrations of the anti-betanodavirus antibodies were tested (Table 6.7). For mouse MAbs four concentrations were tested and for European sea bass three different dilutions of sera were used. The rabbit polyclonal serum assay was optimised with seven different serum dilutions. Antibodies were diluted in DPBS+. A previous run with a randomly chosen peptides revealed MAb 4A12 as the one with more heterogeneous behaviour, showing both very low and very high MFI values. These MFI values revealed MAb 4A12 as the best

antibody for optimisation of the assay, in order to achieve high MFI values without increasing non-specific background due to an excess of mouse-PE. Samples 10 and 15 (SB10 and SB15) were used for the optimisation of the fish serum concentration. These samples were chosen because of the high titre values that they present and the higher amount of sample (as shown in Table 6.3).

The protocol steps were performed as described in Table 6.5.

Table 6.7 – Optimisation of antibody concentration for epitope mapping with SAT.

Step	***Antibody***	***Primary Ab***	***Reporter***	***Sample Incubation***	
				Time	**Temperature**
Sample Incubation	MAb - 4A12 20 μg ml^{-1} 50 μg ml^{-1} 80 μg ml^{-1} 100 μg ml^{-1}	-	Mouse IgG PE 7.5 μg ml^{-1}	Overnight	4°C
	Polyclonal 1/10 1/100 1/500 1/1000 1/2500 1/5000 1/10000	-	Rabbit IgG PE 25 μg ml^{-1}	Overnight	4°C
	Fish sera Sample 10 1/10 1/50 Sample 15 1/50 1/100	Sea Bass IgM 6 μg ml^{-1}	Mouse IgG PE 25 μg ml^{-1}	Overnight	4°C

6.2.5.3 - Reporter antibody concentration

The optimum reporter antibody concentration was determined as indicated in Table 6.8. Mouse IgG PE was optimised with MAb 4A12 (20 μg ml^{-1}) and rabbit IgG PE was optimised with polyclonal antibodies (1/250 v/v). The protocol steps were performed as described in Table 6.5.

Table 6.8 – Optimisation of the reporter antibody concentration used in SAT.

Step	***Antibody***	***Reporter***	***Sample Incubation***	
			Time	**Temperature**
Reporter antibodies	MAb 4A12 20 μg ml^{-1}	Mouse IgG PE 7.5 μg ml^{-1} 15 μg ml^{-1} 25 μg ml^{-1} 35 μg ml^{-1}	Overnight	4°C
	Polyclonal 1/250 (v/v)	Rabbit IgG PE 7.5 μg ml^{-1} 10 μg ml^{-1} 15 μg ml^{-1} 25 μg ml^{-1}	Overnight	4°C

6.2.5.4 - Concentration of anti-European sea bass monoclonal antibodies

Primary antibody was used at a concentration (6 μg ml^{-1}) as recommended by the supplier (Aquatic Diagnostics Ltd) for use in ELISA (section 6.2.5.2). Several different concentrations of anti-European sea bass IgM were tested with fish sample 10 (Table 6.9). The protocol was carried out as described in Table 6.5.

Table 6.9 – Optimisation of primary antibody concentration used for SAT.

Step	***Antibody***	***Primary Ab***	***Reporter***	***Sample Incubation***	
				Time	**Temperature**
Primary Antibody	Fish sera Sample 10 1/10 (v/v)	Anti-sea bass MAb 4 μg ml^{-1} 6 μg ml^{-1} 10 μg ml^{-1} 20 μg ml^{-1}	Mouse IgG PE 25 μg ml^{-1}	Overnight	4°C

6.2.6 - Data Analysis

Data analysis is comprised of the following steps;

① Subtraction of the blank from the raw sample data. The blank was obtained by running a simultaneous assay using the same procedure where antibody samples were replaced with DPBS+.

② Background elimination. According to Carter (1994) the background should be the mean of 10-25 % of the lowest values read. Opalka *et al.* (2004) considered all values obtained below 50 MFI as background. During this study both of these concepts of background were employed. The mean of the 25 % lowest values read was determined and subtracted from the data obtained. Then all readings below 50 MFI were rejected.

③ Sensitivity may be a problem when analysing serum samples where background is a problem (Johansson and Andersson, 2003). To reduce the influence of the background readings in the analysis of fish serum samples an additional step was incorporated. The values obtained for each peptide with the negative serum sample (Machrihanish fish farm sample) were subtracted from the corresponding peptide MFI value for the naturally infected fish serum samples. After this subtraction the average of the 25 % lower readings was determined and subtracted from the data. A further step was performed - all the points below 150 MFI were rejected and not analysed.

The betanodavirus coat protein amino acid sequence was submitted into www.compbio.dundee.ac.uk/~www-jpred/jnet/ for prediction of the secondary structure of the protein using the Jnet secondary structure prediction method (Cuff and Barton, 1999). Jnet is a neural network prediction algorithm which applies multiple sequence alignments against PSIBLAST and HMM profiles. This secondary structure prediction program achieves an average accuracy of 76.4 %.

The 3D structure of the Betanodavirus coat protein has not been determined using X-ray crystallography. Consequently, in this study, CPHmodels 2.0 homology-modelling server

(Lund *et al.* 2002) was used to predict the 3D-structure. This algorithm is available on: www.cbs.dtu.dk/services/CPHmodels/. The program searches for homologies between a target protein sequence and the sequences of proteins contained in three databases (NCBI, SWISS-PROT and RCSB). The betanodavirus sequence was aligned with the template and modelled according to Lund *et al.* (2002) recommendations. Regions of protein molecules that are modelled at less than 95 % accuracy are excluded from the predicted structural model.

Amino acids may be classified as either hydrophobic or hydrophilic. The hydrophobicity of amino acids contributes to the folding of protein molecules. Hydrophobic radical groups tend to be located on the inside of a protein whereas hydrophilic groups tend to occur on the exterior interacting with water molecules (Kyte and Doolittle, 1982).

The hydropathicity profile of the betanodavirus coat protein was analysed using a method developed by Kyte and Doolittle in 1982. In this procedure, each amino acid is given a hydrophobicity score between -4.5 and 4.5, where 4.5 represents the most hydrophobic, and -4.5 represents the most hydrophilic (Table 6.10).

Table 6.10 – Hydropathicity of each amino acid according to Kyte and Doolittle (1982).

Amino Acid (Hydrophobic)		***Hydropathy Score***		***Amino Acid (Hydrophilic)***		***Hydropathy Score***
Isoleucine	I	4.5		Glycine	G	-0.4
Valine	V	4.2		Threonine	T	-0.7
Leucine	L	3.8		Tryptophan	W	-0.9
Phenylalanine	F	2.8		Serine	S	-0.8
Cysteine	C	2.5		Tyrosine	Y	-1.3
Methionine	M	1.9		Proline	P	-1.6
Alanine	A	1.8		Histidine	H	-3.2
				Glutamic acid	E	-3.5
				Glutamine	Q	-3.5
				Aspartic acid	D	-3.5
				Asparagine	N	-3.5
				Lysine	K	-3.9
				Arginine	R	-4.5

In the present study, hydropathicity profiles were analysed in a window of seven amino acids. Hydrophobicity scores were averaged and assigned to the amino acid located in the centre of each window. The computer program starts with the first window of amino acids and calculates the average of all the hydrophobicity scores in that window. Then the computer program moves down one amino acid and calculates the average of all the hydrophobicity scores in the second window. This pattern continues to the end of the protein, computing the average score for each window and assigning it to the middle amino acid in the window.

Hydropathicity analyses were performed with the programme Pepwindow, contained within the EMBOSS (European Molecular Biology Open Software Suite, http://bioinfo.hku.hk/EMBOSS).

6.3 - Results

The goal of this chapter was identification of B-cell epitopes on the betanodavirus capsid protein. This was approached in two stages: i) optimisation of xMAP technology and ii) epitope mapping with overlapping synthetic peptides.

6.3.1 - Optimisation of the assays

Optimisation assays was performed in three different experiments. These were designed to optimise: sample incubation time, amount of primary antibody, amount of reporter and amount of sample antibody.

6.3.1.1 - Reporter

Sample incubation time was optimised using mouse MAbs. Overnight incubation at 4°C was found to be optimal. This incubation period was used in all subsequent experiments, and also applied to assays using rabbit and fish antibodies.

The concentration of the conjugate (mouse IgG PE) was optimised with MAb 4A12. A concentration of 25 μg ml^{-1} was found to be optimal. This concentration of mouse IgG PE was used in mapping experiments with fish sera.

Experimental analysis of the optimal concentration of reporter molecule was also performed for rabbit IgG PE. A concentration of 25 μg ml^{-1} was also found to be optimal.

6.3.1.2 - Target antibody

For monoclonal antibodies, a concentration of 100 μg ml^{-1} of purified MAb was optimal. For rabbit and fish polyclonal antibodies, optimal results were achieved with serum dilutions of 1:250 and 1:25 respectively.

Optimised incubation times and reactant concentrations used in epitope mapping studies are summarised in Table 6.11.

Table 6.11 – Optimised conditions for mouse MAbs, rabbit polyclonal antibodies and fish sera in epitope mapping with SAT.

Antibody	***Procedure step***				
	Sample Incubation			**Primary Ab**	**Reporter**
	Amount Ab	**Time**	**Temperature**		
MAb	100 μg ml^{-1}	Overnight	4°C	-	Mouse IgG PE 25 μg ml^{-1}
Polyclonal	1/250 (v/v)	Overnight	4°C	-	Rabbit IgG PE 25 μg ml^{-1}
Sea bass sera	1/25 (v/v)	Overnight	4°C	Sea bass IgM 20 μg ml^{-1}	Mouse IgG PE 25 μg ml^{-1}

6.3.2 - Epitope Mapping

B-cell epitope mapping of the betanodavirus coat protein was performed using antibodies or serum samples from three different species; mouse monoclonal antibodies, and polyclonal sera from rabbit and European sea bass.

All the data shown are above the cut off established for the assay.

6.3.2.1 - Monoclonal antibodies

6.3.2.1.1 - Negative control

Epitope mapping with mouse MAbs using the xMAP technology yielded a wide range of Mean Fluorescence Intensity (MFI) values. The antibodies bound to synthetic peptides are themselves bound by the PE-labelled conjugated. The MFI is proportional to the fluorescence of the PE, and thus a measure of the amount of antibody bound to a bead.

During this study an anti-*Tetracapsuloides bryosalmonae* MAb was used as a negative control. This MAb did not show any affinity with the any of the synthetic peptides used in this study.

Table 6.12 – Mean Fluorescence Intensity values for *T. bryosalmonae* and MAbs 1E3.

Peptide	***Monoclonal Antibodies (MFI)***			***Peptide***	***Monoclonal Antibodies (MFI)***	
	1E3	***T. bryosalmonae***			**1E3**	***T. bryosalmonae***
1	0.00	0.0		**19**	3.6	0.0
2	2.0	0.0		**20**	19.3	0.0
3	9.0	0.0		**21**	3.6	0.0
4	0.0	0.0		**22**	0.0	0.0
5	2.3	0.0		**23**	0.3	0.0
6	0.0	0.0		**24**	0.0	0.0
7	0.0	0.0		**25**	0.0	0.0
8	0.0	0.0		**26**	0.0	0.0
9	0.0	0.0		**27**	0.0	0.0
10	0.0	0.0		**28**	0.0	0.0
11	0.0	0.0		**29**	0.0	0.0
12	0.0	0.0		**30**	6.3	0.0
13	0.0	0.0		**31**	0.0	0.0
14	0.0	0.0		**32**	0.0	0.0
15	0.0	0.0		**33**	3.3	0.0
16	8.7	0.0		**34**	3.3	0.0
17	0.0	0.0		**35**	9.7	0.0
18	0.0	0.0		**36**	1.7	0.0

6.3.2.1.2 - MAb 1E3

Fluorescence values obtained with MAb 1E3 were slightly higher than *T. bryosalmonae*, however, these were excluded since they were lower than the cut-off value of 50 MFI. Results obtained with the Mabs raised against *T. bryosalmonae* and 1E3 are shown in Table 6.12.

6.3.2.1.3 - MAb 5G10

MAb 5G10 showed lower MFI values than 4A12, 4C3 and 3B10, however approximately 1/3 of these were greater than the threshold of 50 MFI, and thus considered to be valid. MAb 5G10 recognised peptides 16 (190 MFI), and 20 (110 MFI) most strongly Figure 6.6).

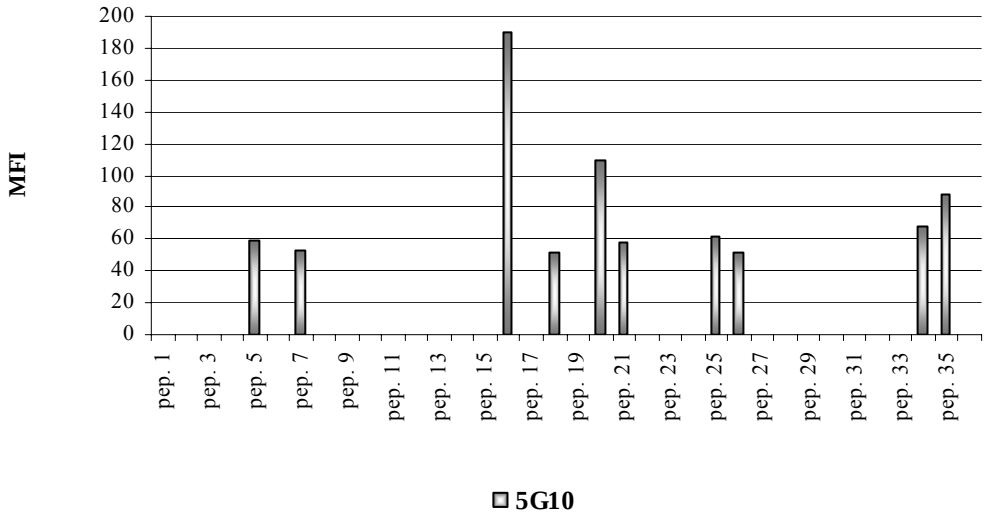


Figure 6.6 –MAb 5G10 binding to synthetic peptides.

The majority of the synthetic peptides recognised by 5G10 are located on the same region of the coat protein, between peptides 16-21 (amino acids 151-212 of the capsid

protein). Peptide 35 was also recognised (88 MFI). This peptide was synthesised to act as negative control.

6.3.2.1.4 - Mab 3B10

MAb 3B10 recognised peptides 19 and 20 most strongly (MFIs 1399 and 2138 respectively) (Figure 6.7).

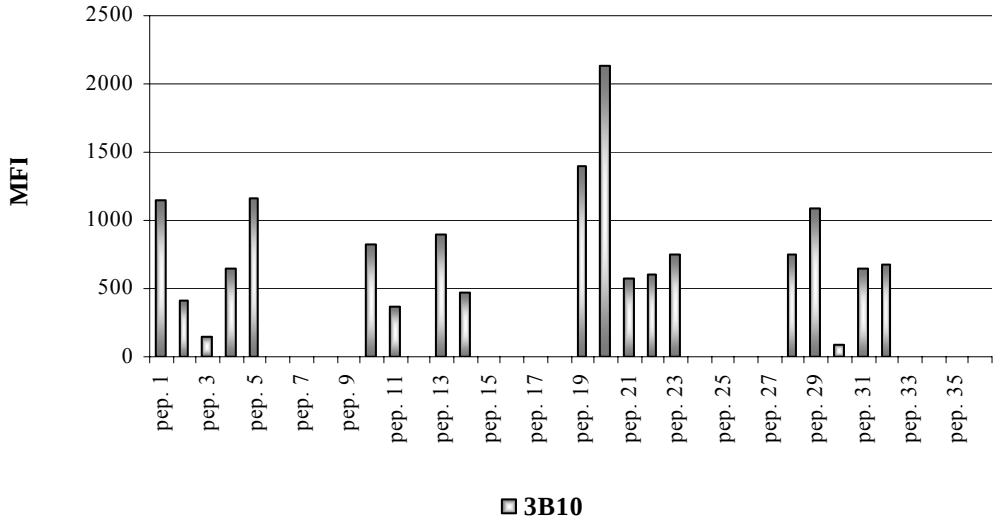


Figure 6.7 – MAb 3B10 binding to synthetic peptides.

The peptides recognised by MAb 3B10 were located in four different regions of the betanodavirus coat protein. Peptide 5 (MFI 1162) and peptide 1 (MFI 1153) were also recognised. These are located in the N-terminus of the coat protein. A further region was recognised by MAb 3B10 comprising peptides 28 - 32, situated near the C terminus of the capsid protein.

6.3.2.1.5 - MAb 4A12

MAb 4A12 exhibited the highest MFI values of the MAbs analysed (peptide 3, MFI 3704). This MAb strongly recognised the three consecutive peptides, 19, 20 and 21 with very high MFI (Figure 6.8).

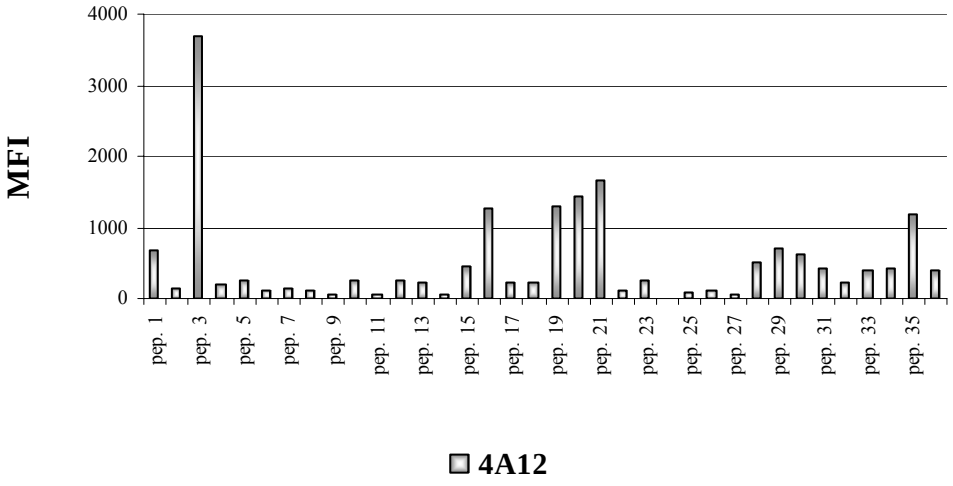


Figure 6.8– MAb 4A12 binding to synthetic peptides.

MAb 4A12 recognised three regions of the coat protein. Two of these are located in the N and the C termini of the coat protein, and the third region is situated in the central region of the protein. This antibody recognised both control peptides (35 and 36).

6.3.2.1.6 - MAb 4C3

MAb 4C3 exhibited lower binding to the synthetic peptides than either MAb 4A12 or 3B10 (Figure 6.9). This MAb recognised peptides situated in the centre of the coat protein most strongly, together with peptide 35 (control peptide). These results were obtained for two

groups of consecutive peptides, peptide 15-16 and peptide 19-20. Peptide 20 revealed the highest binding values (842 MFI) follow by peptide 35 (669 MFI).

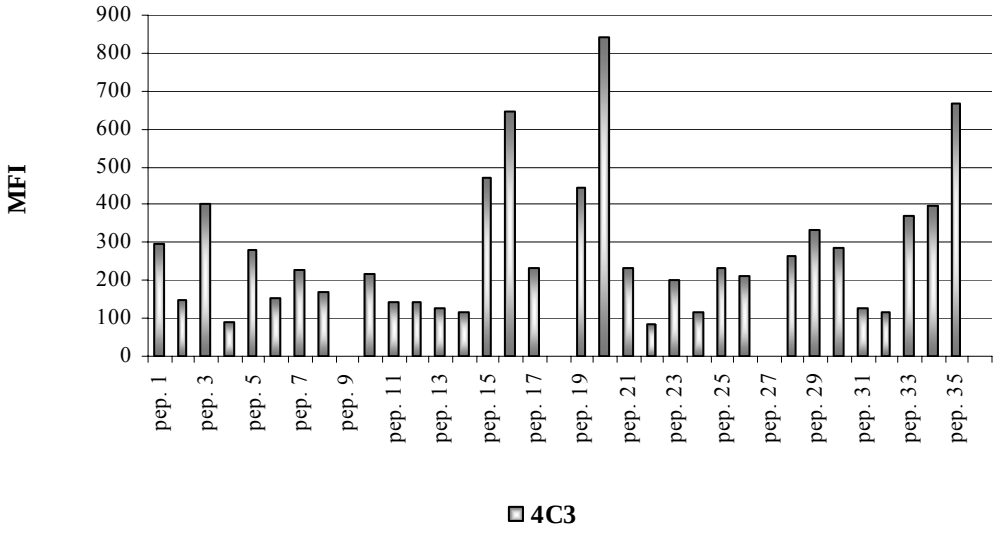


Figure 6.9 – MAb 4C3 binding to synthetic peptides.

