UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Division of Clinical and Experimental Sciences

Human and Murine Noroviruses: Detection, Replication and Epidemiology

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For the degree of Doctor of Philosophy

July 2013

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE
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HUMAN AND MURINE NOROVIRUSES: DETECTION, REPLICATION AND EPIDEMIOLOGY

By Simone Stacie Hadley

Noroviruses are members of the *Caliciviridae* family of positive strand RNA viruses and are one of the leading causes of non-bacterial gastroenteritis in humans. Currently no *in vitro* cell culture method has been reliably described, which has hindered the progress of norovirus research, leading to little understanding of the replication of these viruses.

The purpose of this study was to produce a reliable and sensitive detection assay for noroviruses in oysters, by incorporating a broadly cross-reactive monoclonal antibody. Bivalve shellfish are one of the main transmission routes of noroviruses from the environment to humans, especially when consumed raw. Noroviruses concentrate in the digestive tissue of bivalve shellfish, such as oysters as the shellfish filter feed.

In order to develop a detection assay, monoclonal antibodies against the current norovirus surrogate Murine norovirus (MNV, Genogroup V) and prevalent genogroup II noroviruses were produced. Five monoclonal antibodies detected MNV capsid protein and eleven detected GII.4 capsid protein by direct ELISA. Two of the GII.4 monoclonal antibodies showed cross-reactivity, CM160 to Southampton virus (GI) and both CM160 and CM162 detected Desert Shield virus (GII) in ELISA. No broadly cross reactive monoclonal antibody which detected a linear epitope in the capsid protein could be isolated.

Alongside development of monoclonal antibodies, chimeric MNV containing a pre-characterised human norovirus epitope was created. Monoclonal antibody CM54 had been previously isolated, which detected a linear epitope LEDVRN. This epitope was incorporated into the P2 domain of MNV. LEDVRN is a common epitope to GI capsid proteins but is not present in GII capsid proteins. This was an attempt to side-step the need for MNV/human norovirus cross-reactive monoclonal antibodies, which detected a linear epitope in the P domain of the capsid protein. No viable chimeric virus could be detected after transfection of Raw 264.7 and 293T cells.

Detection assays, ELISA, DELFIA and qRT-PCR were compared in the sensitivity of detecting norovirus in oyster digestive tissue, using a Lordsdale (LV) GII.4 monoclonal antibody and GII specific qPCR primers described by Kageyama *et al* (118). An antigen capture ELISA and DELFIA was developed using hyper-immune rabbit antiserum raised to recombinant LV capsid protein. Both assays were specific for LV in faecal samples. Preliminary experiments indicated that the DELFIA was four fold more sensitive than the ELISA.

Ten pacific oysters were analysed, all ten were negative by ELISA, seven positive in the DELFIA and all ten positive by qRT-PCR. This suggests that the majority (70%) of the oysters contained pre-existing norovirus protein or RNA; however these data provide no indication of infectivity. DELFIA demonstrates promise as a more sensitive alternative to the ELISA, while significantly cheaper than qRT-PCR.

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DECLARATION OF AUTHORSHIP

I, Simone Stacie Hadley

declare that the thesis entitled

Human and Murine Noroviruses: Detection, Replication and Epidemiology

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
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- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission.

Signed: ……..

Date:……

Acknowledgements

I would like to show my appreciation to my supervisors Professor Ian Clarke and Dr Paul Lambden for their advice, support, encouragement and belief in me throughout my PhD studentship.

I would like to thank the Food Standards Agency for funding this project.

My thanks go out to Shirley Sawers for providing a continued supply of sterile tips, glassware and other materials. As well as Lesley Cutcliffe, Rachel Skilton, Cynthia Prince, Clare Labrian, Colette O'Neill and Morgan Herod for their constant support, advice, listening ear, laughs and help in the lab during the course of my PhD; I couldn't have done it without your help, thank you!

Thank to my mum, Valerie; dad, David; grandparents, Jean and Ernest, Irene and Ken; my brother, Nic; friends and family for their love and unwavering support over the last four years.

Finally to my husband Christian and our beautiful children, Rhys and Eliza for all their love, hugs, kisses and willingness to proof read constantly, I'm not sure I would have made it this far without you. Thank you for everything.

Abbreviations

3’	3 Prime
5’	5 Prime
aa	Amino Acid
Amp	Ampicillian
APS	Ammonium persulphate
A_{550}	Absorbance at 550nm
bp(s)	Base Pairs
BSA	Bovine Serum Album
cDNA	Complementary deoxyribonucleic acid
cm	centimetre
CO_2	Carbon Dioxide
dH_2O	Deionised water
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Ds	double stranded
dATP	2’ deoxy adenosine5’ triphosphate
dCTP	2’ deoxy cytidine5’ triphosphate
dGTP	2’ deoxy guanosine5’ triphosphate
dNTP(s)	Deoxyribonucleoside(s)
ddNTP(s)	Dideoxyribonucleoside(s)
E.Coli	*Escherichia coli*
EDTA	Ethylenediaminetertra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
FCS	Foetal Calf Serum
FBS	Foetal Bovine system
FCV	Feline Calicivirus
g	Gram
H_2O	Water
H_2O_2	Hydrogen peroxide
Ig (G/A/M)	Immunoglobulin (G/A/M)
IMS	Industrial methylated spirits
IPTG	Isopropyl-D-thio-galactoside
Kb(s)	Kilobase(s)
Kbp(s)	Kilobase pair(s)
kDa	Kilodaltons
L	Litre
LB	Luria Bertani
LV	Lordsdale Virus
M	Molar
MCS	Multiple cloning site
MNV-1	Murine Norovirus
MOI	Multiplicity of Infection

mg(s)	milligram(s)
ml(s)	millilitre(s)
mM	millimolar
mm	millimetre
M_r	Relative molecular mass
mRNA	Messenger ribonucleic acid
MW	Molecular Weight
ng(s)	nanogram(s)
NV	Norwalk virus
nt(s)	nucleotide(s)
No:	Number
OD_{450}	Optical Density at 450 nanometres
ORF(s)	Open Reading Frame(s)
P Domain	Protruding Domain
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pi	post infection
psi	pounds per square inch
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcription PCR
S Domain	Shell domain
SDS	Sodium dodecyl sulphate
ss	Single stranded
SRSV	Small round structured virus
SV	Southampton Virus
T=3	Triangulation number 3
Temed	N, N, N'N' tetramethyl-ethylenediamine
T_m	Melting Temperature
Tris	Tris (hydroxymethyl)-methylamine
UHQ	Ultra high quality (water)
UV	Ultraviolet
V	Volts
VLPs	Virus-like particles
v/v	volume per unit volume
w/v	weight per unit volume
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometre

Chapter 1 Introduction

1.1 Historical Perspectives

Non-bacterial gastroenteritis is a major health concern worldwide, with the first documented report in 1929. Initially described as 'winter vomiting disease', this infection caused epidemics of severe vomiting and diarrhoea lasting between 2-4 weeks, mostly occurring in infants and young children (260). The duration of the symptoms varied from one to three days, with no deaths reported. This disease was suspected to be of viral origin since none of the known bacterial causes of gastroenteritis could be isolated from the clinical samples, despite the suspected viral cause; standard viral cell culture methods were unsuccessful in attempts to isolate and identify the virus. Since 1929, epidemics of non-bacterial gastroenteritis have been reported in the UK, across Europe and worldwide (22,239,246).

Between 1940 and 1970 several volunteer studies were performed using bacteria free filtrate obtained from patients with clinical symptoms. The filtrate was administered orally to the volunteers and was shown to cause the transmission of gastroenteritis (66).

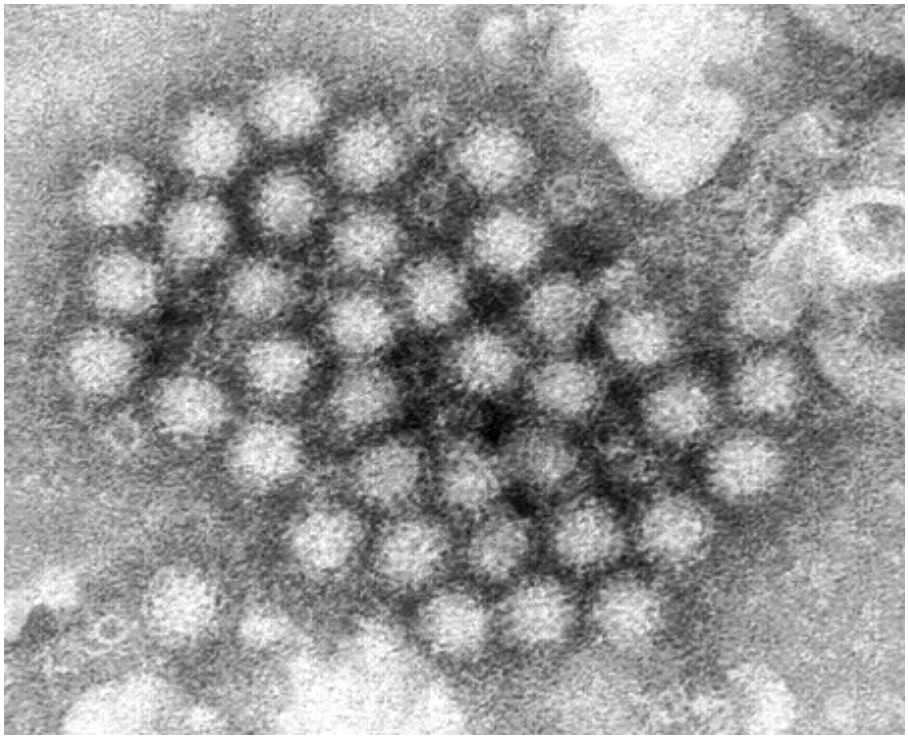
In 1972 a virus was found in faecal specimens collected during an outbreak of gastroenteritis in Norwalk, Ohio (120). It was reported that fifty percent of students and staff experienced symptoms of nausea, vomiting and abdominal pains for 12 -24 hours. A stool sample was obtained from an infected individual and serially passaged through volunteers. This filtrate induced symptoms of gastroenteritis in 6 out of the 10 volunteers (120).

Immune electron microscopy methods were used to reveal the causative agent, a virus which was measured as 27nm in diameter and became known as Norwalk Virus (NV). Norwalk virus has now been established as the prototypical member of the genus

Norovirus. Subsequent attempts to grow human noroviruses in cell culture failed, despite this the Norwalk virus was successfully cloned and characterised in 1990 from human stool samples (256). Further outbreaks of this virus were confirmed worldwide, the first norovirus outbreak in Australia was reported in 1978 and was associated with oyster consumption (180).

Figure 1 shows norovirus particles visualised by electron microscopy.

Figure 1. Electron microscope picture of norovirus particles. Source: CDC/Charles D. Humphrey



Until 2002, these viruses were known as 'Norwalk-like viruses' (NLV) and small round structured viruses (SRSV). After this period, norovirus became the official genus name, approved by an international committee after investigations into viral taxonomy.

Noroviruses belong to the *Caliciviridae* family of positive strand RNA viruses, due to their phylogenetic variation and genome organisation (71,150). Other members of this family include lagoviruses, vesiviruses, neboviruses and sapoviruses, with only sapoviruses and noroviruses causing gastroenteritis in humans. Vesiviruses include animal viruses, Primate

calicivirus (Pan-I), Vesicular exanthema of swine virus (VESV), San Miguel sea lion virus (SMSV) and Feline calicivirus (FCV). Lagoviruses are comprised of animal viruses, Rabbit hemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHSV). The genus Nebovirus contains a single species, Newbury-1 virus.

Calici is derived from the Latin word Calyx, or cup, this feature is common to the calicivirus group of viruses. Characteristic cup-shaped depressions can be seen on the virions when viewed by negative stain electron microscopy (37). One of the main features of the viral genome in this family is a 5' non-structural polyprotein preceding the single viral structural capsid protein (35,37).

Noroviruses are non-enveloped viruses with a ragged outline; they lack the distinctive structure of other Caliciviruses e.g. FCV and RHDV. These viruses have a buoyant density of 1.34 – 1.41g/cm^3 on caesium chloride gradient (80) which is similar to sapoviruses (1.33 – 1.38 g/cm^3) which also infect humans (231). Regardless of the fact that noroviruses lack the classical cup-shaped surface structure, genome analysis has confirmed these viruses are members of the *Caliciviridae* family (114,133).

A reliable *in vitro* cell culture method for human noroviruses has so far eluded researchers despite continued efforts (37). This has meant the replication and the characteristics of infection has stemmed predominately from related animal caliciviruses or from human volunteers infected with faecal filtrates. The discovery in 2004 of the murine norovirus (MNV) has provided an experimental system for the study of noroviruses. MNV remains the only member of the norovirus genus which can be propagated in *in vitro* cell culture (222). Until the discovery of MNV, Feline Calicivirus (FCV) was used almost exclusively as a surrogate for environmental studies on human noroviruses. FCV causes a respiratory illness in cats, so its use as a surrogate for human noroviruses (which cause gastroenteritis) is limited (11). Another surrogate was rabbit haemorrhagic disease (RHD) in the genus

Lagovirus *(19))* .This virus only affects rabbits and is characterised by high mortality rates. It was first recognised in 1984 in China where it caused symptoms of fever, diarrhoea and coma before death within 1-2 days so its use in investigations of human gastroenteritis are also limited (29). As a result of a lack of a cell culture method, molecular characterisation has formed the basis of studies into norovirus replication and mechanisms of infection.

It has been recently described that Tulane virus (TV), which is genetically closely related to human noroviruses and recognises the same receptors, may be a more suitable surrogate than MNV for human noroviruses. TV has been proposed to represent a novel calicivirus genus, Recovirus. TV is more sensitive to pressure treatment when seeded into oyster tissue than MNV, but shows the same sensitivity to pH being more stable in acidic conditions than neutral pH (151). However MNV still persists over a greater range of pH values (98).

1.2 Norovirus genus

The norovirus genus has been separated into five different genogroups; viruses in genogroups I, II and IV are associated with humans, genogroup III viruses with bovines and sheep while the recently discovered genogroup V viruses are associated with mice (15). The murine norovirus (genogroup V) has been shown to have the greatest phylogenetic distance from viruses in other genogroups, I-IV (261). The genogroups are based on phylogenetic variation of the sequences and genome organisation (>60% identity in VP1) (73,261). Each of these genogroups can be further sub-divided into genoclusters based on sequence similarity and phylogenetic analysis (>80% identity of VP1). Genogroup I (GI) contains 8 clusters and Genogroup II (GII) contains 17 clusters, both of these genogroups contain most of the diverse and common human noroviruses (261). Genotypes are further separated into strains based on specific amino acid changes in the P2 domain of VP1.

Figure 2 shows the phylogenetics of the norovirus capsid protein, demonstrating the wide diversity present across the genogroups of the norovirus genus.

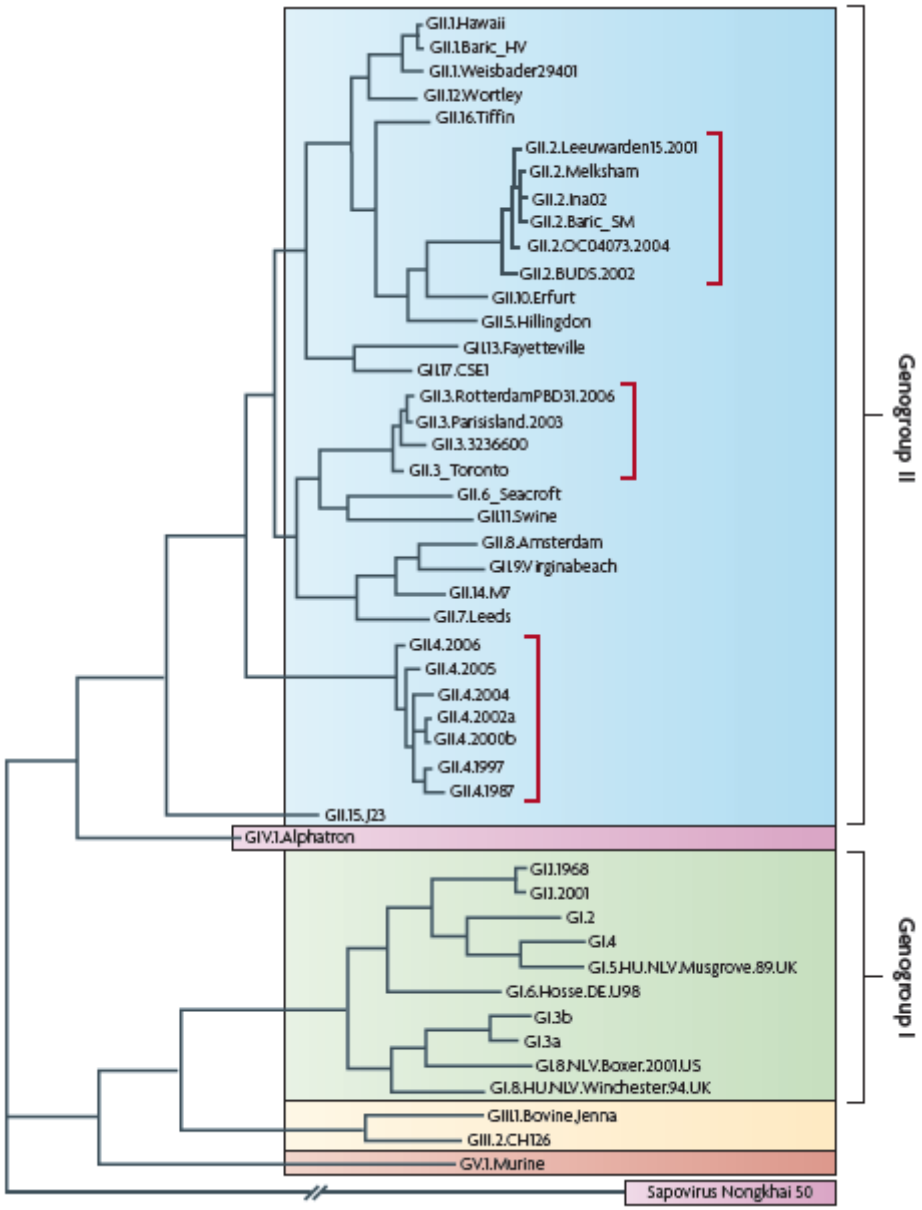


Figure 2. A Bayesian phylogenetic tree of norovirus capsid protein sequences from all major genogroups and genotypes. Image from E.Donaldson *et al* (47).

The different viruses are named after the geographical locations where the outbreaks occurred, such as Norwalk and Southampton viruses (GI).

1.3 Genome Organisation

Molecular characterisation of the norovirus genome was first undertaken in 1990 when Jiang and Estes cloned and partially sequenced the Norwalk virus genome (112). Then in 1993, Southampton virus was described and the complete genomic nucleotide sequence was determined (133). This new information led to dramatic progress in understanding of the genome organisation, diversity, epidemiology and pathogenesis of noroviruses.

Southampton virus (SV) was recovered from a diarrhoeic stool of a two year old child during a family outbreak of gastroenteritis in 1991 (133). Viral RNA was retrieved from the stool sample; subsequent synthesis of cDNA identified the absence of the conserved motif MMMASKD at the 5' terminus of the protein translation of SV ORF1. The SV genome has 7708 nucleotides and was the first GI norovirus to be fully sequenced (133). SV and Norwalk virus share 93% and 79% amino acid sequence similarity in the polymerase and capsid regions respectively. SV is rarely reported as causing infections, however GI noroviruses which are closely related SV are still detected (64,70,166).

Studies on the Southampton virus (GI) showed that the norovirus genome is a single-stranded, positive-sense RNA molecule; the virions are non-enveloped and approximately 27 to 35nm in diameter. The RNA genome is covalently linked to a virus protein (VPg) at the 5' end, polyadenylated at the 3' end and approximately 7.5 – 7.7kb in length (18).

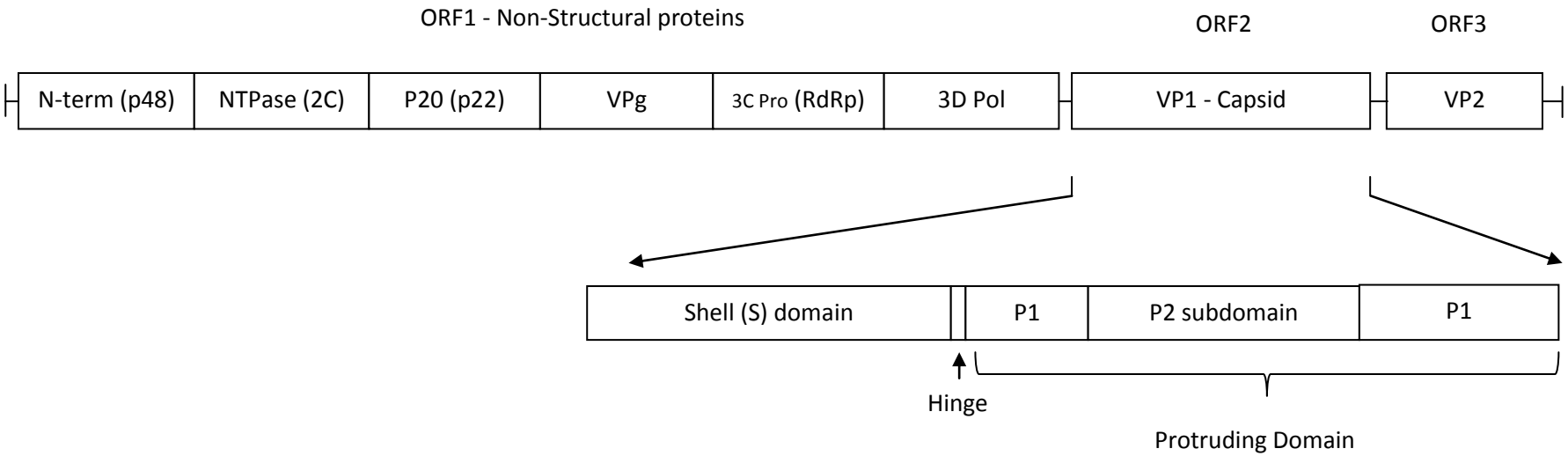
The genomic structure of the norovirus classifies it within the *Caliciviridae* family, as shown in Figure 3. It encodes three linear open reading frames (ORF). A polyprotein expressed from ORF1 is cleaved by a '3C-like' viral protease during a proteolytic cascade into six non-structural proteins; N-term, 2C (an NTPase), p22, VPg, 3C protease and 3D RNA-dependent

RNA polymerase (RdRp). The VPg protein is covalently linked to the 5’ end of the viral RNA and has been suggested to play a role in translation initiation (32,76,222). ORF1 in noroviruses is located at nucleotide 20 encoding a long polyprotein of 1763 amino acids (114).

A comparison of GI and GII noroviruses has shown that their genomes differ in size; GI noroviruses are 7.7kb whilst GII are 7.5kb in length. This difference in size is a result of GII viruses having a smaller ORF1 than GI noroviruses (156). Alongside this GII noroviruses also show significant sequence diversity at the 5’ end when compared to the sequence of GI viruses. It has been reported that this is the cause of differences in secondary structure and regulatory signals present in the GII viruses (36).

Many of the non-structural proteins have been named because of sequence similarities with the non-structural proteins of picornaviruses (54,189). There are significant differences between picornaviruses and caliciviruses in the way in which they encode their structural proteins. *Caliciviridae* genomes encode non-structural proteins at the 5’ end of the genome and structural proteins at the 3’ end whereas picornaviruses encode these the opposite way round (114). *Caliciviridae* genomes have multiple ORFs and the picornaviruses have a single ORF that encodes all the structural and non-structural proteins.

Figure 3. The norovirus genomic structure, displaying the non-structural proteins and capsid domains (46)



ORF1 overlaps with the 5' end of ORF2 and this junction is highly conserved within the norovirus genome. ORF2 encodes the major capsid protein VP1 of approximately 60 kDa and ORF3 encodes the small basic protein VP2. VP2 has been associated with capsid stability and is required for the production of infectious particles (94,221). A VPg-linked 2.3kb sub genomic RNA containing ORF2, ORF3 and the 3' untranslated region (UTR) is produced in abundance during norovirus replication (4,123,255). It is this sub genomic RNA, rather than the genomic RNA which is used as the template for the capsid protein (4,76). The 5' end of the genomic and sub genomic RNA contains a conserved motif upstream from the initial start codon, which is thought to be the site for the initiation of transcription (100).

As shown in Figure 3, VP1 folds into the two major domains of the capsid, the shell (S) domain connected by a flexible hinge to a protruding (P) domain (198). The P domain is divided further into P1 and P2, with the P2 domain forming the surface exposed region. Studies in immune-compromised patients, who chronically shed norovirus, have shown that the majority of mutations occur within the P2 region (43). The S domain forms the inner part of the capsid that surrounds the RNA genome and is involved in forming the icosahedral shell. The S domain consists of the N terminal 225 amino acid residues and is a highly conserved area in the norovirus genome (124,198,261). The X-ray crystallographic structure of the norovirus capsid region has been resolved to show that it consists of 90 dimers of a single protein with a T=3 icosahedral symmetry (15). This crystal data demonstrated that the capsid protein was comprised of two principal domains, the shell and protruding domains.

Surface exposed residues such as in the P2 domain (which forms the hyper-variable region), mutate frequently, this is most likely because of immune surveillance by the host. The hyper-variable sequence of the P2 domain is thought to be responsible for determinants of

strain specificity (198). It has been demonstrated that a few amino acid changes within the P1 or P2 sub-domains can result in a substantial change in receptor usage and antigenicity, allowing the noroviruses to persist within the human population (46).

The capsid sequence varies by up to 60% between the five genogroups and up to 57% in noroviruses which infect humans. This illustrates the broad diversity amongst the noroviruses in the capsid region of the genome. It also suggests mutations in the capsid protein (particularly the P2 domain) are vital for noroviruses to persist in the human population and successfully avoid the host immune system. This level of diversity is much higher than that seen in other positive single sense stranded RNA viruses making it difficult for the immune system to detect the viral capsid of the different noroviruses (261,262,264).

The development of the reverse transcription-polymerase chain reaction (RT-PCR) and genomic sequencing has demonstrated that noroviruses are genetically diverse (193). Due to the great diversity of nucleotide sequences throughout the entire virus genome and extreme variation in the capsid protein, currently no molecular techniques can detect all types of norovirus in a single diagnostic test (257).

The genetic diversity is likely to stem from the norovirus RNA polymerase which lacks proof-reading ability, meaning during rapid replication mutations frequently occur (43). Most RNA viruses evolve at a rate of approximately 10^{-3} nucleotide substitutions/site/year (50) however the rate of evolution for GII.4 noroviruses is 4.3×10^{-3} nucleotide substitutions/site/year (18). This high level of mutation gives rise to norovirus diversity; this means that further infection of the same individual after a short period of time with a new variant is possible despite previous norovirus infection (46,251).

1.4 Histo-blood group antigen receptors

Norwalk virus (GI) virus-like particles (VLPs) have been found to bind to histo-blood group antigens (HBGAs) in intestinal tissues expressing H antigen (108) and have been indicated to function as receptors or co-receptors for a productive norovirus infection (93,152,202). There has been no direct evidence that viral binding to HBGAs facilitates entry. HBGAs are complex carbohydrates which are linked to glycoprotein's present on both red blood cells and mucosal epithelial cells; these include H type, the ABO blood group and Lewis carbohydrates. HBGAs are also expressed in a secreted form in saliva, milk and intestinal contents. Due to the high genetic diversity noroviruses display in the P domain, it has been shown that a single amino acid change in this region can result in HBGA binding pattern changes (229). The wide genetic diversity of noroviruses means there is also a large variation in the way noroviruses bind to HBGA (104).

HBGAs are markers of genetic susceptibility and resistance for norovirus infection (107,152). Individuals with no HBGAs on their intestinal mucosa are referred to as non-secretors and have been shown to be resistant to norovirus infection (107,152). It has been reported that approximately 80% of the European and North American population are secretors. Secretors carry the fucosyltransferase2 gene (FUT2) which is responsible for encoding the H1 and H3 antigens, which when present on the mucosal surface have been shown to bind GI.1 norovirus VLPs (167).

The discovery of HBGAs has led to development of receptor-binding assays in which noroviruses or norovirus VLPs bind to synthetic immobilised HBGAs, allowing infectivity studies to be undertaken (90,91,103).

Recent studies have suggested that people with blood group O are more susceptible to GI.1 norovirus infection than those of other blood groups (107). The recent co-crystallisation of the norovirus P domain with HBGAs has provided further evidence of the receptor

molecules in the P2 domain of the capsid protein (28). These advances are hoped to contribute to a full understanding of the method of replication and infection of these viruses.

1.5 Clinical Features

Noroviruses are the world's leading cause of epidemic, acute, non-bacterial gastroenteritis and are transmitted directly from person to person via the faecal – oral route or indirectly by contaminated food, water or environmental surfaces. They regularly cause outbreaks in semi-closed communities such as cruise ships, hospitals, nursing homes and affect people of all ages. They are able to cause these outbreaks within the communities due to their high infectivity (<10 virions per individual) and their stability in the environment (112,255). Noroviruses contribute to more than 95% of non-bacterial gastroenteritis (55,56). Norovirus infection can be separated from bacterial gastroenteritis by the lack of blood, mucus or high white cell content in the diarrhoea.

Following infection, virus replication occurs in the mucosal epithelium of the small intestine leading to broadening and blunting of the villi, as well as shortening of the microvilli and subsequent reduction in water absorption while the mucosa remains intact (211). These effects do not seem to be present in the large intestine. The onset of symptoms occurs between 12 – 24 hours after exposure. Symptoms of diarrhoea and vomiting usually resolve within 2-3 days, vomiting is more common in children, while diarrhoea is often seen in adults (1,67,121). These viruses are self-limiting although human norovirus can be shed for up to three weeks post infection (135,255). Other symptoms can include fever, headaches and abdominal pain (190). Despite the lessening of symptoms after 3 days, the prolonged viral shedding is thought to contribute to the spread of the virus within communities (68). The different strains of human noroviruses have clinically indistinguishable symptoms as observed in outbreaks and human volunteer studies (177).