6.3.2.1.7 - MAbs - analysis of the peptides recognised most strongly

None of the MAbs analysed bound to all the peptides. However all peptides were recognised by at least one MAb, although in some cases, the MFI values were low. For each MAb studied, the three peptides recognised with the highest MFI value were located in three regions of the coat protein: peptide 1 to 5; peptide 15 to 16 and peptide 19 to 21 (Table 6.13).

Within the peptides that were recognised most strongly, i.e. greatest MFI values, peptide 20 was most frequently recognised, showing high MFI with all five MAbs studied. Peptides 16 and 35 were recognised by MAbs 5G10 and 4C3. These two MAbs showed similar binding affinities, and recognised the same peptides. The other peptides recognised with high MFI values were recognised by a single MAb.

Table 6.13 – The three peptides with highest MFI for each of the MAbs analysed.

	Peptide		*MFI*
5G10	16	KWWESRTVRPQY	190
	20	NTDVVNVSVLCR	110
	35	KSVPADTRNSRR	88
3B10	20	NTDVVNVSVLCR	2138
	19	GRLILLCVGNNT	1399
	5	KASTVTGFGRGT	1162
4A12	3	QPRRRANNRRRS	3704
	21	CRWSVRLSVPSL	1658
	20	NTDVVNVSVLCR	1440
4C3	20	NTDVVNVSVLCR	842
	35	KSVPADTRNSRR	669
	16	KWWESRTVRPQY	648

The six peptides showing the highest MFI values were aligned with the predicted secondary structure of the betanodavirus coat protein (Figure 6.10). All peptides showing high MFI values could be mapped to regions of the coat protein that form areas of β-sheet conformation.

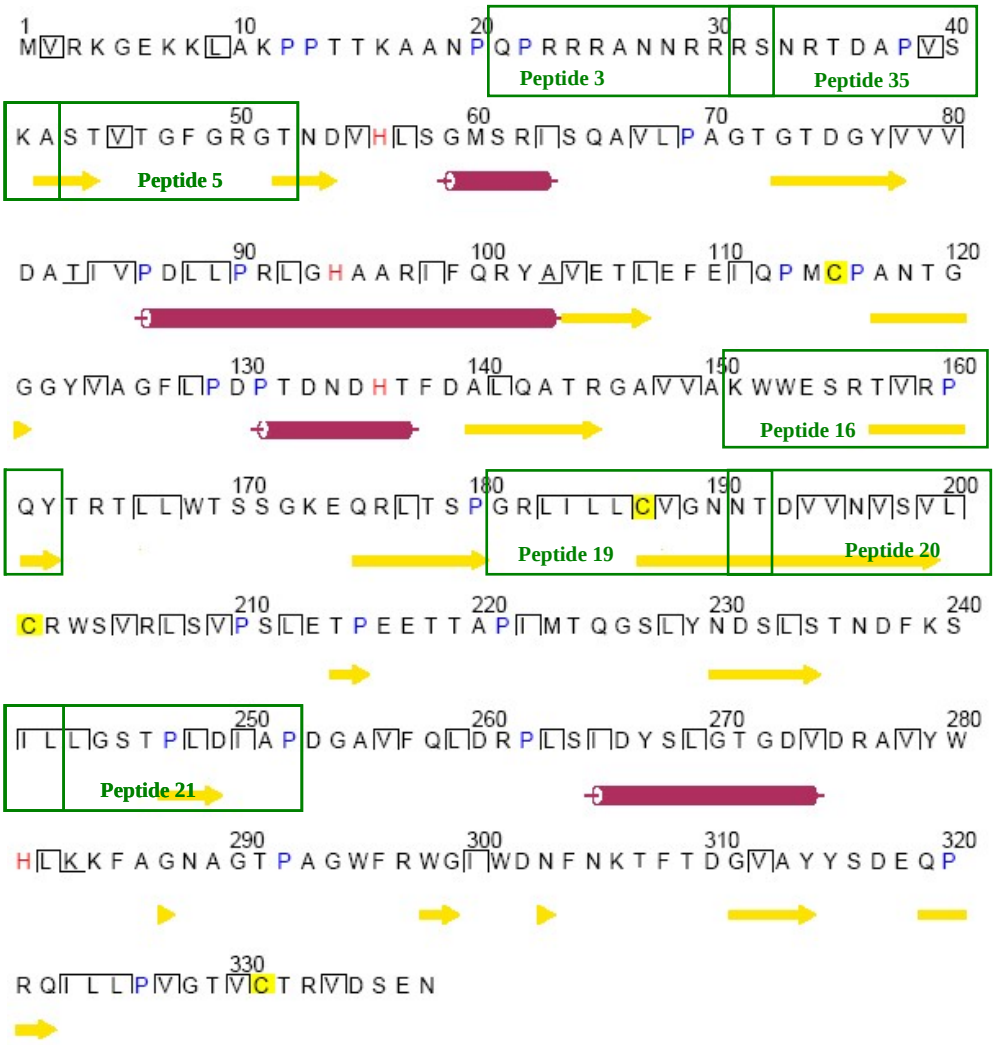


Figure 6.10 – Synthetic peptides recognised by MAbs, aligned with the secondary structure of DFNNV coat protein (Jnet secondary structure prediction method). is α-helix regions and is β-sheet regions.

6.3.2.2 - Polyclonal Antibodies

The xMAP procedure was also used to map regions of the betanodavirus coat protein recognised by polyclonal antibodies from rabbit immunised with European sea bass betanodavirus. The results of this study are presented in Figure 6.11. Peptide 20 (1290 MFI) followed by peptide 13 (679 MFI) were recognised most strongly.

Four different regions of the coat protein were recognised by rabbit polyclonal antibodies: peptides 1 to 4; peptides 10 to 13; peptides 19 to 21 and peptides 28 to 30.

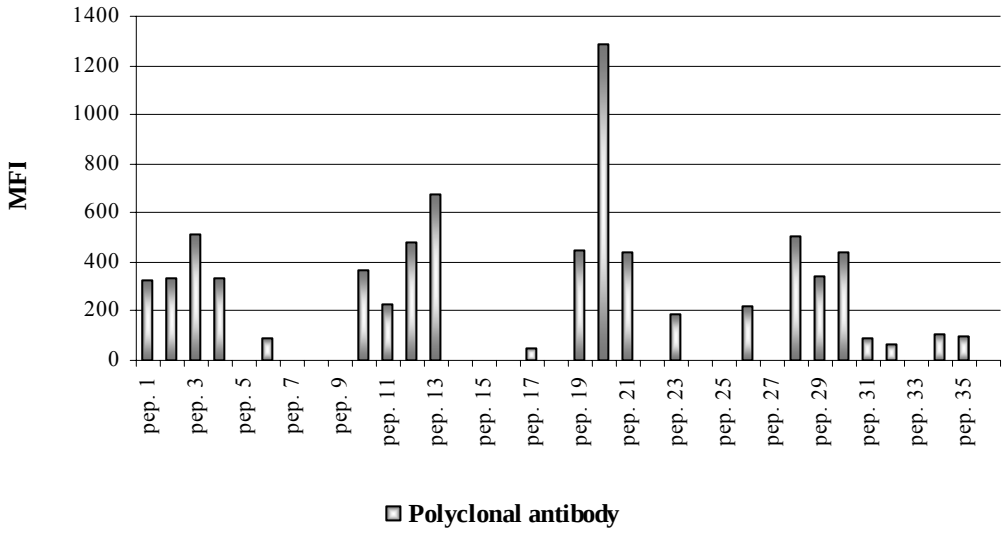


Figure 6.11 – Rabbit polyclonal antibodies binding to synthetic peptides.

The three peptides showing the highest MFI values (peptides 10, 13 and 20) were located in three different regions of the coat protein (Table 6.14).

Table 6.14 - The three peptides with highest MFI to rabbit polyclonal antibodies.

Polyclonal antibodies		
Peptide		***MFI***
20	NTDVVNVSVLCR	1290
13	GGYVAGFLPDPT	679
3	QPRRRANNRRRS	511

After aligning these peptides with the predicted secondary structure of the betanodavirus coat protein, peptide 20 was found to correspond to region with B-sheet structure. Peptide 13 has a mixture of α-helix and β-sheet regions (Figure 6.12).

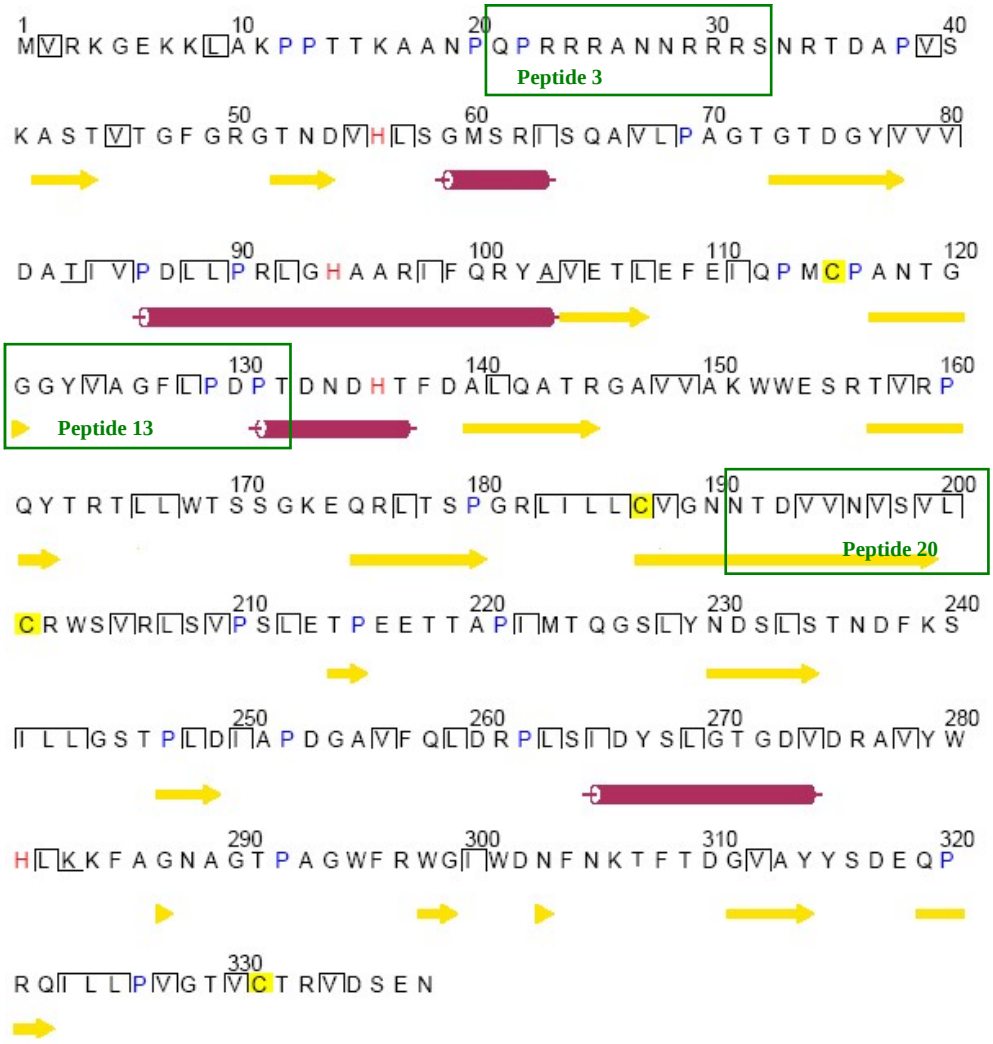


Figure 6.12 – Synthetic peptides recognised by polyclonal antibodies aligned with the secondary structure of DFVVN coat protein (Jnet secondary structure prediction method). is α-helix regions and is β-sheet regions.

6.3.2.3 - European sea bass sera

The xMAP procedure was also used to study the interaction between fish (European sea bass) antibodies and the coat protein of betanodavirus. Nine serum samples, obtained from a natural VNN outbreak in Greece, were used in this study.

6.3.2.3.1 - Negative control serum samples

For negative controls, fish sera were obtained from European sea bass held at Machrihanish fish farm. This fish have never been exposed to a betanodavirus infection. All the peptides showed a degree of non-specific binding to the fish serum (Table 6.15).

Table 6.15 – Negative control, European sea bass sera, binding to synthetic peptides.

Machrihanish fish serum				
Peptide	**MFI**		**Peptide**	**MFI**
1	44		**19**	53
2	50		**20**	168.5
3	256		**21**	175.5
4	65		**22**	72.5
5	60		**23**	85
6	76		**24**	31.5
7	30		**25**	42.5
8	40.5		**26**	69
9	50		**27**	70.5
10	83.5		**28**	66.5
11	89		**29**	211.5
12	125.5		**30**	214.5
13	107		**31**	93
14	44.5		**32**	77.5
15	41		**33**	78.5
16	198.5		**34**	60
17	72.5		**35**	144.5
18	67		**36**	86

This was considered a result of natural binding capacity of fish serum to the synthetic peptides being used as background. This background value obtained per peptide was subtracted from the corresponding peptide for infected European sea bass (Sb).

6.3.2.3.2 - Serum samples betanodavirus infected European sea bass

European sea bass serum sample 1 (SB1) showed high MFI towards peptides 15 and 16 (1113 MFI and 1030 MFI, respectively), however the highest value was obtained with peptide 21 (2108 MFI) (Figure 6.13). These peptides mapped to the two main regions of the coat protein (peptide 15-16 and 19-21). Other regions are comprised by singular peptides as number 3 and 12.

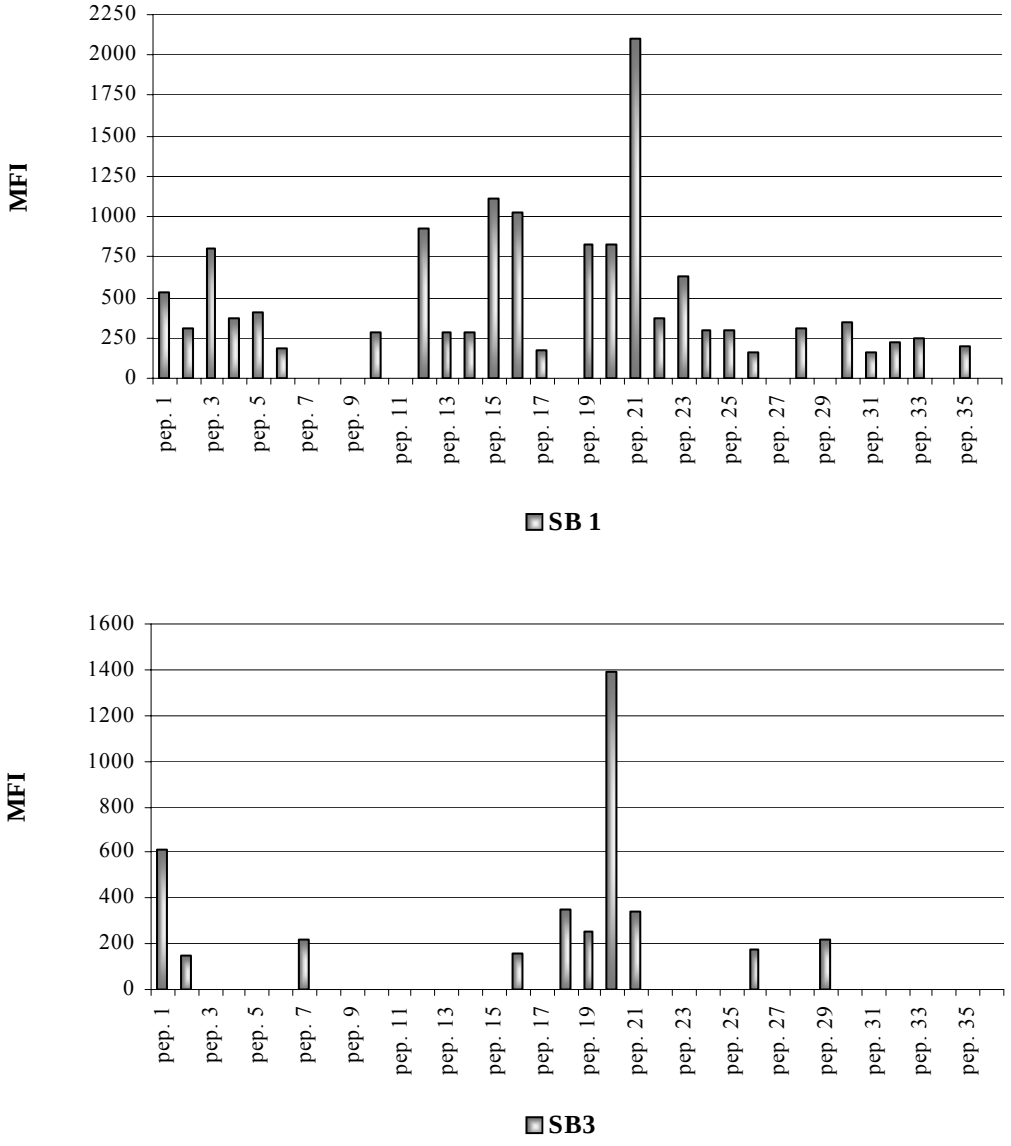


Figure 6.13 – European sea bass antibodies binding to synthetic peptides (① sample 1 and ② sample 3).

Sample 3 (SB3) strongly identified peptide 20 (1394 MFI) and peptide 1 (612 MFI) (Figure 6.14).

Serum sample 4 (SB4) revealed similar affinities for peptide 1 and peptide 16, (1224 MFI and 1207 MFI respectively) (Figure 6.14). Peptide 16 belongs to a region, defined by amino acid residues 141-212 (peptides 15-21) that reveal high binding (MFI) values.

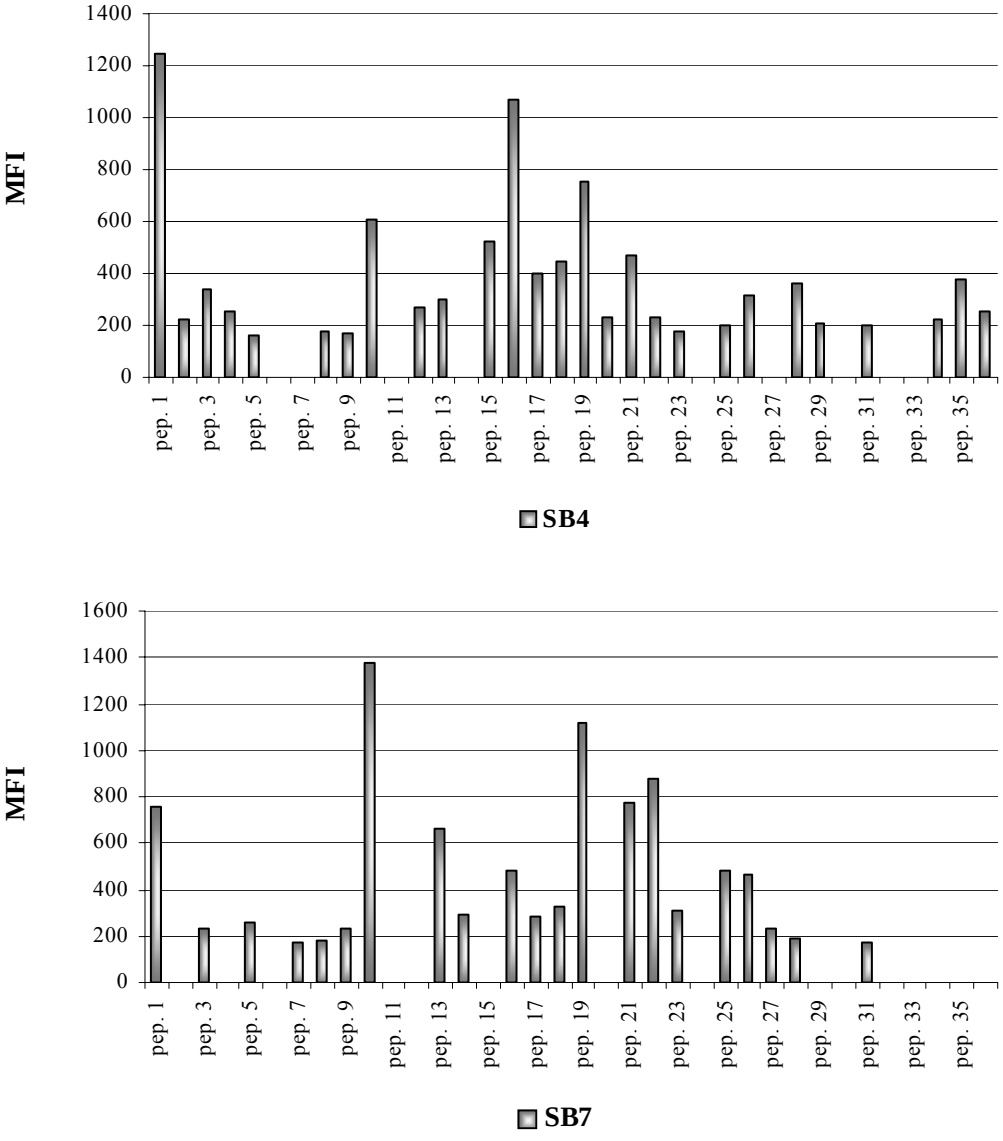


Figure 6.14 - European sea bass antibodies binding to synthetic peptides (① sample 4 and ② sample 7).

SB7 bound most strongly to peptide 10 (1297 MFI), followed by peptide 19 (1107 MFI). Peptides 21 and 22 have similar affinities to SB7 with an MFI of 883 and 888, respectively. These two peptides with peptide 19 comprise a regions of the coat protein recognised by SB7 antibodies. Other regions are not so easy to define because the peptides are located throughout the coat protein (Figure 6.14).

Serum sample 10 (SB10) showed similar binding affinities to peptides 1, 22 and 10 (891, 879 and 802 MFI, respectively) (Figure 6.15).

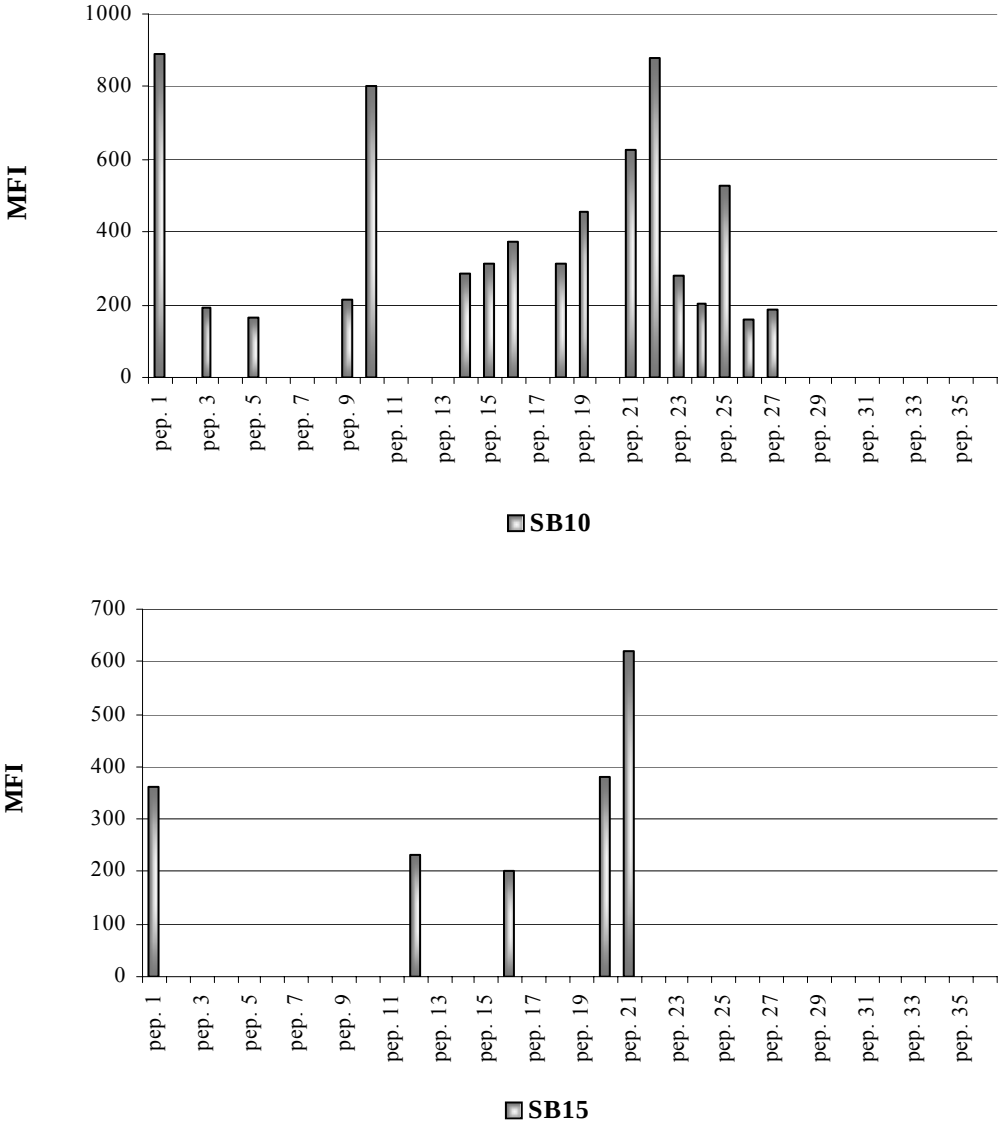


Figure 6.15 – European sea bass antibodies binding to synthetic peptides (① sample 10 and ② sample 15).

Fish serum sample 15 (SB15) showed relatively higher specificity for the synthetic peptides (Figure 6.16). This sample yielded MFI values that were lower than those recorded for other serum samples. Peptides 20-21 were recognised with the highest MFI (621 MFI and 381 MFI, respectively). A further region was recognised by sample 15. This comprised peptide 1, which is located near the N terminus of the coat protein.

Serum sample 17 (SB17) recognised one region comprising peptides 20 and 21 (MFI values with 745 and 1161 respectively). Peptide 1 and peptide 12 were also recognised with high MFI values (Figure 6.16).

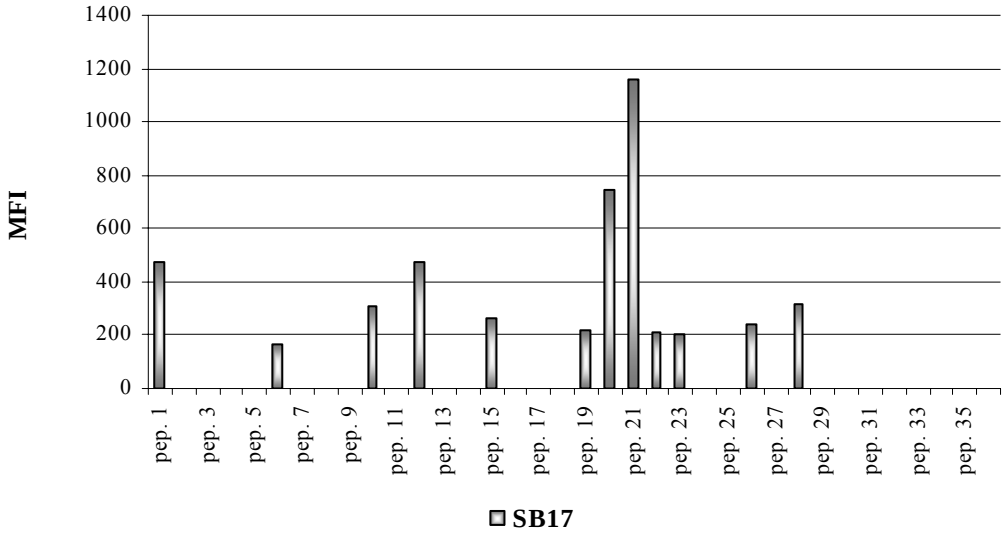


Figure 6.16 – European sea bass antibodies binding to synthetic peptides (sample 17).

6.3.2.3.2.1 SB2 and SB9

Serum samples SB2 and SB9 did not recognised betanodavirus when assessed by ELISA. These samples were used as negative controls, both SB2 and SB9 showed similar levels of MFI to the peptides as the samples from infected fish (Figure 6.17).

European sea bass serum sample SB2 exhibited the greatest level of binding to any peptide (peptide 10, 2239 MFI). This fish serum sample recognised two consecutive peptides, 15 and 16, with the second and the third highest binding value with 2168 MFI and 1435, respectively. With this sample it is possible to distinguish several peptides or group of peptides of the coat protein with high MFI values. The peptides/regions are peptide 3, 10 and peptides 15-16 and 20-23.

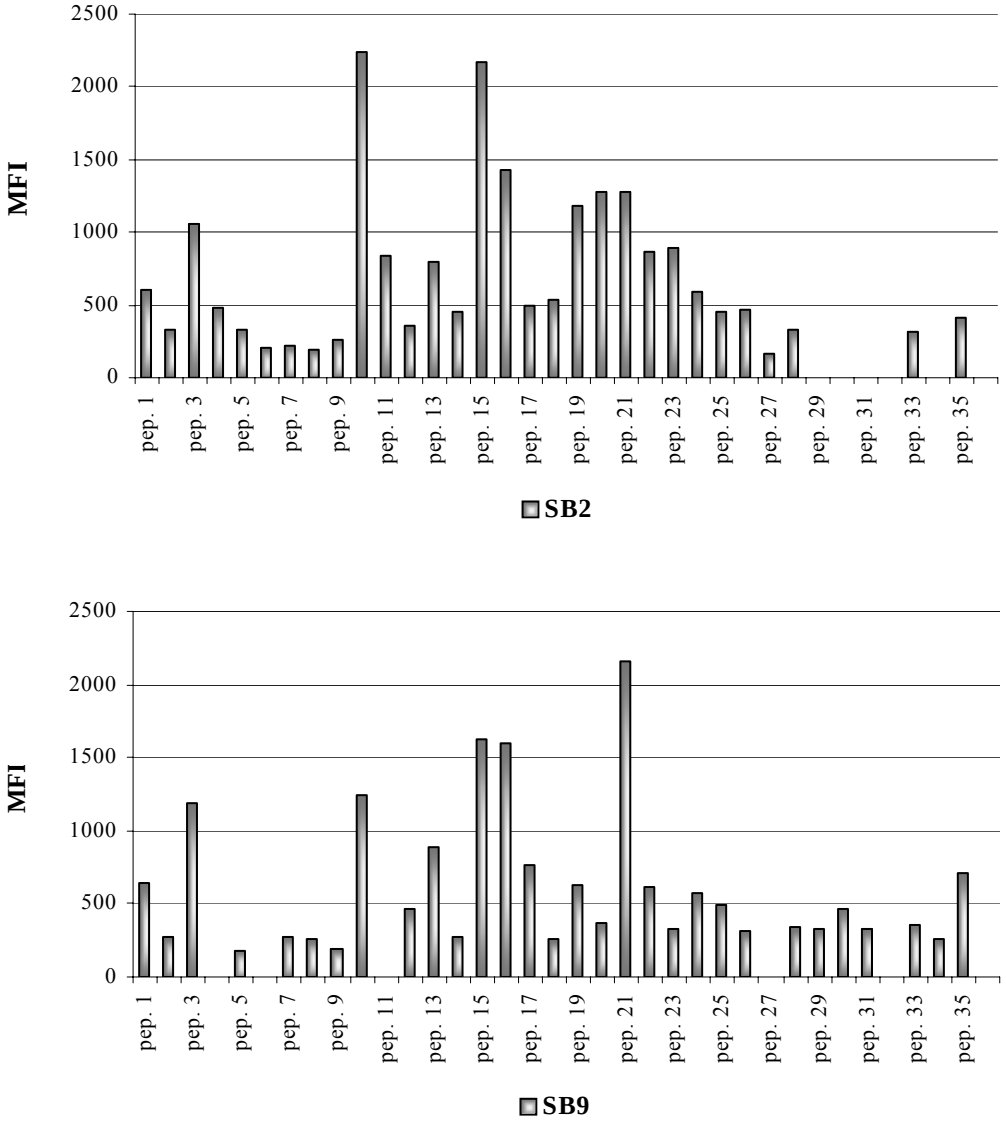


Figure 6.17 – European sea bass antibodies binding to the synthetic peptides (① sample 2 and ② sample 9).

Serum sample 9 (SB9) showed the greatest level of binding to peptide 21 (2164 MFI). As was observed for sample SB2, sample SB9 bound to two consecutive peptides, 15 and 16, (1620 MFI and 1595 MFI respectively). Sample SB9 also strongly recognised peptide 3 (1210 MFI) and peptide 10 (1273 MFI).

6.3.2.3.3 - European sea bass sera – analysis of the peptides recognised most strongly

The three peptides recognised by the serum samples with the greatest MFI values were submitted to further analysis. The three peptides recognised most strongly (i.e. three highest MFI values) by each fish serum sample are shown in Table 6.16. These peptides map to two major regions of the coat protein defined by peptides 15-16 and peptides 18-22. The region comprising peptides 15-16 (aa 141-162) was recognised by five sera, and the region comprising peptides 18-22 (aa 171-222) was recognised by eight sera. Additionally, peptide 1 (aa 1-12) was recognised by four serum samples.

Using this approach the most commonly recognised peptides were peptides 1, 16 and 21. These peptides were recognised by four of the nine fish serum samples analysed. One third of the fish serum antibodies recognised peptides 10, 15 and 21. Peptides 19 and 22 were recognised by two of the fish samples, and a single serum sample recognised peptide 18 and peptide 12.

The same three peptides were recognised by SB1 and SB9 (15, 16 and 21). These peptides were identified by serum sample SB2, however, this sample recognised peptide 10 as opposed to peptide 21. Both samples SB7 and SB10 bound strongly to peptides 10 and 22. SB15 and SB17 both showed high binding levels to two peptides, 20 and 21.

Table 6.16 - The three peptides showing the highest MFI values to each of the European sea bass samples analysed.

	Peptide		*MFI*		*Peptide*		*MFI*
SB 1	21	CRWSVRLSVPSL	2108	***SB 9***	21	CRWSVRLSVPSL	2164
	15	LQATRGAVVAKW	1113		15	LQATRGAVVAKW	1620
	16	KWWESRTVRPQY	1030		16	KWWESRTVRPQY	1595
SB 2	10	RLGHAARIFQRY	2239	***SB 10***	1	MVRKGEKKLAKP	891
	15	LQATRGAVVAKW	2168		22	SLETPEETTAPI	879
	16	KWWESRTVRPQY	1435		10	RLGHAARIFQRY	802
SB 3	20	NTDVVNVSVLCR	1394	***SB 15***	21	CRWSVRLSVPSL	621
	1	MVRKGEKKLAKP	612		20	NTDVVNVSVLCR	381
	18	SGKEQRLTSPGR	349		1	MVRKGEKKLAKP	361
SB 4	1	MVRKGEKKLAKP	1243	***SB 17***	21	CRWSVRLSVPSL	1161
	16	KWWESRTVRPQY	1071		20	NTDVVNVSVLCR	745
	19	GRLILLCVGNNT	754		12	IQPMCPANTGGG	474
SB 7	10	RLGHAARIFQRY	1377				
	19	GRLILLCVGNNT	1117				
	22	SLETPEETTAPI	879				

The peptides showing the three highest binding levels for each fish serum sample were mapped onto a computer predicted model of the secondary structure of the beta-nodavirus coat protein. (Figure 6.18). One peptide (#10) mapped to a region predicted to form an α-helix. The other peptides mapped to regions of the coat protein that form β-sheets.

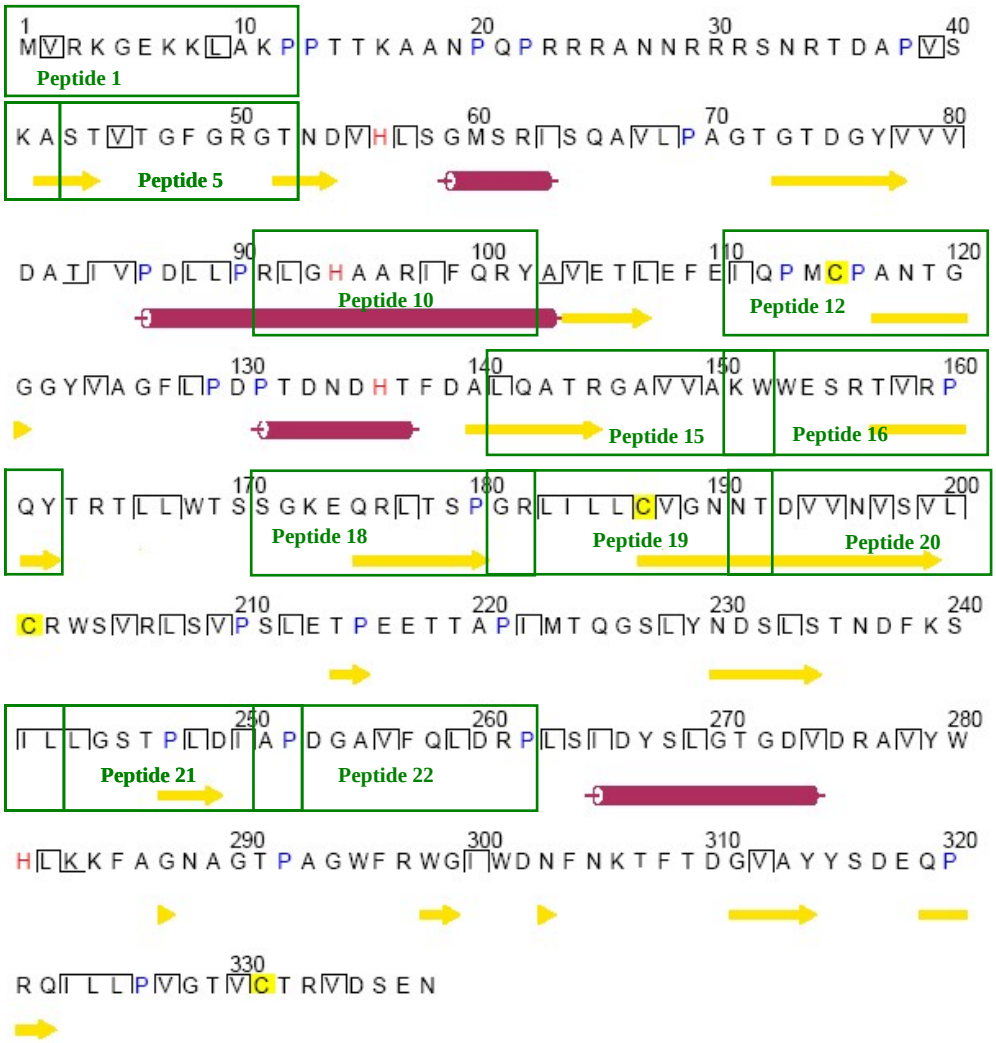


Figure 6.18 – Synthetic peptides recognised by fish antibodies aligned with secondary structure of DFNNV coat protein (Jnet secondary structure prediction method). is α-helix regions and is β-sheet regions.