Medical intervention, in the majority of cases is not required, however in the very young or elderly, fluid replacement and additional care maybe required. The majority of outbreaks within the UK are found to be related to Lordsdale virus, GII.4, this genotype has been associated with outbreaks as early as 1974 (57). Worldwide GII.4 epidemics are widespread for example; Farmington Hills virus caused epidemics of norovirus across Europe and the United States from 2002 (43,159,252), Hunter virus was prevalent across Australia, New Zealand and Japan from 2004 (24) and subsequent descendants 2006a and 2006b viruses have become widespread in Australia and Europe in recent years (239). The centres for Disease Control and Prevention have reported that noroviruses account for 73% of all viral gastroenteritis cases within the United States (53). Seroprevalance studies in humans have shown that in over 90% of the people analysed in certain areas had antibodies towards norovirus, indicating the worldwide prevalence of these viruses (160,184). Short term immunity has been described in volunteers infected with norovirus experimentally and re-challenged with the homologous virus after 6 – 14 weeks, which failed to induce illness (44,116,168,192). Long term immunity could not be induced even after challenge with the same norovirus 27 – 42 months after the initial infection (168,194).

Since the report of short term immunity, it has been put forward that a rapid mucosal IgA response may play a part in preventing infection (152). Attempts to develop a vaccination programme using VLPs to prevent norovirus infection are on-going (12,52,81,92).

Recent data have shown that GII noroviruses have been the predominant genogroup for several decades in the human population (20,219). Two mechanisms give rise to GII.4 viruses persisting in the human population. Firstly, changes in and around the receptor-binding domain can lead to altered histo-blood group antigen (HBGA) binding. Secondly, changes on the outer capsid (P2) surface can alter antigenicity, allowing the virus to escape from pre-existing herd immunity (46).

1.6 Stability and disinfection of noroviruses

The ease at which these viruses spread despite continued and aggressive cleaning, demonstrates the stability of these viruses. Reports of repeated norovirus infections in a hotel over a six month period and cruise ship over six consecutive cruises (34,82,109,250) further suggests at the stability and low infectious dose of these viruses.

Norovirus surrogate Feline Calicivirus (FCV) and MNV have been used extensively in *in vitro* disinfection studies, due to a reliable cell culture method. It was shown that Sodium hypochlorite concentrations of 5000 ppm for 1 minute or 3000 ppm for 10 minutes were able to inactivate FCV (49,51). MNV has been shown to be inactivated at temperatures above 63°C (27). It is important to note that it is difficult to translate studies using surrogates to human noroviruses as they cause different clinical symptoms and FCV has a different genomic structure. Studies using human noroviruses have shown that standard chlorination levels in drinking water do not prevent infection (175). Clinical samples positive for norovirus exposed to either a pH 2.7 for 3 hours at room temperature or 60°C for 30 minutes could still cause and infection in human volunteers (45).

1.7 Norovirus concentration within Oyster tissue

A number of foods have been implicated in outbreaks of norovirus gastroenteritis, such as shellfish, raspberries, sandwiches and salads. Norovirus is thought to contribute to an estimated 30 – 50% of all foodborne outbreaks of gastroenteritis (253). As shellfish are primarily grown on the coast in shallow water and feed by filtration of several litres of sea water daily (which could potentially be contaminated by untreated sewage), they are a common vehicle for norovirus transmission (25). The risk of contamination is particularly high following heavy rain fall but can also occur due to contamination by an infectious food handler (135). Shellfish have been shown to be one of the most common vehicles of outbreaks involving multiple norovirus strains at the same time (21,119,135,139,241).

Bivalve molluscan shellfish are the only shellfish which have consistently been proven to concentrate norovirus within their tissues (142). Viral contamination of water can occur by release of inadequately treated sewage water or sewage into the water pool, as well as exposure to vomit soiled hands, soiled clothing or contaminated surfaces. The uptake of virus by shellfish in sewage-contaminated waters occurs rapidly, within 24 hours of exposure (25,48,142,210). In 1998 the first clear link was established between clinical and environmental samples in a Californian outbreak in which the same norovirus sequence was retrieved in two separate shellfish samples and one clinical sample (139). Viral contamination in shellfish can persist for several weeks in the harvest area before disappearing which can lead to multiple shellfish harvests accumulating multiple noroviruses (139). Only noroviruses and hepatitis A virus (HAV) have been linked to outbreaks involving shellfish consumption (41,134,226).

Norovirus particles can survive in shellfish tissue for several months either by ionic binding or specific attachment (25,137). The accumulation of viruses within the shellfish tissue during feeding is due to the ionic binding of viral particles to the mucopolysaccharide moiety of shellfish mucus. Several factors such as water temperature, mucus production and glycogen content of connective tissue can affect virus accumulation in oysters (25).

Shellfish are regularly eaten raw or at best lightly cooked; this increases the risk of infectious illness by bacteria or viruses which would have been inactivated by high temperatures. The absence of any cooking has been shown to be the contributing factor for greater than 90% of shellfish outbreaks (25,249).

The number of illnesses which occur due to foodborne sources are often underestimated, as not all outbreaks are recognized or reported due to the short period of illness (249). Seafood is involved in an estimated 11% of foodborne outbreaks in the United States, 20%

in Australia and over 70% in Japan. The high level of outbreaks in Japan is due to the Japanese tradition of eating raw seafood (212).

1.8 Oyster Structure

Oysters produce their own shell secreting a composition of 95% calcium carbonate. Within this double hinged shell is a soft-bodied invertebrate, growing in predominately coastal waters.

Organs involved in the ingestion and digestion of food and the elimination of faeces include the mouth, a short oesophagus, stomach, crystalline style sac, digestive diverticula, midgut, rectum and anus; these are collectively known as the digestive tissues (164). These organs are covered by a mantle, consisting of a sheet of connective tissue and muscle. The mantle receives sensory stimuli in conjunction with the nervous system and is part of the process of shedding and dispensing eggs. Figure 4 shows the digestive system of an oyster. In the digestive diverticula, noroviruses accumulate during feeding in sewage contaminated waters.

Intracellular digestion takes place in the stomach and surrounding hepatopancreas, also known as the digestive diverticula in shellfish. The digestive diverticula are made of several blind tubules which lead to a number of large ducts and empty into the interior of the stomach. The stomach itself is entirely surrounded by the digestive gland.

Selective filter feeding involves several mechanisms including; particle retention, pre-ingestive selection and differential absorption. Oysters in particular use particle retention to distinguish between particle sizes and can capture particles which range in size from 100-200 μm. This range includes small organisms such as phytoplankton, zooplankton, and protists. The oyster gills are responsible for differentiating between organic and inorganic particles before passage onto the gut which absorbs microalgal species.

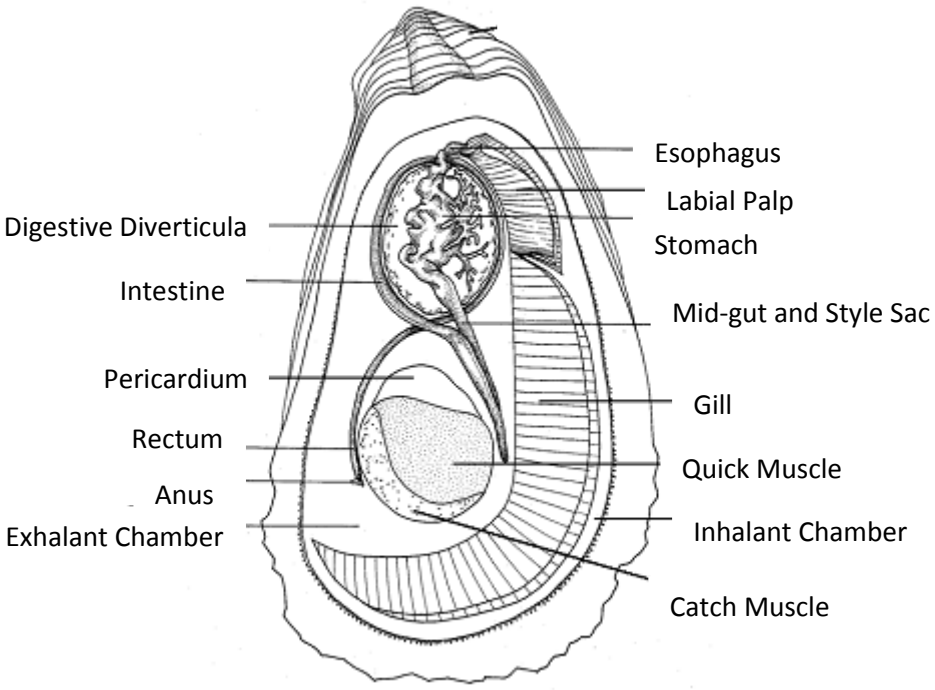


Figure 4. Digestive system of the oyster, C.virginica taken from Galtsoff, P.S., 1964. The American oyster, Crassostrea virginica Gmelin. US Fish. Wildlife Ser, Fish. Bull 64:1-480.

1.9 Laboratory Diagnosis

Development of diagnostic assays have been hampered by the lack of a simple cell culture system in which viable norovirus can be detected (51). These problems are exacerbated by the absence of an animal species which can be experimentally infected with the human virus.

Immuno incompetent newborn pigtail macaques (*Macaca nemestrina*) did display characteristic symptoms of diarrhoea, dehydration and vomiting when infected with a faecal filtrate of Toronto virus. Viral RNA could be detected in all three monkeys, and the virus transmitted to a further two monkeys by administration of faecal filtrate from the previously infected animals (225).

Until a suitable culture or animal model can be described, molecular diagnostic methods are the greatest tool in detecting norovirus infection. Early biopsy on norovirus infection indicated that norovirus infection targeted the small intestine (45,207,224). It has been recently described by Straub *et al* that noroviruses can infect and replicate in a 3D model of the human small intestinal epithelium. Demonstration of infection was shown by microscopy, PCR and fluorescent in situ hybridization. The human intestinal epithelium cells were maintained on collagen coated micro carrier beads in rotating bioreactors (224).

Other attempts to cultivate noroviruses have not been successful (51) and the model described by Straub *et al* has not been successfully validated in other laboratories despite numerous efforts.

1.9.1.1 Electron Microscopy

For many years electron microscopy (EM) was the only technique available for the diagnosis of norovirus infections. Electron microscopy is relatively insensitive as virus particles can be difficult to visualise due to their small irregular size and low abundance in

stool samples (57,142). The sensitivity of EM detection is low as it requires at least 10^6 viral particles per ml of stool to detect viruses (118). Immune EM attempted to improve sensitivity; however it was still unable to detect norovirus in many experimentally infected volunteers (234).

1.9.1.2 Reverse Transcription PCR (RT-PCR)

The description of the Southampton and Norwalk genomes expanded the range of molecular diagnostic tests available for norovirus exponentially. However RT-PCR is unable to detect all genogroups or genotypes of norovirus in one assay due to the high level of diversity in the norovirus genome (257). RT-PCR is currently one of the most sensitive and broadly reactive diagnostic tests for noroviruses, because it is able to detect very low levels of viral nucleic acid in a sample (57).

The majority of RT-PCR assays target the RNA polymerase gene or the capsid protein sequences (in particular the shell domain) as these regions are well conserved amongst the different strains of norovirus. RT-PCR is important for the detection of noroviruses in clinical and environmental specimens as it is able to detect viral nucleic acids from food, water and faecal samples (144,209,247). RT-PCR followed by nucleotide sequencing can identify the point-source of infection which is important in preventing further outbreaks (193). Despite the sensitivity of RT-PCR, the long extraction procedure, relative expense and technical expertise required has meant that it does not fit into the routine diagnostic screening of noroviruses within small scale laboratories (57). A faster and more sensitive Real-Time quantitative RT-PCR (qRT-PCR) has recently been developed for rapid detection of norovirus in large numbers of stool samples during epidemics (193). This method is capable of quantifying the number of virus copies in a particular sample and has become the gold standard of diagnostic assays for norovirus. Trujillo *et al* demonstrated that an

adapted TaqMan assay had a higher sensitivity compared to conventional RT-PCR assays with the ability to detect virus in water previously found negative by RT-PCR (238).

qRT-PCR is sensitive, due to the ability of designing specific primers for a specific norovirus; despite this, currently it has not been possible to detect both GI and GII viruses within the same reaction.

1.9.1.3 Enzyme linked immunosorbent assay (ELISA)

Prior to the development of enzyme linked immunosorbent assays, radioimmune assays were employed for the detection of noroviruses in clinical samples but despite this being a highly sensitive and simple procedure, the use of radioisotopes and special equipment has limited its widespread application (182). An ELISA by comparison is inexpensive and simple to perform, while maintaining equivalent sensitivity to the radioimmune assays (182). ELISAs have been used extensively in epidemiological studies of noroviruses (96,165,182).

Advances in expression of norovirus capsid proteins using the baculovirus expression system has led to improved diagnostic ELISAs; using polyclonal sera for detection in clinical samples. Many noroviruses have now been expressed using this system, leading to an unlimited supply of capsid antigens, known as virus like particles (VLPs). VLPs can be used as an immunogen to obtain polyclonal antisera, which is used to develop antigen capture ELISAs for noroviruses. Polyclonal antiserum does have the disadvantage of being batch dependent. Therefore the production of monoclonal antibodies is important in generating cross-reacting antibodies directed towards a common norovirus epitope, which will produce the same results in different laboratories. Monoclonal antibodies can be expanded and stored to supply a continuous reliable antibody source.

ELISAs have been utilized as a broad routine diagnostic tool, used to determine norovirus infection and to determine serum antibody concentrations such as in the HIV and West Nile

virus tests. ELISAs have been developed to detect a broad spectrum of diseases such as Malaria, Rotavirus, Hepatitis B, Chagas disease and enterotoxin of *E.Coli.* It is also used in the food industry for detecting potential food allergens such as milk, peanuts, walnuts and eggs.

There are several commercially available ELISA kits which have been developed for detecting Norwalk-like virus antigen in faecal samples, such as IDEIA NLV ELISA (Dako, Cytomation, Ely, UK). When compared with RT-PCR the ELISA had a sensitivity and specificity of 55.5 and 98.8% respectively (200). There is also RIDASCREEN Norovirus (R-Biopharm) which has been shown to detect 80.3% of norovirus-infected faeces samples compared to 60.6% by IDEIA NLV ELISA (30). The RIDASCREEN was the first test permitted for marketing for norovirus detection by the U.S Food and Drug administration (FDA).

Two different types of ELISA have been developed for use in this project; antigen capture and direct ELISA. The antigen capture (sandwich) ELISA utilises hyper-immune sera which is immobilised on the plate surface and binds to the required antigen in the sample. Without hyper-immune sera, any proteins present in the sample competitively absorb to the plate surface, lowering the quantity of antigen immobilised. This type of assay is useful when the antigen is in complex mixtures, particularly faecal samples as it eliminates the need to purify the antigen. Bound antigen is detected using either a murine or human norovirus specific monoclonal antibody. The antibody-antigen complex is detected using a horseradish peroxidise conjugated antibody directed against mouse IgG. The enzyme substrate (TMB/H_2O_2) is added to the wells to give a coloured reaction which could be colourimetrically quantified at a wavelength of 450nm and compared directly with other samples. Positive and negative controls are easily incorporated into the assay.

In a direct ELISA, the antigen such as human VLPs or cell lysates bind directly to the plate surface, the detection antibody then forms an antibody-antigen complex which can be

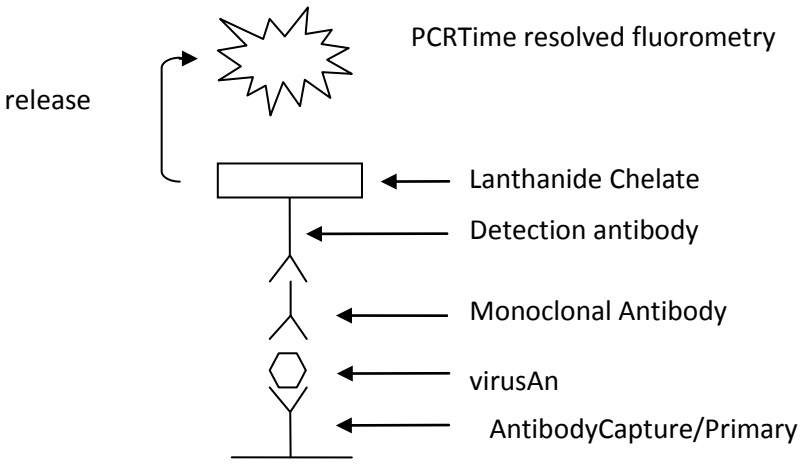
detected. This assay is designed for use with purified antigen samples and can be used to measure either antigen or antibody binding capabilities.

1.9.1.4 Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA)

The DELFIA assay follows a similar principle to the antigen capture ELISA until the addition of a HRP labelled conjugate antibody. Instead a lanthanide chelate is used which allows highly specific labelling activity without decreasing affinity or immunoreactivity. It is this chelate which is the main difference between the ELISA and DELFIA. The lanthanide is labelled with a Europium (Eu) molecule and is practically non-fluorescent; however after binding with the detection antibody is complete, fluorescence is developed with the addition of an enhancer solution. The low pH of the enhancer dissociates the fluorescent molecule, Eu allowing it to rapidly form a fluorescent chelate which can be quantified on a plate reader at an excitation wavelength of 615nm. The lack of an enzymatic reaction contributes to an increase in sensitivity seen in the DELFIA when compared to ELISA. The set-up of the DELFIA assay is shown in Figure 5.

DELFIA is currently used as a diagnostic test for thyroid, cancer and growth hormone screening. There are commercially available automated systems for the DELFIA on 96 well plates, such as AutoDELFIA (Perkin Elmer) which is an automatic immunoassay system designed for routine diagnostics or screening laboratories. This system performs all sample and reagent handling including measurement of fluorescence automatically.

Figure 5. The DELFIA components. This figure indicates the composition of the antibodies and antigen within the wells on the assay plate.



1.10 Murine Norovirus (MNV)

The discovery of murine norovirus has opened new avenues into the research of replication strategy of norovirus developing new diagnostic assays. MNV was discovered in immuno-compromised mice, which lacked the recombination activity gene 2 (RAG2) as well as the signal transducer and activator of transcription 1 (STAT-1); these are known as (RAG 2/STAT-1 $^{-/-}$) mice. The (RAG2/STAT-1 $^{-/-}$) mice succumbed to a pathogen which could be serially passed by intracerebral inoculation. The brain tissue from these infected mice was discovered to be negative for known human and murine pathogens, which suggested that the RAG/STAT $^{-/-}$ mice were succumbing to a previously uncharacterised IFN sensitive virus. Karst *et al* retrieved sequence data from this virus which were homologous to regions of previously published Calicivirus genomes (122). This pathogen was more virulent in mice lacking both the interferon αβ and interferon γ receptors (IFN αβγR$^{-/-}$) compared to wild-type mice and was subsequently named MNV-1 (122).

The recombination activating gene (RAG) is essential for the generation of mature B and T lymphocytes, which form components of the adaptive immune system. These genes encode proteins RAG1 and RAG2 which enable the recombination of VDJ genes, part of the generation of pre B and T cells.

The signal transducer and activator of transcription (STAT) proteins are a family of latent cytoplasmic proteins, involved in the development and function of the immune system and are important in maintaining immune tolerance, demonstrated through many gene knockout studies in mice. These proteins are activated when cells encounter numerous extracellular polypeptides.

In 2003, Wobus *et al* demonstrated that MNV-1 was able to infect macrophage-like cells *in vivo* and replicate in cultured primary dendritic cells and bone-marrow derived macrophages (254). The murine cell line, RAW 264.7 is a macrophage cell line which, when

infected with MNV-1, showed a visible cytopathic effect (CPE). When MNV-infected RAW 264.7 monolayers were maintained under agarose, plaques could be observed which then allowed the development of assays to quantify live virus titres. The research by Wobus *et al*, has suggested that the role of the innate immune system during MNV infection could go some way to explaining the short clinical symptoms of the human norovirus disease, due to the rapid clearance of MNV-1 infection in immune-competent mice (255). The MNV infection was cleared before the initiation of the adaptive immunity (179). Further studies have shown that MDA5 (part of the RIG-I like receptor (RLR) family) is the predominant sensor of MNV-1 and initiates the innate immune response (170). Mice have been shown to have prolonged faecal shedding and can become persistently infected with MNV (101).

MDA5 (Melanoma Differentiation associated protein 5) functions as a pattern recognition receptor and a sensor for viruses. MDA5 is a cytoplasmic DEx(D/H) box helicase that can detect intracellular viral products such as genomic RNA and signal the downstream production of IFN – α/β in infected cells (259). Homotypic capsid activation and recruitment domain (CARD) interactions with the interferon promoter-stimulating factor (IPS-1) adaptor protein (127,176,214) recruit RIG-1 and MDA5 to the outer membrane of the mitochondria which aims to activate downstream interferon-regulatory factors (IRFs) that induce IFN-α/β and ISG expression (115). Signalling by MDA5 is triggered during picornavirus infections or in the presence of a synthetic RNA polymer consisting of annealing strands of inosine and cytosine, poly (I:C) (63,125).

MNV has a similar positive-sense single stranded RNA genome to human noroviruses, encoding three major ORFs with a minor ORF4 recently discovered, though its function is still unknown (232,251). The similarity in the capsid structure, genomic organisation and replication cycle to the human norovirus has made it a useful surrogate in research (16,222). The P domain differs between MNV and other Calicivirus structures as it is lifted

off the S domain by ~16Å and rotated ~40° in a clockwise direction, creating a new interaction at the P1 base forming a cage-like structure engulfing the shell domains (126). MNV can be productively replicated in cultured macrophages and dendritic cells (86). At the time of writing, over forty murine norovirus strains have been isolated from laboratory mice (13,101,130).

1.11 Aims

Currently, many widespread norovirus outbreaks are due to food that is contaminated before distribution and can go undetected because assays for detecting noroviruses in food are insensitive and not widely used (193). Methods for detecting noroviruses in food are crucial for both preventing infection and to investigate the cause and point source of the contamination.

The overall aim of this project is to develop a sensitive antigen detection system for noroviruses in shellfish; there were a series of objectives designed to help achieve this aim;

- To produce a broadly-reactive monoclonal antibody with cross-reacting properties to human and murine VLPs which would be incorporated into developing an immunoassay to give an indication of the risk of norovirus exposure in shellfish to the human population.
- If broadly cross reactive monoclonal antibodies could not be isolated, an existing GI norovirus monoclonal antibody epitope will be incorporated into MNV capsid sequence. This would side step the requirement for MNV monoclonal antibodies as an existing antibody can be utilised. It is hoped that viable chimeric virus will be propagated.
- To compare and refine existing detection techniques; enzyme-linked immunosorbent assay (ELISA), quantitative RT-PCR (qRT-PCR) and dissociation

enhanced lanthanide fluorescent-immunoassay (DELFIA) for detecting and typing human noroviruses seeded into shellfish tissue.

- To develop a cost effective and simple colourimetric test in the form of an ELISA or DELFIA which may be used in a routine diagnostic food laboratory to detect human norovirus in contaminated shellfish.

Chapter 2 Materials and Methods

2.1 Chemicals and Solutions

2.1.1 Water

General purpose solutions were prepared using deionised water (dH_2O) produced by reverse osmosis using the SG reverse-osmosis-system euRO^{TM} (Triple Red Ltd). Solutions for nucleic acid manipulations were prepared using the Barnstead/Thermolyne NANOpure® Diamond^{TM} Life Science (UV/UF) ultrapure water system (Triple Red Ltd). This Ultra High Quality water (UHQ H_2O) was produced by a four stage deionisation process combined with a UV lamp, an ultrafilter and a 0.2 micron filter. The UHQ H_2O was guaranteed to be nuclease-free and DNA-free by the manufacturer.

2.1.2 Chemicals and Enzymes

Chemicals were purchased as Merck grade AnalaR® or equivalent unless otherwise stated. Bacterial culture medium was purchased from Difco laboratories, Detroit, USA. Nucleic acid modification enzymes, including restriction endonucleases, were purchased from Sigma-Aldrich (Poole), Life Technologies (Middlesex [Gibco BRL/Invitrogen]), Promega (Southampton), New England Biolabs Inc (Beverly, USA) and Bioline (London).

All chemicals and enzymes were stored and handled as recommended by the manufacturer.

2.1.3 Sterilisation

Bacterial growth media and solutions were sterilised by autoclaving at 15psi for 15minutes. Antibiotics, vitamins and other heat sensitive solutions were sterilised by filtration using a 0.22µm Millipore filter.

2.1.4 Plastics and Glassware

Disposable polypropylene 1.5ml tubes were purchased from Sarstedt Inc (UK) and autoclaved at 18psi for 30 minutes to minimise the possibility of contamination by nucleases.

Nuclease free 200µl PCR tubes were purchased from Axygen (Thistle, UK). Non-sterile 200µl and 1000µl pipette tips were purchased from Alpha Laboratories Ltd. Sterile, nuclease-free, DNase-free, and Pyrogen free filtered pipette tips (10µl, 20µl, 200µl, 1000µl tips) were purchased from Axygen (Thistle, UK).

Glassware was sterilised by heating to 160°C for at least 1 hour. Sterile plastics such as universals, bijoux and Petri dishes were purchased from Sterilin (UK).

2.2 Bacterial Growth Medium and Solutions

2.2.1 Luria Bertani (LB) medium

LB medium was prepared for the growth of *Escherichia coli* cultures. Bacto-tryptone (10g/l), Bacto-yeast extract (5g/l) and NaCl (10g/l) were dissolved in dH_2O and the pH adjusted to 7.4 with 5M NaOH. The medium was autoclaved immediately and stored at room temperature.

2.2.2 LB Agar Plates

LB agar plates were used for the growth and maintenance of *E.coli* strains listed in Table 1. 1.5% (w/v) Bacto-agar (Difco) was added to LB media, autoclaved, cooled to 50°C and poured into sterile plastic Petri-dishes of 90mm diameter. The agar plates were allowed to set at room temperature and then the agar surface was air-dried in an incubator at 45-50^{o}C. Unused plates were stored inverted at 4^{o}C for a maximum of two weeks.

2.2.3 Antibiotics

Ampicillin (Sodium salt, Sigma-Aldrich, (Poole)) was dissolved in UHQ H_2O to a concentration of 25mg/ml, sterilised by filtration through a 0.2 micron filter and aliquoted out into 1.5ml sterile eppendorf tubes and stored at -20°C until use.

2.2.4 LB Agar and Medium with Antibiotics

LB-Ampicillin medium was used to maintain the growth of Ampicillin resistant *E.coli.* Ampicillin (2.2.3) was added to molten LB agar (2.2.2) at final concentrations of 50µg/ml immediately prior to use. All agar plates were poured, dried and stored as described previously.

2.3 Bacteria, Bacteriophage and Plasmids

2.3.1 Growth and storage of E.coli

Strains were kept as working stocks on LB agar at 4°C. *E.coli* strains carrying plasmid encoded resistance were maintained at 4°C in LB agar plates containing the required antibiotic.

Liquid cultures of *E.coli* were grown in LB broth (2.2.1) overnight at 37°C at 200 x g on a shaker. For long term storage, a suspension of each strain was stored at -80°C in LB medium containing 10% (v/v) sterile glycerol.

Various methods required a static overnight starter culture of the appropriate *E.coli* strain. The starter culture was prepared by inoculating 5ml of LB broth with the required *E.coli* strain and incubated at 37°C overnight with shaking at 200 x g.

2.3.2 Preparation of Competent E.coli

The transformation efficiency of *E.coli* is dependent upon the maintenance of all reagents and materials at 4°C. A 5ml static LB broth pre-culture of the recipient *E.coli* strain was

grown overnight at 37°C. A 1ml aliquot of the starter culture was used to inoculate 25ml of LB broth and incubated on a shaker at 200 x g at 37°C until the A_{550nm} value reached 0.4-0.5. The culture was then placed on ice for 20 minutes and all subsequent procedures performed at 4°C.

Cells were pelleted by centrifugation at 3000 x g for 10 minutes and the supernatant discarded. The bacterial pellet was suspended in 12ml of ice-cold 0.1M $MgCl_2$ and centrifuged as before. The supernatant was discarded and the bacterial pellet resuspended in 1ml of ice-cold 0.1M $CaCl_2$. The *E.coli* was kept on ice for a minimum of 1 hour before use as competent cells.

For long term storage, sterile glycerol (15% w/v) was added to the competent cells. Aliquots (300µl) of competent cells were stored at -80°C and were thawed on ice prior to transformation.

2.3.3 Transformation of Competent Cells

Competent *E.coli* was distributed into 100µl aliquots in ice-cold Eppendorf tubes for each transformation. Ice-cold DNA was added to each tube of competent cells and left on ice for 30 minutes with occasional shaking. The competent *E.coli* cells were heat shocked at 42°C for 45 seconds to enable the uptake of DNA. Cells were allowed to recover on ice for a further 5 minutes. 500µl of fresh LB broth (2.2.1) was added to each tube and incubated at 37°C for 1 hour to confer antibiotic resistance to the *E.coli* by the plasmid DNA. The bacterial cultures were then harvested by centrifugation at 5000 x g for 1 minute and the supernatant discarded. The cells were suspended in 100µl of LB broth prior to spreading on LB agar plates containing the appropriate selective antibiotic (2.2.4) before incubating overnight at 37°C.

Competent *E.coli* strains are shown in Table 1.