6.3.3 - Comparison of peptides recognised by mouse, rabbit and fish antibodies

The three peptides recognised most strongly by each monoclonal antibody or serum sample are presented in Figure 6.19 and Table 6.17.

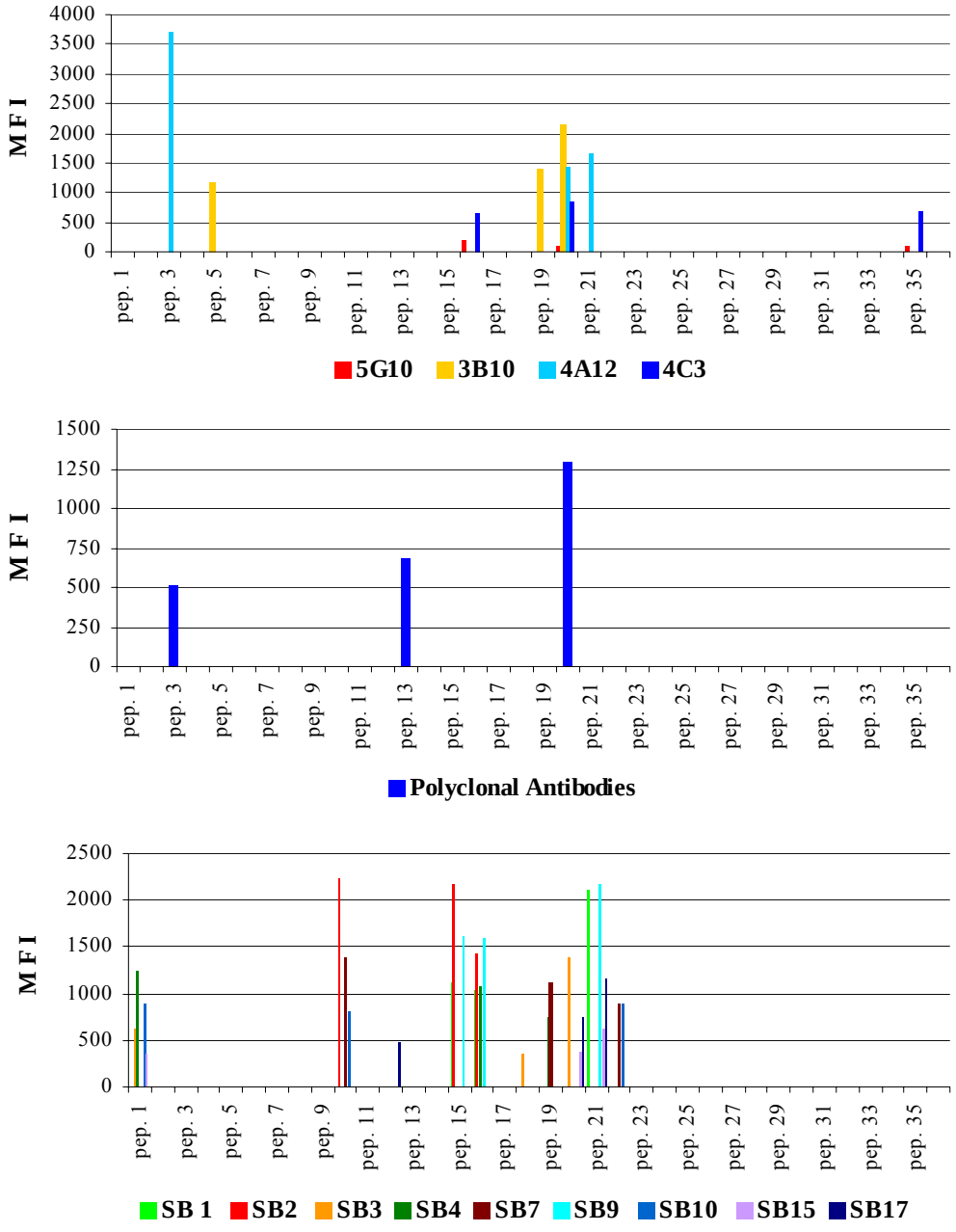


Figure 6.19– The three peptides recognised most strongly (i.e. highest MFI) by ① mouse MAbs, ② rabbit polyclonal antibodies, ③ European sea bass antibodies).

Antibodies from mouse, rabbit, and European sea bass recognised peptide 20. All of the mouse MAbs, the rabbit polyclonal sera, and one third of the fish serum samples identified this peptide, which mapped to amino-acid residues 191-202 of the coat protein. Peptide 16 was recognised by four fish sera and two MAbs. Peptide 21 was recognised by one MAb (4A12) and four fish serum samples. Peptide 19 was also strongly recognised by one MAb, but only two fish serum samples. The region defined by peptides 18-22 contained almost half of the peptides that were recognised most strongly.

Table 6.17 – The three peptides recognised most strongly by Mabs and serum samples.

	Peptide	***Coat protein residue***	***Antibody***
1	MVRKGEKKLAKP	1-12	SB3, SB4, SB10, SB15
3	QPRRRANNRRRS	21-32	4A12, Poly
5	KASTVTGFGRGT	41-52	3B10
10	RLGHAARIFQRY	91-102	SB2, SB7, SB10
12	IQPMCPANTGGG	111-122	SB17
13	GGYVAGFLPDPT	121-132	Poly
15	LQATRGAVVAKW	141-152	SB1, SB2; SB9
16	KWWESRTVRPQY	151-162	4C3, 5G10, SB1, SB2, SB4, SB9
18	SGKEQRLTSPGR	171-182	SB3
19	GRLILLCVGNNT	181-192	3B10, SB4, SB7
20	NTDVVNVSVLCR	191-202	3B10, 4A12, 4C3, 5G10, Poly, SB3, SB15, SB17
21	CRWSVRLSVPSL	201-212	4A12, SB1, SB9, SB15, SB17
22	SLETPEETTAPI	211-222	SB7, SB10
35	KSVPADTRNSRR	41-30	4C3, 5G10

Note - MAbs: 3B10, 4A12, 4C3 and 5G10
- Polyclonal antibodies: Poly
- European sea bass: SB1, SB2, SB3, SB4, SB7, SB9, SB10, SB15 and SB17

Some peptides were recognised by antibodies from only one of the species studied. Peptides 5 and 35 were only identified by mouse antibodies. Peptide 13 was only recognised by rabbit polyclonal antibodies, and peptides 1, 10, 12, 15, 18 and 22 by fish antibodies.

The warm blooded species, mouse and rabbit, were the only ones that strongly recognised peptide 3.

The three peptides recognised most strongly by each antibody or serum sample were mapped onto a hydropathicity profile of the betanodavirus coat protein produced using the method described by Kyte and Doolittle (1982).

As can be observed in Figure 6.20, peptide 3 mapped to the most hydrophilic region of the coat protein and was only identified by MAb 4A12 and rabbit polyclonal sera. Peptide 19 which is strongly recognised by one mouse MAb and two European sea bass sera maps to the most hydrophobic region. Peptide 1 is hydrophilic and peptide 15 can be considered neutral. These peptides were recognised only by European sea bass sera. The first residues of peptides 5, 13, 10, 16 and 35 are hydrophobic but most of the peptide sequence is hydrophilic. Peptides 12, 20, 21 and 22 have different behaviour in the plot but they are all hydrophobic.

The most hydrophobic region (peptide 19-22) is recognised by all the mouse MAbs, the rabbit polyclonal and eight of nine fish sera samples.

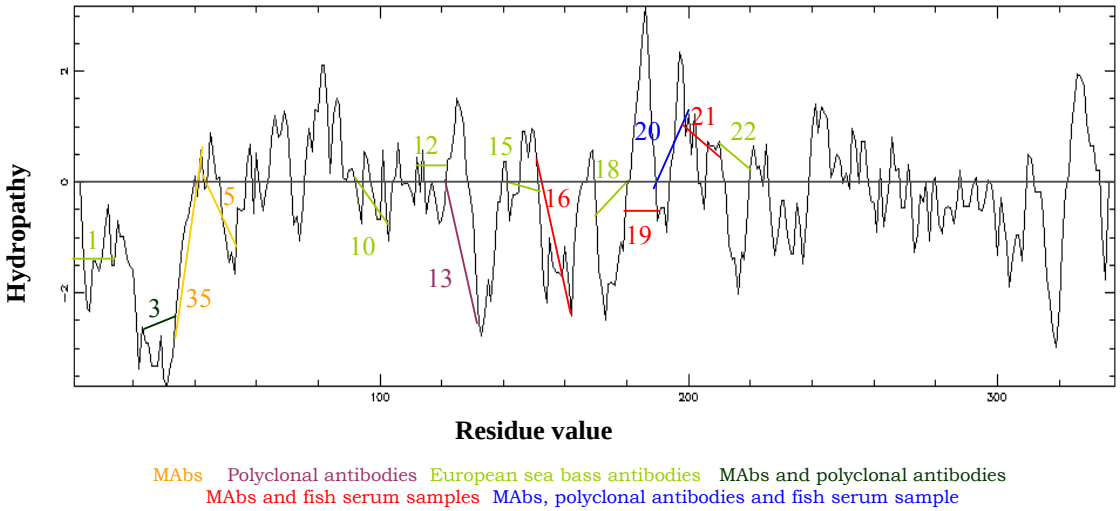


Figure 6.20 – Positions of the most strongly recognised peptides mapped onto a hydropathicity plot of the betanodavirus coat protein.

Mapping of peptides that were most strongly recognised by antibodies onto the predicted secondary structure of the coat protein indicates that the majority of these peptides belong to regions comprised of β-sheet (Figure 6.21). Only peptide 10 mapped to a region of

the coat protein that possessed an α-helical structure. A mixture of β-sheet and α-helix region can be observed in peptide 13 mapped to a region containing both β-sheet and α-helix structure.

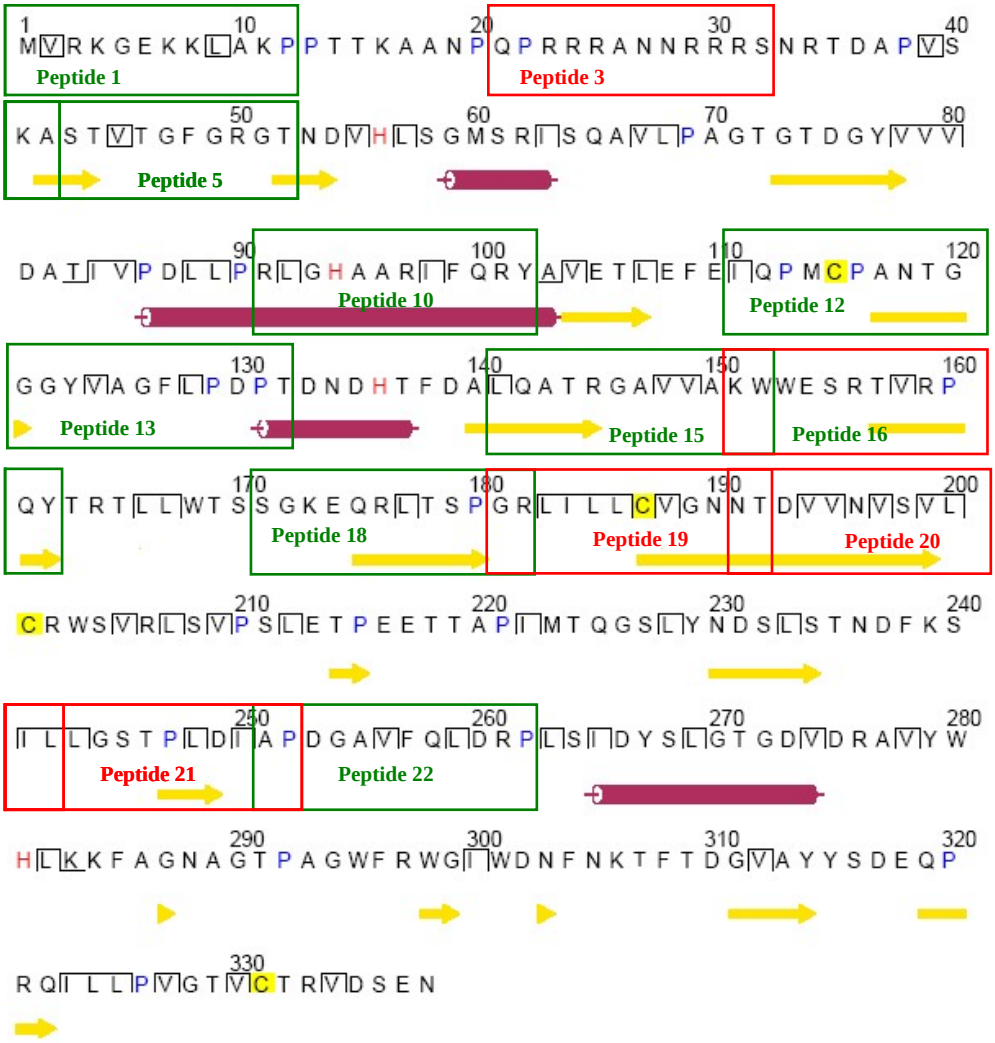


Figure 6.21 – The most strongly recognised peptides aligned with the secondary structure of DFNNV coat protein. The red boxes show the peptides that were recognised by more than 1 species. is α-helix regions and is β-sheet regions.

6.4 - Discussion

This discussion is divided into four sections. Section one focuses on the use of SAT technology and synthetic peptides for epitope mapping studies. Section two deals with the validity of the experimental results. In section three, the peptides predicted to be of greatest significance in epitope mapping studies are highlighted and mapped onto the native structure of the betanodavirus coat protein. Finally in section four, the results of the present study are compared and contrasted with those obtained from other epitope mapping studies of betanodaviruses.

Section 1 - SAT technology and synthetic peptides

In the first part of this section, assay optimisation and accuracy are discussed. This is followed by a discussion of the use of synthetic peptides in epitope mapping studies.

Optimisation

The epitope mapping procedure used in this thesis was based on two principles: ① - the ability of flow cytometry to distinguish separate sets of labelled beads based on their unique optical properties. Results are expressed as fluorescence intensities for each microsphere subset. This value is proportional to the concentration of analyte contained in individual samples (Yingyongnarongkul *et al.* 2003); ② - the identification of peptide fragments derived from a protein molecule that are able to react with antibodies raised against the intact protein (Arnon and van Regenmortel, 1992).

A Bio-Plex™ system utilising xMAP technology was used for flow cytometric measurements, and a panel of synthetic peptides 12 amino-acid residues in length that mimicked the betanodavirus coat protein were used to identify antibody binding sites.

Antibodies to betanodavirus from three different species were studied: mouse (monoclonal antibodies), rabbit (polyclonal antibodies) and European sea bass (polyclonal antibodies).

As with any equipment the accuracy and reproducibility of the results obtained using the Bioplex™ may be influenced by a number of parameters. Validation assays were employed to optimise the equipment, which was also calibrated on a daily basis using materials supplied by the manufacturer.

SAT assays are dependent on the consistent and predictable performance of the microspheres and the properties of the antibody molecules used in conjugates (Mandy *et al.* 2001). High quality microspheres supplied by the manufacturer were used throughout the present study, ensuring full compatibility between the detection system and software. Antibody conjugates produced by Molecular Probes and recommended for SAT assays were also used to ensure efficient analyses.

Optimisation of the experimental protocol was performed prior to epitope mapping studies. The parameters studied were sample incubation period, and concentrations of sample, primary antibody and reporter molecule (Table 6.11). The optimal incubation times for use in Bio-Plex™ based assays is currently undefined. Whilst some data suggest that short incubation periods are advantageous (Pickering *et al.* 2002b, Seideman and Peritt, 2002, Kellar and Douglass, 2003) other studies suggest that the opposite is true (Vignali, 2000) and that short sample incubation periods can reduce assay sensitivity up to 100 fold. Two incubation periods were used in the present study: 1 hour at room temperature and overnight at 4°C. Overnight incubation yielded superior results. For logistical reasons (costs; time) extensive analyses of the effects of incubation temperature and duration were not performed. However, it is possible that shorter incubation times could be employed in combination with different incubation temperatures as described by Dasso *et al.* (2002) and Prabhakar *et al.* (2002).

The optimal concentration of antibodies used in SAT assays was found to vary according to the host species. Polyclonal rabbit and fish antibodies were used at a dilution of 1:250 and 1:25 (v/v), respectively. Purified MAbs were used at a concentration of 100 μg ml^{-1}.

This is a relatively high value in comparison to the detection levels that can be obtained for cytokines (1.6 to 32000 pg ml^{-1}) and pesticides (0.1 to 300 ng ml^{-1}) (Jenmalm *et al.* 2003, Biagini *et al.* 2004b). This may be due to the fact that antibodies raised against a native protein antigen usually show lower affinities for binding to fragments of the antigen (Sachs *et al.* 1972). This lower affinity has been related to the absence in the peptide of a portion of a determinant residue; conformational differences between peptides and the native molecule; and the absence in the peptide of long-range effects (e.g. electrostatic) that may exist in the native protein (Al Moudallal *et al.* 1982).

The sensitivity of SAT assays may be influenced by ratio of reporter molecules per microsphere and the number of microspheres with the same classification address. As long as both of these values exceed one hundred, statistical limitations imposed by the Poisson distribution have a nominal effect (Mandy *et al.* 2001). These limitations were avoided by careful assay optimisation, and the use of a concentration of 25 μg ml^{-1} of PE-conjugated antibody in all assays. Furthermore, a minimum of 100 beads per analyte were used as recommended. The requirement for just 100 readings is due to the fact that reaction on the microsphere surface is uniform between microspheres (McHugh, 1994).

Synthetic peptides

The length and overlap of synthetic peptides are significant factors that can influence epitope mapping analyses. In the present study, synthetic peptides twelve amino acid residues in length were chosen. Adjacent peptides overlapped by two amino acid residues. The ideal length of synthetic peptides used in epitope mapping studies is not known with certainty. Sumar (2001) suggested that the ideal overlapping can be calculated using the formula:

$$X = [n-(n-1)]$$

where: *X* is the number of overlapping residues and n is the number of residues of the protein.

As a first approach synthetic peptides should not be shorter then eight residues and with an overlap of five residues (Tribbick and Rodda, 2002), instead of the seven residues recommended when using the formula mentioned above. However, several works have been performed using different peptide sizes with good results. Peptides 15 residues in length were used for epitope mapping of the fish virus Viral Haemorrhagic Septicaemia Virus (VHSV) (Estepa and Coll, 1996) and peptides 13 residues in length were used for epitope mapping of the G glycoprotein of Herpes Simplex Virus type 2 (Liljeqvist *et al.* 2002). Decapeptides have also been widely used for epitope mapping many different proteins, for instance anti-troponin (Filatov *et al.* 1998), Beet Necrosis Yellow Vein Virus (BNYVV) (Commandeur *et al.* 1994) and interleukin-10 (Reineke *et al.* 1998). The epitope mapping of interleukin-10 by these authors was also successful when peptides 6 residues in length were used. Thus previous studies suggested that synthetic peptides 12-residues in length could be used successfully and represented a balance between maximising the efficacy of epitope mapping and the funding available for the project.

However, the use of such peptides should be borne in mind during interpretation of results. For example, use of longer peptides could explain why several peptides were recognised by many of the antibodies analysed. An increase in the peptide length has been associated with an increase in the number of peptides identified (Geysen *et al.* 1987a). Furthermore, the detection of some epitopes may be compromised by the use of synthetic peptides that overlap by only two residues. (Sumar, 2001). The use of greater numbers of synthetic peptides of shorter length was not possible in the present study due to financial and logistical constraints. However, in future studies, the use of a set of synthetic peptides of reduced length and with greater overlap could facilitate the identification of epitopes on the betanodavirus capsid protein with greater resolution, especially within the regions that are predicted to be of significance based upon the present study.

Section 2 - Validity of the assay

In this section, the precautions taken to ensure assay validity are discussed.