Table 1. *Escherichia coli* strains

Strain:	Relevant Genotype:	Reference:
DH5α	F-, Φ*80d lacZ*ΔM15*, rec*A1, *end*A1, *gyr*A96,*thi*1, *hsd*R17 (rK-, mK+), *Sup*E44, *rel*A1, *deo*R, Δ(*lac*ZY A-*arg*F)U169	Hanahan, (1983)
BL21 (DE3)	F-*omp*T *hsdS*$_B$ (rB- mB-) *gal dcm*	Novagen

2.4 Isolation and Purification of Nucleic Acids

2.4.1 Ethanol precipitation of DNA

Sodium acetate was added to aqueous DNA solutions to a final concentration of 0.3M by the addition of 0.1 volume of a 3M stock. Two volumes of absolute ethanol were added and the DNA precipitated at -20°C for 16 hours. The precipitated DNA was pelleted by centrifugation (12,000 x g) for 5 minutes and washed with 70% ethanol to remove excess salts, followed by centrifugation as described previously. The DNA pellet was dried under vacuum for 2-5 minutes and resuspended in the required volume of sterile UHQ H_2O.

2.4.2 Isolation of Plasmid DNA

2.4.2.1 Wizard®Plus SV Miniprep DNA purification system (Promega)

Small scale purification of plasmid DNA was achieved using a miniprep kit (Promega). Bacteria from an overnight culture (2ml) were pelleted by centrifugation (12,000 x g, 1 minute). The cell pellet was then suspended in 250µl supplied resuspension solution. After cell lysis (250µl cell lysis solution), alkaline protease was added (10µl of supplied solution) to inactivate endonucleases and other proteins released during lysis which could affect DNA quality. The mixture was then incubated at room temperature for 5 minutes. Following cell neutralization (350µl Neutralization solution) cellular debris and chromosomal DNA were removed by centrifugation (12,000 x g, 10 minutes).

The plasmid DNA lysate was applied to the miniprep binding column and bound by centrifugation (12,000 x g, 1 minute). The column was washed by adding wash solution (750µl of supplied solution) and then placing the column in the centrifuge (12,000 x g, 1 minute). This was then repeated with another wash (250µl) and centrifuged as previous. The column was dried under vacuum for 5 minutes. The purified DNA was eluted in 30µl H_2O (room temperature for 1 minute, then centrifuged, 12,000 x g, 1 minute). The purified DNA was analysed by agarose gel electrophoresis or stored at 4°C.

2.4.2.2 PureYield™ Plasmid Midiprep DNA purification system (Promega)

Large scale purification of plasmid DNA was achieved using a Midiprep kit (Promega). Bacteria from 50 – 100ml of an overnight culture in LB (2.2.1) containing antibiotics were pelleted by centrifugation (5000 x g, 10 minutes). The bacteria pellet was suspended in 3ml of supplied cell resuspension solution. Following cell lysis and neutralisation (3ml Cell lysis solution, incubation for 3 minutes then addition of 5ml neutralisation solution) cellular debris and chromosomal DNA were removed by centrifugation (15,000 x g, 15 minutes).

Supernatant was applied to the midi binding column stack and vacuum applied until all liquid had passed through both the clearing and binding columns. The clearing column was then discarded and 5ml of endotoxin removal wash was added to the binding column. The vacuum was applied, until all liquid had passed through. This step was repeated with 20ml of endotoxin removal wash. The column membrane was dried with the application of the vacuum for an additional minute.

A 1.5ml eppendorf tube was placed into the base of the Eluator™ vacuum elution device (Promega); the binding column was then inserted above the eppendorf tube. This assembly was placed on the vacuum manifold, 400µl of sterile UHQ H_2O was added to the binding membrane and incubated at room temperature for 1 minute, and DNA was eluted by

vacuum. The DNA was concentrated into 50µl UHQ H_2O by ethanol precipitation (2.4.1). The purified DNA was analysed by agarose gel electrophoresis (2.5.4).

2.4.2.3 Alkaline Lysis Preparation of Crude Plasmid DNA for PCR Screening

An inoculating loop full of *E.coli* from a LB agar plate culture of a single transformant was resuspended in 10µl UHQ H_2O. KOH (10µl of 0.25M) was added to the suspension which was then boiled for 5 minutes. HCl (10µl of 0.25M) was added to neutralise the lysate. The volume was made up to 300µl with UHQ H_2O. An aliquot (1µl) of diluted lysate was used as crude template for PCR.

2.5 Analysis of Nucleic Acids

2.5.1 TAE Buffer

A 50x TAE stock (2M Tris, 0.1M EDTA, pH 8.2-8.4) was purchased (Promega, UK) and a 1x working dilution was used as the electrophoresis buffer for horizontal submerged agarose gels.

2.5.2 Agarose Gel Loading Buffer

Agarose gel loading buffer is used for DNA samples analysed by horizontal submerged agarose gel electrophoresis as it facilitates the loading and tracking of samples through the gel. Loading dye Orange G™ (6x Sigma-Aldrich) was used as it migrates faster (50bp) than other dyes such as Bromophenol Blue (300bp) and does not interfere with the visualisation of nucleic acid under UV.

2.5.3 Sybr® Safe

Sybr® Safe DNA gel stain (Invitrogen) was used to view DNA run on submerged agarose gels and can be viewed directly in a UV Trans-illuminator. SYBR® Safe DNA gel stain has been specifically developed for reduced mutagenicity, making it safer than ethidium bromide for staining DNA in agarose or acrylamide gels.

Concentrated Sybr® safe, 10,000x was diluted 1:20,000 in agarose gel buffer (1 x TAE [2.5.1]). The buffer/stain solution was added to the powdered agarose and heated briefly in a microwave oven until dissolved. 15µl Sybr®Safe was used in 300ml TAE, 40ml of TAE/Sybr® Safe solution was used to cast a small gel. 20µl Sybr® Safe was used in 400ml TAE, 100ml of TAE/Sybr® Safe solution was used to cast a large gel. The gel was then cast in Perspex trays (Bio-Rad) of various sizes, as described (2.5.4).

SYBR® Safe DNA gel stain was stored at room temperature and protected from light.

2.5.4 Agarose Gel Electrophoresis of Nucleic Acids

Agarose gel electrophoresis was used for the separation, quantification and characterisation of nucleic acid fragments, as well as to enable the purification of specific DNA fragments. The concentration of agarose used to prepare gels for the horizontal submerged electrophoresis was determined by the size of the nucleic acid to be resolved. High strength ultra-pure analytical grade agarose (Fisher, UK) was used for all gels.

Agarose gels were cast in Perspex trays (Bio-Rad) of an appropriate size. The tray was sealed using the gel case and placed on a level surface. A comb was used to form the sample wells, which were 2mm above the base of the tray. The comb was placed at approximately 1cm from the edge of the gel. Agarose was melted in 1 x TAE/Sybr® Safe by boiling in a microwave oven.

The melted agarose and buffer was cooled to 50°C, poured into the gel tray and allowed to set for at least 20 minutes before use. Once set, the comb was removed, the tray rotated 90° and the gel was submerged under 1 x TAE buffer. Samples containing 1 x gel loading buffer were loaded into the wells under the 1 x TAE buffer. A 5µl aliquot of a Hyperladder I or II (Bioline) was used as a marker for both size and quantity of the analysed DNA. Samples underwent electrophoresis at 90V.

Once the loading dye front had migrated to 1cm from the end of the gel, the electrophoresis was stopped. The Perspex tray and gel were removed. The nucleic acid was visualised on a UV transilluminator at 302nm. A photographic image of the gel was obtained using the camera contained within the UV transilluminator and printed out.

2.5.5 Denaturing Agarose Gel Electrophoresis of Nucleic Acids

Denaturing gel electrophoresis was used for the separation and visualisation of *in vitro* RNA generated by the T7 RNA polymerase and mMessenger mMachine methods (2.7.6.1 and 2.7.6.2 respectively).

The agarose concentration used was 1% (w/v) for all electrophoresis performed with RNA. High strength ultra-pure analytical grade agarose (Fisher, UK) was used. Agarose gels were cast in Perspex trays as described previously (2.5.4). Agarose was melted in 1x MOPS (Fisher, UK)/DEPC treated UHQ H_2O in a total volume of 37.8ml by boiling in a microwave oven. The melted agarose and buffer was cooled to 50°C, in a fume hood before adding 2.2ml of 37% formaldehyde. The mixture was then poured into the gel tray and allowed to set for at least 20 minutes before use. Once set, the comb was removed, the tray rotated 90° and the gel was submerged under 1 x MOPS buffer. RNA samples were heated with 10µl RNA loading buffer (240µl Foramide, 50µl 10 x MOPS buffer, 87µl 37% formaldehyde, 15µl 0.1% bromophenol blue, 1µl Ethidium Bromide (5mg/ml) and 25µl glycerol, prepared in a fume hood, in an RNase and DNase free eppendorf) at 85°C for 10 minutes and placed on ice prior to being loaded into the wells under 1 x MOPS buffer.

An aliquot (5µl) of ssRNA ladder (New England Biolabs) was heated with RNA loading buffer as described above and used as a marker for the approximate size of the analysed RNA. Samples underwent electrophoresis at 50V for approximately one hour. The RNA loading dye contained Ethidium Bromide; therefore no post-staining of the gel was required. Nucleic acids were visualised on a UV transilluminator at 302nm. A photographic image of

the gel was obtained using the camera contained within the UV transilluminator and printed out.

2.6 Manipulation of Nucleic Acids

2.6.1 Modification of DNA

2.6.1.1 Restriction Enzyme Digests

Enzymes and buffers (10x concentrates) were stored at -20°C. Digests contained 1-2µg of DNA, 5-10 units of enzyme per µg DNA, 10 x restriction digest buffer and 10x BSA at the appropriate concentrations in a total reaction volume of 10-50µl. Reactions were incubated at 37°C (unless otherwise stated by the manufacturer) for 2 hours. Care was taken to ensure that the final glycerol concentration of the reaction did not exceed 10% (w/v) to prevent the reduction of specificity of the enzyme (star activity).

2.6.1.2 Dephosphorylation of DNA

Linearised vector DNA was dephosphorylated prior to cloning to prevent the plasmids from recircularising during ligation. The dephosphorylation reaction contained remaining DNA from restriction enzyme digest, alkaline phosphatase, 10x buffer and 10x BSA at the appropriate concentrations in a total reaction volume of 50-80µl.

Reactions were incubated at 37°C for 30 minutes and then 56°C for 15 minutes. The 37°C incubation step caused the ends of the DNA duplex to separate slightly allowing the enzyme access to the 5' terminal phosphate groups, the 56°C step inactivates the enzyme. Purification with a PCR Clean up kit (2.7.3.1) was performed prior to cloning.

2.6.1.3 Ligation of DNA

Inserts generated by PCR (2.7) were digested using restriction endonucleases to facilitate their ligation to the vector, as well as ensuring phosphorylation of the 5' termini. Rapid

ligations were also performed using T4 ligase. The 10µl reaction consisted of 10ng dephosphorylated vector (2.6.1.2), a 3 fold molar excess of insert DNA, 1µl T4 DNA ligase and 5µl 2x rapid ligation buffer (Promega) at the appropriate concentrations. Rapid ligation reactions were incubated at room temperature for 15 minutes. Ligated plasmids were then transformed into competent *E.coli* cells (2.3.3).

2.7 Amplification of DNA by Polymerase Chain Reaction (PCR)

The thermostable properties of Taq polymerase have been exploited in the development of an amplification procedure for DNA using the polymerase chain reaction (PCR) (205).

Phusion™ Flash High Fidelity PCR master mix (New England Biolabs) is a 2 x master mix based on modified Phusion™ Hot Start DNA Polymerase. Phusion™ Flash PCR master mix contains all reagents required for PCR except for the DNA template and primers. Phusion™ Flash is a proof reading polymerase that contains a unique processivity-enhancing domain, making this polymerase accurate and rapid. It also posses 5'→3' DNA polymerase activity and 3'→5' exonuclease activity. The error rate is approximately 25-fold lower than that of *Thermus aquaticus* DNA polymerase.

BioMix™ Red (Bioline) is a 2x pre optimized master mix containing Taq DNA polymerase. BioMix™ Red contains all reagents required for PCR except for DNA template and primers. Also included is an inert red dye that permits easy visualisation and direct loading onto the gel.

Phusion™ was used to amplify DNA for cloning and sequencing purposes and BioMix™ Red was used in general PCR applications including amplification to screen for possible recombinant clones.

2.7.1 Reaction Conditions

Samples were amplified in a Veriti™ Thermal Cycler (Applied Biosystems) or GeneAmp® PCR 9700 system (Applied Biosystems). Amplification of template DNA was achieved in cycles comprising of 3 steps:

- Template DNA denaturation
- Primer annealing
- Primer extension/polymerisation

Each step within a cycle was carried out at a defined temperature for a specified period of time with a rapid thermal ramp between temperatures. Each cycle was repeated 30-40 times. Denaturation of template DNA was generally for 30 seconds at 94°C or 98°C. The annealing temperature was selected based on the melting temperature (T_m) of the two primers, usually T_m°C -5°C for 30 seconds [where T_m°C=4(G+C) +2(A+T)]. The samples were exposed to an extension temperature of 72°C for all reactions, for 15 seconds per 1000 bases amplified for Phusion™ Master Mix and 15 seconds per 100 base pairs for Bio Mix Red. After completion of the thermal cycling, all samples were held at 10°C until removed from the thermal cycler.

A typical 25µl PCR sample comprised of:

General PCR:

12.5µl	BioMix™ Red
2µl	Template DNA
1.25µl	Forward Primer
1.25µl	Reverse Primer
8µl	H_2O

Cloning and Sequencing PCR:

10µl	Phusion™ Flash Mix
2µl	Template DNA
2.5µl	Forward Primer
2.5µl	Reverse Primer
8µl	H_2O

2.7.2 Primer Design

Oligonucleotides used for amplification of DNA by PCR were custom made from Eurogentec. The sequence selection was determined using the following criteria:

1. Primers should not contain palindromic sequences. For example when the 5' sequence is the same as the 3' sequence on the same strand.
2. Absence of dimerization capability.
3. The primers should be between 17 and 40 bases in length.
4. Neither termini of the primers should be complementary.
5. The primers of each pair should have similar T_m values.

PCR primers containing restriction sites were used to introduce enzyme recognition sites into certain amplification products to facilitate cloning.

2.7.3 Purification of amplified products

Following amplification and before sequencing or cloning, the DNA fragments were purified to remove contaminating primers, nucleotides and template DNA by performing either gel electrophoresis or undergoing PCR clean-up (2.7.3.1).

2.7.3.1 Wizard® SV Gel and PCR Clean-Up System (Promega)

Purification of DNA fragments from agarose gels was achieved using a clean-up system (Promega). Following electrophoresis, the DNA band was removed from the agarose gel using a scalpel and the gel slice placed in a pre-weighed 2ml eppendorf tube. 10µl of membrane binding solution (supplied) was added per 10mg of gel slice and incubated at 50-65°C till the gel slice was completely dissolved.

The dissolved gel mixture was transferred to the minicolumn and incubated at room temperature for 1 minute. The mixture was centrifuged in the minicolumn (12,000 x g, 1 minute) then an additional 700µl membrane wash solution (supplied) was added and centrifuged (12,000 x g, 1 minute). The wash was repeated with 500µl membrane wash solution (supplied) and centrifuged (12,000 x g, 5 minutes). The purified DNA was eluted in

50µl UHQ H_2O (incubate at room temperature, 1 minute and then centrifuged at 12,000 x g for 1 minute). Purified DNA was stored at -20°C.

2.7.4 DNA sequencing

All DNA sequencing was sent to Geneservice (Source Bioscience, Oxford) who use the platforms Illumina Genome Analyser 2 and Applied Biosystems 3730 DNA Analyzers. DNA sent to Geneservice is required to be 1ng/µl per 100bp for PCR products, 100ng/µl for plasmids and 3.2pmol/µl for oligonucleotide primers.

PCR templates for sequencing were purified using Promega clean-up kit to remove unincorporated reactants before sequencing (2.7.3.1).

2.7.5 Reverse Transcription by PCR (RT-PCR)

2.7.5.1 cDNA synthesis

Transcriptor reverse transcriptase (Roche) was used to perform reverse transcription PCR (RT-PCR) amplification of RNA to first strand cDNA for use in subsequent PCR reactions. The enzyme has RNA-directed DNA polymerase activity, DNA-dependent DNA polymerase activity and helicase activity. Transcriptor reverse transcriptase was used for RT-PCR as it is both highly sensitive and very thermostable. The enzyme uses two different types of primer for cDNA synthesis, Oligo $(dT)_{15}$ primer and random hexamer primer which binds along the length of the RNA.

The reaction comprised of 10µM Oligo $(dT)_{15}$, 3.2µg Random Hexamer and 1µg template RNA, with the addition of UHQ H_2O to increase the volume to 13µl. The solution was incubated at 65°C for 10 minutes, to ensure denaturation of RNA secondary structures. To this reaction, 5x Transcriptor RT reaction buffer (supplied) was added at a 1:5 dilution, 1mM of each dNTP and 10U Transcriptor reverse transcriptase was added. This was then incubated for 10 minutes at 25°C and then 30 minutes at 55°C. This ensured efficient

annealing of the primers to the RNA. The Transcriptor reverse transcriptase was inactivated by heating to 85°C for 5 minutes. cDNA was stored at -20°C until required.

2.7.5.2 SuperScript™ III Platinum® One-Step Quantitative RT-PCR System

The SuperScript™ III Platinum® One-Step Quantitative RT-PCR System is a quantitative RT-PCR (qRT-PCR) kit which detects and quantifies RNA using Real-Time detection instruments. This system combines SuperScript™ III Reverse Transcriptase (RT) and Platinum® *Taq* DNA polymerase in a single enzyme mix. This allows cDNA synthesis and PCR to be performed in a single tube using gene-specific primers and RNA.

For quantitative RT-PCR the reaction comprises of 1µg of purified RNA, 3µM forward and 3µM reverse primers, 2µM fluorogenic probe, 12.5µl 2x Reaction mix (supplied), 0.8µl SuperScript™ III RT/Platinum® *Taq* Mix (supplied) and UHQ H_2O to equal 25µl total reaction volume. All reactions were assembled on ice.

The reaction conditions for cDNA synthesis were a 45°C hold for 20 minutes to allow cDNA synthesis, then 95°C for 10 minutes to inactivate SuperScript™ III. The cDNA synthesis was immediately followed by PCR amplification of 40 cycles of 90°C for 10 seconds and 56°C for 1 minute. Data was collected at each 56°C stage in the cycle and quantified using a dilution series of known standards.

2.7.6 Production and purification of RNA

2.7.6.1 Production of in vitro RNA using T7 RNA polymerase kit (Promega, UK)

T7 RNA polymerase was used in the production of non-labelled *in vitro* RNA. The following reagents were added to an RNase and DNase free eppendorf using RNase and DNase free pipettes at room temperature;

20µl Transcription optimised 5x buffer (supplied)
10µl DTT 100mM (supplied)
2µl DNA template, linearised (in UHQ H_2O at 2 – 5µg)

2.5µl RNA Safe™ (AllianceBio)
44.5µl Nuclease free H_2O

The reaction was heated at 60°C for 10 minutes then placed on ice. RNA safe™ ribonuclease inhibitor is a recombinant protein which helps maintain the integrity of RNA. It binds to a broad range of RNases at a 1:1 ratio and does not interfere with the performance of other enzymes present in the reaction. To the reaction mixture above the following components were added;

1µl (100 units) Recombinant RNasin® Ribonuclease inhibitor (supplied)
20µl rNTP mix (2.5mM of each rATP, rGTP, rUTP and rCTP in nuclease free H_2O)
2µl (40 units) T7 RNA polymerase (supplied)

Reaction was incubated overnight at 26°C. 3µl DNase was added to the reaction and incubated at 37°C for 30 minutes to remove any remaining template DNA. RNA was then purified using the Purelink RNA purification kit (2.7.6.3) and visualised on a denaturing agarose gel (2.5.5).

2.7.6.2 mMessenger mMachine® T7 ULTRA kit (Ambion)

In vitro RNA incorporating an addition poly(A) tail on the 3' end was required for transfection into Raw 264.7 cells using the neon system and generated using the mMessenger mMachine® T7 ULTRA kit. The poly(A) tail serves a role in enhancing translation over untailed mRNAs due to increased mRNA stability and translation efficiency. This kit also generates RNA transcripts which produce higher protein yields using the ARCA technology (anti-reverse Cap analog).

The following reaction was assembled at room temperature in an RNase and DNase free eppendorf, using RNase and DNase free pipettes;

2µl 10x T7 reaction buffer (supplied)
2µl DNA template, linearised (in UHQ H_2O at 2-5µg)
2.5µl RNA Safe™
0.5µl Nuclease free H_2O

The eppendorf was heated at 60°C for 10 minutes then placed on ice. To the reaction mix the following components were added;

10µl 2x NTP/ARCA (supplied)
2µl T7 enzyme mix (supplied)
1µl GTP (supplied)

Giving a total volume of 20µl. The tube was mixed thoroughly and incubated at 37°C for 2 hours. After incubation 1µl TURBO DNase (supplied) was added and incubated for a further 15 minutes at 37°C. The DNase treatment removed the original DNA template to prevent detection in downstream applications.

To add a poly(A) tail to the transcripted RNA the following reagents were added to the 20µl reaction mix;

36µl Nuclease free H_2O
20µl 5x E-PAP buffer (supplied)
10µl 25mM $MgCl_2$ (supplied)
10µl ATP solution (supplied)

2.5µl of this mixture was removed before adding the E-PAP enzyme as a minus enzyme control. Finally 4µl E-PAP is mixed gently, with the reaction to give a total volume of 100µl and incubated at 37°C for 45 minutes; the reaction was then placed on ice. RNA was purified using the PureLink™ kit (2.7.6.3) and visualised on a denaturing agarose gel (2.5.5). The RNA concentration was quantified using the Nanodrop™.

2.7.6.3 PureLink™ RNA mini kit

Concentration and purification of the RNA was performed using the PureLink™ RNA mini kit (Invitrogen).

Liquid stool (100-300µl) was added to 500µl TRIzol® and incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. 100µl of chloroform was added and the mixture was vortexed for 30 seconds to ensure the sample was resuspended before incubating at room temperature for a further 3 minutes. The

sample was centrifuged for 10 minutes, 12,000 x g at 4°C and the top aqueous layer which contained RNA, free from DNA and protein was transferred to a fresh Eppendorf tube. An equal volume of 70% ethanol was added to the RNA sample to obtain a final concentration of 35% ethanol which was then vortexed.

700µl of the RNA sample was transferred to the spin cartridge and bound with centrifugation (12, 000 x g, 15 seconds). This was repeated for the rest of the liquid until the entire sample had been processed. The column was washed with 700µl Wash Buffer I (supplied) and centrifuged (12,000 x g, 15 seconds). The wash was repeated with 500µl Wash Buffer II (supplied) and centrifuged (12,000 x g for 15 seconds). The spin column was centrifuged again (12,000 x g, 1 minute) to dry the membrane with attached RNA.

The purified RNA was eluted in 30µl UHQ H_2O by incubating at room temperature for 1 minute and then centrifuged at 12,000 x g for 2 minutes. Purified RNA was stored at -80°C until required.

2.8 Protein Analysis

2.8.1 BCA Protein Assay Kit (Pierce)

To determine the concentration of protein in a sample, the BCA protein assay kit (Pierce) was used. A set of eight BCA concentration standards (2000, 1500, 1000, 750, 500, 250, 125, and 62.5µg/ml^{-1}) were prepared from 2mg/ml. 10µl of sample or standard was added to 200µl of working reagent (50:1 of reagent A and B [supplied]) on a 96 well tray. The plate was incubated for 30 minutes at 37°C and optical density recorded on the imark® plate reader at 570nm.

2.8.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE separates linear polypeptide-SDS complexes by electrophoretic molecular sieving in polyacrylamide gels. The proteins are denatured by SDS and β-mercaptoethanol which

destroy secondary and tertiary structures and inter- and intra-chain disulphide bonds respectively. The treated proteins have approximately equal charge-to-mass ratios and are separated solely on the basis of molecular size.

All glass plates and spacers (0.75mm) used to form the gel mould were cleaned and degreased with Industrial Methylated Spirits (IMS). All buffers and solutions were prepared using UHQ H_2O as shown in Table 2. SDS-PAGE was performed with either a 12% or 14% separating and 5.3% stacking acrylamide gels, unless otherwise stated and prepared as shown in Table 3. Polymerisation of acrylamide and the cross-linker, N, N,-Methylenebisacrylamide which formed the gels was accelerated by free radicals from N, N, N'-N'-tetramethylethylenediamine (TEMED) generated by the action of the initiator Ammonium Persulphate (APS).

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Table 2. Solutions for SDS-PAGE

Solution	Contents
Acrylamide monomer Solution	40% Stock Solution
SDS Stock Solution	10% (w/v)
5x Separating Buffer	36.33g Tris dissolved in 200ml UHQ H_2O pH 8.8 with 1M HCl
5x Stacking Buffer	12.11g Tris dissolved in 200ml UHQ H_2O pH 6.8 with 1M HCl
N,N,N'-N'-tetramethylenediamine	TEMED
Ammonium Persulphate solution	10% (w/v)
Electrode Buffer	20mM Tris, 192mM glycine, 0.1% (w/v) SDS
Sample Buffer	Tris-HCl pH6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol, 10µg Bromophenol blue

Table 3. Components for SDS-PAGE

Regents	12% Gel		14% Gel	
	Separating Gel	Stacking Gel	Separating Gel	Stacking Gel
Acrylamide	1.5ml	225µl	1.75ml	225µl
5 x Separating Buffer	1.9ml	-	1.9ml	-
5 x Stacking Buffer	-	1ml	-	1ml
H_2O	1.5ml	675µl	1.25ml	675µl
TEMED	5µl	2µl	5µl	2µl
Ammonium Persulphate	20µl	10µl	20µl	10µl
10% SDS	50µl	20µl	50µl	20µl

The separating gel was cast first, allowing room for the 5.3% stacking gel to be cast on top. The separating gel was overlaid with H_2O to ensure the gel surface was flat and air excluded. Once the separating gel had set, the H_2O was removed. The stacking gel was poured and the well comb carefully inserted. Polymerisation was complete after approximately 30 minutes.

The required volume of electrode buffer (Table 3) was freshly prepared. The well forming comb was removed, the gel assembled in the electrophoresis tank and the reservoirs were filled with electrode buffer. Protein samples were diluted in SDS sample buffer, denatured by boiling for 5 minutes and loaded onto the stacking gel. Electrophoresis was carried out at a constant 200 volts per gel. On completion, the proteins within the gels were stained using PAGE blue 83 (Coomassie) stain or transferred via Semi-Wet Blot for Immunoblot.

2.8.3 SDS-PAGE Protein Markers

Molecular weights and concentrations of proteins were estimated by comparing electrophoretic mobility with proteins of known molecular weight. The pre-stained protein marker used was Page Ruler (Fermentas) which was stored at -20°C.

2.8.4 Staining of SDS-PAGE Gels

The plastic container used for the staining procedure was cleaned and degreased with IMS and the gel was placed in staining solution [0.1 %(w/v) PAGE Blue 83, 20% (v/v) isopropanol, 10% (v/v) acetic acid] for 1 hour. Excess stain was removed by repeated washes in a destain solution [20% (v/v) isopropanol, 10% (v/v) acetic acid] until the gel background was clear. The gel was placed in gel drying solution [25 %(v/v) methanol, 5% (w/v) glycerol] for 1 hour and dried between two sheets of gel drying film (Promega gel drying kit). Once dry, the gel becomes clear and the proteins can then be visualised as blue bands.

2.8.5 Electrophoretic transfer of Proteins

Protein samples were separated by SDS-PAGE and transferred on to nitrocellulose Hybond-C membrane in the presence of transfer buffer [Tris – HCl 25mM; Glycine 192mM; SDS 0.1% (w/v); methanol 20% (v/v)] using a Trans-blot 'semi-dry' blotter (Bio-rad Laboratories Ltd, Hertfordshire).

2.8.6 Immunological Detection of Proteins

2.8.6.1 Immuno blot - ECL™ Western Blotting Analysis System

Following electrophoretic transfer of proteins the Hybond-C membrane was treated with blocking solution [5% Marvel + PBS/0.05% Tween-20 (PBST)] for 1 hour at room temperature. The Marvel blocks all free protein binding sites to avoid non-specific binding of antibodies. The membrane was incubated with primary antibody diluted in PBS. Unbound antibody was removed by three x 5 minute washes in PBS-T (PBS/0.05% Tween-20). The primary antibody was detected by incubation for 2 hours at room temperature with a secondary antibody-horse radish peroxidase (HRP) conjugate diluted 1:3000 in PBS-T. Unbound antibody was removed as previously described.

An ECL™ Western Blotting Analysis System (GE Healthcare) solution was made of 1ml Reagent 1 and 1ml Reagent 2 (supplied). The membrane was removed from PBS-T and placed in a shallow tray. ECL™ solution was added and incubated at room temperature for 1 minute. The membrane was then developed onto photographic film (Kodak, Biomax XAR film, scientific imaging film, Sigma-Aldrich) in a development box, for 2 minutes in a dark room. After this the photographic film was placed in developing solution [100ml developer, black and white (Champion Histochemistry, UK)/400ml water] for 2 minutes, then washed in water, before being placed in fixing solution of [100ml fixer developer (Champion Histochemistry, UK), 400ml water, 25ml hardener developer (Champion Histochemistry, UK)] for 2 minutes. The photographic film was then washed under running water before

being left to dry. Detected proteins can be visualised as black bands on the photographic film.

2.8.7 Immunoblot- NBT/BCIP system

Following electrophoretic transfer of proteins the Hybond-C membrane was treated with blocking solution [5% Marvel + TTBS (NaCl 0.5M, Tris – HCl 20mM; pH 7.5 +0.05% Tween-20 (v/v)] for 1 hour at room temperature and then washed in TTBS until clear. The membrane was cut into strips for the subsequent immuno detection protocol.

The strips were incubated with primary antibody diluted in 10% Normal Goat Serum for two hours, shaking at room temperature. Unbound antibody was removed by three x 5 minute washes in TTBS. The primary antibody was detected by incubation for 1 hour at room temperature with a secondary antibody-alkaline phosphatase conjugate diluted in 1% Marvel + TTBS. Unbound antibody was removed as previously described, with an extra wash in TBS to remove all traces of Tween 20, which could interfere with the enzyme activity of the alkaline phosphatase. Bound antibody reactions were detected by incubation with 2ml per membrane strip of 20ml carbonate buffer ($NaHCO_3$0.1mM; $MgCl_2$ 1mM; pH 9.8) containing 200µl NBT (nitro-blue tetrazolium chloride) [30mg/ml in 70% (v/v) DMF (Dimethylformamide)] and 200µl BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) (15mg/ml in 100% DMF) for no longer than 10 minutes at room temperature. The transferred proteins were indirectly detected by the action of the alkaline phosphatase-conjugate on the NBT/BCIP substrate resulting in a visible colour change. When the colour of the reaction was sufficient the enzyme reaction was stopped by two x 5 minute washes in UHQ H_2O. The membranes were then air dried, mounted on chromatography paper and sealed between plastic sheets.

2.8.8 Direct Enzyme Linked ImmunoSorbant Assay (ELISA)

Poly-vinyl 96 well plates were coated directly (100µl/well) with the appropriate antigen in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6/0.02% NaN_3) at an optimum concentration of 2µg/ml. Plates were incubated overnight in a humid environment at 4°C. The wells were washed three times in PBS/0.05% Tween-20 (PBST) and blocked with 200µl of PBS/5% (w/v) skimmed milk (Marvel) for 30 minutes at 37°C. The wells were emptied and an appropriate dilution of monoclonal antibody or antiserum in PBS/1% (w/v) skimmed milk was added. The plates were incubated for 1-1 ½ hours at 37°C before the wells were emptied and washed four times in PBST. The appropriate species specific horseradish peroxidise conjugate (Sigma Aldrich, Poole) diluted in PBS /1% skimmed milk (1:3000) was applied to each well (100µl/well) and incubated for a further 1-1 ½ hours at 37°C. The plate was then washed four times with PBST before development with 100µl TMB substrate (0.1% sodium acetate, pH 6, 0.1% tetra methyl benzidine, 0.01% H_2O_2 or TMB solution (eBioscience)) at room temperature for 15 minutes in the dark. The reaction was stopped with the addition of 50µl 2M H_2SO_4 and the optical density at OD_{450}nm of each well was read using an imark® microplate absorbance reader (Bio-Rad). Optical density is the measure of the amount of light absorbed by the sample when measured with the use of an imark® microplate absorbance reader (Bio-Rad).

When the colorimetric substrate (tetramethyl benzidine (TMB)) binds to the HRP conjugate it catalyzes a reaction which produces a quantifiable colorimetric change (208) which can be measured spectrophotometrically at 450nm and the results expressed as an optical density value (OD).

2.8.9 Antigen capture ELISA (Sandwich ELISA)

Poly-vinyl 96 well plates were coated directly (100µl/well) with the appropriate capture antibody in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6/0.02% NaN_3) at an

optimum concentration of 2µg/ml. The plates were incubated overnight in a humid environment at 4°C. The wells were washed three times in PBS/0.05% Tween 20 (PBST) and blocked with 200µl of PBS/5% (w/v) skimmed milk (Marvel) for 1 hour at 37°C. The wells were emptied and a 100µl of antigen sample was loaded into each well. Plates were incubated for 2 hours at 37°C before the wells were emptied and washed three times in PBS-T. Detection antibody against the antigen of interest was added to each well at an appropriate dilution in PBS/1% (w/v) Marvel, total volume 100µl. The plates were incubated for 90 minutes at 37°C before the wells were emptied and washed four times in PBST. 100µl of the appropriate species specific horseradish peroxidise conjugate diluted in PBS-T/1% (Marvel) was applied to each well (100µl/well) and incubated for a further 90 minutes at 37°C. The plate was then washed three times with PBST before development with 100µl substrate (0.1% sodium acetate, pH 6, 0.1% tetra methyl benzidine, 0.01% H_2O_2 or TMB solution (eBioscience)) at room temperature for 15 minutes in the dark. The reaction was stopped with the addition of 50µl 2M H_2SO_4 and the optical density at OD_{450}nm of each well was read using an imark® microplate absorbance reader (Bio-Rad).

2.8.10 Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA)

Fluronunc microplates (NUNC) were coated directly (100µl/well) with the appropriate capture antibody in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6/0.02% NaN_3) at an optimum concentration of 2µg/ml.

The plates were incubated overnight in a humid environment at 4°C. The wells were washed three times in PBS/0.05% Tween 20 (PBS-T) and blocked with 100µl of PBS/5% (w/v) skimmed milk (Marvel) for 30 minutes at 37°C. The wells were emptied and a 100µl of antigen sample was loaded into each well. Plates were incubated for 2 hours at 37°C before the wells were emptied and washed three times in PBS-T. Detection antibody against the antigen of interest was added to each well at an appropriate dilution in DELFIA

assay buffer (Perkin Elmer). The plates were incubated for 1 hour at 37°C before the wells were emptied and washed three times in PBS-T. The secondary antibody was detected by the addition of a europium-labelled species specific antibody (Rabbit anti-mouse –Eu, 100ng/ml (Perkin Elmer)) incubated at 37°C for 1 hour. The plate was washed three times in PBS-T. A DELFIA enhancement solution (100µl/well) was used to dissociate Eu^{3+} from the Eu chelate labelled detector antibody. Plates were allowed to incubate for 10 minutes at 37°C whilst shaking. Fluorescence was read using a Wallac mulitlabel counter. The results were captured as TRF (time resolved fluorometry) counts at 615nm after excitation of the samples at 340nm.

2.8.11 Antibody Isotyping using the Mouse Typer®Sub-Isotyping kit

Polyvinyl 96 well plates were coated directly (100µl/well) with the appropriate antigen in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6/0.02% NaN_3) at an optimum concentration of 2µg/ml. The plates were incubated overnight in a humid environment at 4°C. The wells were washed three times in PBS/0.05% Tween 20 (PBS-T) and blocked with 200µl of PBS/5% (w/v) skimmed milk (Marvel) for 1 hour at 37°C. The wells were emptied and 100µl of 1:5 dilution of hybridoma supernatant in PBS/1% (w/v) marvel was added to the wells. This was incubated for 1 hour at 37°C and washed three times in PBST. The appropriate rabbit anti-mouse Ig panel were added to the appropriate well (100µl/well) and incubated for a further hour at 37°C. The wells were washed three times in PBS-T and incubated with 1:3000 dilution of goat anti-rabbit HRP conjugate in PBS/1% (w/v) marvel for one hour at 37°C. The plate was washed three times in PBS-T before development with 100µl TMB solution (eBioscience) at room temperature for 15 minutes in the dark. The reaction was stopped with the addition of 50µl 2M H_2SO_4 and the optical density at OD_{450}nm of each well was read using an imark® microplate absorbance reader (Bio-Rad).

2.9 Cell Culture

2.9.1 Cell Culture Medium

Raw 264.7 cells are a mouse monocyte macrophage cell line, established from an ascites of a tumour induced in a male mouse by intraperitoneal injection of Abelson Leukaemia Virus (A-MuLV).

The RAW 264.7 cell line was maintained using sterile Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) and supplemented with 4.5g/L D-Glucose, 25mM HEPES, 10% low endotoxin foetal bovine serum, 100 U/L penicillin and 100 µg/L streptomycin.

HEK293T cells originate from Human embryonic kidney cells, SV40 transformed. The 293T cell line was created by transfection of a sub-line of adenovirus-immortalized human embryonic kidney cells with a gene encoding the SV40T-antigen and a neomycin resistance gene.

The HEK293T cell line was maintained using sterile Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) and supplemented with 4.5g/L D-Glucose, 10% low endotoxin foetal bovine serum, 100 U/L penicillin and 100 µg/L streptomycin.

2.9.2 Transfection of HEK293T cells

The FuGENE transfection reagent (Promega, UK) was used to transfect DNA into HEK293T cells with a high efficiency and low toxicity in a lipid based system. The cationic headgroup of the lipid compound associates with the anionic phosphates on the nucleic acid. DNA transfection is essential for the study of gene function and regulation.

FuGENE was allowed to reach room temperature prior to use. Plasmid DNA for transfection was prepared in UHQ H_2O at a final concentration of 0.2 – 1 mg/ml. HEK293T cells (an

adherent cell line) were plated onto a 6 well plate one day before transfection so that cells are approximately 80% confluent on the day of transfection.

In a 96 well tray, the following components were assembled, 200µl pre-warmed Opti-mem (Invitrogen), 20µl (1µg) plasmid DNA and 10µl FuGENE in a single well. The well was incubated at room temperature for 15 minutes prior to the addition to the plated HEK293T cells; 110µl was added to each well for transfection. The plates were incubated for 48 hours at 37°C, 5% CO_2.

One well of each plasmid DNA sample was harvested before undergoing two cycles of freeze/thaw and centrifugation at 1300 x g to pellet the cellular debris. Supernatant was used to inoculate one well in a 6 well tray of 80% confluent Raw 264.7 cells for 2 hours. The inoculum was then removed and replaced with 3ml fresh DMEM media containing 10% FBS and 25mM HEPES. Inoculated Raw 264.7 cells were incubated for a further 48 hours at 37°C, 5% CO_2. The remaining wells of each sample on the original 6 well tray were harvested and spun 12,000 x g for 1 minute. The supernatant was removed and stored at -80°C for downstream experiments; the pellet was suspended in 300µl RIPA buffer and stored at -20°C.

2.9.3 Neon Transfection System (Invitrogen)

The Neon Transfection system (Invitrogen) is based on electroporation technology and can be utilised to deliver nucleic acids, proteins and siRNA into mammalian cell types with a high cell survival rate. The Neon system aims to expose the sample to a uniform electric field, minimal pH change, less ion formation and negligible heat generation.

Purified RNA was used at a concentration of 5ug/µl in UHQ H_2O, purity was ensured by analysing the $A_{260/280}$ ratio using the Nanodrop, and a value of at least 1.8 was required.

Prior to transfection Raw 264.7 were seeded into a T75 flask to ensure the cells are 70 – 90% confluent on the day of the experiment.

On the day of the experiment Raw 264.7 cells were harvested and washed in PBS twice by centrifugation at 400 x g for 5 minutes at room temperature. The PBS was then discarded and the cell pellet gently suspended in resuspension buffer R (supplied) to obtain a single cell suspension. 6 well plates were prepared by pre-filling with 2ml Raw 264.7 growth media without antibiotics and incubated at 37°C/5% CO_2 until required.

The Neon tube was filled with 3ml Electrolytic buffer (E2, supplied) and inserted into the Neon pipette station. The purified RNA is added to the required volume of Raw 264.7 cells and 100µl drawn into the Neon pipette. It is important to ensure that no bubbles are present in the tip which could affect the transfection efficiency. The pipette containing RNA and Raw 264.7 cells is subjected to pulse conditions of 1720V, 10ms and 1 pulse.

The sample is transferred to one well of pre-warmed growth media in a 6 well plate. Plates were rocked to ensure even distribution of the cells and incubated at 37°C/5% CO_2. Samples were assessed for the presence of CPE and transfection efficiency by a variety of downstream methods.

2.9.4 Immunofluorescence of transfected or infected Raw 264.7 cells

Identification of individual proteins synthesised *in vivo* following transfection/infection was performed using undiluted antisera or monoclonal antibodies. The immune complexes were visualised by using an antibody, directed against the detection antibody, conjugated to a fluorescent compound, Florescein Isothiocynate (FITC). Fluorescence was observed using a DMRB fluorescent microscope (Leica).

Cells were grown on sterile coverslips, following transfection or infection and fixed by incubation with 500µl of 4% formaldehyde in PBS for 10 minutes. The cells were rinsed

three times in PBS, then incubated for 1 hour at 4°C in 1ml Saponin buffer (180ml PBS, 20ml FCS, 0.2g Saponin and 0.2g Sodium Astade). The detection antibody was then added at a 1:5 dilution in saponin buffer to each coverslip and incubated at 4°C for 1 hour. The cells were rinsed three times in saponin buffer; species specific FITC conjugate antibody was added (1:50 conjugate in saponin buffer) and incubated for 30 minutes at 37°C. The cells were washed in saponin buffer as described previously and placed cells down on 8µl of Vector safe on a clean microslide. Cells were observed by fluorescence microscopy at an excitation wavelength of 488nm.

2.9.5 Murine norovirus plaque assay

Plaque assays were performed using monolayers of RAW 264.7 cells. Sub-confluent RAW 264.7 cells were resuspended in 30ml of DMEM; 3ml of cells were added per well in a 6 well tray and then incubated at 37°C and 5% CO_2 until the cells formed a monolayer (approximately 3 hours). Sub-confluent monolayers of RAW 264.7 cells were inoculated with 1ml of 10-fold serially diluted virus and the trays incubated at 37°C, 5% CO_2 for 90 minutes. After incubation 3ml of complete DMEM containing 1.25% (w/v) low melting point (LMP) agarose was added. The agarose was set by incubation at 4°C for 15 minutes, before being transferred to 37°C, 5% CO_2 for 72 hours.

The wells were stained with 2ml Neutral Red Stain (3%) and incubated at 37°C, 5% CO_2 for 2 hours. Plaques were visualised over a light box to quantify virus titre.

2.10 Production and characterisation of monoclonal antibodies

2.10.1 Materials

2.10.1.1 SP2/O-Ag14 cell line

The SP2/O-Ag14 cell line is a non-Ig secreting or synthesising line, isolated as a re-clone of Sp2/HL-Arg, which was derived from Sp2/HLGK, created by the fusion of a BALB/c spleen cell and the myeloma cell line X63-Arg8 (216).

2.10.1.2 SP2 medium (DMEM)

Sterile Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) 500ml, supplemented with 20% low endotoxin foetal bovine serum, 1mM Sodium Pyruvate, 4.5g L-glutamine, 100 U/L penicillin and 100µg/L Streptomycin is required for the growth of SP2 cells and is stored at 4°C.

2.10.1.3 DMEM-HAT medium

HAT (Hypoxanthine, Aminopterin, Thymidine) media supplement (50x) Hybri-Max™ (Sigma-Aldrich) was required for the maintenance of hybridomas after fusion. 10ml HAT supplement was used per 500ml of SP2 medium (2.10.1.2).

HAT is required after the fusion procedure for the selection of hybridomas. After the fusion of mouse spleen cells with SP2 cells, the SP2 cells provide myeloma growth properties so the hybridomas can divide continuously, while the lymphocytes provide the capacity to secrete antibody. Unfused cells will not continue to grow in the presence of HAT. The selection occurs as only cells fused to lymphocytes can utilise the salvage pathways required for growth in HAT. It achieves this by supplying the required enzyme hypoxanthine (guanine) phosphoribosyl transferase (HGPRT) to synthesis the purines and pyrimidines supplied by hypoxanthine and thymidine necessary for growth.

HAT contains aminopterin which blocks biosynthesis pathways for purine and pyrimidine synthesis by inhibiting the dihydrofolate reducatase enzyme. This makes the cells dependent on the HGPRT enzyme which is absent in unfused cells.

2.10.1.4 DMEM-HT medium

HT (Hypoxanthine, Thymidine) supplement (Sigma-Aldrich, Poole) is required once the selection of hybridomas has been completed. Fused lymphocyte and myeloma cells are selected and transferred to a fresh well on a 96 well plate.

The supplement provides purines and pyrimidines to overcome the effects of HAT media which could result in residual intracellular aminopterin that is toxic to cells over a long period of time. HT medium allows the cells to reinstate the alternative biosynthesis pathway to the HGPRT enzyme for purine and pyrimidine synthesis.

2.10.2 Cell viability count

SP2 cells were resuspended in a 1:3 dilution of DMEM (2.10.1.2) and left to grow. To release cells from the flask surface, the cells were washed three times with sterile PBS. This is to remove any remaining traces of DMEM which may interfere with the effectiveness of the Versene dissociation method. The cells are then incubated at 37°C, 5% CO_2 with 1ml Versene (Invitrogen). Dissociation was stopped with the addition of 5ml DMEM. After 2-3 days a cell viability count was performed by diluting 100µl of suspended cells with 900µl 0.4% Trypan blue stain. Trypan blue stains non-viable cells making viable cells easy to count. The number of viable cells was determined with a Fast Read, disposable counting chamber (ISL, Immune Systems).

Spleen cells were resuspended in 10ml serum free DMEM and 100µl was removed and diluted with 900µl 0.4% Trypan blue stain and the number of viable cells was determined as previously described.

2.10.3 Immunisation regime

All immunisations were performed by a certified technician in accordance with home office legislation and the institutions' ethical guidelines.

Prior to immunisation a pre-immunisation tail bleed was obtained and screened by direct ELISA (2.8.8) for pre-existing cross-reacting antibodies. The blood was clotted for 1 hour at room temperature and then placed at 4°C overnight. The blood was centrifuged at 4000 x g and the serum was removed and stored with 50% glycerol at -20°C. A negative result in an ELISA and immunoblot indicates the animal has no pre-existing antibodies towards the intended immunising antigen and therefore is suitable for immunisation.

The immune response with a low dose antigen can be enhanced by the use of adjuvants. The half-life of the antigen is prolonged because the adjuvant helps protect it from degradation. Adjuvants minimise any direct toxic effects and increase the efficiency of phagocytosis of the antigen by macrophages by allowing it to be slowly released from the site of immunisation.

The first immunisation used Freund's complete adjuvant which contains heat killed and dried *Mycobacterium tuberculosis* and was administered subcutaneously. Subsequent injections were with Freund's incomplete adjuvant (paraffin oil and mannide monooleate). Only the first immunisation can be given in Freund's complete adjuvant as the presence of *Mycobacterium tuberculosis* can induce a granulomatous response which, if severe, could kill the animal being immunised. The mycobacteria contained within the complete Freund's adjuvant stimulate the immune system by lengthening the antibody production time and allowing the B cells to become memory cells. Freund's complete adjuvant is particularly potent as it stimulates both cell-mediated and humoral immunity.

Subsequent injections are generally subcutaneous and given at roughly ten day intervals.

The second tail bleed was taken after the last immunisation (day 35) and screened by direct ELISA (2.8.8) for the immunising antigen. This is to check that the immunisation procedure has been successful and that antibodies to the antigen have been produced. A positive result is required before the mouse was considered ready for fusion. A subcutaneous boost of antigen was administered four days prior to fusion.

2.10.4 Hybridoma production

Six tissue culture flasks with a base surface area of 175cm^2 (T175) of confluent SP2 cells (2.10.1.1) were pelleted at 524 x g for 5 minutes and washed in unsupplemented DMEM. The cells were resuspended in 10ml unsupplemented DMEM.

The immunised mouse was sacrificed and its spleen was dissected, avoiding damage to the intestine, by a certified technician.

The spleen was transferred to a Petri dish containing 10ml of unsupplemented DMEM and gently teased apart into a single-cell suspension to release as many lymphocytes as possible. Tissue debris was removed by passage through a 100µm nylon pore cell strainer (Becton Dickinson, New Jersey). The volume was increased to 20ml with unsupplemented medium and then pelleted at 524 x g for 5 minutes. The pellet was then washed twice in unsupplemented DMEM, before being resuspended in 10ml unsupplemented DMEM. Cell viability counts were performed on both cell types (2.10.2). A ratio of 1:1 (SP2: Spleen cells) was used for the fusion procedure.

The cells were mixed and pelleted together at 524 x g for 10 minutes. The medium was poured off and the tube inverted on sterile filter paper to remove final traces of medium. The pellet was loosened by tapping to avoid splashing up the side of the tube. Over a period of one minute 1ml of warm PEG [50% polyethylene glycol (PEG), 10% DMSO in PBS Hybri-Max^{TM} (Sigma-Aldrich), 37°C] was added to the cells. PEG makes the cell membranes

porous to induce fusion, however they do become fragile. The cells were transferred to a 37°C water bath and swirled gently for 90 seconds before 20ml of warm unsupplemented DMEM was added over the following time frame:

1ml in 30 seconds
3ml in the following 30 seconds
8ml in the following 30 seconds
8ml in the following 30 seconds

The tube was then slowly inverted to ensure an even suspension and placed in a water bath (37°C) for 5 minutes. The cells were then pelleted at 149 x g for 10 minutes and the medium was removed. The cells were then resuspended in 10ml warm, (37°C) DMEM with HAT (2.10.1.3) and pelleted 524 x g for 10 minutes. The pellet was then resuspended in 10ml DMEM with HAT and 5ml aliquots added to two 120ml warm, (37°C) DMEM with HAT aliquots, with 2.4ml Hybridomas, Fusion and Cloning Supplement (HFCS) (Roche, Applied Science).

HFCS is used as a supplement to culture medium to support the growth of B-cell hybridomas after fusion and during cloning. Prior to the use of HFCS, mouse feeder cells were used to maximise the yield of hybridomas from fusion and cloning procedures. Mouse peritoneal cells, of which most are macrophages have been found to be convenient and effective feeder cells. The feeder cells are a source of soluble growth factors for hybridoma cells (199).

Separating the suspension into two aliquots is a precaution taken to avoid potential contamination of the material. The fusion mixture can then be distributed across twelve, 96 well trays (200µl/well) using a multichannel pipette and wide end sterile tips to minimise damage to the cells.

The trays were then incubated in a box with ventilation at 37°C and 5% CO_2. The trays are left undisturbed for 10-14 days to allow the hybridomas to grow. The trays were assayed by a direct ELISA (2.8.8) against the required antigen to detect hybridomas secreting the desired antibodies.

2.10.5 Purification and maintenance of hybridomas

Hybridomas secreting the desired antibody were detected by direct ELISA (2.8.8) in the primary screen. Hybridomas in the positive wells were picked (8-10µl) to fresh 96 well trays and allowed to re-grow in 200µl DMEM with HAT (2.10.1.3). These were re-screened by direct ELISA (2.8.8) and positive hybridomas were again picked and diluted across a 96 well tray with 200µl fresh DMEM with HT, to obtain single hybridomas by terminal dilution. Positive hybridomas were then incubated at 37°C until there were enough cells to permit re-screening. When a well with a single positive hybridoma was obtained this was assumed to be purified. It was still however, subjected to another round of cloning to ensure purity. The hybridoma was then picked and expanded to obtain enough cell culture supernatant containing the monoclonal antibody for analysis and long term storage.

For long term storage of hybridoma trays and monoclonal antibodies, 20% Dimethyl Sulfoxide (DMSO) DMEM containing HT and 10% DMSO DMEM containing HT respectively was used and cells were slowly frozen to -80°C. 20% DMSO was added directly to hybridoma trays due to the difficulty in releasing cells from the well surface in a small surface area. For this reason 100µl 20% DMSO was added to the existing 100µl media in the well to achieve 10% DMSO per well. Frozen hybridoma preparations were then transferred to liquid nitrogen (-135°C).

2.10.6 MNV CW1 viral stock

Confluent T75cm^2 RAW 264.7 cells were infected with MNV CW1 (available at the onset of this project) at a multiplicity of infection (MOI) of 1. After 18 hours the cells were harvested

and spun at 524 x g for 5 minutes to pellet the cells. The supernatant was aliquoted into cyrotubes and stored at -80C. Viral stocks underwent a plaque assay to determine viral titre.

2.10.7 MNV CW1 Lysate

Briefly, to produce MNV CW1 lysate, confluent T75cm^2 RAW 264.7 cells were infected with MNV CW1 at a multiplicity of infection (MOI) of 1. After 18 hours the cells were harvested and spun at 524 x g for 5 minutes to pellet the cells. The supernatant was removed and the pellet washed in PBS. The cells were pelleted again at 524 x g for 5 minutes. The supernatant was removed and the pellet was resuspended in ELISA coating buffer (2.8.8) and sonicated to ensure that the cells were thoroughly resuspended.

2.10.8 RAW 264.7 and SF9 cell lysate

To produce RAW 264.7 and SF9 cell lysate, confluent non-infected cells were harvested from a T75cm^2 and spun at 524 x g for 5 minutes to pellet the cells. The supernatant was removed and the pellet washed in PBS. The cells were pelleted again, at 524 x g for 5 minutes. The supernatant was removed and the pellet was resuspended in ELISA coating buffer (2.8.8) and sonicated to ensure that the cells were thoroughly resuspended.

2.11 Oysters

Samples were collected from a local fishmonger in Southampton, Hampshire. Native pacific rock oysters (*Crassostrea gigas)* were used in these investigations, grown at several oyster farms located across Scotland.

2.11.1 Oyster Processing and virus extraction and purification

Oysters were shucked and the stomachs and the hepatopancreas removed from the shellfish by dissection.

2.11.1.1 Viral protein extraction

Extraction and purification of virus particles from oyster tissue was adapted from a procedure to detect norovirus from shellfish using RT-PCR (7). In seeding experiments 100µl virus containing stool or 100µl PBS was added to the digestive tissue and incubated for 1 hour at room temperature. ¾ of the sample was used for protein extraction and the remaining ¼ was used for viral RNA extraction. The solution was homogenised in 10ml PBS containing 0.2ml anti-foam B (Sigma-Aldrich) for 60 seconds in a 15ml non-sterile tube on an IKA homogeniser (Fisher Scientific). The solution was transferred to a 50ml apex tube and chloroform/butanol (6ml 1:1 v/v) was then added. The solution was vortexed for 30 seconds; to the homogenate 173µl of Cat-Floc T (Sigma-Aldrich) was then added and rocked for 5 minutes at room temperature. The solution was allowed to settle for 15 minutes at room temperature and then centrifuged at 7000 x g for 15 minutes at 4°C. The aqueous phase was collected and added to 6.5ml of polyethylene glycol 6000 (24% w/v in 1.2M NaCl) and rocked for 1 hour at 4°C. To concentrate the virus particles the solution was then centrifuged for 20 minutes at 7000 x g and the supernatant removed. The pellet was suspended in 400µl UHQ H_2O and stored at 4°C. 100µl of the preparation was analysed by ELISA or DELFIA.

2.11.1.2 Viral RNA extraction

¼ of the extracted digestive tissue, dissected from oyster tissue (2.11.1.1) was used in extraction of viral RNA. 2.5ml TRIzol was added to the digestive tissue in a 15ml homogeniser tube, the solution was homogenised using an IKA homogeniser (Fisher Scientific) for 60 seconds and incubated at room temperature for 5 minutes. 500µl chloroform was added; the mixture was vortexed for 30 seconds and then incubated at room temperature for 3 minutes. The sample was centrifuged for 10 minutes, 12,000 x g at 4°C and the top aqueous layer containing RNA, free from DNA and protein was transferred

to a fresh Eppendorf tube. An equal volume of 70% ethanol was added to the sample to obtain a final concentration of 35% ethanol which was then vortexed.

Extracted RNA was purified using the Purelink RNA mini kit as described previously (2.7.6.3). The RNA was eluted in 20µl of UHQ H_2O and stored at -80°C.

2.12 Computer Analyses

DNA sequence editing, restriction site analyses, translations, open reading frame locations, alignments, protein molecular weight predictions and amino acid compositions were performed using DNA start LaserGene software. qRT-PCR data analysis, Ct value determination and quantification of RNA genomes per ml were performed using Applied Bio systems Software, 7500 v2.0.5.

Chapter 3 Results: Isolation of monoclonal antibodies to the norovirus capsid protein

3.1 Introduction

At the time of this research, no cell culture method has been reliably described for human noroviruses. The current human norovirus 'surrogate', murine norovirus (MNV) can be propagated in mouse macrophages and is used as a research aid for investigating the basic properties of noroviruses (replication, polyprotein processing and effectiveness of disinfectants (16,255). Murine norovirus does not produce similar clinical symptoms in mice to those seen in humans when infected with norovirus; therefore only limited conclusions can be drawn from research into virulence of MNV for comparison with human norovirus.

In 2003, MNV was discovered as the cause of lethal infections in immune-compromised mice. This pathogen could be serially passaged by intra-cerebral (i.c.) inoculation (255) and was identified using representational difference analysis showing it contained strings of sequence that were homologous to regions in the genomes of members of the *Caliciviridae* family. Lethal infection in the mice was due to encephalitis (irritation and swelling of the brain), hepatitis, cerebral vasculitis and pneumonia (122). In comparison, human norovirus infection is lethal only in vulnerable population groups and is characterised by its quick onset with symptoms of diarrhoea and vomiting lasting between 24 – 48 hours. The symptoms of infection subside before the adaptive immune system can be employed. Studies involving MNV have suggested that the innate immunity, in particular interferon (IFN) or STAT-1 dependent immune responses, may control the clearance of norovirus infections from the host (255).

It was discovered that while MNV does not cause clinical symptoms in immune-competent mice; in mice lacking intact IFN signalling pathways, either type I or type II IFN receptors or lack of STATI molecule, the infection was lethal (122). Immuno-competent mice have prolonged faecal shedding of virus and can become persistently infected with MNV without displaying any clinical symptoms (101). STATI $^{(-/-)}$ mice which were infected with MNV displayed rapid viral replication, with virus present in the spleen, liver, lungs and distal lymph nodes (179). The spread and severity of infection in mice lacking intact IFN and STATI $^{(-/-)}$ indicates the importance of these pathways for protection against MNV. IFNs in particular are critical to the innate immune response in the presence of viral infection (65,147,195,206). IFNs inhibit the replication of viruses through the induction of an antiviral state (206).