Several precautions were taken to ensure validity of the assay. Negative controls were incorporated to confirm the specificity of results. These comprised a Betanodavirus-negative sea bass serum sample, and a mouse MAb; and two control peptides (with the same amino acid composition as two test-peptides, but synthesised in the reverse sequence (i.e. C terminus - N terminus). The latter were designated peptides 35 and 36.

The specificity of the interaction between antibodies or serum samples with synthetic peptides was supported by the lack of affinity of MAbs raised against the fish parasite *Tetracapsuloides bryosalmonae* towards the panel of peptides. The lack of reaction between an unrelated antibody (anti-*Tetracapsuloides bryosalmonae* MAb) and the panel of peptides reinforced confidence in the specificity of the nodavirus epitope mapping data.

In order to assess the specificity of European sea bass sera to synthetic peptides two different types of serum samples were used. The first type comprised two European sea bass serum samples that exhibited no activity to betanodavirus as determined by ELISA. However, in SAT assays, these serum samples demonstrated strong binding to the panel of synthetic peptides. This could be due to the relatively greater sensitivity of SAT assays as compared to ELISA (Carson and Vignali, 1999, Biagini *et al.* 2004a, Biagini *et al.* 2004b). Furthermore, at lower concentrations of analyte, SAT microspheres have been demonstrated to be four times more sensitive than assays performed using the microtitre plate format (McDade and Spain, 1997).

The second type of negative control serum was obtained from European sea bass that had never been exposed to betanodavirus. This European sea bass serum showed non-specific binding to synthetic peptides. This fluorescence was considered to represent non-specific background, and on this basis was subtracted from all the fluorescence values of all test samples.

The use of peptides synthesised in the reverse sequence as controls yielded contradictory results. None of the serum samples analysed recognised control peptide 36 but MAbs 4C3 and 5G10 recognised control peptide 35, showing high MFI values. The ability of these MAbs to recognise control peptide 35 could be due to the presence of common residues between this peptide and the recognition target of the MAbs studied (Saul and Alzari, 1996). The recognition of this peptide indicates that epitope mapping with synthetic peptides requires careful interpretation. Pepscan results are presented such that all of the peptides recognised by a given MAb or serum sample are evident.

Section 3 – Discussion of pepscan results

Discussion of the pepscan results is divided into three sub-sections. The first sub-section compares and contrasts the results obtained with antibodies from different host species. The second sub-section comprises an analysis of the synthetic peptides that were most strongly recognised by antibodies/serum samples. In the final sub-section, the peptides recognised by antibodies/serum samples are discussed with reference to the structure of the betanodavirus coat protein.

Comparation of pepscan data obtain with antibodies from different species

The immune systems of different species may react to different sets of epitopes in a given antigen (Geysen *et al.* 1984). In this thesis, the recognition sites of mouse MAbs, rabbit antibodies and European sea bass sera were characterised using synthetic peptides that mimicked the betanodavirus coat protein. Antibodies and serum samples from all species studied exhibited similar levels of binding to synthetic peptides. Maximum MFI values were 3704, 2138, 842 and 190 for the four mouse MAbs; 1290 for rabbit polyclonal antibodies; and

2239, 2164, 2108, 1394, 1377, 1243, 1161, 891 and 621 for the nine European sea bass serum samples.

Mice and rabbit were immunised with a standardised betanodavirus preparation facilitating a comparison of the immune responses in these species. Both mouse and rabbit B-cell immune responses were directed against two principal regions of the betanodavirus coat protein defined by peptides 1-5 and 19-21. Additionally, mouse MAbs recognised another region represented by peptide 15-16. Rabbit polyclonal antibodies also recognised two further regions (peptides 10-13 and 28-30).

Binding of rabbit polyclonal antibodies to several peptides is in agreement with previous studies. Valle *et al.* (1999) mapped the bacteriophage ϕ29 connector protein recognised by rabbit polyclonal antibodies and identified 11 regions. Although polyclonal antibody responses are frequently directed against several epitopes, in many cases, one epitope is recognised more strongly than the others (Carter, 1994).

The results of the present study suggest that peptide 20 may contain a major epitope. This peptide was the most strongly recognised by half of the mouse MAbs analysed (3B10 and 4C3) and by the rabbit antibodies. Peptide 20 was also amongst those most strongly recognised by the other two MAbs.

When the three peptides that were recognised most strongly by each antibody/serum sample are compared, peptide 3 was recognised by mouse MAb 4A12 and rabbit antibodies, but not by the natural host (European sea bass). Similar findings have been reported in studies of immune responses to African Horse Sickness Virus (AHSV) (Martínez-Torrecuadrada *et al.* 2001). Further similarities in the patterns of epitopes recognised by MAbs and polyclonal antibodies were reported by Quesniaux *et al.* (1990). These studies conflict with the hypothesis that MAbs tend to be directed towards minor epitopes that are not immunodominant and are not detected by polyclonal antisera (van Regenmortel, 2000).

The mouse MAbs studied recognised several epitopes. At first sight, this is a surprising result since a MAb should only recognise a single target sequence (Carter, 1994). Short peptides are believed only to be capable of mimicking linear or continuous epitopes. Thus antibodies recognising short synthetic peptides are believed to be directed against linear epitopes (van Regenmortel, 1990a). However, a linear epitope defined by a single peptide may represent a part of a larger discontinuous epitope present on the native antigen (van Regenmortel, 1989b). Carter (1994) has hypothesised that an antibody recognition site may be comprised of two or three discontinuous regions of a protein sequence that fold into a discrete conformation. In this thesis, mice were immunised with a live betanodavirus for production of MAbs. Thus, B-cell clones selected during the production of MAbs may be directed against portions of discontinuous epitopes that are created by the molecular folding which forms the three dimensional structure of a protein antigen. Consequently, individual synthetic peptides may represent fractions of a discontinuous epitope present on the native virus coat protein. Accordingly, it is possible for MAbs derived from an immunisation with intact virus to recognise more than one synthetic peptide in epitope mapping studies as occurred in the present study. Similar findings have been described in epitope mapping studies of β-factor XIIa (a component of human blood coagulation factor XII), where multiple peptides located throughout the protein were recognised by mouse (Gao and Esnouf, 1996).

The spectrum of epitopes recognised in antiviral immune responses varies between individuals of the same host species, the route of infection, and the strain of virus (de Vegvar and Robinson, 2004). Idiosyncrasies are evident in immune responses by individual European sea bass. The peptides that were recognised most strongly (20 % highest MFI) by each European sea bass serum are located in two large regions that span almost all of the coat protein. This type of immune response may be a reflection of unique characteristics of fish immune systems. It is known that the major immunoglobulin class (IgM) of fish that exhibits neutralising activity towards bacteria and viruses is similar in structure to IgM of higher vertebrates (Shelton and Smith, 1970). In fish of the class Actinopterygii, IgM forms a

tetramer instead of the pentamer structure displayed by IgM in higher vertebrates ([Pilstrøm 1998]). These structurally diverse and flexible IgM tetramers may easily provide binding capabilities through cross linking not available to higher vertebrates (Kaattari *et al.* 1999). These authors suggest that the flexibility in accommodating differentially spaced epitopes could serve to increase the avidity of binding over a more rigid, fully-polymerised form. A more flexible array of Fabs would be able to accommodate more epitopes by physically adjusting in orientation, enabling an antibody to bind a wider range of epitopes (Kaattari *et al.* 1999). The versatility of fish IgM might compensate for the lack of a diverse antibody response such as that found in higher vertebrates.

When the three peptides recognised most strongly by European sea bass sera are taken into consideration, the majority of samples were found to be directed against four regions of the coat protein defined by: a) peptide 1; b) peptide 10-12; c) peptide 15-16; and d) peptide 18-22. Mouse MAbs and rabbit polyclonal antibodies also identified two of these four regions, suggesting that there are similarities in antibody responses between these species. Similar findings have been reported for immune responses to varicella-zoster virus by Fowler *et al.* (1995), who suggested that some of the differences in immune responses by mice and humans may have been due to differences in the form of antigen presented to the immune systems of these species. In the present study, European sea bass were naturally infected with betanodavirus, whereas mice and rabbits received a virus/adjuvant preparation.

All the antibody samples analysed showed reactivity to several peptides with different amino acid sequences. This may be due to the fact that these peptides contain a sufficient proportion of the amino acids that comprise the recognition site of a given antibody (van Regenmortel, 1999). Some of the residues in a linear epitope can be replaced by any of the other 19 amino acids without impairing antigenicity, and the linear fragment may in fact be antigenically discontinuous (Geysen *et al.* 1988). Thus, peptides of differing primary amino acid sequence may be antigenically similar. In the absence of crystallographic data on the peptide-antibody complex, it is not clear whether such replaceable residues actually interact with the antibody only through their main chain or whether they play merely an indirect role

by acting as a scaffold to bring the neighbouring essential residues at the required location for contacting the antibody (van Regenmortel, 2001).

Comparation of pepscan data with respect to betanodavirus coat protein structure

The recognition of a synthetic peptide by an antibody does not necessarily establish the presence of a continuous epitope in the corresponding region of the protein (van Regenmortel, 1992). Consequently the interpretation of epitope mapping studies such as those reported in this thesis is not straightforward.

Rabbit antibodies, mouse MAbs and 5/9 of the fish serum analysed identified the region comprised by peptides 20 and 21 (coat protein amino acids 191-212, see Table 6.17). The identification of this region by many diverse antibodies/sera suggests that it may represent a significant immunogenic domain. When peptides 20 and 21 are aligned together, it is evident that they posses similarities in amino acid sequence. This comprises two residues (CR) flanked by SV residues on the N and C termini, suggesting that the amino acid sequence SVxCRxSV is potentially an important epitope that is recognised by the majority of the antibody/serum samples analysed. This hypothesis is experimentally testable, and has important implications for the design of immunodiagnostic reagents and vaccines to counter betanodavirus infections.

The region represented by peptides 15 and 16 (amino acids 141-162) was recognised by three European sea bass serum samples (SB1, SB2 and SB9). When peptides 15 and 16 are aligned, it is possible to observe a common motif, in which, lysine-tryptophan residues, overlapped residues between the peptides (Figure 6.22, residues labelled in red), are flanked by glutamine residues at a spacing of 8 residues. Arginine residues also flank the tryptophan at a spacing of 6 residues (QxxRxxxxxKWxxxxxxRxQ).

A similar motif is also present in peptide 21 (amino acids 201-212) recognised by serum samples SB1 and SB9. Arginine (R) proline (P) residues occur at the same spacing from a tryptophan (W) residue (Figure 6.22, residues labelled in blue). Valine (V) and serine (S) residues also occur at the same positions in peptide 16 and peptide 21 (Figure 6.22, residues labelled in green).

Peptide 15/16	LQATRGAVVAKWWESRTVRPQY
Peptide 21	CRWSVRLSVPSL

Figure 6.22 – Alignment of peptides 15, 16 and 21 recognised by SB1 and SB9. In red can be visualised the overlapping residues of peptide 15 and 16. In bold are the amino acids with the same spacing to KW. In blue are the consensus amino acids of peptide 16 and 21 and in green the similar residues between the two sequences.

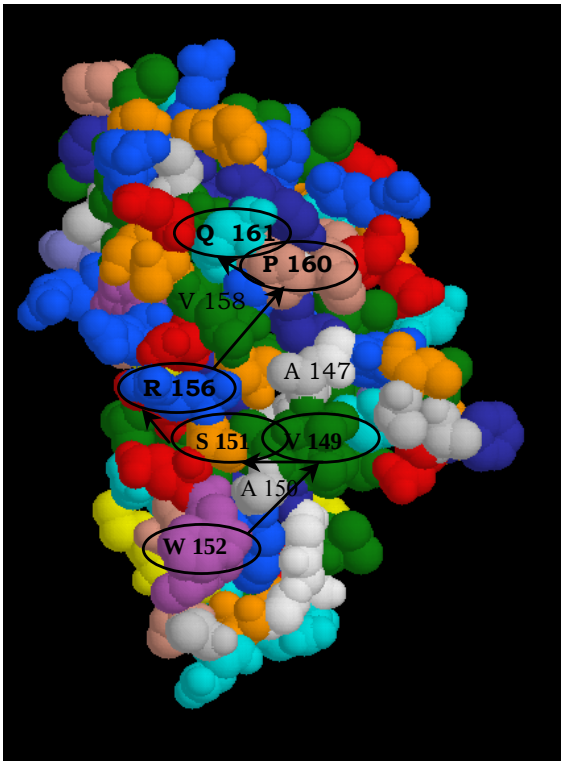


Figure 6.23 – The 3D view of the nodavirus coat protein showing the amino acid residues identified by SB1 and SB9.

When all of these peptide sequences are taken into consideration, it is possible that residues W, V, S, R, P and Q are involved in the recognition site of serum samples SB1 and SB9. This conclusion is supported by the predicted three-dimensional structure of the betanodavirus coat protein (Figure 6.23). Protein folding brings peptides 20 and 21 into proximity, and shows that peptide 16 shares residues in common with this region.

Comparison of peptide 10 (amino acids 91-102) with peptides 15 and 16 suggests that serum sample SB2 recognises a different epitope (Figure 6.24). The overlapping residues, lysine and tryptophan (KW) are flanked by an alanine residue (A) and an aromatic amino acid (W). An arginine (R) occurs in the fifth positions before the alanine (Figure 6.24, residues labelled in blue). The same motif occurs, RxxxxAxxF is also present in peptide 10.

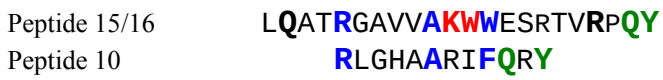


Figure 6.24 – Alignment of peptides 15, 16 and 10 recognised by SB2. In red can be visualised the overlapping residues of peptide 15 and 16. In bold the amino acids that with the same distance to KW. In blue the consensus amino acids of peptide 10 and peptides 15-16 and in green the similar residues between the two sequences.

Both peptides 16 and 10 contain glutamine (Q) and tyrosine (Y) residues near their C termini.

Based on analysis of the amino acid sequences of the peptides recognised by serum sample SB2, it is possible that the target epitope of this serum comprises K, A, W/F, Q and Y. The 3D structure of this sample does not confirm the epitope content, because the folding of the protein does not bring together peptide 10 and peptide 15-16 region (Figure 6.25).

Only serum samples SB1, SB2, and SB9 contained aromatic amino acid residues (W, F and Y). Relative scarcity of aromatic amino acids in B cell epitopes has also been identified in lysozyme (Davies and Cohen, 1996).

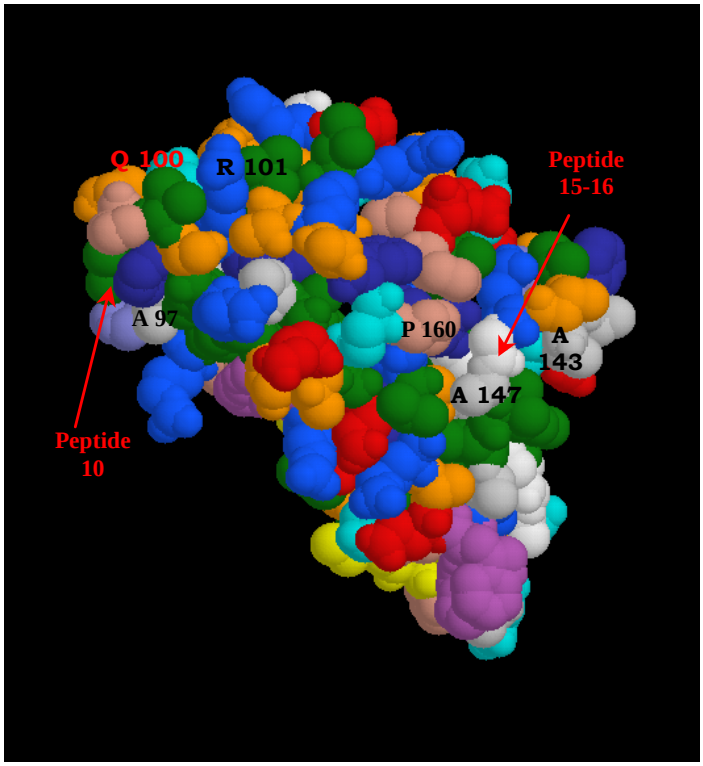


Figure 6.25 – The 3D view of the nodavirus coat protein showing the amino acid residues identified by SB2.

The same three peptides (16, 20 and 35) were recognised most strongly (i.e. highest MFI values) by mouse MAbs 4C3 and 5G10. Peptide 35 was used as a negative control. If the MFI obtained with the control peptide 35 is considered to represent background fluorescence and used as a cut-off value, MAb 4C3 only binds to peptide 20 and MAb 5G10 binds to peptides 16 and 20. Analysis of the amino acid sequence of these peptides and 3D structure of the coat protein suggest that Serine (S), Valine (V), and Arginine (R) are present within the epitope recognised by these Mabs (Figures 6.26 and 6.27).

Peptide 16 KWWE**SR**T**V**R**R**PQY
Peptide 20 NTDVVNV**S**V**V**LC**R**

Figure 6.26 – Alignment of peptides 16 and 20 recognised by MAb 5G10. In blue the consensus amino acids of peptide 16 and 20. In bold the common residues between the two peptides

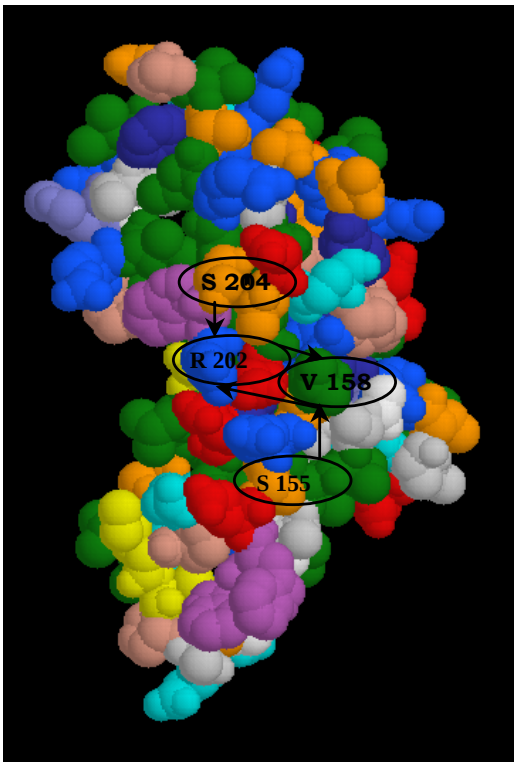


Figure 6.27 - The 3D view of the nodavirus coat protein showing the amino acid residues identified by MAbs 4C3 and 5G10.

However, the amino acid residues (S, V and R) that are predicted to be present in the epitopes recognised by MAbs 4C3 and 5G10 may not be the crucial residues for the binding of these MAbs. This is because in neutralisation and cross-reactivity assays, 4C3 and 5G10 do

not exhibit equivalent binding patterns which suggests that the epitopes recognised by these two MAbs may not be composed of the same residues. The MAbs were produced by immunisation of mouse with a European sea bass nodavirus strain, but 5G10 is also cross-reacts by ELISA (Chapter 3) with Atlantic cod and halibut strains, and is capable of neutralising the European sea bass and Atlantic cod strains.

Epitope mapping studies of the glycoprotein of Viral Haemorrhagic Septicemia Virus (VHSV) glycoprotein also identified conformation constraints. In this case, peptide recognition only occurred with non-neutralising MAbs (Fernandéz-Alonso *et al.* 1998). These authors concluded that a glycoprotein neutralising epitope was only available when the protein was in the native form and situated on the surface of the infected cells.

The results of the present study which suggest B-cell epitopes can be comprised of several regions of a polypeptide molecule are supported by X-ray crystallographic analysis of protein structure. The surface area of the interaction between an antibody and an antigen varies from 600-900 $Å^2$, involves 14-20 amino acid residues, is discontinuous, and is formed from two to five separate stretches of the antigen polypeptide chain (Laver *et al.* 1990, Saul and Alzari, 1996, van Regenmortel, 2000). Calculations suggest that a smaller sub-set of 3 to 6 of these residues contributes most of the binding energy, with the surrounding residues merely "indulging in complementarity" (Laver *et al.* 1990). The disproportionate contribution of such a small number of residues to the overall binding energy makes the functional epitope much smaller than the structural epitope (van Regenmortel, 2000).