Once cells are infected with the virus they produce type I IFNs (IFN α/β), these bind to a common receptor in neighbouring uninfected cells and initiate a paracrine signalling cascade. IFN signalling produces the induction of IFN-stimulated genes (ISGs), which in turn generate an anti-viral state. Specific ISGs have been shown to directly inhibit viral replication, such as double-stranded RNA activated protein kinase (PKR), dsRNA-dependent 2'-5' oligoadenylate synthetase and RNase L (62,175,265). These downstream signalling molecules allow protection from viral infection, the effects of removing these factors can be seen in mice lacking IFN and STATI. Complete understanding of the mechanisms of inhibition has still not been achieved either with MNV or human noroviruses.

After the discovery of Murine Norovirus -1 (MNV) other isolates, MNV 2, 3 and 4 were discovered in laboratory mice and there have now been over forty strains of MNV isolated globally from laboratory mice (13,101,130).

In the absence of a cell culture system for human noroviruses and the lack of a similar surrogate, recombinant expression systems such as the baculovirus replicon system; which

enables expression of the capsid protein in insect cells from recombinant baculovirus have been developed. This system can generate large quantities of norovirus capsid protein which spontaneously self-assemble into Virus like Particles (VLPs). The baculovirus replicon system produces intact human norovirus viral particles (lacking the non-structural proteins) by utilising recombinant insect viruses to introduce foreign genes into insect cells. The foreign DNA is incorporated by co-transfection of insect cells with a transfer vector and a modified virus genome. Insect cells act as cellular factories to produce large quantities of recombinant capsid protein which can be purified and characterised. VLPs mimic the capsid protein structure of a norovirus whilst not containing any genetic material required for replication making them non-infectious as well as morphologically and antigenically indistinguishable from native noroviruses (75,113). VLPs have been important in research to study virus-ligand interactions (such as the CD40 ligand) and immune responses to human noroviruses (47,227).

Norovirus VLPs have been used extensively as immunising antigens for the production of monoclonal antibodies. Quantities of between 500µg – 1mg VLPs are required for the immunising of the animals and subsequent screening of isolated antibodies, therefore sufficient quantities are required before commencing experiments. Monoclonal antibodies are so named as they originate from identical B cells which are clones of a unique parent cell. All antibodies produced from the immune cells have monovalent affinity and therefore bind to the same epitope.

The earliest report of a monoclonal antibody reacting to a norovirus epitope was published in 1988. This antibody was specific to Snow Mountain virus, produced by *in vitro* immunisation of murine spleen cells (237). Monoclonal antibodies are produced by the fusion of a mouse B cell with an immortal myeloma cell. B cells are lymphocytes and are an essential component of the adaptive immune system, producing antibodies to a specific

antigen which is determined (by the immune system) to pose a threat to the host. The fused B and myeloma cells are known as hybridomas which can be grown indefinitely and so a replenishable supply of monoclonal antibodies can be produced. This constant supply allows the monoclonal antibody to be utilised as a standardised reagent in multiple experiments in different laboratories to produce consistent results.

The development of broadly reactive monoclonal antibodies is vital in detecting a broad range of norovirus capsid proteins. The great diversity of the amino acid sequence throughout the norovirus genome, in particular the capsid protein, has made attempts to detect multiple noroviruses in a single assay a slow process. Currently neither ELISA nor RT-PCR can detect all norovirus sequences in a single assay. The production of broadly reactive monoclonal antibodies should decrease the number of ELISAs that would have to be performed in a diagnostic laboratory to detect all genogroups and genotypes of norovirus.

The hypothesis was that by immunising mice with MNV VLPs it might be possible to produce a broadly reactive monoclonal antibody with properties directed towards a common norovirus epitope shared by MNV and human norovirus genogroups I and II. Previous hybridoma productions in our laboratory have demonstrated that after immunisation with a single human norovirus genotype, cross-reactive antibodies could be isolated across an entire genotype (C.Batten, PhD thesis). Therefore by using MNV it is hoped that a monoclonal antibody with a common epitope to all noroviruses can be isolated. Cross-reactive monoclonal antibodies would enable in-depth epidemiological studies of both murine and human norovirus infections. Monoclonal antibodies able to detect MNV in environmental samples would provide the ability to draw a limited number of conclusions on the levels of infectivity caused by particular detection limits seen in the ELISA. Despite the lack of similar clinical symptoms between the two viruses in their respective hosts, MNV is currently the closest surrogate to human noroviruses which can

be propagated and used in these types of experiments (102). Until a surrogate can be isolated which has identical properties, MNV is used to investigate the infection and replication properties of human noroviruses.

Should it not prove possible to produce a broadly reactive monoclonal antibody, it is hoped to isolate a MNV capsid specific monoclonal antibody which can be incorporated into an ELISA immunoassay, to detect intact MNV virions seeded into environmental samples, such as oysters. Using a model system of MNV seeded oysters; an indication of the risk of norovirus exposure in shellfish to the human population may be gained by comparing antigen capture and live virus techniques. This is based on the assumption that MNV will bind to shellfish tissue and be concentrated in a similar way to human noroviruses.

As part of forming a tool kit of antibodies towards MNV protein in another project, colleagues at Otago University, New Zealand provided MNV VLPs to use as immunising and screening antigen in this project. VLPs were produced at Otago University using a baculovirus-expression system. Thus the aim of this work was to immunise mice with MNV VLPs to produce hybridomas secreting capsid specific monoclonal antibodies and to screen these for cross-reactivity with human capsid proteins. The aspirational goal was to find a cross-reacting monoclonal antibody that could detect both human and murine norovirus.

MNV, a GV norovirus, was chosen as the immunogen. Three immunisations of MNV VLPs were followed by a single boost immunisation, ensuring successful stimulation of the immune system. Once isolation of purified hybridomas was achieved, characterisation of the secreted monoclonal antibodies will be determined through a series of direct ELISAs against human VLPs. It is also important to determine the exact location of the amino acid epitope which the isolated monoclonal antibodies recognise; achieved by ELISA, immunoblot and immunofluorescence analysis. Purified antibodies are hoped to detect an epitope in the P domain and potentially enable the capture of intact virions from complex

matrices and quantification of genomic RNA by qRT-PCR. Quantification of genomic RNA from intact virions would provide a massive step forward into the research of infectivity posed by noroviruses to the human population.

3.2 Characterisation of murine and human VLPs.

The quantity of protein present within the MNV VLP preparations obtained from Otago University was determined to be 664 $\mu g/ml^{-1}$ before commencing the immunisation regime; using the BCA protein assay kit [2.8.1 (Pierce)]. 50µg of immunising antigen (VLPs) is recommended for each immunisation per animal in a volume of 50µl (C.Batten, PhD Thesis).

MNV VLPs were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot (2.8.5 and 2.8.6) to assess purity before the antigen was used in the immunisation schedule. A band was visualised at 58kDa, the described size of the MNV capsid protein (31,254).

The immunoblot of MNV VLPs and MNV lysate generated from MNV infected RAW 264.7 cells (2.10.6) is shown in Figure 6. Both antigens were detected using a rabbit polyclonal anti-MNV capsid antibody, a gift from Prof. Veron Ward, Otago University. In Figure 6, a high number of background bands can be seen for MNV VLPs indicating that proteins from baculovirus-infected insect cells were detected by the polyclonal antibody. The non-specific bands seen in the VLP preparation are most likely remaining cellular components due to the lack of purification. The polyclonal serum used as the detection antibody was generated from the immunisation of rabbits with MNV VLPs generated in New Zealand. Therefore the polyclonal MNV capsid antibody is likely to be reacting to components of the preparation they were immunised with, as well as the immunogen MNV.

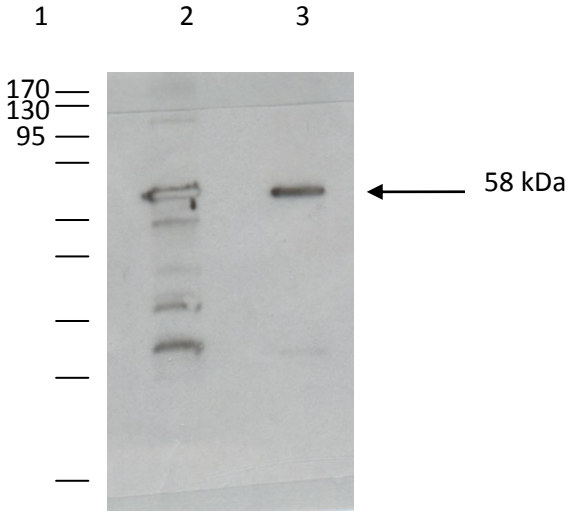


Figure 6. Immunoblot of MNV VLP in Lane 2 and MNV Lysate in Lane 3 detected with rabbit polyclonal anti-MNV capsid antibody. The protein ladder (kDa) is marked alongside.

MNV VLPs produced by colleagues in New Zealand were not purified by a Cesium chloride (CsCl) gradient, which is the traditional purification procedure in VLP production (9,105), as this was shown to drastically reduce the quantity of VLP recovered (V. Ward, personal communication). Under high centrifugal force, a solution of CsCl molecules will dissociate, resulting in the heavy Cs^+ molecules being forced to the edges of the tube. This forms a shallow density gradient. Within this gradient the VLPs will migrate to the region of the same density, therefore separating particles with differing densities. It is for this reason that there are insect (SF9) cellular components present in the VLP preparation.

The immunoblot in Figure 6, displays a single clear band for MNV infected cell lysate produced from MNV infected RAW 264.7 cells (2.10.6) thus MNV lysate was chosen to screen for MNV monoclonal antibodies produced from the fusion as antigen in a direct ELISA, as it was shown to be a purer preparation than that of the MNV VLPs.

For determining the reactivity profile of isolated monoclonal antibodies, a direct ELISA utilising human norovirus VLPs was used. Human VLPs were a kind gift from Dr. K.Y Green, USA. The VLPs included, Desert Shield, Toronto, Maryland, Hawaii and Snow Mountain which were analysed by SDS-PAGE to determine purity. Bands were visualised for each antigen at the previously reported size for each capsid protein.

In human noroviruses the capsid protein varies in size across genogroups. Toronto virus (GII.3) has a smaller capsid of 58kDa (145) than Snow Mountain virus (GII.2) 59.2kDa (128). Desert shield virus capsid protein is 59kDa (181), MD-145 is 58kDa and the Hawaii virus capsid protein is 57kDa (74). The purity of the human VLPs ensures that results generated are towards the coating antigen rather than other components of the preparation.

3.3 Immunisation Regime

Four Balb/c mice, (female, date of birth 10/1/2010, HO. batch no. 1/10/67) were used for the production of hybridomas (2.10.4). Balb/c is a commonly used species for monoclonal antibody production due to the large spleen to body ratio, which yield a higher number of B cells that are ideal for fusions. A direct ELISA (2.8.8) was performed with pre-immunisation serum as the primary antibody, at a range of dilutions from 1 in 100 to 1600 against MNV VLPs (the proposed immunising antigen). Results indicate if the mice have pre-existing antibodies towards the proposed immunising antigen.

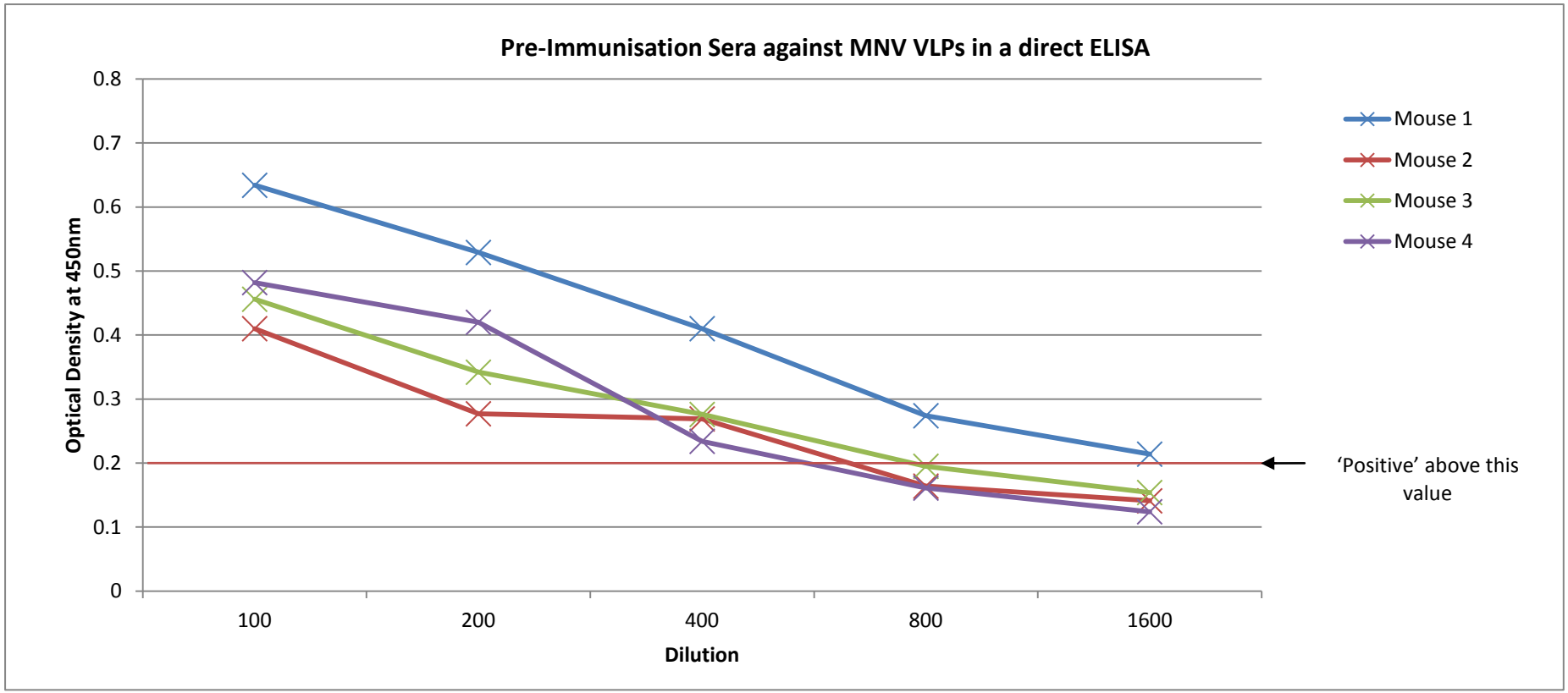


Figure 7. Direct ELISA results of the pre-immunisation serum against MNV VLPs. The negative control of H_2O gave a OD_{450} value of 0.070 (not shown).

All four mice had pre-existing antibodies towards MNV as shown in Figure 7, a positive result was taken as a value >0.2 OD_{450nm} this was triple the negative control value of UHQ H_2O 0.070. There was a low level of pre-existing MNV infection in the animal house; therefore presence of antibodies towards MNV in the mice was not unexpected as MNV is endemic throughout many animal houses (102). It was intended that the proposed immunisation regime would stimulate the immune response of these animals towards MNV capsid protein for the production of monoclonal antibodies. Pre-existing MNV antibodies present in the mice were not guaranteed to be towards the MNV capsid protein. The immunisation regime with MNV VLPs increases the possibility of generating antibodies towards the desired region, the capsid protein, rather than any non-structural proteins. Monoclonal antibodies binding non-structural proteins cannot be incorporated into assays to fulfil the study aims and therefore are unsuitable for this project. The MNV infection that the four immuno-competent mice were exposed to was not considered a problem to the design of this experimental strategy and it was unlikely that mice could be obtained in a sufficient time-scale which had not either been infected or exposed to MNV.

Four mice were immunised (2.10.3) following the standard regime shown in Table 4 with MNV VLPs; only one mouse would be used for hybridoma production. This regime has been previously successful in our laboratory at producing a high yield of monoclonal antibodies directed towards norovirus VLPs (C. Battern, PhD thesis).

The immunisation and selection regime was performed as described by Kohler and Milstein (132) and Oliver *et al* (187) with the following modifications. The two subsequent immunisations containing Freund's incomplete adjuvant were administered at 10 day intervals and culture medium of hybridomas resulting from successful fusion was screened for reactivity by direct ELISA. This regime has been cited extensively for the production of

monoclonal antibodies towards noroviruses, with various modifications in the timing of the immunisations and quantity of antigen administered (89,95,131)).

Since the original description of this technique by Kohler and Milstein (132) the use of polyethylene glycol (PEG) as the fusogenic agent and use of screening procedures such as ELISA have increased the reliability of the technique (59).

Table 4. Immunisation regime for the production of MNV monoclonal antibodies.

Day	Details
0	Pre immunisation bleeds
1	Subcutaneous Immunisation : 50µg MNV VLPs + 50% Freund's Complete Adjuvant
11	Subcutaneous Immunisation: 50µg MNV VLPs + 50% Freund's Incomplete Adjuvant
21	Subcutaneous Immunisation: 50µg MNV VLPs + 50% Freund's Incomplete Adjuvant
35	Test Bleeds Subcutaneous Immunisation: Boost with 50µg MNV VLPs
39	Hybridoma Production

The second tail (test) bleed was taken after the last immunisation at day 35 and screened by direct ELISA against MNV VLPs, the immunising antigen, ensuring that the antibody response to MNV had been stimulated sufficiently. A direct ELISA was performed (2.8.8) comparing the pre immunisation (day 0) and second tail bleeds (day 35) for each mouse.

After the immunisation regime, mouse 1 died. At day 35, mouse 3 was chosen for hybridoma production following advice from the animal house technicians; this mouse had signs of small tumours across its body. Mice 2 and 4 were kept at the animal house for future potential use in alternative experimental strategies.

Despite the pre-existing level of MNV antibodies present in all four mice, it was expected that the immunisation with MNV VLPs would increase the immune response against the desired protein. The immunisation regime resulted in a greater than two fold increase in serum reactivity against MNV VLPs when the second tail bleed serum was compared to the pre-immunisation serum for mouse 3. The increase in OD values after the immunisation regime are seen in Figure 8, these values were determined to be sufficient to produce a high number of monoclonal antibodies with the ability to detect MNV VLPs. Analysis of these results contributed to the decision to use mouse 3 for hybridoma production.

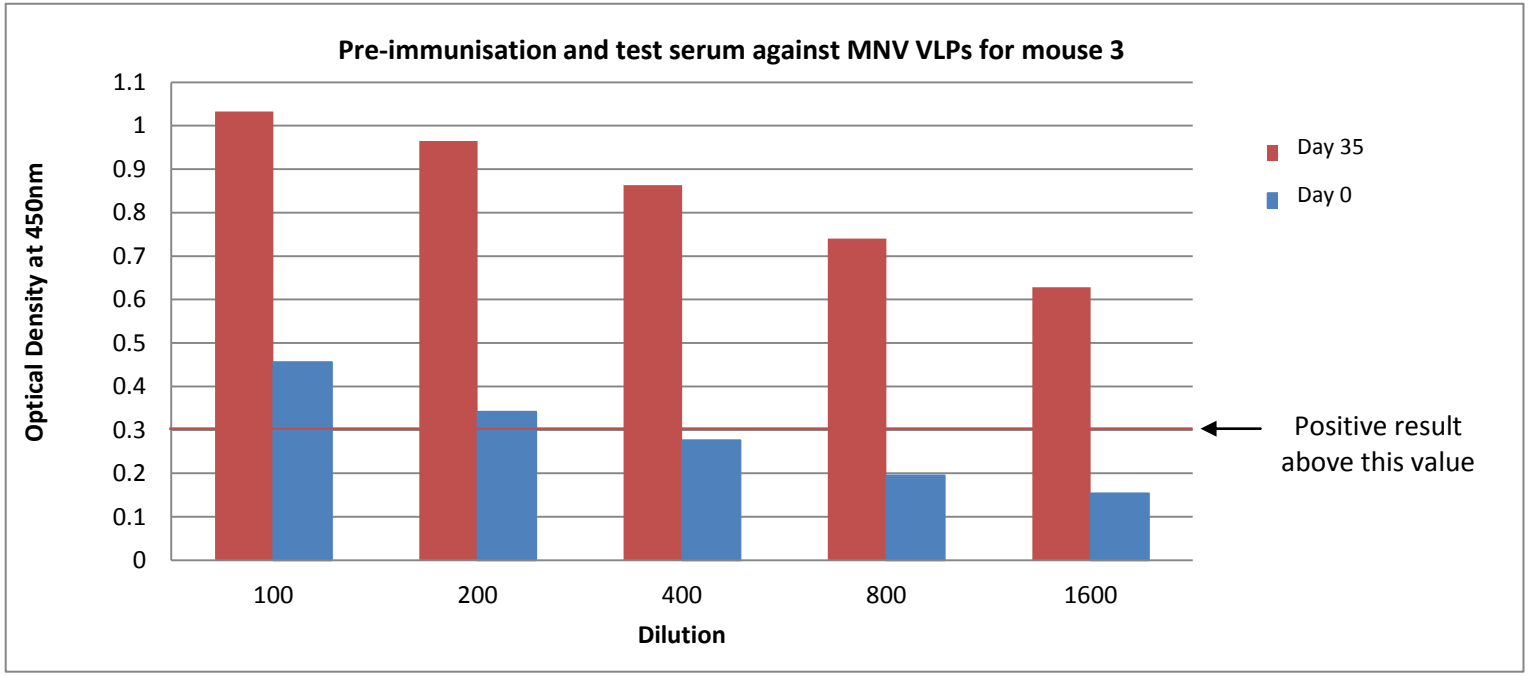


Figure 8. Direct ELISA performed on mouse 3 pre-immunisation (day 0) and test serum (day 35) against MNV VLPs. A 'positive' value was taken as >0.3 OD_{450nm}.

The second tail (test) bleed serum had increased OD values across the same dilution range when compared to the pre-immunisation serum. The OD values obtained demonstrate that immunising mouse 3 with MNV VLPs did result in an increase in MNV antibody production against the MNV capsid protein.

To determine the immunoreactivity of pre-immunisation (day 0) and second tail bleed serum (day 35) an immunoblot was performed. Using antigen strips (2.8.5 and 2.8.6) the sera from the pre-immunisation and second tail (test) bleed was added as the primary antibody, the results obtained are displayed in Figure 9. Duplicates were performed for both the pre-immunisation and second tail bleeds to confirm the results, only one strip is shown in Figure 9.

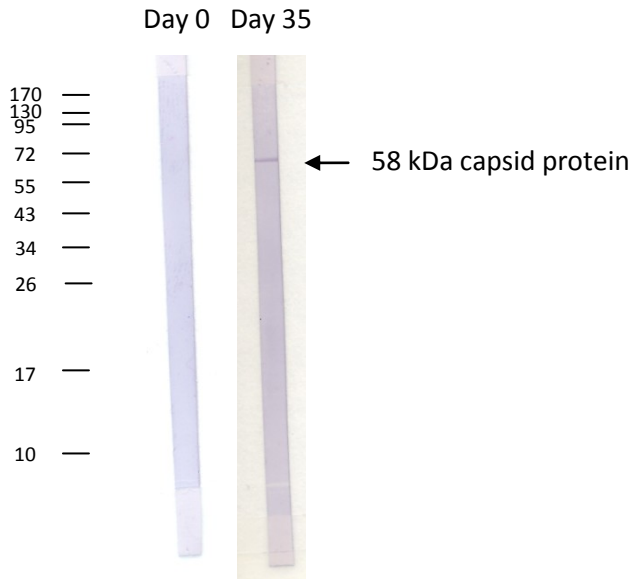


Figure 9. Immunoblot of day 0 and 35 serum against MNV VLPs for mouse 3. The protein ladder is displayed with the molecular weights (kDa) alongside.

The pre-immunisation serum (day 0) for mouse 3 indicated no visible bands in the immunoblot against MNV VLPs. In comparison the second tail bleeds taken (day 35) produced a visible band at 58 kDa, the predicted size of the MNV capsid protein, indicating that post-immunisation mouse 3 produced an immune response to MNV capsid protein. Despite the pre immunisation serum having a high OD value in the direct ELISA against MNV VLPs, the immunoblot method enables more specific results against a particular antigen. This technique can determine if the epitope recognised by the antibodies present in the second tail bleed are linear or conformational due to the denaturating of the MNV capsid protein, after subjection to high temperatures and maintaining the denaturation throughout the SDS-PAGE gel. The denaturing properties of the SDS gel guarantees that all of the protein which runs through the gel is linear and has a negative charge, ensuring the proteins are separated by size alone. After establishing that the second tail bleed (taken at day 35) yielded an antibody response to MNV capsid protein by direct ELISA and immunoblot, a boost immunisation with 50µg of MNV VLP was administered to mouse 3.

3.4 Hybridoma selection and characterisation to MNV

Mouse 3 was selected for hybridoma production, despite pre-existing antibodies being detected by direct ELISA in both the pre-immunisation and second tail bleeds. This decision was taken following advice from the animal house and analysis of the results obtained in the direct ELISA and immunoblot experiments.

A fusion of mouse B cell (spleen) and myeloma (SP2) cells (2.10.1.1) was performed as described (2.10.4) at a 1:1 ratio. Hybridoma supernatant (20µl) was screened against MNV infected cell lysate in a direct ELISA (2.8.8). Wells were considered positive if they had a reading > 0.3 OD_{450nm}. This cut-off was an arbitrary figure above the background level of reactivity and was used to ensure that only a manageable number of hybridomas were selected for further cloning and characterisation.

Fifty-three individual hybridomas were generated from the fusion, all of which produced an OD value above 0.3 against MNV infected cell lysate in a direct ELISA. Individual hybridomas were picked and passed through two rounds of limiting dilution to ensure clonal purity prior to amplifying individual hybridoma clones in bulk for antibody production. Hybridomas were screened by direct ELISA against RAW 264.7 and insect (SF9) cell lysate (2.10.8); the SF9 cell lysate was chosen because it did not contain MNV or mouse cell components. Screening with SF9 cell lysates ensured that the antibodies were not detecting a background cellular component of the cell line rather than MNV which was generated by infection of RAW 264.7 cells with MNV CW1. SF9 cells are an insect cell line from which the MNV VLPs were generated, using the recombinant baculovirus system by colleagues in New Zealand. The direct ELISA was performed as described previously (2.8.8) with both cell lysates. Forty one of the fifty three hybridomas produced antibodies which were able to detect a component of the RAW 264.7 or SF9 cell lysate. All forty one hybridomas were discarded as the monoclonal antibodies they were producing could not be recognising solely MNV capsid protein.

The remaining twelve hybridomas, which were assumed to detect only MNV in infected cell lysate were picked and transferred to fresh trays and cloned by terminal dilution (2.10.5). All twelve were expanded and stored at -135°C in DMEM and 10% DMSO.

3.5 Characterisation of the twelve monoclonal antibodies to human norovirus capsid protein (VLPs)

The twelve clonally purified hybridomas which secreted antibodies able to detect MNV infected cell lysate, but not RAW 264.7 and SF9 cell lysates, were further characterised to obtain a full reactivity profile. Characterisation was achieved by performing a direct ELISA against a panel of seven human norovirus VLPs (5 x GII and 2 x GI) available in our laboratory shown in Table 5, a kind gift from Dr. K.Green *et al* (USA).

Table 5. Panel of seven human norovirus VLPs indicating genogroup, genotype, name of virus and accession number.

Genogroup/Genotype	Name of virus	Accession Number
GII.I	Hawaii	U07611
GII.2	Snow Mountain	U70059
GII.3	Toronto	U02030
GII.4	MD-145	AY032605
GII.4	Lordsdale	X86557
GI.2	Southampton	L07418
GI.3	Desert Shield	U04469

These data generated from the direct ELISAs against human norovirus VLPs were normalised to allow the comparison of the data from different ELISA experiments, performed on different days and with different coating antigens as shown in Figure 10. To achieve this, the values obtained from the positive and negative controls which were performed on each plate, were allocated 100% and 0% reactivity respectively. The positive control was MNV infected cell lysate with detection antibody 6.2.1, a monoclonal antibody directed towards MNV capsid protein.

Antibody 6.2.1 was a kind gift from C.Wobus *et al*, alongside monoclonal antibodies 6.2.2 and 2H6.1. All three of these monoclonal antibodies were generated by the immunisation of mice with brain homogenate containing MNV-1. Antibodies were isolated which detected MNV-1 capsid protein in ELISA; however they were unable to detect MNV-1 capsid protein by immunoblot (126,254). The negative control was CM64, a monoclonal antibody directed towards Manchester virus, a Sapovirus, accession number X86560 (155). Sapoviruses are a member of the *Caliciviridae* family, causing mild gastroenteritis in young children.

The values for each monoclonal antibody were calculated using the OD_{450nm} values to generate a percentage of the control values. A summary of the results are shown in Figure 10, the twelve hybridomas were allocated numbers CM 99 – 102, 121, 125, 128 – 131, 139 and 140. Numbers were generated for all fifty-three hybridomas isolated, CM 99-152, hybridomas kept their original numbering even when forty-one were removed for background cross-reactivity.