The fact that different peptides are recognised by MAbs is not surprising, and supports the idea that almost any part of the surface of a protein may be antigenic (Benjamin *et al.* 1984). The capacity for immune recognition of several sites of a pathogen is advantageous to the host, and facilitates effective immune responses against pathogens. This is particularly true for RNA viruses, which exhibit high rates of evolution and variability. Similar findings have been described in mouse humoral immune responses to influenza A virus (Hatta *et al.* 2000). Involvement of different amino acid residues within an epitope in

antibody binding has been reported in immune responses to human epithelial mucins (MCU1) protein core, molecules associated with secretory epithelia and with carcinoma of breast, ovary and other tissues (Petrakou *et al.* 1998).

Comparasion of pepscan data with respect to betanodavirus coat protein regions

In the present study mouse MAbs, rabbit polyclonal antibodies and European sea bass antibodies recognised different regions of the betanodavirus coat protein. Within a given region, different amino acid residues can be involved in the binding of antibodies to the coat protein.

The abundance of charged amino acids residues in the N terminal region of the betanodavirus coat protein, which possesses nine arginine residues and six lysine residues within the first 50 amino acids, could explain the recognition of this region by the majority of the antibodies studied. Basic/charged amino acids have been identified in antibody recognition sites in many proteins (Shi *et al.* 1984). A similar abundance of arginine and lysine residues is present in the coat protein of insect nodaviruses and is believed to be involved in the interaction between the coat protein and viral RNA genome required for encapsidation (Sideris, 1997). This region of the coat protein plays an essential role in replication and thus represents a "logical" target for protective immune responses. In fact all of the MAbs studied that showed neutralising activity (Chapter 3) recognised a region comprising coat protein amino acid residues 29-52, that contains five arginine residues and one lysine residue. Studies performed with the betanodavirus dragon grouper NNV show the triple arginine motif at 29-31 to be critical for virus particles formation (Lu and Chan-Shing, 2003). Similar studies in the alphanodavirus Flock House Virus (FHV) indicate that the region of the coat protein between amino acids 31 and 50 is critical for the assembly of the coat proteins into virus particles, and the deletion of this region completely blocks particle

formation (Schneemann *et al.* 1998). The amino acid residues 23-31 have been identified as a nucleolus localisation signal for greasy grouper NNV (Guo *et al.* 2003). Deletion of this region prevents the accumulation of viral particles in the nucleolus of the cells.

These results suggest that amino acid residues of the N terminal region of the coat protein potentially represent an immunogenic domain.

The region of the coat protein containing amino acids 181-212 contains only two arginine residues and no lysine residues. However, this region was recognised by all of the antibodies analysed with the exception of SB2 and SB10. This region is rich in leucine (n = 6) and valine residues (n = 7). These amino acids are very hydrophobic. The region of the betanodavirus coat protein within residues 181-212 is the most hydrophobic portion of the coat protein (Figure 6.20). The high hydrophobicity suggests that this region is situated in the interior of the coat protein molecule, and not available on the surface for antibody binding. However, the involvement of hydrophobic residues in antibody binding has been demonstrated in, for example, VP1 of Foot and Mouth Diseases Virus (FMDV), in which one epitope not predicted to be surface-presented is largely dependent on two leucine residues for antibody binding (Geysen *et al.* 1984). The interaction of antibody and antigen involves conformational changes in both, that can range from insignificant to considerable (Davies and Cohen, 1996). The amino acid residues flanking an epitope may influence the three dimensional structure of the latter, or alter physicochemical properties such as hydrophillicity/hydrophobicity or van der Waals forces, bulkiness or charge, (van Regenmortel, 1992). An epitope of Herpes Simplex Virus type 2 (HSV-2) has been found to contain predominantly positively charged arginines and residues that are hydrophobic (Liljeqvist *et al.* 2002). This finding is in agreement with X-ray crystallographic analysis of antibody-antigen interactions that show that discontinuous epitopes are usually composed of highly charged amino acids flanking a central core of hydrophobic residues (Smith *et al.* 1996, Kwong *et al.* 1998). This pattern occurs in the region comprising residues 181-212 of the betanodavirus coat protein, where the first leucine residue (L) is flanked by an arginine

residue (R). Two further arginines and an aspartic acid (D) are contained in this region, and the final leucine residue is flanked by another charged amino acid – glutamic acid (E). The prominence of hydrophobic residues in the most frequently recognised region suggests that the surface structure of the betanodavirus coat protein when combined with antibody differs from the native structure. This phenomenon has been identified in FMDV by Geysen *et al.* (1988). These results suggest that region (aa 181-212) is highly antigenic, and contains epitopes recognised by many serum samples studied in this thesis.

The C terminus of the betanodavirus coat protein is predicted to be located at the outer surface of the virus capsid, and the region comprised by amino acids 83-216 forms the inner shell of the capsid (Lin *et al.* 2001, Tang *et al.* 2002). This is in marked contrast to the localisation of the C terminus of insect nodavirus coat proteins, which is on the inside of the folded molecule (Wery *et al.* 1994, Lin *et al.* 2001). A surface location of the C terminus would be predicted to make this region a primary target for antibody binding, yet this prediction is in conflict with the results of the present study. None of the antibodies studied recognised the C-terminal region. A lack of recognition of the C-terminal region of the nodavirus coat protein by European sea bass sera was reported by Coeurdacier *et al.* (2003).

The short C-terminal segment of the insect nodavirus coat protein is cleaved to yield mature infectious virus particles, and is believed to play a role in the delivery of viral RNA into host cells (Fisher and Johnson, 1993). Encapsidation of insect nodavirus (BBV) is mediated through the C terminus of the coat protein (Schneemann and Marshal, 1998). European sea bass nodavirus coat protein is shorter and is believed to lack a critical proteolytic cleavage site situated at C terminal residues Asn363-Ala364 (Delsert *et al.* 1997a). The lack of this functional site would confer less importance with respect to immune control. However, the essential role of the C-terminal region for virus particle formation has been demonstrated for dragon grouper NNV (Lu and Chan-Shing, 2003). The deflection of humoral immune responses to less important areas of this protein may be an important strategy for survival and propagation.

Section 4 - Comparison with previous studies of betanodavirus epitopes

In this section results are discussed in relation to the two previous studies aimed at the identification of neutralising epitopes in the betanodavirus coat protein. The first of these studies was performed by Nishizawa *et al.* (1999), who identified an amino acid motif, PAN , in the coat protein as immunogenic. The second study performed by Coeurdacier *et al.* (2003) suggested that the first 14 amino acids of the coat protein contain neutralising determinants.

A putative major neutralising epitope, with the amino acid sequence PAN, has been predicted to occur at amino acid residues 254-256 of the coat protein of the betanodavirus SJNNV (Nishizawa *et al.* 1999). PAN is encoded by a fragment of the coat protein gene referred to as T4. The MAbs utilised by Nishizawa *et al.* (1999) also recognised a target situated in the T2 region of the coat protein gene of three other nodavirus genotypes. The cross-reactivity was mapped to a region of the coat protein located between amino acids 83-216. This region is highly conserved (> 93 % sequence identity), whereas the T4 region is located in a variable region (sequence identity 62 %) that encodes amino acids 235-315 (Nishizawa *et al.* 1995a). In the T2 region, a PAN sequence is situated between amino acid 116-118. In the present study, European sea bass serum sample SB17 was the only antibody sample that recognised this region (contained within peptide 12).

In the nodavirus coat protein peptides 12, 20 and 21 recognised by SB17 are brought together by the tertiary protein folding. It is known that antigenic sites are often complex conformations dependent on the tertiary folding of the protein chain (Geysen *et al.* 1987b). The software used, in the present study, to predict the betanodavirus 3D coat protein structure was unable to incorporate the amino acid residues 1 to 87 and 213 to 336 of the nodavirus coat protein. The lack of these residues was revealed by the incomplete appearance of the protein and the presence of a gap between residues N 191 and N 118 as shown in Figure 6.28.

The three peptides sequences recognised by SB17 were compared and amino acids were located in the 3D structure. This analysis suggests that the amino acid residues involved

in SB 17 epitope are C, P, V and S. The analysis of the peptide sequences and 3D structure of the protein suggests that the amino acids involved in the SB17 epitope are not the linear "PAN" sequence as described by Nishizawa *et al.* (1999). However, both the PAN sequence and the putative epitope containing residues identified in the present study may represent components of a single immunogenic domain that is the target of neutralising immune response. This is of considerable relevance to the design of betanodavirus vaccines.

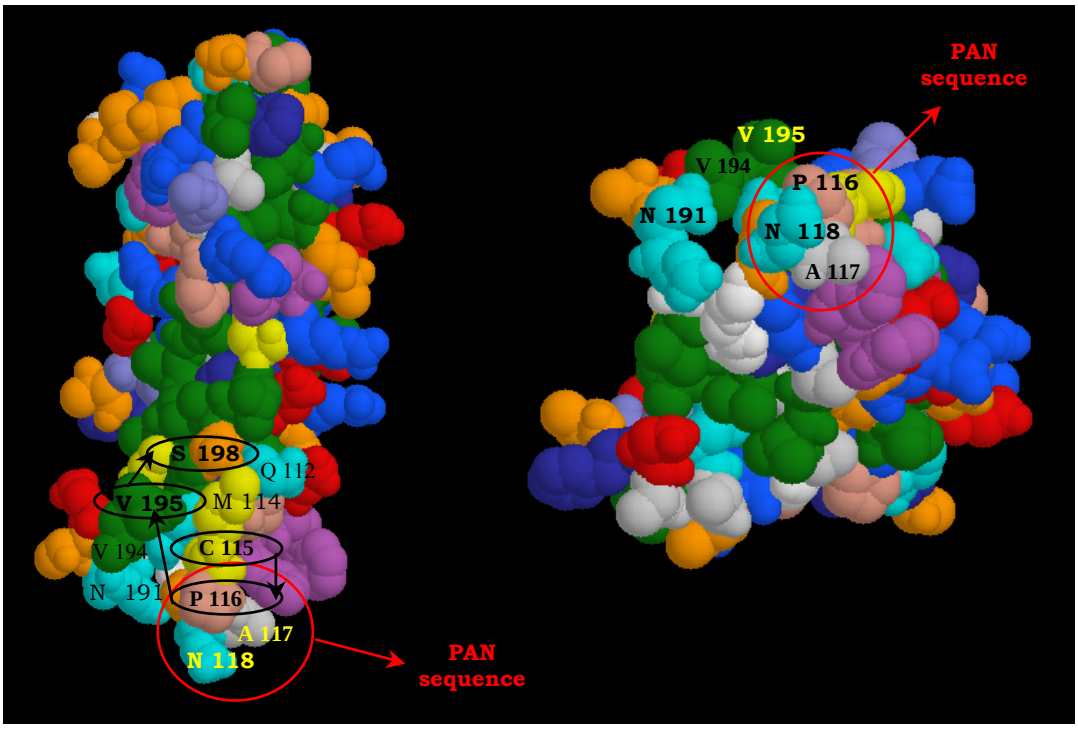


Figure 6.28 – The 3D view of the nodavirus coat protein showing the amino acid residues identified by SB17.

The region of the coat protein between amino acid 1 and 12 (peptide 1) has been postulated to represent a potential vaccine target in European sea bass by Coeurdacier *et al.* (2003). These authors evaluated the degree of protection conferred by immunisation with synthetic peptides mimicking the N-terminus (amino acids 1-14) and C terminus (amino acids 325-338). The strong antigenicity of terminal segments of proteins is believed to arise from their location on cell surfaces (Thornton and Sibanda, 1983). Surface orientation of the coat

protein N-terminus is not observed in insect nodaviruses, in which the N and C termini of the coat protein are situated within the interior of the molecule (Schneemann *et al.* 1998).

In the present study, this region of the N-termini was strongly recognised by four of the European sea bass serum samples analysed suggesting that region comprising peptide 30-50 is potentially immunogenic. The region comprised by peptide 1 (coat protein residues 1-12) has been suggested to represent a putative immunogenic region in European sea bass (Coeurdacier *et al.* 2003) . The region represented by peptide 1 is rich in charged amino acids, containing four lysines and one arginine. It is known that the addition of a single lysine residue to a peptide can result in a ten-fold increase in antibody binding (Shi *et al.* 1984). This high content may have led to over production of antibody. However the recognition of this region by four European sea bass serum samples confirms that it is probably important in fish humoral immune response to betanodavirus. Future work on the development of vaccines against betanodavirus should consider the N-terminal as a potentially important immunogenic site.

6.4.1 - Final reflection

It is believed that this is the first time that SAT technology has been used for epitope mapping of a fish viral pathogen. At the time of writing, a single publication exists describing the use of synthetic peptides coupled to microspheres for epitope mapping of the gp41 protein of HIV-1 (Opalka *et al.* 2004) by SAT.

The combination of SAT technology and synthetic peptides has proved to be a useful technique for epitope mapping of the betanodavirus coat protein but was compromised by background fluorescence as evident in the results obtained with peptide 35 and European sea bass serum.

Further optimisation of the SAT protocol is possible. The use of different incubation periods and temperatures for each incubation step might yield better results. Optimisation of

the incubation steps may also permit smaller volumes of antibodies to be used in assays. The requirement for all washing steps could be confirmed, since their omission would simplify the protocol.

It was possible to verify that all the antibodies analysed, mouse MAbs, rabbit polyclonal antibodies and European sea bass sera identified multiple peptides, which may have been linear fragments of discontinuous epitopes.

Analysis of the three peptides with highest MFI values identified by each antibody or serum sample resulted in the identification of two regions of the coat protein which may represent important immunogenic domains. These are located at amino acid residues 1-50 and 181-212, in agreement with previous studies performed by Nishizawa *et al.* (1999) and Coeurdacier *et al.* (2003). A further potentially immunogenic region was identified between amino acids 141-162 based on analysis of European sea bass sera. Further work is required to confirm the role of these regions as sites that are of importance in protective immune responses to betanodaviruses. The precise location of residues within these regions and their involvement in antibody binding should also be determined. Furthermore, the ability of synthetic peptides mimicking these regions to serve as vaccines could be investigated. The development of such a synthetic vaccine that could be synthesised inexpensively and reproducibly would be of great benefit to the aquaculture industry.

Chapter 7 - General discussion and future prospects

The B-cell epitopes of a virus represent the regions of virus encoded proteins that are specifically recognised by the antigen binding sites of B-cell immunoglobulin receptors and antibody molecules (van Regenmortel, 1990a).

Betanodavirus epitopes are poorly characterised. Nishizawa *et al*. (1999) suggested that a region defined by the amino acid sequence PAN within the coat protein was a putative epitope. This conclusion was based on a comparison of MAbs to recognise different betanodavirus genotypes with defined coat protein amino acid sequence. In 2003 Couerdacier *et al*. suggested that the first fourteen N-terminal amino acids of the coat protein constitute an immunogenic region. Fish were immunised with synthetic peptides mimicking both N and C terminal regions, but only the fish immunised with the N-terminal region produced an immune response. However, this study did not examine the full-length of the coat protein and other regions may be involved in the immune response.

The aim of this thesis was to map B-cell epitopes in betanodavirus. The first step in the identification of these regions involved the production of betanodavirus monoclonal antibodies. Two fusions were required in order to obtain monoclonal antibodies directed against European sea bass NNV. Successful production of MAbs was crucial to the thesis as these were to be utilised to produce neutralisation escape mutants, in phage display and pepscan methodology in epitope mapping experiments.

Selection and characterisation of neutralisation escape mutants was facilitated by the capacity of the MAbs produced in the project to neutralise betanodaviruses. This technique is applicable to the identification of discontinuous epitopes and is based on the isolation of neutralisation resistant viral clones through plaque purification. However, the betanodaviruses studied were refractory to plaque production, and consequently, a limiting dilution procedure established by Borrego *et al.* (2002) for swine vesicular disease virus was used. In this procedure, cDNA sequence analysis of putative neutralisation-resistant clones is performed

after an ELISA that is used to confirm that isolated virus variants are not recognised by the selecting monoclonal antibody. In the present study, all betanodaviruses obtained by limiting dilution were recognised by MAbs in the confirmatory ELISA, and so no sequencing analyses were performed, based on the assumption that escape mutant selection was not successful. It is possible that strict adherence to the protocol of Borrego *et al.* (2002) may have resulted in an inability to detect escape mutants. This is because virus *recognised* by a MAb in the screening ELISA may not necessarily be *neutralised* by the same antibody. Thus, the characterisation of potential escape mutants by neutralisation tests with the selecting antibody may increase the effectiveness of the procedure. This could be incorporated into future studies. Nucleotide sequencing of viruses recognised by MAbs in the confirmatory ELISA could also be performed. Financial and time constraints ruled out this approach in the present study.

Monoclonal antibodies that bind to proteins in Western blot assays are almost always directed against linear epitopes (Morris, 1996a). The MAbs used in phage display and pepscan studies were shown to bind to the betanodavirus coat protein in Western Blots, indicating their suitability for use in these epitope mapping procedures as these techniques are more suitable for the identification of linear epitopes. However, a number of proteins that are completely denatured in Western blots have been found to renature during or after electroblotting (Wang and Yu, 2004). Thus it is potentially possible for epitopes recognised by MAbs in Western blots to possess conformational structure (Morris, 1996a, Wang and Yu, 2004). The majority of epitopes recognised by host antiviral responses are usually conformational (Hadlock *et al.* 2000). This is of relevance to the present study as the results obtained by epitope mapping with synthetic peptides, suggests that the MAbs recognised a discontinuous epitope on the betanodavirus coat protein.

This hypothesis may explain the results obtained using phage display. This technique depends on the affinity between randomly expressed peptides on the phage surface and the antigen recognition site of a MAb. Usually antibodies against discontinuous epitopes do bind linear and constrained phage libraries efficiently (Bonnycastle *et al.* 1996). When the spatial

presentation of random peptides does not match the original three-dimensional structure of the natural protein, binding affinity is lost (Rodi and Makowsi, 1999). This could explain why a clear consensus sequence was not obtained in phage display experiments. It is difficult to obtain epitope mapping data for discontinuous epitopes with phage display (Williams *et al.* 2001). However in some cases antibodies that are directed against discontinuous sequences recognise phage displayed peptides (Balass *et al.* 1993, Luzzago *et al.* 1993, Birkenmeier *et al.* 1997).

When analysing the data obtained from the phage display system Ph.D.-7™ and from pepscan analyses, it is possible to align the sequence of the phage clones with the synthetic peptides recognised in pepscan (Figure 7.1). This alignment strengthens confidence in the results obtained with the pepscan procedure, that identified three major epitope containing regions in the betanodavirus coat protein: amino acid residues 1-50 (peptide 1-5), 141-162 (peptide 15-16) and 181-212 (peptide 19-21).

As discussed in Chapter 5 in studies employing the Ph.D.™-7 library the high occurrence of phage clones containing double inserts may be due to the relatively short insert size (i.e. 7 residues). Confidence in this explanation is increased when the phage display results are considered together with the data from pepscan analysis (e.g. phage clones 1a and 1b selected with MAb 4A12). Unfortunately the use of a phage library with a longer insert (12 amino acid residues, Ph.D.-12™) did not produce conclusive results.

Analysis of phage clones with double inserts also enables identification of some of the amino acid residues that may potentially be involved in the interaction between MAbs and betanodavirus. For MAb 3B10 the critical binding residues (CBRs) are represented by the sequence VxxxNTxV(T)xxRSVLxR. For MAbs 4A12 and 4C3 the identification of CBRs and their localisation in the betanodavirus coat protein amino acid sequence is less clear. However, for MAb 4A12, possible CBRs are SVLxRWxRLxSxPS and PrxxRAxxRxxS; and for MAb 4C3 NxSVLxR and KW(S)xSR. The identification of the negative control peptide 35 by MAb 4C3 may be explained by the presence of the CBRs TxNxSR. This number of amino acid residues can be sufficient to allowing antibody binding, once it is been

demonstrated that a sub-set of 3 to 6 of these residues contributes to most of the binding energy (Laver *et al.* 1990).