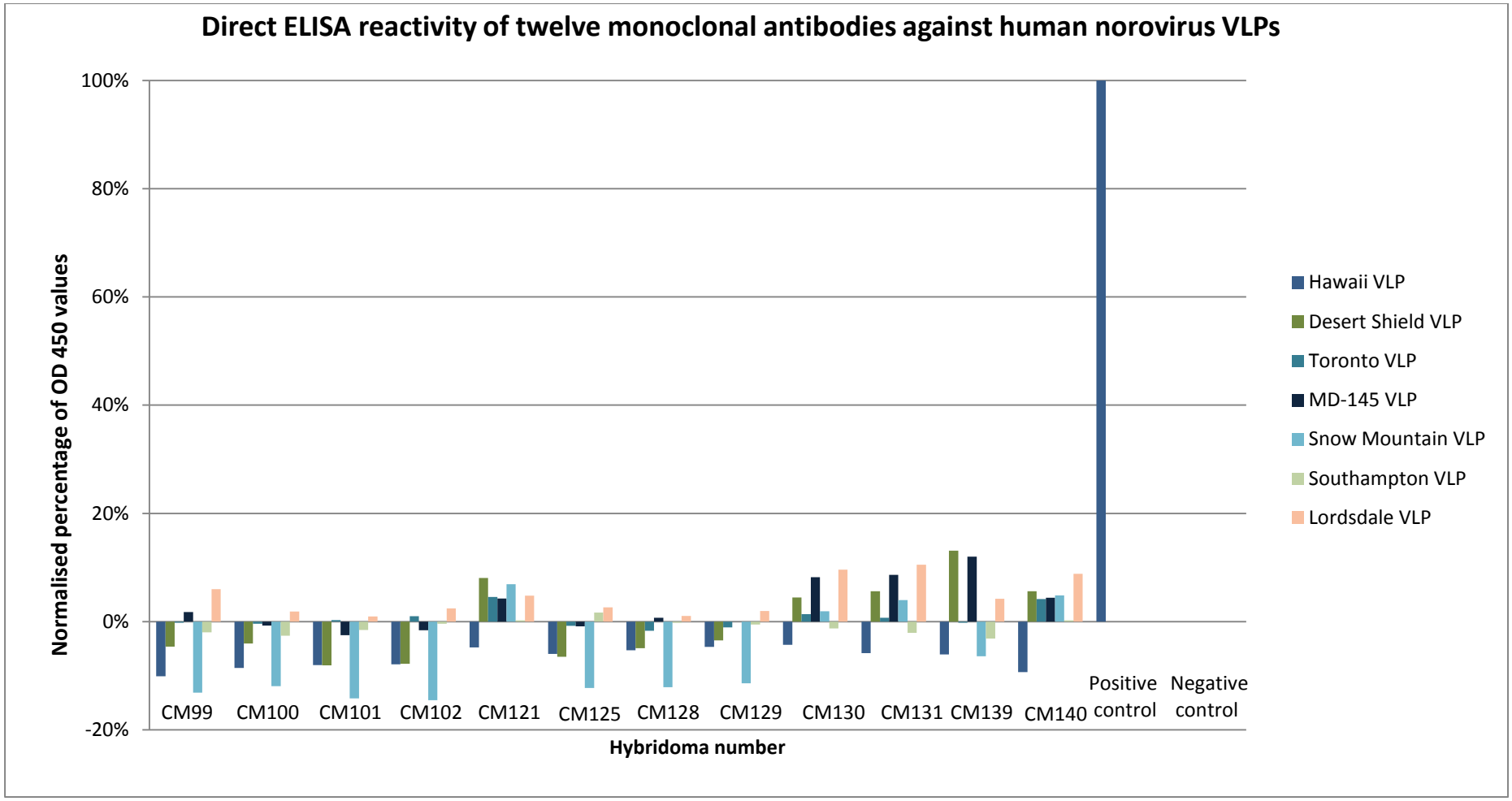


Figure 10. Direct ELISA of the 12 MNV monoclonal antibodies against 7 human norovirus VLPs. This data has been normalised using antibody 6.2.1 as the positive control (100%) and CM64 as the negative control (0%).

Table 6 displays the OD_{450nm} values obtained for each of the twelve monoclonal antibodies against the panel of seven human norovirus VLPs, shown in the previous graph, Figure 10. The data generated from the direct ELISAs indicate all twelve monoclonal antibodies, had values against MNV infected Raw 264.7 cell lysate, above the cut off of $OD_{450nm} > 0.3$. This cut-off was double the negative control, CM64 in each assay. None of the monoclonal antibodies showed cross-reaction to human norovirus VLPs.

		MNV	Cell Lines		Human Noroviruses						
Hybridoma No.	**Hybridoma Name**	**MNV lysate**	**RAW cell lysate**	**Sf9 cell lysate**	**Lordsdale VLP**	**Southampton VLP**	**Hawaii VLP**	**Desert Shield VLP**	**Snow Mountain VLP**	**Toronto VLP**	**MD-145 VLP**
99	13A2.i	0.606	0.125	0.163	0.136	0.11	0.114	0.071	0.069	0.113	0.087
100	13A2.ii	0.581	0.158	0.195	0.092	0.104	0.126	0.075	0.076	0.111	0.070
101	4G9	0.516	0.151	0.144	0.082	0.114	0.130	0.047	0.063	0.118	0.058
102	7A7.i	0.483	0.203	0.23	0.098	0.125	0.131	0.049	0.061	0.125	0.064
121	7A7.ii.iii.iii	0.356	0.171	0.256	0.123	0.131	0.155	0.159	0.185	0.161	0.104
125	10C8.ii.ii	0.511	0.112	0.122	0.100	0.145	0.146	0.058	0.074	0.107	0.069
128	10G6.i.iii	0.389	0.240	0.222	0.083	0.127	0.151	0.069	0.075	0.098	0.080
129	10G6.i.iv	0.367	0.188	0.209	0.093	0.124	0.156	0.079	0.079	0.104	0.075
130	11A8.ii.i.i	0.449	0.185	0.206	0.174	0.117	0.159	0.134	0.156	0.129	0.131
131	11A8.ii.i.ii	0.485	0.204	0.163	0.184	0.109	0.147	0.142	0.168	0.122	0.134
139	11A8.iii.ii.ii	0.308	0.109	0.298	0.117	0.099	0.145	0.194	0.108	0.113	0.157
140	11A8.iii.ii.iii	0.409	0.294	0.292	0.166	0.131	0.120	0.142	0.173	0.157	0.105
Positive Control	Ab 6.2.1	0.964									
Negative Control	CM64	0.192									

Table 6. Direct ELISA results for the 12 monoclonal antibodies against MNV capsid protein, displaying their OD_{450nm} values against a panel of 7 human norovirus VLPs, MNV infected cell lysate, RAW 264.7 and SF9 cell lysate. The values in green indicate hybridomas which produced a value > 0.3.

3.6 Analysis of MNV detecting monoclonal antibodies

Whilst the data suggests that the twelve monoclonal antibodies react with MNV capsid protein, it was unclear which part of the protein was involved in this interaction. Therefore the antibodies were characterised to determine where the epitope was located in the capsid protein. A direct ELISA against MNV VLPs was performed using hybridoma supernatant, five monoclonal antibodies detected MNV VLPs; with values > 0.3 OD_{450nm}, a summary is provided in Table 7.

MNV VLPs are comprised solely of capsid protein, both the shell and protruding domains; either intact or fragmented. Monoclonal antibodies with OD_{450nm} values > 0.3 against MNV VLPs are detecting an epitope located in either domain of the capsid protein. As demonstrated in Figure 6, the MNV VLP preparation used in these experiments was not as pure as would have been hoped, with the presence of many proteins which could be detected by an anti-MNV capsid polyclonal antibody. In comparison the MNV infected cell lysate preparation contains intact and fragmented structural and non-structural proteins. The lack of non-structural proteins in the VLP preparation guarantees that any antibodies with an OD value >0.3 are binding to the capsid protein, having been previously screened to ensure they do not detect cellular SF9 or Raw 264.7 proteins.

Monoclonal antibodies were screened against MNV infected cell lysate during the purification process in a series of direct ELISAs. Mouse 3 already had antibodies detecting MNV prior to immunisation, which may have led to isolation and purification of monoclonal antibodies directed towards an MNV non-structural protein. This is demonstrated with only five of the twelve antibodies detecting MNV VLPs in the direct ELISA. It was possible that the remaining monoclonal antibodies detected non-structural proteins and were discounted from the study.

Table 7. 12 monoclonal antibodies, tested in a direct ELISA against MNV VLPs. OD values >0.3 are shown in bold.

Monoclonal antibody	OD_{450nm} Value	ELISA Designation
99	**0.762**	**+**
100	**0.750**	**+**
101	**0.743**	**+**
102	**0.731**	**+**
121	0.142	-
125	**0.817**	**+**
128	0.090	-
129	0.088	-
130	0.100	-
131	0.100	-
139	0.098	-
140	0.126	-
CM64 (negative control)	0.101	-
6.2.1 (positive control)	**0.829**	**+**

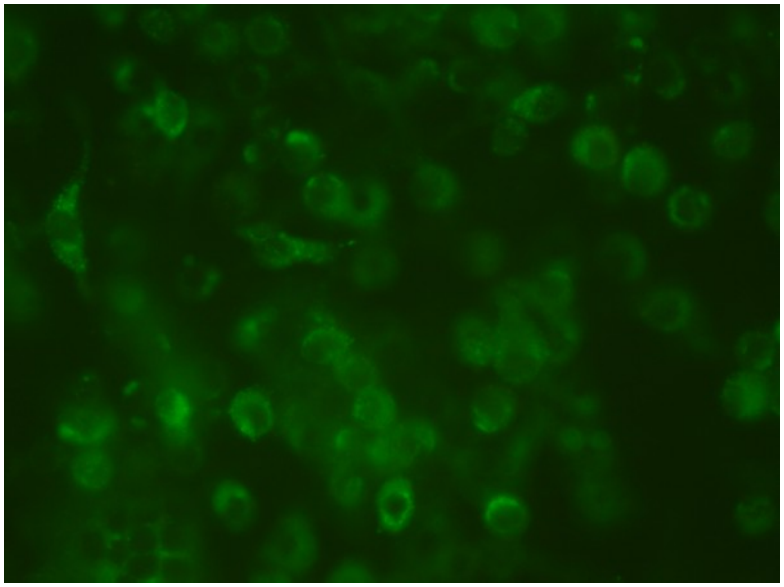
To test the hypothesis that the five monoclonal antibodies may bind a linear epitope, they were analysed by immunoblot against MNV infected cell lysate (2.10.6). None of the monoclonal antibodies detected MNV infected cell lysate (Data not shown, as no bands were present on the strips). Therefore it can be assumed that the five antibodies detect a conformation dependent epitope in the capsid protein.

To further investigate the assumption that these antibodies could detect an epitope in the MNV capsid protein, immunofluorescence was performed with MNV infected cells. Coverslips of Raw 264.7 cells were inoculated with MNV at a MOI of 1 and fixed with 4% formaldehyde (2.9.4). Hybridoma supernatant was added to the coverslip and detected using a secondary antibody, goat anti-mouse IgG Fluorescein isothiocyanate (FITC). FITC is a derivative of fluorescein, functionalised with an isothiocyanate reactive group. The isothiocyanate group reacts with amino terminal and primary amines in the proteins.

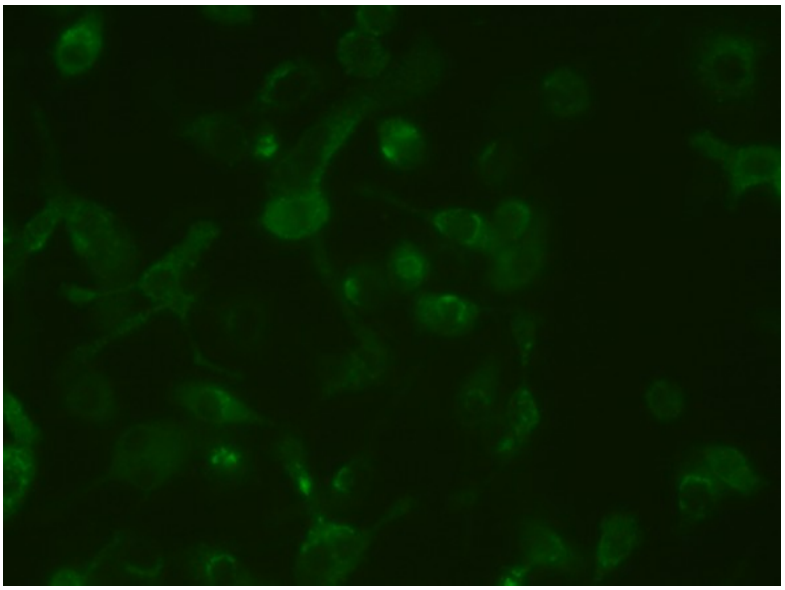
Images of each monoclonal antibody with MNV infected cells are shown in Figure 11. The negative control, CM64 showed no reactivity against MNV; positive control polyclonal rabbit anti MNV capsid (a kind gift from colleagues at Otago University, NZ) showed detection of MNV by visualisation of the FITC, fluorescent green. All five antibodies were able to detect MNV in infected cells confirming the location of the epitope in the capsid protein, by the detection of MNV virions. Localisation of fluorescence was visualised in a pattern similar to that reported by Ward *et al* when Raw 264.7 cells were infected with MNV and detected using an anti-capsid antibody (251). This reference supported the assumption that these antibodies were detecting solely capsid protein. MNV virions are intact, capable of causing infection of Raw 264.7 cells, no non-structural proteins present and the RNA genome is contained within the capsid. Non-structural proteins may be localised to a specific location within the Raw 264.7 cells, therefore antibodies which detect non-structural proteins would result in specific localised fluorescence (251).

Monoclonal antibodies which detect a linear epitope provide the greatest opportunity for incorporation into diagnostic tests. The disadvantage of a conformational epitope is that it requires the 3D structure of the protein to remain intact which is not guaranteed in degraded virions and are therefore limited to intact or functional proteins. Ideally a linear epitope can detect protein in both intact and degraded virus. A linear epitope also provides opportunity for epitope mapping using a peptide array, so the exact recognised amino acid sequence can be determined.

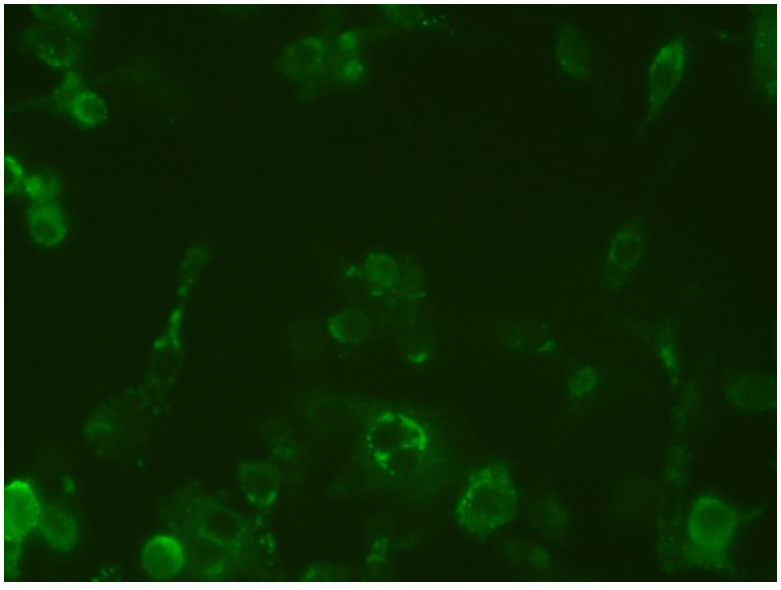
CM99



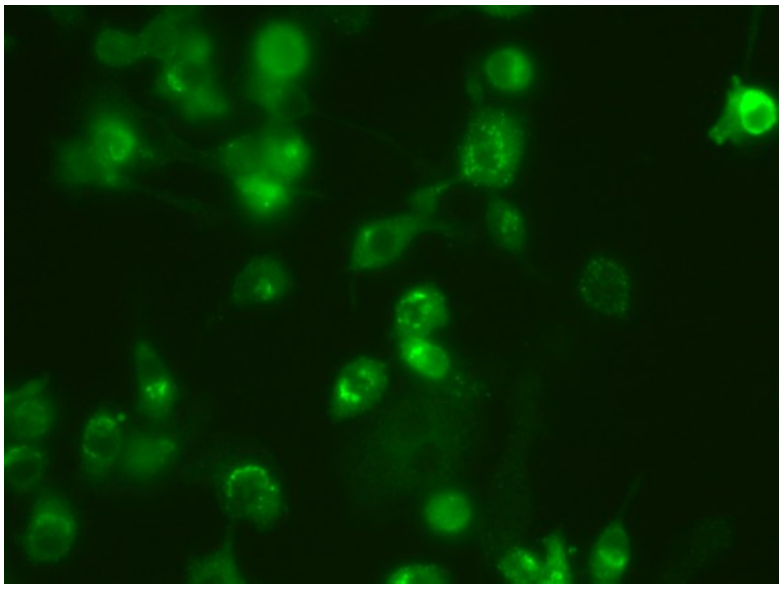
CM100



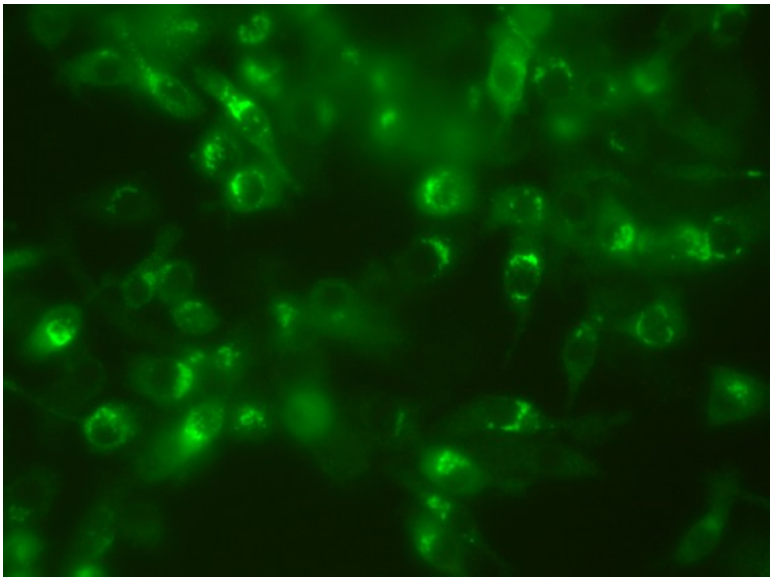
CM101



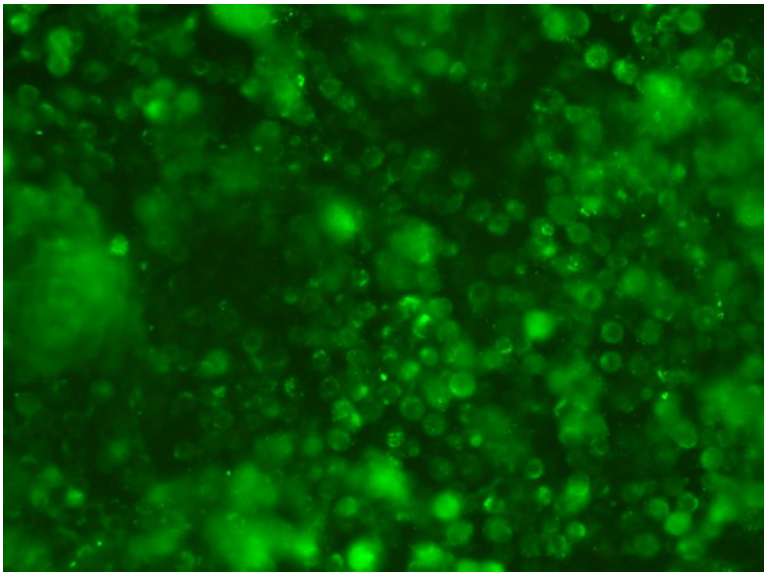
CM102



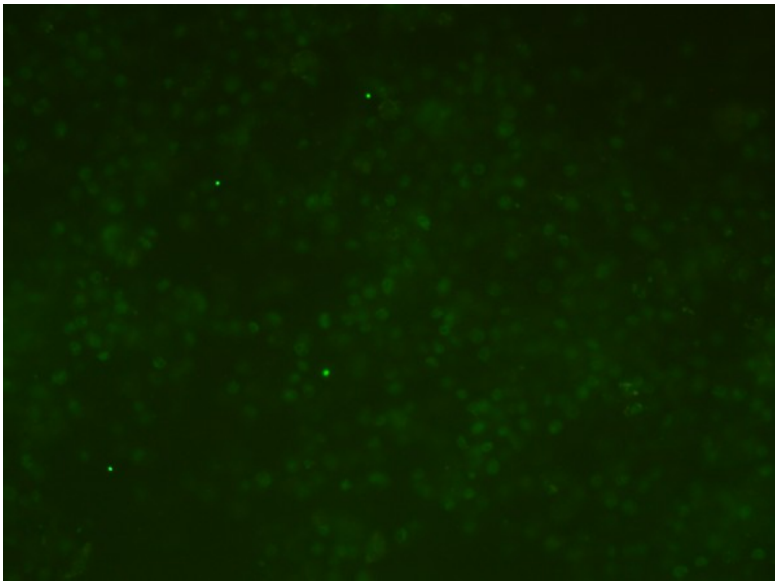
CM125



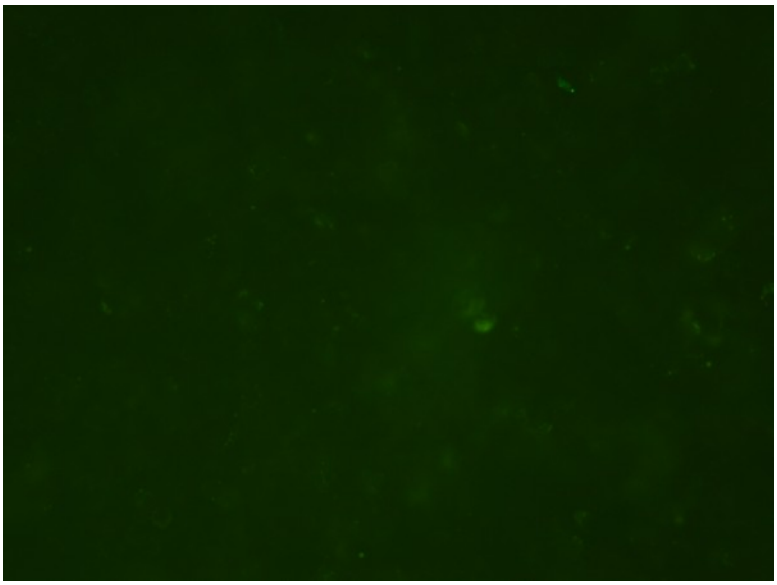
Polyclonal Anti-MNV capsid



CM64



Uninfected Raw Cells



Uninfected Raw Cells DAPI

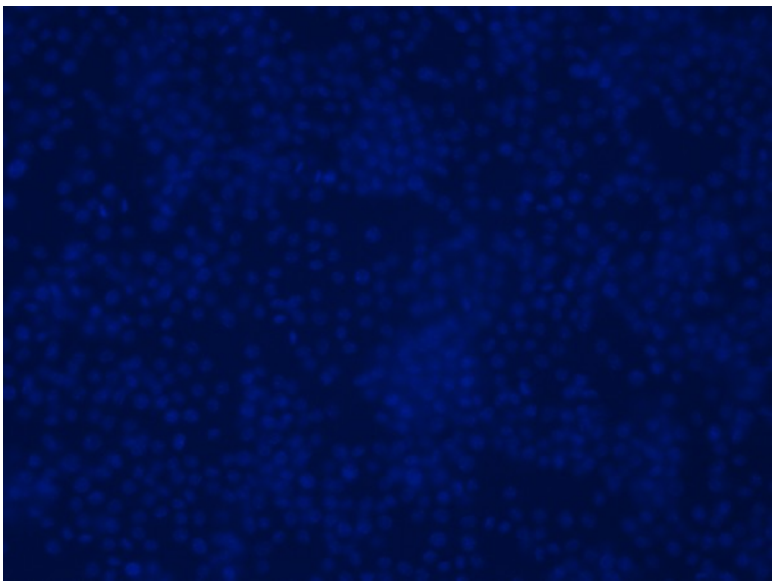


Figure 11. Immunofluorescence images, of five monoclonal antibodies against MNV infected Raw 264.7 cells.

Isotyping of the monoclonal antibodies was performed using the Mouse Typer Sub-Isotyping kit (2.8.11). The type of heavy chain present in the antibody determines the class and subclass of an immunoglobulin molecule. There are five antibody isotypes, IgA, IgD, IgE, IgG and IgM. Each isotype has different properties so it is important to determine the isotype of each monoclonal antibody.

IgG is the most common immunoglobulin type, in mice there are four IgG subclasses, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3. IgG consists of 2 gamma heavy chains that are disulphide linked with 2 light chains that are disulphide linked, one to each heavy chain. IgA is found as a monomer and is the main immunoglobulin in mucous secretions and is secreted in saliva and milk. IgM is a pentamer of the basic IgG structure; this is the first antibody that is made in response to an antigenic challenge. IgD and IgE are present in very low levels in the serum, the functions of these isotypes is less defined than the previously described isotypes.

The mouse Isotyping kit can detect isotypes, IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and which immunoglobin light chain the antibody has; either lambda (λ) or kappa (κ).

The summary of the monoclonal antibody isotypes is shown in Table 8. This summary when combined with information of the master well the hybridomas were picked from in the original fusion 96 well plates indicated that there are four unique hybridomas. These unique hybridomas may have different properties and detect epitopes located in different regions of the norovirus capsid protein. It was therefore important to fully characterise each antibody to determine if any have similar properties, which may indicate they are identical.

Table 8. Isotypes of the five monoclonal antibodies which detect MNV capsid protein**.**

Monoclonal antibody	Isotype	Light Chain
99	IgG_{2b}	λ
100	IgG_1	λ
101	IgG_3	κ
102	IgA	λ
125	IgG_{2b}	λ

3.7 Development of an MNV capture ELISA and qRT-PCR

After full characterisation of the five monoclonal antibodies, a capture ELISA and qRT-PCR was developed to determine if the antibodies could capture intact virions. Due to the lack of a method for quantifying infectious human norovirus virions; a capture ELISA/qRT-PCR provides a way of achieving this aim using MNV. This method incorporates MNV monoclonal antibodies which are hypothesised to bind intact MNV virions due to the identification that they are capable of binding MNV VLPs and qRT-PCR which enables quantification of infectious norovirus. Despite the lack of cross-reactivity with human noroviruses when using these antibodies, this method still provides an invaluable insight into the infectivity potential of these viruses.

Previous experiments supported the assumption that the epitope detected by the five monoclonal antibodies was located in the MNV capsid protein, however they were unable to conclude if the epitope was located in the protruding or shell domain. A capture ELISA would enable RNA extraction after removal of the capsid protein which would expose the genomic RNA. This genomic RNA could be quantified using a MNV VPg nucleic acid specific qRT-PCR, previously developed in our laboratory.

The proposed capture ELISA strategy is shown in Figure 12; it utilises an anti-Fc antibody to capture the monoclonal antibody, ensuring the binding properties of the monoclonal antibodies can be preserved and providing maximum detection of MNV virions. It is hypothesised that as the monoclonal antibodies can bind MNV VLPs, they may also be capable of binding intact MNV virions. Once the virions have been captured by the monoclonal antibody, the capsid protein will be removed using proteinase K, releasing the genomic RNA which will be quantified in a qRT-PCR. This would provide proof that these monoclonal antibodies are capable of capturing intact virus and may also be able to capture intact virions in environmental samples.

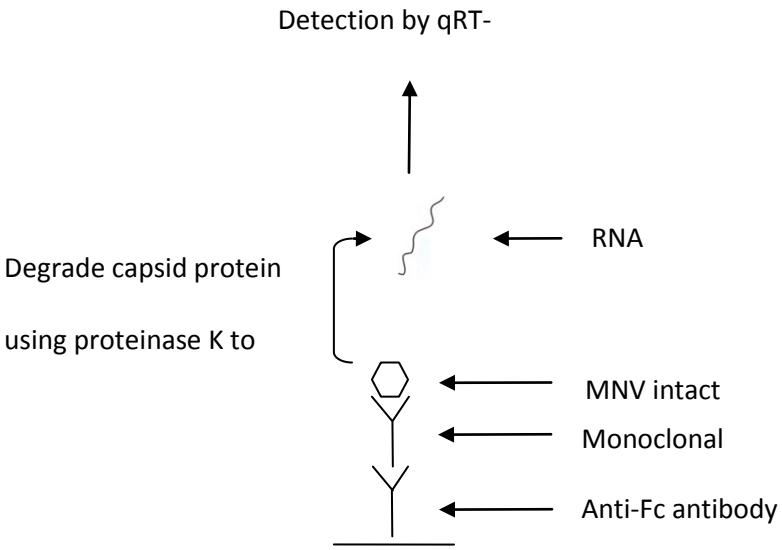


Figure 12. Proposed capture ELISA and qRT-PCR experimental strategy, with the aim of quantifying genomic RNA from captured virions.

MNV CW1 viral stock was a kind gift from C.Wobus *et al* in America. Viral stocks were prepared as described previously (2.10.6) and the titre determined by plaque assay (2.9.5). To ensure all viral stocks used in capture ELISA experiments were of sufficient quality they had to have a titre of >1 x 10^7 plaque forming units (pfu). This titre was used to ensure the maximum number of virions were available for detection and capture.

Viral stocks are capable of infecting Raw 264.7 cells indicated by obtaining a sufficient titre in a plaque assay. To infect the Raw 264.7 cells in this assay the virions need to remain intact, ensuring they contain functional viral RNA within the capsid. As the viral stock is capable of causing an infection it can be assumed that it is mostly intact capsid protein and contains a minimal quantity of fragmented capsid and non-structural proteins.

Initial experiments aimed at developing a method to capture virions and quantify genomic RNA, involved performing a direct ELISA using all five monoclonal antibodies (2.8.8). These antibodies could detect MNV VLPs, MNV infected cell lysate but they could not detect MNV viral stock which contains intact virions, as shown in Table 9.

This experiment did not utilise the anti-Fc antibody as the capture antibody. The inability to capture intact virus via a direct ELISA may have been due to the binding properties of the monoclonal anti-MNV antibodies being disrupted by binding directly to the plate surface. For this reason an anti-Fc antibody was incorporated into future experimental plans to overcome these obstacles.