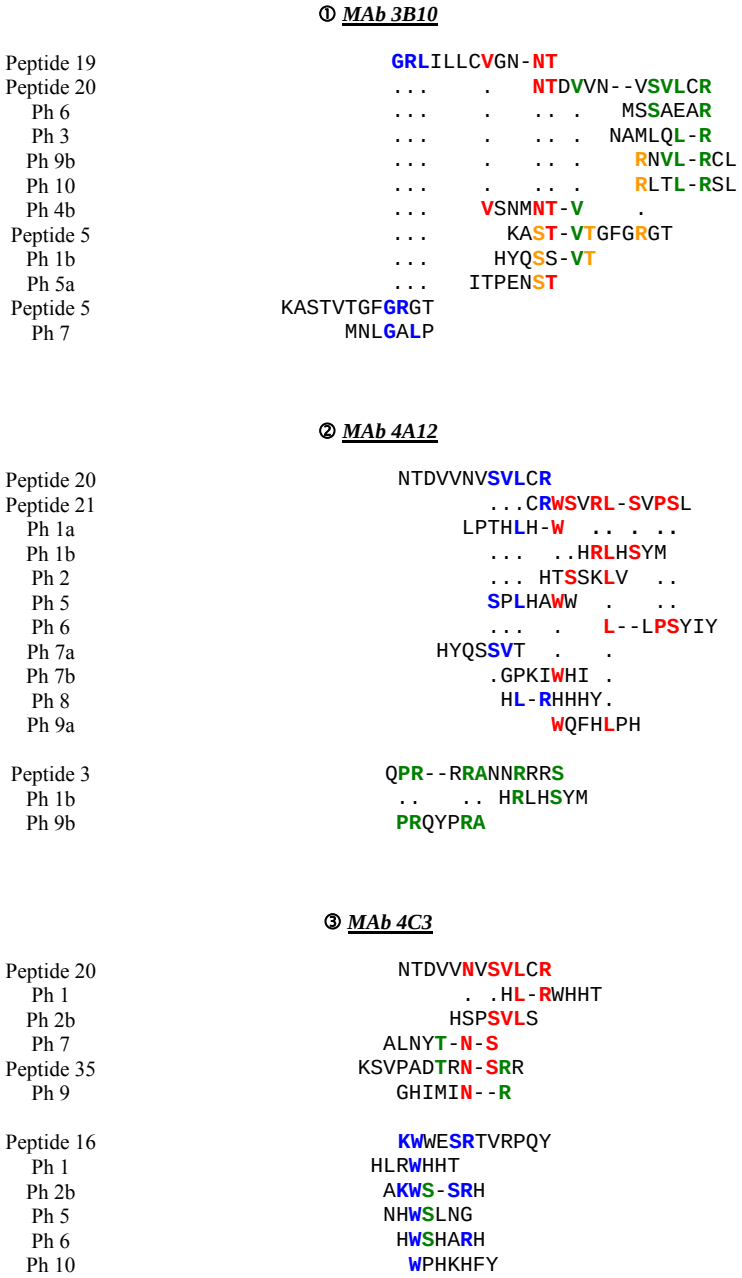


Figure 7.1 - Alignment of the peptides and phage clones selected with ① MAb 3B10, ② MAb 4A12 and ③ MAb 4C3. Ph stands for phage display clone selected with Ph.D.-7™.

Recommendations for future research

The identification of potential epitopes within the betanodavirus coat protein is of significance to the development of diagnostic reagents and vaccines.

Betanodavirus vaccines

In the last four years, a number of studies on the development of betanodavirus vaccines have been published, including recombinant protein and DNA vaccines. Thus far the most encouraging results have been achieved with recombinant protein vaccines using the betanodavirus coat protein as the immunogen (Húsgarð *et al.* 2001, Tanaka *et al.* 2001, Yuasa *et al.* 2002). However, even the most efficient vaccine constructs were unable to achieve complete protection. Mortalities reached forty percent in vaccinated fish populations. This is inadequate for a commercial fish vaccine. Furthermore vaccines based on DNA constructs or recombinant proteins are problematic in relation to licensing. Public perception of these vaccines also may affect the marketability of vaccinated stocks.

Against this background, there are advantages associated with the use of peptide based vaccines. These are chemically defined and not produced using genetic modified organisms, like recombinant protein or DNA vaccines. They also do not pose risks to wild fish associated with live-attenuated vaccines (Arnon and van Regenmortel, 1992).

A knowledge of protective epitopes is required to develop peptide based vaccines. The work of Coeurdacier *et al*. (2003), indicates the potential use of synthetic peptides for fish vaccines. Recently developed techniques permit the presentation to the immune system of multiple peptides through the use of an engineered bacterial protein as a scaffolding construct (Laplagne *et al*. 2004). Use of this system would facilitate the development of vaccine constructs containing several copies of one or more betanodavirus epitopes, or copies of

betanodavirus epitopes combined with T-cell epitopes (if and when these are identified and their role in fish immune responses elucidated) which may improve immunogenicity.

Peptide vaccines have been developed for a number of viruses including respiratory syncitial virus (Steward, 2001) and canine parvovirus (CPV) (Langeveld *et al.* 1994). A peptide vaccine developed against CPV demonstrated good protective efficacy.

Whilst peptide vaccines represent one logical continuation of the work reported in this thesis, the putative epitopes identified through pepscan studies could also be used to inform the development of different types of vaccines, such as those based on the use of recombinant proteins, or DNA / RNA vaccines. In these instances, candidate vaccines could be trialled for induction of humoral immune responses.

The results of the present study suggest three candidate regions (amino acids 1-50, 141-162 and 181-212) for a vaccine, although the optimum region of the coat protein could be further defined using epitope mapping of greater resolution. A series of overlapping synthetic peptides could be synthesised using an overlap of a single amino acid. A peptide set of shorter length (i.e. n = 7) could also be used, permitting a more accurate mapping of the residues recognised by neutralising antibodies. Once these regions are identified then amino acid replacement could be used to identify the critical binding regions. Certain amino acids are more difficult and expensive to synthesise than others. Thus the use of peptides that do not contain such residues in vaccines would simplify the chemical manipulation involved in vaccine synthesis. Thus, these two steps would help to produce a more effective and inexpensive vaccine.

An effective vaccine should be able to induce protection against several betanodavirus strains or ideally against the four betanodavirus genotypes (RJNNV, SJNNV, BFNNV and TPNNV). The importance of multi-genotype vaccines was highlighted by Thiéry *et al.* (2004) who showed that three betanodavirus genotypes could be isolated from a single country. European sea bass were found to be infected with betanodavirus viruses belonging to the RJNNV and BFNNV groups, and *Solea senegalensis* were infected with viruses from the SJNNV clade. S*olea senegalensis* is a novel species in aquaculture and in Portugal and Spain

this species is produced in polyculture systems together with European sea bass and gilthead sea bream.

To produce a vaccine to protect against all or at least several strains of betanodavirus, it would be necessary to investigate whether antibodies from other fish species infected with different betanodavirus strains recognise the same regions identified in this study.

In preliminary vaccine development studies, the regions of the coat protein identified as containing putative epitopes could be synthesised and used to immunise fish. The production of anti-peptide antibodies could be confirmed by ELISA or SAT and the protective efficacy of the peptides could be confirmed by challenge studies.

Further studies are clearly needed to facilitate the development of betanodavirus vaccines. In this respect, a number of research themes require investigation, including the functions of the betanodavirus proteins, the mechanisms of virus replication and accurate definition of betanodavirus structure using X-ray crystallography. The mechanisms through which the virus infects host cells are not fully understood. It is not known whether betanodavirus inhibits post-transcriptional gene silencing or other methods of immune escape. The mechanism of virus recognition by the host immune system also requires research.

Diagnostic tests

Non-invasive sensitive diagnostic methods are essential for screening broodstock, ongrowing stock or fish stock prior to introduction into a fish-farm. Screening methods should ideally be fast and not technically complex. ELISA is a widely used method. However, ELISA tests for betanodavirus are compromised by antigenic variation between different betanodavirus strains. None of the MAbs produced during this study were able to recognise three genotypes groups of betanodavirus (BFNNV, RGNNV and SJNNV). Nevertheless two MAbs, 3B10 and 4C3, are specific to European sea bass betanodavirus, a RGNNV strain used to immunise the mice for their production. The remaining MAbs (4A12, 1E3 and 5G10) also

recognised BFNNV. This same specificity was observed by Chi *et al.* (2003), with MAbs produced against grouper betanodavirus (RGNNV). This lack of ability of the MAbs to recognised SJNNV strain may be associated with the phylogenetic distances observed between the clades. Such phylogenetic distances reflected differences in the coat protein amino acid sequence, and may lead to differences in the 3-dimentional structure of the betanodavirus coat protein that invalidate the binding of the MAbs to SJNNV. Iwamoto *et al.* (2004) identified viral RNA2 and/or encoded coat protein as being responsible for host specificity in SJNNV and SGNNV.

Taking to account the restrictions on their ability to identify the multiple genotypes of betanodavirus mentioned above, the MAbs developed during this project could still be used for the detection of betanodavirus. As been demonstrated MAb 4A12 is suitable for confirmation of the virus in histological samples by immunohistochemistry and this MAb is now commercially available from Aquatic Diagnostic Ltd (University of Stirling, Stirling Scotland) as product number P09 (www.aquaticdiagnostics.com). However, other diagnostic techniques such as sandwich ELISA could be developed and optimised for the detection of betanodavirus. Different MAbs could be used as capture and detection antibodies. For validating ELISA as a betanodavirus diagnostic tool the sensitivity of the assay should be compared with nested-PCR or NASBA. These molecular techniques have been indicated to be more sensitive for the detection of betanodavirus and ideally suitable for diagnostics (Dalla Valle *et al.* 2000, Starkey *et al.* 2004). The use of anti-SJNNV polyclonal antibody revealed to be less sensitive than RT-PCR (Mushiake *et al.* 1994). However, in several studies it has been demonstrated that ELISA can be as sensitive as PCR (*Mycoplasma bovis*) or RT-PCR (hepatitis C virus) (Attallah *et al.* 2003, Ghadersohi *et al.* 2005), ans ELISA possesses the advantage of being a high-throughput screening technique.

Alternatively the ELISA could be set up to detect fish antibodies against betanodavirus. This would have the advantage of being non-destructive, only requiring a serum sample. The use of multiple peptides mimicking several regions of the betanodavirus coat protein to coat the ELISA plate could permit generic assays to be developed, capable of

detecting fish antibodies against all the betanodaviruses. The results obtained from pepscan analyses in this project could be exploited to develop such an ELISA test to detect anti-betanodavirus fish antibodies. The use of synthetic peptides has been applied in serodiagnostics for the detection of antibodies to parasites, such as *Leishmania donovani* (Fargeas *et al.* 1996) or viruses, for example SARS - coronavirus (Chan *et al.* 2004). These ELISA tests demonstrated high sensitivity and specificity. Optimal results were achieved by use of a mixture of peptides to avoid epitope restrictions (Soto *et al.* 1998, Noya *et al.* 2003). Since synthetic peptides are used as the target antigen, assays are easy to standardise, less subject to the variation between batches of antigen that may occur with cell culture grown material, cheaper and ideal for large-scale screening. The use of synthetic peptides in serodiagnostic assays may also be less labour intensive, and confers several advantages since there is also no requirement for infectious virus.

Synthetic peptides could also be linked to beads allowing for sensitive and rapid multiplex testing with high throughput. The SAT is also less labour intensive than ELISA. The use of beads would allowed the multiplexing of the assay and the possibility for screening for antibodies against other pathogens simultaneously.

The main achievement of this work was the identification of putative epitope containing domains in the betanodavirus capsid. It is believed that this is the first time that SAT technology has been used for antigenic epitope mapping of a fish virus. The regions identified in this thesis may have important consequences for vaccine development programs aimed at the control of VNN.

As a complement to the application in vaccine development the epitope regions identified may also be applied in the development of hight-throughput screening ELISA or SAT assays.

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DEPC treatment

Concentration

Add 1ml DEPC per litre of water.

Glass or plastic

- Fill the glass with water
- Add DEPC, mix well and leave overnight in the fume hood
- Following morning autoclave
- Water in the glass can be thrown away in the normal drain
- Glass is autoclaved

Water

Same procedure mentioned for the glass. The flask where the water is kept needs to have been DEPC treated previously.

TNE-buffer

Prepare a stock solution of NaCl (1 M) and EDTA (0.1 M) in distilled water and filter sterilise (0.2 μm filter).

All the procedures should be done in a sterile environment.

- Add 50 ml of 1 M NaCl and 5 ml of 0.1 M EDTA to a 500 ml DEPC treated bottle that has been scored at 500 ml.
- Add 400 ml of DEPC treated distilled water and mix well
- Remove a small aliquot and check the pH with an indicator stick
- Adjust the pH until ± pH 7 with NaOH or HCL. Mix well
- Remove a small aliquot and measure in pH meter
- Adjust pH to 7.4-7.6 using NaOH or HCl
- Add 500 μl of DEPC and leave overnight
- Autoclave to remove any last trace of DEPC
- Allow the solution to cool down
- Add 5 ml sterile, RNAse-free Tris (pH 7.5, 1M)
- Adjust the volume until 500 ml with DEPC treated water
- Remove a small aliquot and check the pH.

Hi Trap protein G HP

All the solutions are made on the day of using.

Binding buffer

(20 mM sodium phosphate, pH 7.0)

	150 ml
Na_2HPO_4 (20 mM)	0.534 g
NaH_2PO_4 (20 mM)	0.468 g

Both solutions are mixed until pH 7.0 is reached. Filter through a sterile 0.45 μm filter.

Elution buffer

(0.1 M Glycine-HCl, pH 2.7)

Glycine - 0.375 g in 50 ml of distilled water.

Add HCl until pH 2.7 is reached. Filter through a sterile 0.45 μm filter.

Tris-Hcl

(1 M Tris, pH 9.0)

Trizma base - 6.057 g in 50 ml of distilled water.

Add HCl until pH 9.0 is obtained. Filter through a sterile 0.45 μm filter.

ELISA

Poly-L-lysine

(Solution at 0.01 %)

1.5 ml of solution dissolved in 13.5 ml of coating buffer.

Coating Buffer

(Carbonate-bicarbonate solution, pH 9.6)

	1000 ml
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Dissolve in distilled water and adjust pH to 9.6 with HCl.

OR

Dissolved 1 tablet of carbonate-bicarbonate buffer (Sigma) in 100 ml of distilled water. Must be made fresh

Glutaraldehyde

(Solution at 0.05%)

Add 10 µl of glutaraldehyde to 10 ml of PBS.

Low Salt Wash Buffer (LSWB)

(0.02 M Tris; 0.38 M NaCl; 0.05 % Tween 20; pH 7.3)

This solution is 10x concentrate.

	1000 ml	500 ml
Trisma base	24.2 g	12.1 g
NaCl	222.2 g	111.1 g
Tween 20	5 ml	2.5 ml

Dissolve in distilled water and adjust pH to 7.3 with HCl.

Phosphate Buffered Saline (PBS)

(0.02 M phosphate; 0.15 M NaCl)

	1000 ml	500 ml
NaCl	8.77 g	4.385 g
$NaH_2PO_4.\ 2H_2O$	0.876 g	0.438 g
$Na_2HPO_4.\ 2H_2O$	2.56 g	1.28

Dissolve in distilled water and adjust pH to 7.2 with HCl.

Blocking buffer
(3 % casein)

Add 3 g of skimmed milk to 100 ml of distilled water.

Antibody Buffer
(solution at 1 %)

Add 1 g of BSA to 100 ml PBS.

Must be made fresh

Conjugate Buffer
(solution at 1 %)

Add 1g of BSA to 100 ml LSWB.

Must be made fresh.

High Salt Wash Buffer (HSWB)
(0.02M Tris; 0.5 M NaCl; 0.1% Tween 20)

This solution is 10x concentrated.

	1000 ml	500 ml
Trisma base	24.2 g	12.1 g
NaCl	292.2 g	146.1 g
Tween 20	10 ml	5 ml

Dissolved in distilled water and adjust pH to 7.7 with HCl.

Substrate

To 15 ml of substrate buffer add 150 µl of substrate solution and 5 µl of hydrogen peroxidase.

Substrate buffer
(0.1 M Citric acid; 0.1 M Sodium acetate)

	1000 ml	500 ml
Citric acid	21 g	10.5 g
Sodium acetate	8.2 g	4.1 g

Dissolved in distilled water and adjust pH to 5.4 with NaOH.

Substrate Solution

(42 mM TMB, 3'3'5'5' – tetramethylbenzidine dihydrochloride)

Prepare the TMB:

0.07896 g to 6 ml
0.0658 g to 5 ml
0.03948 g to 3 ml

Dissolved in distilled water. Add acetic acid in a proportion 1:2 (acid:water). Must be made fresh.

Stop Reagent

(H_2SO_4 2 M)

For 500 ml of solution add 55.5 ml of sulphuric acid (98%) to 444.5 ml of distilled water.

Western Blot

Separating Gel Buffer

(1.5 M Tris; 0.4 % (w/v) Sodium Dodecyl Sulphate (SDS))

	1000 ml	500 ml
Trisma base	182 g	91 g
SDS	4 g	2 g

Dissolve in distilled water and adjust pH to 8.7 with HCl.

Stacking Gel Buffer

(0.5M Tris; 0.4% (w/v) Sodium Dodecyl Sulphate (SDS))

	1000 ml	500 ml
Trisma base	60.5 g	30.25 g
SDS	4 g	2 g

Dissolve in distilled water and adjust pH to 6.8 with HCl.

Ammonium Persulphate Solution

(10 % v/v ammonium persulphate)

Dissolve 0.1 g ammonium persulphate solution in 1 ml distilled water. Must be done fresh every time

Sample Buffer

(100 mM Tris; 4% SDS; 2mM DTT; 0.02 % Bromophenol blue)

This solution is 5x concentrated

Tris HCl 0.5 M (pH 6.8)	2.5 ml
Glycerol	2 ml
SDS (10% (w/v))	4 ml
DTT	0.31 ml
Bromophenol blue	2 mg
Distilled water	0.9 ml

Reservoir Buffer

This solution is 5x concentrated

	1000 ml	500 ml
Tris	15 g	7.5 g
Glycine	43.2 g	21.6 g
SDS	5 g	2.5 g

Dissolved in distilled water and adjust pH to 8.3 with HCl.

Separating Gel (12 % Acrylamide)

Separating buffer	5 ml
Acrylamide (30 %)	8 ml
TEMED	15 µl
Ammonium persulphate (10 %)	7 µl
Distilled water	7 ml

The TEMED should be the last reagent to be added.

Stacking Gel

Distilled water	2.7 ml
SDS (10%)	40 µl
Tris-HCl (1M, pH 6.8)	0.5 ml
Acrylamide (30 %)	0.67 ml
Ammonium persulphate (10 %)	40 µl
TEMED	4 µl

The TEMED should be the last reagent to be added.

Tris Buffered Buffer (TBS)

(0.02 M Tris; 0.5% NaCl)

	1000 ml	500 ml
Trisma base	2.42 g	1.21 g
NaCl	29.24 g	14.62 g

Dissolved in distilled water and adjust pH to 7.5 with HCl.

TBS with Tween (TTBS)

(1.5M Tris; 0.4% (w/v) SDS)

Tween 20	0.5 ml
TBS	1000 ml

Adjust pH to 7.5.

Transblot Buffer

	1000 ml	500 ml
Glycine	14.4 g	7.2 g
Trizma base	3.03 g	1.515 g

Adjust pH to 8.3.