Monoclonal antibody	MNV VLPs	MNV infected cell lysate	MNV virus
99	**+ (0.806)**	**+ (0.338)**	-(0.109)
100	**+ (0.793)**	**+ (0.332)**	-(0.108)
101	**+ (0.802)**	**+ (0.286)**	-(0.106)
102	**+ (0.790)**	**+ (0.278)**	-(0.095)
125	**+ (0.916)**	**+ (0.424)**	-(0.104)
6.1.1	**+ (1.009)**	**+ (0.617)**	-(0.193)
CM64	- (0.085)	- (0.099)	- (0.099)

Table 9. Direct ELISA values for monoclonal antibodies, 99, 100, 101, 102, 125 and positive and negative controls 6.1.1 and CM64 respectively. Positive results (>0.2 OD_{450nm}) are highlighted in bold.

The successful detection of MNV VLPs and MNV infected cell lysate in the direct ELISA led to the development of a capture ELISA with the incorporation of anti-Fc antibody as the coating antibody, hypothesised to preserve the binding properties of the anti-MNV capsid monoclonal antibodies. The capture ELISA utilised a similar strategy to that shown in Figure 12, without subsequent qRT-PCR detection of genomic RNA. The experiment was performed as described (2.8.9) with the following modifications; the wash step of PBST was replaced with DMEM. The use of DMEM was hypothesised to prevent degradation of the intact virions and therefore maximising detection compared to the use of detergent Tween-20, which had been used in the direct ELISA.

All five monoclonal antibodies, positive against MNV VLPs in a direct ELISA, were tested alongside positive control 6.1.1 and two negative controls, capture antibody anti-FC with MNV viral stock and CM125 with Raw 264.7 cell lysate. The antibodies were analysed as the coating antibody and using anti-Fc antibody as the coating antibody, to determine if binding of virions can be improved using an additional capture antibody. A polyclonal anti-MNV capsid antibody (a kind gift from colleagues in New Zealand) was used as the detection antibody in both experiments.

A summary of the results obtained are shown in Table 10. The positive cut-off was taken as OD_{450nm} values >0.3, double the negative control, anti-FC with MNV viral stock.

Monoclonal antibody	MNV viral stock, no anti-FC antibody	MNV viral stock with anti-FC antibody
99	- (0.255)	-(0.074)
100	- (0.182)	-(0.080)
101	- (0.231)	-(0.081)
102	- (0.217)	-(0.094)
125	+ (0.298)	**+(2.469)**
6.1.1	**+ (2.984)**	**+ (2.776)**
Anti-FC with MNV viral stock		- (0.153)
125 with Raw 264.7 cell lysate		- (0.091)

Table 10. Capture ELISA values for monoclonal antibodies, 99, 100, 101, 102, 125 and positive and negative controls. Positive results are highlighted in bold.

None of the five monoclonal antibodies could bind MNV virions when used as the coating antibody. However, the addition of the anti-Fc antibody enabled the detection of MNV viral stock with CM125. This experiment alone does not indicate if the epitope detected by this antibody is located in the P domain or if the antibody is capturing intact virions. To verify this assumption it was important to perform the experiment with subsequent quantification by qRT-PCR, shown in Figure 12.

A capture qRT-PCR was performed as described (2.8.9) with modifications; the wash step of PBST was replaced with DMEM and prior to the addition of a detection antibody, followed by addition of Tris-HCl buffer to each well, apart from the positive control, MNV viral stock with no capture antibody. Proteinase K was applied to each well for one hour at 60^{o}C and the reaction stopped with 0.1mM of PMSF, incubated for 10 minutes at 95^{o}C. 5µl of this sample was then used in a MNV VPg nucleotide specific qRT-PCR. All five antibodies were analysed by capture qRT-PCR.

No amplification of RNA was seen for the five antibodies analysed or the negative control, amplification was seen for the positive control MNV viral stock with no capture antibody. The lack of amplification of viral RNA in the qRT-PCR while providing evidence that the epitope is likely to be located in the S domain may also be due to other factors. The proteinase K digestion of the capsid may not have completed, leading to a reduced quantity of available RNA, alternatively the RNA could have degraded upon exposure to heat when released from the capsid. The successful amplification of viral RNA from the viral stock which was not captured by the monoclonal antibody, does suggest that this was not the case in this instance.

3.8 Discussion

Murine norovirus VLPs were used to immunise four Balb/c mice, with the aim of generating broadly cross-reactive monoclonal antibodies towards a range of human noroviruses. If this

approach was successful it was hoped that these antibodies could be incorporated into a capture ELISA, enabling extensive epidemiological and diagnostic studies to be undertaken with both murine and human noroviruses.

MNV detecting antibodies could be used to detect MNV in complex matrices often found in environmental samples, to gain an insight into the relation between positive results in an immunoassay to the potential risk of infection. The same sample which is detected in a capture ELISA can also be grown in cell culture and the titre of virus determined using a plaque assay. In these experiments; MNV would be used as a surrogate for human noroviruses, as there is no assay which is capable of differentiating between infectious and non-infectious virions.

From immunisation of four mice and subsequent hybridoma production, fifty three purified hybridomas secreting antibodies able to detect MNV infected cell lysate were obtained. This was reduced to twelve after removal of hybridomas which displayed cross-reactivity with RAW 264.7 and SF9 cell lysates. The properties of these antibodies were characterised further through a series of direct ELISAs, immunoblot and immunofluorescence experiments.

Results obtained from screening the twelve antibodies by direct ELISA against a range of antigens (MNV, RAW 264.7and SF9 cell lysates and a panel of seven human norovirus VLPs), suggested that it may not be possible to produce broadly cross-reactive monoclonal antibodies against human and MNV norovirus VLPs using this experimental strategy.

Due to the lack of human norovirus cross-reactivity further characterisation was performed solely with MNV. MNV could still be utilised as an indicator for the potential risk of infection from human noroviruses by seeding of environmental samples with MNV and comparing the immunoassay results to infectivity observed in *in vitro* cell culture. To

determine if these monoclonal antibodies could be used in this application, they would require a linear epitope located in the P sub-domain of the capsid protein.

To determine the location of the epitope, a direct ELISA using MNV VLPs was performed. Only five of the twelve antibodies could detect MNV VLPs, the high number which could not detect MNV VLPs is most likely due to the number of pre-existing MNV B cells in the mice, produced by the mouse's immune system against a previous infection or exposure to murine norovirus. As the monoclonal antibodies were screened using MNV infected cell lysate, containing both capsid and non-structural proteins, it enabled the picking and purification of antibodies which may not detect an epitope in the capsid protein.

Five of the twelve antibodies detected an epitope located in the MNV capsid protein, the remaining seven were assumed to be directed towards non-structural proteins encoded by ORF1; these antibodies were not suitable for use in this project and not characterised further.

In future work, further characterisation of the seven discarded antibodies could be performed to determine if they are detecting non-structural proteins. However, to obtain this information a method of obtaining a preparation of only non-structural proteins would be required. From previous characterisation experiments it was shown that the seven antibodies did not detect uninfected Raw 264.7 or SF9 cells, however could detect MNV infected Raw 264.7 cell lysate. MNV infected Raw 264.7 cell lysate contains both structural and non-structural proteins due to the method in which the lysate is generated (2.10.7). The lack of detection of MNV VLPs demonstrated that the antibodies did not detect capsid protein.

Within our laboratory a R1 cell line was developed which when induced, expresses solely non-structural MNV proteins. This cell line provides the opportunity of performing an

immunoblot of induced R1 cell lysate against the individual monoclonal antibodies. If the seven antibodies are capable of detecting an epitope in the non-structural proteins a band will be visible at the appropriate size for one of the following proteins, N- term, NTPase, p20, VPg, 3C pro and 3D pol. These results would provide evidence that these antibodies could detect a linear epitope, present in one of the non-structural proteins.

An alternative route to obtaining non-structural protein samples is to insert the appropriate gene into a bacterial plasmid vector and transform the plasmid into competent *E.Coli* cells, such as BL21. This cell line contains a T7 polymerase which can be induced upon the introduction of d-galactopyranoside (IPTG) to produce high level protein expression. An immunoblot of the non-structural protein containing lysate can be performed using the suspected MNV non-structural protein binding monoclonal antibody as the detection antibody. Visualisation of a band at the described size for one of the non-structural proteins would indicate the antibody recognises the inserted non-structural protein.

The ideal monoclonal antibody would detect a linear epitope in the P domain of the MNV capsid protein, increasing the potential of detecting noroviruses in complex matrices such as environmental samples where the virus may be either intact or degraded. The location of the epitope in the P domain would ensure that it was accessible for antibody binding. The P2 domain is located on the outer tip of the capsid protein and is suggested to be under the greatest immunogenic pressure (153), shown by the exposed hyper variable region which mutates rapidly and varies extensively between different genotypes (2,23). Therefore it is unlikely that a broadly cross-reactive antibody could detect a sequence common to both GI and GII viruses in this region.

To investigate the reactivity pattern of the five selected antibodies towards MNV capsid protein, an immunoblot analysis was performed to confirm the direct ELISA results. None of the five antibodies showed reactivity to MNV infected cell lysate, suggesting that the

antibodies are detecting a discontinuous epitope in the MNV capsid. This assumption was taken as the protein sample is degraded by heat and β-mercapaethanol prior to running through an SDS gel, destroying the 3D protein conformation, producing linear protein. By linearising the protein, all conformation dependent epitopes are removed, permitting only linear epitopes to be detected. A linear epitope would be beneficial for further research, as a conformational epitope requires the antigen to remain intact so that discontinuous amino acids come together in a 3D conformation which can be recognised by the antibody binding regions. Antigen in environmental samples cannot be guaranteed to not degrade over time; therefore antibodies binding conformational epitopes are not routinely used in these types of diagnostic assays.

Analysis by immunofluorescence against MNV infected Raw 264.7 cells showed fluorescence could be visualised with the application of the five antibodies, further supporting the assumption that all five antibodies recognise an epitope in the MNV capsid protein.

Despite the five antibodies detecting MNV VLPs and lysate in a direct ELISA, they could not be incorporated into a capture qRT-PCR for the detection of native virus. A virus capture method would enable capture of MNV virions in complex matrices such as faecal specimens and the subsequent detection of RNA by removal of the capsid protein and quantification by qRT-PCR. The lack of detection when using the MNV antibodies alludes to the epitope being located in the semi-conserved S domain which is not located on the surface of the virion. The antibodies isolated likely detect a conformational epitope (determined by immunoblot) in the interior shell domain, rather than the external protruding domain. The location of the epitope is drawn from analysing the characterisation results, including direct and capture ELISA, immunoblot and immunofluorescence. Detection of MNV VLPs and MNV infected cell lysate in the ELISA and

MNV virus in the immunofluorescence provided evidence that the epitope was located in the capsid protein, the lack of detection in the immunoblot indicated that the epitope was conformation dependent. No amplification of genomic RNA in the capture qRT-PCR demonstrated that it is likely that the epitope is within the shell domain resulting in an inability to capture intact virions. MNV VLPs and MNV infected cell lysate both contain capsid protein fragments, where the shell domain is exposed. In intact virions the shell domain is beneath the protruding domain, preventing monoclonal antibodies which detect a shell domain epitope from binding.

It was possible for antibodies to be isolated which bound an epitope in the S domain rather than the desired P domain as during immunisation and purification as MNV VLPs were used as the immunising antigen and MNV infected cell lysate was used as the screening antigen. These antigens could contain both intact and fragmented capsid protein; fragmented protein enables the S domain to be exposed for antibody recognition. The choice of immunisation route has been suggested to influence the location of the epitope the isolated monoclonal antibodies detect. Monoclonal antibodies produced from mice immunised subcutaneously with boosts administered intrapentoneally regardless of the antigen used, detect predominately epitopes in the N terminus of the capsid (95,237,258). While mice immunised intraperitoneally or orally produce antibodies directed towards the C-terminal domain (89,131).

For a capture ELISA to detect intact MNV virions using monoclonal antibodies, an epitope in the P domain is required. The P domain is easily accessible for antibody binding, located on the outer surface of the virion. However the P domain is highly variable in comparison to the more stable and conserved S domain. Antibodies binding to epitopes in the P domain would permit capture of intact virions in the assay, from which the capsid protein could then be removed, releasing the genomic RNA for quantification by qRT-PCR; providing a

method of relating ELISA values to the number of RNA copies per ml of sample and therefore the number of intact virions present in a sample.

The lack of cross-reactivity of the isolated monoclonal antibodies against a panel of human noroviruses was not surprising, mice were immunised only with MNV VLPs, contained within a separate genogroup (GV) to the human norovirus VLPs (GI and II). Murine norovirus has the furthest divergence, when viewed phylogenetically to any other norovirus genogroup (219).

When the genomes both of murine norovirus, MNV-1 and human norovirus, Norwalk, are aligned they have 50% amino acid sequence similarity, containing suitable regions for potential epitope recognition. These regions require stretches of six or more identical amino acids across the norovirus sequences. A higher proportion of divergence is present in the capsid region Norwalk and MNV-1, sharing only 40% sequence identity when the amino acid sequence is aligned, with stretches of identical amino acids predominately in the S domain. It has previously been reported that the S domain is a highly conserved region amongst different genogroups of noroviruses (84).

Cross-reactive monoclonal antibodies have been isolated for genogroup I and II viruses but as of yet no monoclonal antibody has been isolated which covers all five genogroups (15,191,215). With only 40% amino acid similarity in the capsid protein between Norwalk and MNV viruses there are only a few possible regions of homology which are suitable for antibody detection. For this reason production of a broadly cross-reactive antibody is possible, however it would be a rare occurrence.

It was unfortunate that none of the monoclonal antibodies could be incorporated into an ELISA for the detection of intact MNV virions. As a result these antibodies could not be incorporated into an immunoassay for the detection of MNV seeded into oyster samples

enabling the relationship between ELISA and qRT-PCR results to be evaluated. However isolation of five antibodies which detect an epitope in the shell domain of MNV capsid protein will enable future work into characterisation of MNV. From the results presented in this chapter, conclusions can be drawn that the immunisation of mice with MNV VLPs does not commonly produce cross-reacting monoclonal antibodies detecting genogroups, GI, GII and GV, using the described experimental strategy and screening procedure.

Future experiments must be aimed at overcoming the lack of antibody which can detect a linear epitope in the P domain of MNV capsid, permitting detection of intact and degraded virions. From the immunisation regime described in this work, two mice remained which had been immunised with MNV VLPs. In a bid to obtain cross-reacting antibodies these mice could be boosted with a human norovirus, such as a Lordsdale (LV) VLP preparation. Lordsdale is a GII.4 norovirus, GII.4 viruses are the predominant circulating strain of noroviruses worldwide (18,23,219). This strategy could potentially increase the mouse's immune response to both viruses and resulting in B cells producing the desired antibodies.

The decision was made to boost immunise the two remaining mice with LV VLPs, unfortunately during the immunisation process the animal house which housed these animals was infected with mouse hepatitis virus (MHV). Due to the limited availability of MNV VLPs, the choice was taken to continue with the experimental plan. It was hoped that an MHV PCR could be developed which would enable the detection of MHV infected hybridomas. The results from this experimental plan are discussed in Appendix 1.

A separate potential avenue is producing a chimeric MNV incorporating a known linear epitope sequence detected by previously isolated monoclonal antibody into the P2 domain; this strategy would overcome the problems encountered in the previously described experiments.

Chapter 4 Development of Chimeric Murine Norovirus

4.1 Introduction

The 3D structure of baculovirus-expressed, recombinant Norwalk capsid protein was determined by electron microscopy and computer image processing to a 2.2nm resolution (197). These observations showed empty spherical shells, which were 38nm in diameter with a T=3 icosahedral symmetry. The capsid shell (S) domain was comparable in size to the S domains of TBSV (Tomato Bushy Stunt Virus) and TCV (Turnip Crinkle Virus). The S domains of these plant viruses form an 8 stranded β-barrel structure which has also been shown to be formed by the 170 N-terminal residues of the recombinant Norwalk virus protein by X-ray crystallography (197). These findings have been confirmed by a further study of the X-ray structure of the recombinant Norwalk virus (NV) capsid (198). The shell domain forms the interior shell of the capsid protein, from which the P domain forms arch-like protrusions.

The P domain is comprised of two sub domains, P1 and the P2; of these it is the P2 domain which is most protruding. The P2 domain contains a region of sequence which when multiple noroviruses are aligned, illustrate that it is the most hyper variable region of the genome. Prasad *et al* therefore suggested that this sub domain is highly antigenic and determines strain specificity (198).

Investigations into the hyper variable sequence of the P2 domain have focused on immunosuppressed patients with chronic diarrhoea; it was found that this region had the highest number of cumulative amino acid substitutions (185). Isolated norovirus sequences from these patients taken over time had eleven cumulative amino acid substitutions; eight occurred in the P2 domain, two in the P1 domain and one in the S domain (185).The location of the P2 domain as the most protruding sub-domain and the number of amino

acid substitutions reported in these patients indicated that these mutations are selected by immune pressure.

A primary aim of norovirus research is to establish a reliable *in vitro* tissue culture system from which viable virus can be recovered and passaged. These techniques would enable a quick and easy method of identifying norovirus infection in clinical samples to be developed. Whilst this is still an aspirational long term goal, it is important to continue research focused on preventing outbreaks of norovirus infection in the human population. Current research into norovirus replication has focused predominately on two main surrogates, feline Calicivirus (FCV) and murine norovirus (MNV).

Whilst MNV is not the ideal surrogate, causing differing clinical symptoms in mice to humans, it has similar properties in terms of pH stability, genome organisation and capsid protein structure homology to human noroviruses (27). Due to this homology, MNV can be utilised in research focused at developing methods to study viral concentration in oyster tissue and replication potential, once recovered. Whilst there is no *in vitro* cell culture model for human noroviruses, MNV can be propagated in Raw 264.7 cells (254). The murine norovirus 'model' has provided opportunities to determine the infectivity potential of these viruses in complex matrices such as oyster tissue.

It is proposed that from oyster samples seeded with MNV, MNV can be recovered from the tissue and propagated to quantify the viral titre; this titre can be compared to the initial seeded titre which is currently not possible with human noroviruses. Alongside this, it is hoped a method could be developed, in which MNV seeded into environmental samples could be detected using an antigen capture ELISA/qRT-PCR and simultaneously propagated in cell culture. Results obtained would enable the relationship between 'positive' OD values to be compared to the viral titre and quantity of RNA genomes present, as determined by qRT-PCR providing an insight into the viability of the virus once in oyster tissue.

Unfortunately, neither antigen capture nor qPCR has the capability of detecting and quantifying solely infectious norovirus particles. By linking these data to MNV infectivity it might be possible to extrapolate the results to human norovirus detection. At the start of this project there was no suitable monoclonal antibody directed towards MNV capsid protein, detecting both degraded and intact virions from environmental samples.

Monoclonal antibodies isolated through the work described in Chapter 3 were unable to detect intact virions, leading to the assumption that the epitope was located in the S domain. The location of the epitope and the lack of cross-reactivity with a panel of human noroviruses meant the isolated monoclonal antibodies were unsuitable for use detecting noroviruses in oyster samples.

The MNV virion is composed of the characteristic structural and non-structural proteins seen across all noroviruses. Chimeric MNV, incorporating a previously isolated and characterised monoclonal antibody epitope sequence into the P domain, offers an alternative route to developing monoclonal antibodies which have a linear epitope. These antibodies could be utilised for detection of degraded and intact capsid. Insertion of a known epitope nucleotide sequence into the MNV genome is hypothesised to enable a method of detection of intact and degraded virions by immunoassay techniques; however this is only possible if viable virus can be produced.

Monoclonal antibody CM54 was isolated following immunisation of Balb/C mice with Southampton virus (GI) VLPs and successful fusion of the mouse B cells with NS-1 cells. Antibodies were selected which immunoprecipitated Southampton virus capsid protein in radio immune precipitation assays (C.Batten, PhD Thesis). The epitope sequence detected by CM54 was chosen for incorporation into MNV, this is a linear epitope common to all GI noroviruses, LEDVRN. The location of the epitope in the S domain prevented CM54 from detecting intact virions in environmental samples (C.Batten, PhD Thesis). The insertion of

the LEDVRN epitope into the P2 domain of MNV decreases the possibility of detecting pre-existing GI norovirus in environmental samples as native GI virions cannot be detected with this antibody. GI viruses have been implicated in several shellfish related outbreaks (119,157); however they have been shown to be the cause of less than 10% of outbreaks in clinical settings (188,252).

Previous characterisation of monoclonal antibody CM54, demonstrated that it was capable of binding Southampton virus VLPs and SV-GST fusion protein, as well as cross-reacting with Southampton virus (GIa), Babbacombe (GIa), Norwalk virus (GIb) and Desert Shield virus (GIc) VLPs in a direct ELISA. All of the viruses detected in the direct ELISA were GI. The LEDVRN amino acid sequence is also present in the bovine norovirus, Newbury-2. This sequence is not present in GII noroviruses, bovine norovirus Jena virus or sapoviruses. CM54 did not detect GII and GIII noroviruses or sapoviruses in a direct ELISA nor did it detect native Southampton virus in faecal specimens (C.Batten, PhD Thesis). The lack of detection of virus in faecal specimens further demonstrated that the epitope was not on the surface of the capsid.

Epitope mapping of the linear epitope was performed previously and showed that this sequence corresponds to amino acid residues 162 -167 LEDVRN in the Southampton virus capsid protein (C.Batten, PhD Thesis) located in the S domain. The S domain is involved in forming the icosahedral shell and is thought to be protected from the host's immune system (198).

The experimental strategy undertaken in this work aims to develop chimeric MNV; bypassing the requirement to isolate a monoclonal antibody with the desired properties, a linear epitope located in the P domain. If it is possible to construct a viable chimeric MNV it could be utilised in developing a method of detection in samples which have been implicated in human norovirus infection, such as oysters.

The production of recombinant human norovirus is not uncommon and is a naturally occurring event. Recombinant viruses such as Snow Mountain and Saitama viruses have been reported previously (78). Within the genome of these particular viruses, recombination occurred with the RdRp from cluster GII.4 and a capsid protein which originating from a separate cluster of noroviruses combining to generate a novel virus (85,88,111,124,244). It has been proposed that recombination events and the mutation of regions within the sequence of the genome is a mechanism that noroviruses have evolved to evade detection by the host immune system (153,185).

The description of recombination and mutation events within the norovirus genome led to the hypothesis that the insertion of a novel sequence would not disrupt the formation of viable virus, indeed this is a method utilised by the virus itself to evade the host immune system. The decision to embark upon this experimental strategy was also supported by findings from colleagues in America, C.Wobus *et al*. Through personal communication, these colleagues reported the successful insertion of a Norwalk sequence, NGIGSGN into MNV ORF2. This insertion resulted in production of viable virus, which had a log less pfu/ml than wild type MNV when assessed by plaque assay.

The insertion of the CM54 epitope sequence into MNV is intended to produce viable virus which can be detected using monoclonal antibody CM54. This insertion provides both proofs that the P2 domain in MNV can tolerate such a large mutation and provide a method for detecting MNV seeded into oyster tissue by developing an antigen capture ELISA. The production of chimeric MNV, containing the CM54 epitope within the P domain is intended to overcome the barriers of a lack of a monoclonal antibody towards MNV capsid protein described in Chapter 3.

Development of chimeric MNV would enable understanding of human norovirus replication until such time as a reliable *in vitro* cell culture method is described.

4.2 Construction of Chimeric MNV

4.2.1 Development of Chimeric MNV

The region of the MNV genome chosen for the insertion of the LEDVRN epitope was reported as suitable by colleagues in America (C.Wobus *et al*, personal communication). A section of Norwalk virus (GI) amino acid sequence; NGIGSGN was inserted into the P2 sub domain of MNV, replacing the original MNV amino acid sequence SVTAAAS. The nucleotide substitutions undertaken to insert the NGIGSGN mutation are shown in Figure 13. Colleagues in America reported successfully recovering live virus from the transfection of Raw 264.7 cells with MNV containing the Norwalk mutation. MNV containing the NGIGSGN mutation was shown to multiply and infect Raw 264.7 cells a log less pfu/ml than wild type MNV when assessed by plaque assay.

Insertion of the Norwalk sequence into the MNV P2 sub domain places it in an exposed region on the capsid (185). The region of MNV into which the Norwalk sequence was inserted is shown in Figure 15; this is a 3D image of the P2 sub domain, with the SVTAAAS sequence highlighted in red. These experiments by colleagues in America provided evidence that this sequence of MNV, SVTAAAS could be altered and still result in viable virus.

Wild type MNV sequence:

V	F	A	S	V	T	A	A	A	S	L
Gtg	ttc	gcc	agc	gtc	act	gct	gcg	gcc	tct	ctt

Chimeric MNV sequence with Norwalk Virus insertion highlighted in bold:

V	F	A	**N**	**G**	**I**	**G**	**S**	**G**	**N**	L
Gtg	ttc	gc**a**	a**at**	g**g**c	a**t**t	g**gc**	**agt**	g**gt**	**aa**t	ctt

Figure 13. Norwalk virus sequenue inserted into MNV by C.Wobus *et al*. Nucleotide substitutions are indicated in bold which differ from wild type MNV**.**

Software programmes, LaserGene and VMD (a three-dimensional [3D] modelling program) were used to view the position of the proposed insertion sequence, the capsid protein and P2 domain of Norwalk virus and MNV respectively in a 3D format. Colleagues in America (C.Wobus *et al*) provided the crystal data of the MNV capsid P2 domain which was used to generate the image in Figure 15.

The 3D structure of the norovirus capsid was first resolved by cyro-EM (204) and further analysed by Prasad *et al*, from which the X-ray structure of Norwalk virus capsid was obtained (197) to generate the image in Figure 14.

The location of the NGIGSGN sequence in the Norwalk virus capsid is at the tip of the P2 sub domain shown in Figure 15. The amino acids SVTAAAS are located in the MNV P2 domain on one of the side loops at the tip of the P2 domain shown in Figure 15. The P2 domain is hypothesised to be under immunogenic pressure, which can result in random single amino acids substitutions (185).

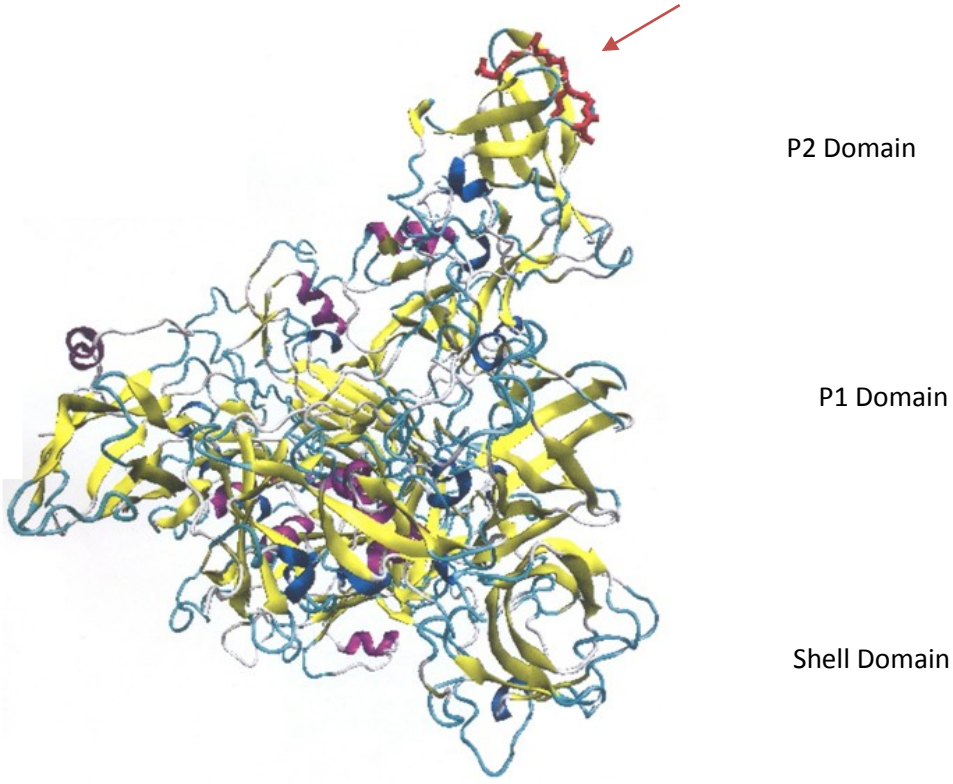


Figure 14. Norwalk virus capsid protein**.** The NGIGSGN sequence located within Norwalk virus which is highlighted by the red arrow.

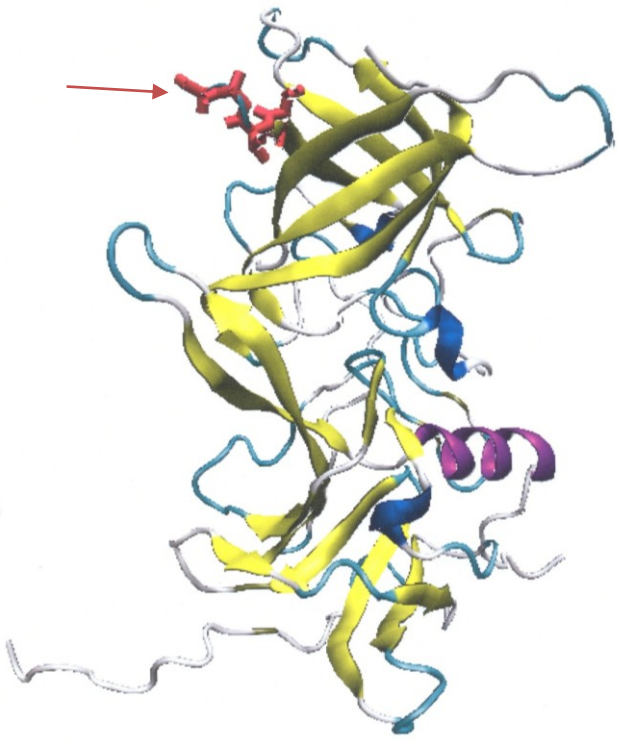


Figure 15. P2 domain of MNV, displaying the location of the SVTAAAS motif, shown by the red arrow**.**

The experimental plan was to replace the SVTAAAS sequence in MNV, with the LEDVRN, CM54 epitope sequence from Newbury and Norwalk virus.