Coomassie Brilliant Blue Staining

Coomassie Brilliant Blue	2.5 g
Methanol	400 ml
Acetic acid	100 ml
Distilled water	500 ml

Immunohistochemistry

TBS

(0.05 M Tris; 0.15 M NaCl)

Tris	6.055 g
NaCl	8.766

Dissolved in 1000 ml of distilled water and adjust the pH to 7.6.

Formic acid

(0.1 M formic acid; 2 % gelatine; pH 6.0)

Gelatine	8 g
Formic acid	1.576 ml
Distilled water	Until 400 ml

Dissolve the gelatine in the microwave and then add the formic acid. Adjust the pH to 6.

Citrate buffer

(0.1 M citric acid; 0.2 M Na_2HPO_4. $2H_2O$)

Na_2HPO_4. $2H_2O$	3.6 g
Citric acid	2.1 g
Distilled water	100 ml

DAB

Dissolve 1 tablet in 6.67 ml of TBS. Aliquot into 0.5 ml and freeze. Keep in the dark until further use.

For use: to the 0.5 ml DAB aliquot add 5 ml TBS and 0.1 ml of H_2O_2 (1 %)

Molecular Biology

Dilution buffer

Mix 200 mM Tris-HCl to 5mM $MgCl_2$ in nanopure water. Adjust the pH to 9.

Precipitation buffer

For each sample mix throughly 50 µl of ethanol 95 % (kept at – 20°C) with 2 µl of NaAc (3 M).

Acrylamide Sequencing gel

Nanopure water	26 ml
Mix beads	0.5 g
Urea	18 g
Long Ranger (acrylamide)	5 ml

Mix well and allow to reach room temperature. Filter through 0.2 µm cellulose nitrate membrane filter and degas for 4 minutes. Just before loading the gel add:

Ammonium persulphate (10%)	250 µl
TEMED	35 µl

TBE

This solution is 10x concentrate

Nanopure water	Bring up to 1000 ml
Tris	108 g
EDTA(Na_2)	8.3 g
Boric acid	55 g

Filter through 0.45 µm filter.

Ficoll loading buffer

Ficoll (10 %) – 0.5 g ficoll in 5 ml deionised water
Blue dextran stock solution – 0.01 g of blue dextran in 1 ml of deionised water

Mix well 4.5 ml of ficoll 10 % with 0.5 ml of blue dextran stock solution. Aliquot and freeze at –20°C.

Agarose gel

Agarose	1 g
TBE (1x)	100 ml

Dissolve in the microwave. Add 50 µl ethidium bromide (1 mg l^{-1}) when the gel temperature < 60°C.

Phage display

Microbiology

LB Medium

	Per liter	*Per 450 ml*
Bacto-Tryptone	10 g	4.5 g
Yeast extract	5 g	2.25 g
NaCl	5 g	2.25 g

Autoclave and store at room temperature.

Agarose Top

	Per liter	*Per 450 ml*
Bacto-Tryptone	10 g	4.5 g
Yeast extract	5 g	2.25 g
NaCl	5 g	2.25 g
Agarose	7 g	3.15 g
$MgCl_2 \cdot 6H_2O$	1 g	0.45 g

Autoclave, dispense into 50 ml aliquots. Store solid at room temperature, melt in microwave as needed.

Tetracycline Stock

Tetracycline	200 mg
Ethanol	10 ml

Store at –20°C in the dark. Vortex before using.

LB-Tet Plates

LB medium	1000 ml	450 ml
Agar	15 g	6.75 g

Autoclave, cool down to < 70°C and add Tetracycline stock solution (0.1 %). Pour and store plates at 4°C in the dark. Do not use plates if brown or black.

LB/IPTG/Xgal Plates

LB medium	1000 ml	450 ml
Agar	15 g	6.75 g

Autoclave, cool down to < 70°C and add IPTG/Xgal (0.1 %). Pour and store plates at 4°C in the dark.

IPTG/Xgal

IPTG (isopropyl β-D-thiogalactoside)	1.25 g	0.5 g
Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)	1 g	0.4 g
dimethyl formamide	25 ml	10 ml

Solution can be stored in glass or polypropylene tube protected from light at -20°.

Panning

Antibody buffer

(0.1 M NaHCO3, pH 8.6)

NaHCO3 - 0.840 g in 100 ml of distilled water. Adjust the pH to 8.6. Autoclave and store at 4°C.

Blocking Buffer

(0.1 M NaHCO3, pH 8.6; 0.5 % BSA; 0.02 % NaN_3)

NaHCO3	0.840 g
BSA	0.5 g
NaN_3	0.02 g
Distilled water	100 ml

Filter sterilize, store at 4°C.

Neutralising Elution Buffer

(1 M Tris-HCl, pH 9.1)

Tris-HCl - 7.88 g in 50 ml of distilled water.

Filter sterilize, store at 4°C.

TBS

(50 mM Tris-HCl, pH 7.5; 150 mM NaCl)

Tris-HCl	0.394 g	0.788 g
NaCl	0.4383 g	0.8766 g
Distilled water	50 ml	100 ml

Prepared Tri-HCl 10x concentrated and filter sterilize. Prepared the NaCl and autoclave.
Mix both solutions, dispense into universals and store at 4°C.

Elution Buffer

(0.2M Glycine-HCL, pH 2.2; 0.1 % BSA)

Glycine-HCL - 1.501 g in 100 ml of distilled water

Adjust pH to 2.2 with HCl and then filter sterilize, store at 4°C.Previously to use add BSA (1 mg/ml). Filter sterilize, store at 4°C until used.

Storage Buffer

(50 mM Tris-HCl, pH 7.5; 0.02 % Sodium azide; 150 mM NaCl)

Tris-HCl (50 mM, pH 7.5)	0.394 g	0.788 g
Sodium azide (0.02%)	0.01 g	0.02 g
NaCl (150 mM)	0.4383 g	0.8766 g
Distilled water	50 ml	100 ml

Prepared Tris 10x concentrated, adjust pH to 7.5 with HCl. Prepared sodium azide 10x concentrated. Mix Tris and sodium azide solutions and filter sterilize. Prepare the NaCl and autoclave.

Mix both solutions, dispense into universals and store at 4°C.

PEG/NaCl

(20 % (w/v) PEG; 2.5 M NaCl)

PEG (polyethylene glycol 8000)	20 g
NaCl	14.61 g
Distilled water	100 ml

Autoclave, dispense into bijoux and universals, store at room temperature.

TBST

(50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1 % or 0.5% Tween-20)

Tris-HCl	3.94 g
NaCl	4.383 g
Tween-20 (0.1 %)	0.5 ml
Tween-20 (0.5 %)	2.5 ml
Distilled water	500 ml

Prepared Tri-HCl 10x concentrated and filter sterilize. Prepared the NaCl and autoclave.

Mix both solutions, dispense into universals and store at 4°C.

TE buffer

(10 mM Tris-HCl, pH 8.0; 1 mM EDTA)

Tris-HCl	0.121 g
EDTA	0.03722 g
Distilled water	100 ml

Filter sterilized and store at 4°C.

Iodide Buffer

(10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 4 M NaI)

Tris-HCl	0.0605 g
EDTA	0.01861 g
NaI	29.978 g
Distilled water	50 ml

Store at room temperature in the dark.

Streptavidin Stock Solution

Dissolved 1.5 mg of Streptavidin (supplied with the Phage display kit) in 1 ml of Na_2PO_4/NaCl/NaN3.

Store at 4°C or –20°C (avoid repeated freezing/thawing).

Na_2PO_4 (10 mM, pH 7.2) /NaCl (100 mM)/NaN_3 (0.02 %)

Na_2HPO_4 (10 mM)	0.0234 g
NaH_2PO_4 (10 mM)	0.0267 g

Dissolve each phosphate in 15 ml of distilled water. Mix until pH 7.2.
Then to 10 ml of sodium phosphate add 0.5844 g of NaCI and 0.002 g of NaN_3.

Reagents/ Chemicals/ Kits/ Consumables

Reagents/ Chemicals

- Acetic Acid, CH_3COOH (100%) – BDH (1001CU)
- Acrylamide/Bis acrylamide stock solution (30 % w/v acrylamide ratio 20:1 –Bis acrylamide) – Severn Biotech Lta (20-2600-05)
- Agar bacteriologic (no. 1) – Oxoid (LP0011)
- Agarose MP – Roche (1388938)
- Albumin standard – Pierce (23209)
- Ammonium persulphate, $(NH_4)_2S_2O_8$ – Sigma (A-3678), *SDS Page gel*
- Ammonium persulphate, $(NH_4)_2S_2O_8$ – amresco® USA (0486) ACS grade, *sequence gel*
- Aquafenol – Qbiogene (AQUAPH01)
- Blue dextran – Sigma (D-5751)
- Boric acid electrophoresis purity reagent – Bio-Rad (161-0750)
- BSA (Albumine Bovine, fraction V) – Sigma (A-9647)
- Carbonate-bicarbonate buffer capsules (0.05 M carbonato-bicarbonato buffer pH 9.6) – Sigma (C-3041)
- Citric Acid (monohydrate), $C_6H_8O_7.H_2O$ – Sigma (C-1909)
- Chlorophorm, CH_4Cl_3 – Sigma (C-2432)
- Contrast Red – KPL (71-00-05)
- DAB (3,3' diaminobenzidine), 3, 3' 4, 4' tetra amino biphenyl tetrahydrochloride – Sigma (D-5905)
- DEPC (diethyl pyrocarbonate), $C_6H_{10}O_5$ – Sigma (D-5758)
- DMSO (dimethyl sulphoxide) – HyBri-max® Sigma (D-2650)
- DTT (DL-Dithiothreitol, Cleland's reagent), $C_4H_{10}O_2S_2$ – Sigma (D-5545)
- Dulbecco's Modified Eagle's Medium – Sigma (D-5671)
- Dulbecco's PBS (DPBS, $CaCl_2$, $MgCl_2$) – Gibco (14190-094)
- EDTA (ethylenediaminetetraacetic acid), $C_{10}H_{14}N_2O_8Na_2.2H_2O$ – Sigma (E-5134)
- EDTA (ethylenediaminetetra acetic acid disodium salt, EDTA(Na_2)), $C_{10}H_{14}N_2O_8Na_2.H_2O$ – AnalaR® BDH (100933 T), *sequence gel*
- Ethanol absolute – Fisher Scientific (E/0650DF/21)
- FBS (fetal bovine serum) – Sigma (F-6178), *hybridoma culture*
- FBS (fetal bovine serum) – Gibco (10106-169), *virus culture*
- Ficoll, type 400 (approx. mol wt 400000) – Sigma (F-4375)
- Formic acid – Sigma (F-4636)
- Gluteraldehyde, $C_5H_8O_2$ (50% solution photographic grade) – Sigma (G-6403)
- Glycerol, $C_3H_8O_3$ – Sigma (G-7757)
- Glycine (amino acetic acid), $C_2H_5NO_2$ – Sigma (G-7126)
- HAT media supplement (50x) – HyBri-max® Sigma (H-0262)
- HBSS ($CaCl_2$, $MgCl_2$) – Gibco (14170-088)
- Heparin sodium salt grade I-A: from porcine intestinal mucosa – Sigma (H-3149)
- HT media supplement (50x) – HyBri-max® Sigma (H-0137)
- Hydrogen peroxide H_2O_2 (30% w/w) – Sigma (H-1009)
- IPTG (isopropyl β-D-thiogalacto-pyranoside), $C_9H_{18}SO_5$ – Sigma (I-6758)
- L-15 (Leibovitz) with GlutaMAX™ - Gibco (31425-029)
- L-Glutamine – HyBri-max® Sigma (G-2150)
- Long Ranger® gel solution (acrylamide 50 % stock solution) – Cambrex Bio Science, USA (50611)
- Magnesium chloride hexahydrate, $MgCl_2.6H_2O$ – BDH (10149)
- Methanol, $CH_3.OH$ – Fisher Scientific (M/3950/21)
- Methyl green - Vector (H-3402)
- Mixed bead resin for molecular biology – Sigma (M-8032)
- Nuclease-free water – Promega (P 119C)
- PEG (polyethylene glycol), Av Mol.Wt. 8000 $C_2H_60_2$ – Signa (p-5413)
- Penicillin-Streptomycin solution -– HyBri-max® Sigma (P-7539)
- Pertex - Cellpath Ltd (UN 1866)
- Phenol (saturated Tris-HCl) – Sigma (P-4557)

- Poly-L-Lysine solution (0,1% w/v in water thimerosal 0,01% added as preservative) – Sigma (P-8920)
- SDS (sodium dodecylsulphate), $C_{12}H_{25}O_2SNa$ – Sigma (3771)
- Sodium acetate (pH 5.2 3M), NaAc – Sigma (S-7899)
- Sodium azide, NaN_3 – Sigma (S-2002)
- Sodium bicarbonate, $NaHCO_3$ – Sigma (S-6014)
- Sodium acetate (anhydrous), $C_2H_3O_2Na$ – Sigma (S-7545)
- Sodium choride, NaCl – Sigma (S-9625)
- Sodium dihydrogen orthophosphate dehydrated, $NaH_2PO_4.2H_2O$ – BDH (301324 Q)
- di-Sodium hydrogen orthophosphate , $NaHPO_4.2H_2O$ – BDH (301517 J)
- Sodium iodine, NaI – BDH (301724 F)
- TBE (tris-borate-EDTA) – Sigma (T-4415)
- TEMED (N,N,N',N'-tetramethyl ethylenediamine), $C_6H_{16}N_2$ – Sigma (T-9281), *SDS Page gel*
- TEMED (N,N,N',N'-tetramethyl ethylenediamine), $C_6H_{16}N_2$ – Sigma (T-8133), *sequence gel*
- TiterMax® Gold – TiterMAx USA, Inc (G-1), CytRx® Corporation
- Tris, electrophoresis purity reagent (tris(hydroximethyl)-aminomethane) – Bio-Rad (161-0719), *sequence gel*
- Trizma® (Tris base), $C_4H_{11}NO_3$ – Sigma (T-1503)
- True blue™ peroxidase substrate – KPL (71-00-64)
- Trypsin-EDTA (1x) in HBSS w/o CA&MG w/EDTA.4NA – Gibco (25300-054)
- Tryptone – Oxoid (LP0042)
- TMB (3,3',5,5' – tetramethylbenzidine dehydrochloride) – Sigma (T-8768)
- Tween 20 (Polyoethylene-sorbitan monolaurate) – Sigma (P-1379)
- Urea electrophoresis purity reagent – Bio-Rad (161-0731)
- Xgal (5-bromo-4chloro-3-indolyl-β-D-galactopyranoside), $C_{14}H_{15}BrClNO_6$ – Sigma (B-4252)
- Yeast extract – Oxoid (LP0021)

Antibodies

- Anti-European Sea Bass (*Dicentrarchus labrax*) IgM monoclonal antibody – Aquatic Diagnostic Ltd (F01)
- Anti-Nodavirus IgG monoclonal antibody – Aquatic Diagnostic Ltd (P09)
- Biotin anti-mouse IgG – Vector (BA-9200)
- Goat Anti-mouse conjugated with HRP – Scotland Diagnostic
- Goat Anti-rabbit conjugated with HRP – Sigma (A-6154)
- Goat Anti-mouse IgG conjugated with PE – Molecular Probes (P-852)
- Goat Anti-rabbit IgG conjugated with PE – Molecular Probes (P-2771)
- Goat Sera - Scotland Diagnostic
- Stretavidin-HRP - Vector (SA-5004)

Kits

- BCA™ protein assay reagent – Pierce (23225)
- Bio-Plex™ Amine Coupling Kit – Bio-Rad (171-406001)
- Bio-Plex™ Calibration Kit – Bio-Rad (171-203060)
- Bio-Plex™ COOH Bead 24 – Bio-Rad (171-506024)
- Bio-Plex™ COOH Bead 28 – Bio-Rad (171-506028)
- Bio-Plex™ COOH Bead 42 – Bio-Rad (171-506042)
- Bio-Plex™ COOH Bead 46 – Bio-Rad (171-506046)
- Bio-Rad™ Validation kit – Bio-Rad (171-203000)
- DYEnamic ET Terminator Cycle Sequencing Kit - Amersham Biosciences (US81050)
- Hi Trap protein G HP – Amersham (17-0404-03)
- Immunotype™ kit – Sigma (ISO-I)
- Ph.D-7™ phage display peptide library kit – New England BioLabs® Inc. (E8100S)
- Ph.D-12™ phage display peptide library kit – New England BioLabs® Inc. (E8110S)

- Vectorstain® ABC kit – Vector (PK-6102)
- Vectors® VIP peroxidase substrate kit – Vector (SK-4600)
- 4CN membrane peroxidase substrate system (2-C) – KPL (50-73-00)

Consumables

- 64 well paper shark tooth comb ABI Prism377 – PE Biosystems (A64-199904-0007)
- Cell culture – flasks of 25 cm^2, 75 cm^2 and 175 cm^2; 12 well plates; 24 well plates, Greiner and Nunc
- Comb membrane unlaminated – The gel company (CAM64)
- Cryotube™ - Nunc
- Hybond™-ECL™ nitrocellulose membrane - Amersham (RPN303D)
- Immulon® 4 HBS flat bottom microtitre 96 well plates, Thermo Labsystems (3855), *ELISA plates*
- MultiScreen HTS™ - Millipore (MSBV1210)
- Nescofilm – Bando Chemical Ind. Ltd, Japan
- PAP pen - Liquid blocker Super pap pen, Agar Scientific
- SDS plates - Hoefer™ glass plates 8x10 cm - Amersham (80-6136-81)
- SDS loading tips – Gel loading tip round – Alpha Laboratoires Lda (LW1255R)
- Syringe 1 ml and 5 ml - Terumo™
- Syringe 1 ml – Norm-Ject Turberkulin, Henke Sass Wolf GMBH, *immunised animals*
- Ultra-Clear™ tubes, Beckman (344ϕ59, 44 x 89 mm; 344ϕ58, 25 x 89 mm)
- Whatman number 1 (1001 917)
- 0.20 μm cellulose nitrate membrane filter – Whatman (7182-004)
- 0.20 μm syringe sterile filter - Sartorius (16532)
- 0.45 μm filter – Whatman (7184-004)
- 0.45 μm syringe sterile filter - Sartorius (16555)
- 15 ml sterile centrifuge tubes – Cellstar®, Greiner bio-one
- 50 ml sterile centrifuge tubes – Bibby Sterilin Ltd

Equipment

Centrifuges
- Beckman L80
- Denville Scientific Inc. Micro 240A
- Eppendorf centrifuge 5804R
- Heraus biofuge pico
- Sanyo Mistral 3000i
- Sanyo MSE 2000R
- Sanyo Micro centaur
- Thermo IEC microlite
- Wifug 500E

Electrophoresis
- Amersham Pharmacia Biotech, electrophoresis power supply EPS 1001, *Western blot and SDS*
- Fisher blotting unit FEB10, *Western blot*
- Hoefer Pharmacia Biotech Inc. SE250, *SDS*
- Pharmacia, gel electrophoresis apparatus GNA-100, *agarose gel*
- Pharmacia, electrophoresis power supply LKS CPS 200/400, *agarose gel*

Molecular biology
- ABI Prisma™ 377 DNA sequencer
- Biometra™ Tgradient

Rocker platforms
- Bibby Gyro-rocker
- Mini Rocking platform, Biometra™

Spectrophotometer
- Cecil CE 2041
- Cecil CE 2021

Others
- Bioplex – Bio-Rad
- Dynex Technologies MRXII, *ELISA reader*
- Dual gel caster, Might™ Small SE245, Hoefer Pharmacia Biotech Inc., *SDS*
- Econo System (Bio-Rad) – 2110 fraction collector, *MAb purification*
- Fraction Recovery System 270-331580, Beckman, *virus purification*
- Jung biocut 2035, *microtome*
- Philips cooktronic 7910, *microwave*
- Refractometer 60-70 ABBE, Bellingham + Stanley Limited, *virus purification*
- Sanyo Super Showerwave, *microwave*
- Techne UB-8, *water bath*
- ThermoShadon citadel 2000, *tissue processor*