The location of the epitope sequence recognised by monoclonal antibody CM54 was modelled onto the 3D structure of the Norwalk capsid protein in previous work by C. Battern (PhD thesis) and Dr Stefan Oliver (Royal Veterinary College, London, UK) based on the work by Prasad (198). The epitope was mapped using a peptide array consisting of overlapping dodecamers, performed by Mimotopes, UK.

The two amino acid sequences, NGIGSGN and LEDVRN are in different locations in the intact Norwalk capsid. NGIGSGN is located in the P2 domain whereas LEDVRN is in the conserved shell domain. The different locations may alter the ability of MNV to produce viable virus; while NGIGSGN in Norwalk is thought to be under immunogenic pressure, the LEDVRN amino acids are not subjected to immune pressure in the same way. In intact virions, the P domain is protruding from the shell domain.

*4.2.2 Synthesis of chimeric pMNV*containing LEDVRN*

The CM54 epitope sequence, LEDVRN was incorporated into pMNV* plasmid using mutagenic PCR (251). Site directed mutagenic PCR incorporates primers which enable the insertion of a large nucleotide fragment into pMNV*; the primer sequences included two convenient restriction sites to enable efficient re-insertion into the MNV genome. PCR amplification of the fragment containing the desired mutation enables easy separation from the original unmutated plasmid by gel electrophoresis.

The pMNV* plasmid was utilised as it contains a cytomegalovirus (CMV) promoter, ampicillin resistance gene, the full length MNV genome and a polyA tail. These characteristics enable colonies containing the correct mutation to be easily isolated using LB/agar plates containing ampicillin (2.2.4). Presence of a CMV promoter enables

production of transcripts for downstream applications such as transfection. The pMNV* plasmid was available at the start of this project; this plasmid had been used previously in the inducible DNA polymerase (pol) II driven reverse genetics system (251).

The development of the murine norovirus reverse genetics system provided an insight into the effects of variation at the genetic level on the pathogenicity of this virus in the host. Until 2007, the lack of a norovirus reverse genetics system had stalled research into this virus. Two systems have been reported for MNV, the inducible DNA polymerase (Pol) II driven (251) and T7 RNA polymerase driven recovery of MNV (33).

The Pol II driven system describes several methods for generating infectious MNV from cDNA. The two component baculovirus expression system may be used, alternatively a single plasmid system whereby RNA expression is under the control of a CMV promoter is also reported. Ward *et al* (171) described successful transfection of HEK 293T cells directly with a bacmid construct which contained the MNV genome under the control of a CMV promoter. This promoter is linked to the tetracycline (tet) operator/repressor system that has previously been described for Hepatitis C (171). The pol II promoter driven expression of MNV cDNA generated infectious virus after the transfection of HEK 293T cells. Recovered virus was successfully replicated in Raw 264.7 cells and typically yielded 5×10^3 pfu/ml of MNV (251).

A separately described reverse genetics system is based on the T7 RNA polymerase promoter driven expression of cDNA clones (33). Eukaryotic cells expressing T7 polymerase were transfected with a cDNA copy of the MNV genome under the control of the T7 RNA polymerase promoter, genome-like viral RNA was generated.

The availability of the reverse genetics system for the study of MNV has provided a previously lacking method for analysing the role of viral sequences in replication and pathogenesis.

18 nucleotide changes are required to change the 7 amino acids, SVTAAAS, to the CM54 epitope sequence LEDVRN. Figure 16 highlights the proposed nucleotides changes to incorporate the LEDVRN sequence into wild type MNV and the previously successful NGIGSGN sequence. While 18 nucleotide mutations are needed to insert LEDVRN, the NGIGSGN sequence required only 13 nucleotide mutations to be incorporated into wild type MNV. The increase in the number of mutated nucleotides to insert LEDVRN could affect the ability of the MNV capsid protein to fold successfully and prevent the production of viable virus.

Wild type MNV sequence (GV)

S	V	T	A	A	A	S
agc	gtc	act	gct	gcg	gcc	tct

LEDVRN Norwalk Virus sequence(GI)

L	E	D	V	R	N	N
ctt	g**ag**	**gat**	g**tc**	**cga**	**aat**	**aa**t

NGIGSGN Norwalk Virus sequence (GI)

N	G	I	G	S	G	N
a**at**	g**g**c	a**t**t	g**gc**	**agt**	g**gt**	**aa**t

Figure 16. Proposed site directed mutagenesis of the MNV SVTAAAS sequence to the LEDVRN and NGIGSGN sequence; mutated nucleotides are highlighted in bold.

To facilitate ease of inserting this novel sequence, mutagenic PCR was used to replace the MNV sequence, SVTAAAS with GI sequence, LEDVRN (2.7**Error! Reference source not found.**, 2.7.3.1). The proposed cloning plan is summarised in Figure 17.

Site directed mutagenesis was achieved by designing two non-mutagenic primers. These included a forward primer, LEDVRN-F, containing the unique restriction site SacII and reverse primer LEDVRN-R, containing the unique restriction site BspEI. Incorporation of restriction sites ensures that the mutated cassette can be ligated back into the pMNV* genome. The SacII and BspEI restriction sites are unique in the pMNV* plasmid and so cutting at these sites enabled re-integration of the insert into the genome. The two mutagenic primers designed for these experiments are shown in Table 11, with the LEDVRN nucleotide epitope sequence highlighted in bold. The mutagenic primers had complimentary sequence either side of the LEDVRN mutation to ensure efficient primer binding. Figure 17 provides a summary of the mutagenic PCR protocol which was used to incorporate the LEDVRN nucleotide sequence into the MNV genome.

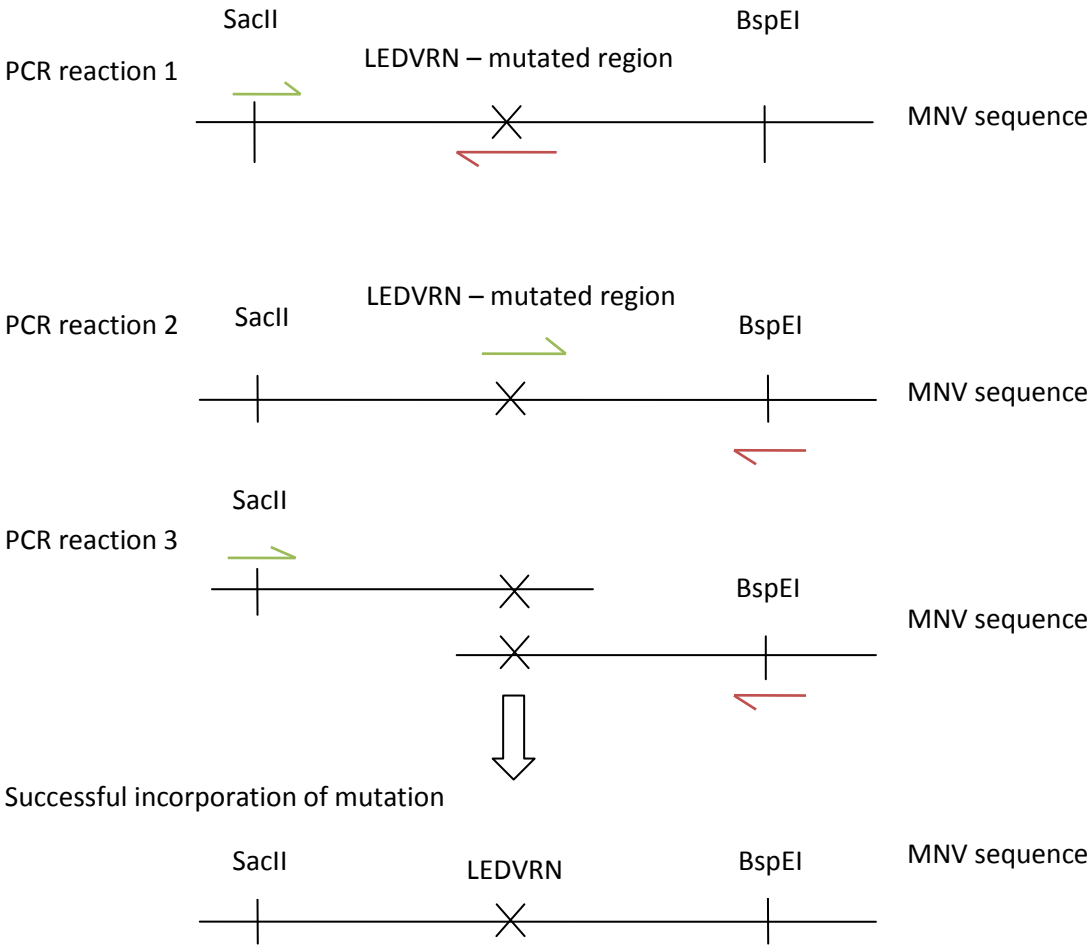


Figure 17. Summary of the mutagenic PCR protocol performed to incorporate the nucelotide sequence of the CM54 epitope, LEDVRN into the MNV genome. The products of two PCR reactions are combined in a further reaction to produce one stretch of MNV sequence containing the mutation.

Table 11. Mutagenic PCR primers designed to insert the LEDVRN amino acid sequence. The LEDVRN nucleotide sequence is highlighted in bold.

Primer Name	Primer Direction	Primer Sequence
LEDVRN_F	3’	ggc-agg-gtg-ttc-gcc-**ctt-gag-gat-gtc-cga-aat-aat** -ctt-gac-ttg-gtg-gat-ggc-agg
LEDVRN_R	5’	cct-gcc-atc-cac-caa-gtc-aag-**att-att-tcg-gac-atc-ctc-aag** -ggc-gaa-cac-cct-gcc

The aim of two component mutagenic PCR is to generate two fragments, the 3' to 5' SacII restriction site to the LEDVRN nucleotide sequence and the 5' to 3' LEDVRN sequence to the BspEI restriction site. These separate fragments then undergo a subsequent PCR to produce one continuous strand of double stranded DNA which runs from restriction site SacII to BspEI, containing the LEDVRN nucleotide sequence at its centre. The mutagenic cassette generated was digested with enzymes SacII and BspEI to produce sticky ends prior to ligation, into SacII and BspEI digested, dephosphorylated pMNV* plasmid to generate a circular chimeric MNV plasmid.

The chimeric MNV plasmid was transformed into competent DH5α cells and grown until visible colonies were present on ampicillin LB/agarose plates (2.3.3 and 2.2.4). Only colonies containing the pMNV* plasmid are able to multiply successfully as they contain the ampicillin resistant gene. Competent cells which have not taken up the pMNV* plasmid will not contain the ampicillin gene. Visible colonies were picked and screened by PCR using the mutagenic primers in Table 11 with non-mutagenic primers, SacII and BspEI to ensure the mutation had been incorporated (2.4.2.3). Two potential plasmids were isolated, which contained DNA of the expected size when analysed by gel electrophoresis. Both plasmids were suspected to contain the LEDVRN amino acid sequence and were sent for sequence conformation at Geneservice (Bioscience, Oxford). Comparison of the two potential chimeric MNV plasmids and the pMNV*protein sequence was performed using software programme LaserGene (2.12) and presented in Figure 18 and Figure 19.

The region of the pMNV* genome where the site directed mutagenesis of the LEDVRN nucleotide and amino acid sequence should be located is highlighted by the red box in both alignments. Alignments of a section of clone 6 and 7 sequence alongside pMNV* indicated that only clone 6 contained the desired sequence. The epitope was incorporated as expected with both nucleotides and amino acids mutated.

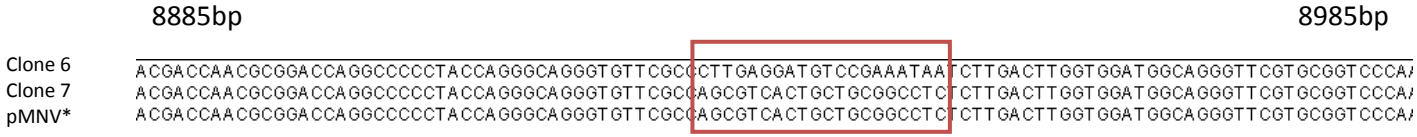


Figure 18. Nucleotide alignment data for Clones 6 and 7 alongside the wild-type pMNV* sequence. The LEDVRN sequence is highlighted by the red box.

Figure 19. Amino acid alignment data for Clones 6 and 7 alongside wild type pMNV* sequence. The LEDVRN sequence is highlighted by the red box.

4.3 Propagation of chimeric MNV

Confirmation that amino acid sequence SVTAAAS was successfully replaced by LEDVRN in clone 6, led to the recombinant MNV from this clone being used in the experiments described in this work.

4.3.1 Production of chimeric MNV using the reverse genetics system

To enable sequence verified chimeric MNV to produce viable virus, it was vital to utilise the reverse genetics system. This system enables intentional genetic mutations to be incorporated into the MNV genome and the effects studied on the replication and infectivity of the virus. DNA purified from clone 6 (2.4.2.1) was used to transfect HEK 293T cells (2.9.2). This system uses the inducible DNA polymerase (pol) II to produce viable MNV.

HEK 293T cells are human embryonic kidney cells, originally generated by the transformation of normal human embryonic kidney cells with sheared adenovirus DNA (69). A variant of this cell line are 293T cells which contain the SV40 large T-antigen. This enables episomal replication of transfected plasmids which contain the SV40 origin of replication.

Transfected HEK 293T cells were harvested and the supernatant used to subsequently transfect Raw 264.7 cells. It was hypothesised that chimeric MNV plasmid would produce viable virus which was capable of infecting the Raw 264.7 cells, with visible cytopathic effect (CPE).

Transfection of chimeric pMNV* into HEK 293T cells was performed using the FuGENE® HD transfection reagent (2.9.2). This reagent is designed to transfect DNA into a variety of cell lines with high efficiency. Raw 264.7 cells are resilient to transfection by electroporation, lipid based delivery and calcium phosphate, for this reason these cells are difficult to transfect. To bypass the difficulties encountered with this cell line, HEK 293T cells were

transfected initially. Noroviruses are positive strand RNA viruses; therefore the DNA has to be transcribed into RNA in order for intact viable virus to be produced by replication of the viral genome. HEK 293T cells were seeded into 6 well cell culture plates and transfected with duplicates of CM54 chimeric pMNV* plasmid alongside negative control UHQ H_2O (displacing DNA). A positive control of wild-type pMNV* DNA was performed on a separate day to prevent contamination, under the same conditions.

After 48 hours of incubation at 37°C, 5% CO_2 the HEK 293T cells were harvested by trypsin and the supernatant stored at 4°C until required. Harvested HEK 293T supernatant contains any viable virus which has been produced from the transfection. Transfected HEK 293T supernatant was applied to sub-confluent Raw 264.7 cells seeded into 6 well cell culture plates and incubated for 72 hours at 37°C, 5% CO_2 and checked regularly for visible CPE.

Transfected Raw 264.7 cells displayed visible CPE for the positive control wild type pMNV* DNA, no CPE was seen in either the negative control UHQ H_2O or for CM54 chimeric pMNV*. Presence of CPE was determined by checking cells under a microscope, daily during the 72 hour period, wells with a lack of CPE after this period were studied for a further 48 hours. The lack of CPE indicated that no viable virus was produced when chimeric MNV DNA was used to transfect HEK 293T cells.

4.3.2 Production of viable chimeric MNV using the neon transfection system (Invitrogen)

Unsuccessful production of chimeric MNV virus after transfection of HEK 293T cells with plasmid DNA may be due to the incompatible location or sequence of the LEDVRN epitope in the P2 sub domain of MNV.

To determine if viable virus could be obtained, the experimental strategy was altered to utilise an alternative method, direct RNA transfection of Raw 264.7 cells using the neon

transfection system (2.9.3 [Invitrogen]). The neon transfection system was developed to enable the transfection of hard to transfect cells directly with RNA. Chimeric pMNV* contains the Cytomegalovirus (CMV) /TET promoter; this is a potent promoter which is commonly used for driving the expression of transgenes. By replacing the CMV promoter with a T7 promoter, when T7 RNA polymerase is applied RNA can be transcribed downstream of the promoter. The T7 RNA polymerase is highly promoter-specific, only transcribing DNA downstream of a T7 promoter. To synthesis RNA, the T7 polymerase requires Mg ions as a cofactor alongside the DNA template.

Following incorporation of the T7 promoter sequence, which was confirmed by sequence analysis performed by Geneservice (Bioscience, Oxford), the chimeric MNV plasmid was linearised. Plasmid preparations were analysed by gel electrophoresis (2.5.4) to ensure complete linearisation. Linear DNA ensured the T7 RNA polymerase did not generate long stretches of nonsense RNA from continuous transcription of the circular DNA.

Linearised chimeric DNA was used to generate RNA using the mMessenger mMachine kit (2.7.6.2). The mMessenger mMachine kit is designed for the synthesis of large quantities of correctly capped RNA. This kit enables efficient poly (A) tailing for use in transfection experiments. Poly (A) tailed RNA has enhanced translation over untailed RNA due to increased mRNA stability and translation efficiency. Incorporation of the anti-reverse cap analogue (ARCA) (223) enables synthesis of functional RNA capped in the correct orientation. Presence of RNase inhibitor in the reaction mix protects the synthesised RNA from degradation by any contaminating ribonucleases.

Synthesised RNA was purified using the RNeasy kit (2.7.6.3) and quantified as 360.21ng/µl. The RNeasy Mini kit enables purification of total RNA from small quantities of initial material, by using guanidine-isothiocyanate lysis and silica –membrane purification.

Purified RNA was used in the transfection of Raw 264.7 cells using the neon transfection system (2.9.3). A positive control of wild type pMNV* RNA was performed on a separate day, under the same conditions as the chimeric MNV RNA, to ensure no contamination could occur between experiments. Negative controls, H_2O (displacing pMNV* RNA) and Raw 264.7 cells (no H_2O or pMNV*RNA) underwent the Neon system conditions to ensure any effects visualised in the cells were not due to the methods used in the neon system. The negative controls were run at the same time as the chimeric RNA experiments. Each experimental control was performed in triplicate. Per experiment, 1 well of a 6 well cell culture plate was allocated for each downstream application, immunoblot, immunofluorescence and recovery of viable virus. 12ng of RNA was added to a 400µl suspension of sub-confluent Raw 264.7 cells; 100µl of the RNA and cell suspension was taken and underwent conditions 1730V, 20ms and 1 pulse through the neon transfection system before addition to the appropriate well, this was repeated for each control (2.9.3). The cells were incubated at 37^{o}C, 5% CO_2 and assessed for CPE daily.

Visible CPE was present in the Raw 264.7 cells after 48 hours in the positive control, wild type pMNV* wells. Lack of CPE was observed in the negative control and chimeric MNV experiments, after being checked daily for one week. To ensure that the transfection of Raw 264.7 cells with positive control, wild type pMNV* was successful and that no viable virus was produced by the chimeric MNV RNA; the Raw 264.7 cells from all transfection experiments were harvested by manual removal and the supernatant was used to infect a separate batch of sub-confluent Raw 264.7 cells. Visible CPE was present after 48 hours as expected for positive control, wild type pMNV*.

Unfortunately no visible CPE was present in Raw 264.7 cells transfected with chimeric CM54 RNA, after visual checks performed daily. Successful visualisation of CPE for the positive control wild type pMNV* and lack of CPE in both the negative control and chimeric

MNV led to the conclusion that the insertion of LEDVRN was not compatible with the formation of intact and viable virions.

To confirm the assumption that no viable virus could be produced by the chimeric MNV RNA, previously transfected Raw 264.7 cells with chimeric CM54 MNV were manually harvested and the supernatant was used as inoculum in a MNV plaque assay, at dilutions ranging from 1×10^{-3} to 1×10^{-7}, alongside positive control, wild type pMNV* (2.9.5**Error! Reference source not found.**). Experiments were performed on separate trays, with the chimeric MNV experiments performed first to prevent cross-contamination. Each tray incorporated a negative control, containing no virus.

Viral titre was determined for wild type pMNV*as 5×10^{5} pfu/ml, no visible plaques were present for chimeric MNV (data not shown, due to the lack of visible plaques).

No viable chimeric MNV virus could be propagated and isolated, using either transfection of HEK 293T cells with DNA in the FuGENE® transfection system or Raw 264.7 cells with RNA in the neon transfection system. Therefore the experimental strategy to use chimeric virus to monitor MNV levels in complex matrices such as environmental samples with detection monoclonal antibody CM54, could not proceed. The mutation of SVTAAAS to LEDVRN, whilst successfully incorporated and verified by sequencing, did not produce viable, intact virus when transfected either as DNA or RNA into HEK 293T or Raw 264.7 cells.

4.4 Discussion

Despite extensive research and many reported attempts to propagate human noroviruses in cell culture, no reliable system has been reported. Current human norovirus surrogates include FCV and MNV, of these MNV has been shown to have many similar properties to human noroviruses and has become the preferred surrogate (27). MNV can be reliably

propagated in Raw 264.7 cells, providing an ideal system for the study of viral infectivity which cannot be performed with the human viruses.

The aim of the work discussed was to generate viable chimeric MNV virions, which contained an epitope sequence in the P domain and could be detected by a previously characterised antibody. Generation of chimeric virus could be used to seed environmental and food samples. This could then be detected using an extensively characterised monoclonal antibody, CM54 which recognises an epitope with the amino acid sequence LEDVRN. This advance would enable an alternative route to producing and isolating monoclonal antibodies, detecting a linear epitope presented in MNV, which has so far proved elusive.

An advantage to using chimeric MNV, instead of simply generating monoclonal antibodies towards MNV capsid, is that OD values obtained from screening environmental samples in an antigen capture ELISA can be directly compared to the same sample when used to infect Raw 264.7 cells to assess the infectivity potential. Analysis of the infectivity potential of the MNV in environmental samples in Raw 264.7 cells and its relation to the potential human norovirus infectivity potential, rely heavily on several assumptions. These assumptions include that murine norovirus is concentrated and stable in environmental samples in a manner which is comparable to the human noroviruses and that MNV infects and propagates in Raw 264.7 cells in similar manner to the infection of humans with the human norovirus; neither of these assumptions have been confirmed at the current time.

Mutation of the SVTAAAS sequence had previously been reported by C.Wobus *et al* (Personal communication), who had inserted a Norwalk virus sequence, NGIGSGN in its place, and generated viable virus. Due to these reports, this region was chosen as suitable for the insertion of the LEDVRN sequence into the P2 domain in MNV. This sequence was

selected as it had been characterised as the epitope detected by monoclonal antibody CM54 (C.Batten, PhD thesis).

Incorporation of the LEDVRN epitope was achieved using mutagenic PCR, into the pMNV* plasmid successful insertion was confirmed by sequence analysis performed by Geneservice (Oxford). Chimeric MNV DNA plasmid was used in the transfection of HEK 293T cells and subsequently Raw 264.7 cells using the FuGENE® transfection reagent, however no visible CPE was present after one week. As an alternative to the DNA transfection of HEK 293T cells, the neon transfection system was utilised to transfect Raw 264.7 cells directly with the chimeric RNA. No CPE was visualised in the Raw 264.7 cells after a period of one week, subsequent use of this transfected cellular supernatant in a MNV plaque assay did not result in visible plaques.

The inability to produce chimeric MNV in either transfection system which could be propagated as intact and viable virus was shown through the experiments described in this chapter. Despite the successful incorporation of the LEDVRN nucleotide sequence in the P2 domain of the MNV genome, the lack of successful isolation of intact chimeric MNV indicates that it was not possible to generate viable chimeric virus containing the LEDVRN sequence in this location. It is clear from this research that further experimentation is required, to achieve the ultimate aim of detecting chimeric virus in environmental samples.

The lack of viable MNV containing the CM54 epitope, LEDVRN may be due to several factors. The CM54 epitope, LEDVRN was originally described as located within the shell domain of Southampton virus and subsequently found to be present in all GI norovirus capsid proteins (C.Batten, PhD Thesis). Insertion of amino acids which have a charged side chain may affect protein-protein interactions and subsequently the formation of a viable capsid, when generating a 3D protein formation. Other factors include hydrophobicity, size and functional groups all of which are important in successful protein structure formation.

During production of intact virions, the LEDVRN amino acid sequence may have generated unfavourable protein interactions, leading to failure to generate a viable 3D structural protein and to produce an intact capsid protein. While the report of the insertion of a Norwalk sequence into this region was shown to be successful, the difference of amino acid side chain charges in this alternative insertion may not have been favourable. These affects need to be studied further to assess which amino acids may not be favourable in the protein folding of the norovirus capsid. This includes undertaking additional work using the 3D structure of the capsid protein to isolate which amino acid from the LEDVRN epitope may be responsible for the failure to fold correctly.

Alternative future work also includes the assessment of potential alternative epitope sequences from previously characterised antibodies which could be inserted into this region of the MNV genome. These epitopes may provide more favourable interactions enabling viable virion formation using the mutagenic capsid.

From the experiments described in this and the previous Chapter 3, there is currently no available monoclonal antibody which detects a linear epitope in the P domain and strategies designed to overcome this, such as chimeric MNV were not successful with the LEDVRN epitope. The ultimate aims to design and develop methods which are able to detect both intact and degraded virions in complex matrices, such as clinical and environmental samples in an indirect ELISA, are still on-going.

Due to these outcomes in future work an alternative approach will be taken. Both strategies described use MNV as either the sole immunising antigen or as the basis for a chimeric virus. The next step in achieving the ultimate aim is to use human norovirus to generate monoclonal antibodies. Using human noroviruses as the initial immunising antigen and boosting with MNV aims to generate cross-reactive human monoclonal antibodies which can be incorporated into an antigen capture ELISA which can detect a

broad range of human and murine viruses. This strategy is similar to the one described in Chapter 3, however coming at the problem from an alternative angle.

Chapter 5 Development of monoclonal antibodies towards GI, GII and GV noroviruses

5.1 Introduction

Noroviruses have a substantial economic cost to society and are a significant health burden on the NHS costing in excess of £100 million each year; a major proportion of these costs come from instigating regular ward closures (HPA press release, 2011, New guidelines for the management of norovirus). These viruses contribute to more than 95% of non-bacterial gastroenteritis in the United States demonstrating the effect these viruses have in the community (55,56). Rapid and sensitive techniques are vital in halting the spread of infection from food to humans and infectious handlers to the food.

GII.4 noroviruses have long been associated with outbreaks and are the predominant circulating genotype worldwide causing between 80-90% of clinical cases (18,23,219). In 2002 an increase of norovirus outbreaks was reported throughout the United Kingdom identified as Lordsdale virus, GII.4 (246). Lordsdale virus was first recognised as a major epidemic strain in the 1990s (153,219,263).

Human noroviruses have been separated into two genogroups by phylogenetic analysis (GI and GII). While GII noroviruses are the predominant genogroup found in outbreak samples, GI noroviruses account for approximately 10% of infections (56). Different genogroups of norovirus collected from outbreaks of gastroenteritis in the UK between 1989 and 1996, showed that the percentage amino acid identity within the capsid protein ranged from 37-44%, in comparison to within genotypes where the amino acid identity within the capsid protein ranged from 61 – 100% (70).

Within GII, the predominance of the GII.4 noroviruses worldwide is supported by reports of substantial outbreaks caused by this genotype, including Farmington Hills virus (252) and

2006b virus (239) in the United States, Hunter virus in Australia, Europe and Asia (24,196) and Lordsdale virus in the UK. Main contributors to the rapid and vast spread of noroviruses are lack of food handler hygiene and the virus's persistence in the environment. Food handlers play an important role in the spread of noroviruses causing both domestic and commercial related outbreaks, many of which have been widely reported in the media.

Genotype II.4 has continued to evolve and the regular emergence of new variants of this strain has contributed to an increase in the number of global epidemics reported (18,153,217). When compared to other norovirus genotypes, GII.4 is linked with an increase in the number of hospitalisations and deaths during outbreaks (40). New GII.4 strains have emerged every 2-3 years in the past decades. In the United Kingdom the most recent outbreak causing norovirus strain is Sydney, replacing the previous predominant strain, New Orleans. The emergence of this new strain has been reported to be associated with an early onset of the 2012 winter norovirus outbreak season.

Current research focused on detection of human noroviruses has relied heavily on molecular techniques such as qRT-PCR, which is both expensive and time consuming. This molecular assay is restricted to well-equipped scientific laboratories with highly trained technicians required to perform the assays and analyse the results. In comparison development of a diagnostic ELISA-based method may offer a cost-effective alternative with the ability to be performed in basic on site set-ups increasing the potential to catch contaminated produce. Previously it has been shown that a Lordsdale specific ELISA could detect 71% of norovirus GII clinical samples (247).

The work performed in this chapter focuses on the importance of developing and isolating monoclonal antibodies which can be used as part of a reliable method for detecting noroviruses. Immunising mice with Lordsdale and MNV VLPs should result in the isolation

of antibodies which can bind both human and murine norovirus capsid protein. B cells produced by the mice will be obtained from the spleen and fused to immortal cells, SP2 to produce hybridomas. Hybridomas produce a constant supply of antibodies which can be used in a broad range of diagnostic immunoassays.

Previous attempts in this project, discussed in Chapter 3, aimed to generate a cross-reactive monoclonal antibody were unsuccessful when using the experimental strategy of MNV VLPs as the sole immunising antigen. Attempts to generate a viable chimeric MNV containing a previously characterised GI monoclonal antibody epitope, described in Chapter 4 were unsuccessful. As an alternative approach to making a chimera it was considered that MNV and human noroviruses might share some conserved epitopes. Thus an immunisation regime was devised that may lead to the generation of monoclonal antibodies able to detect capsid protein across all five genotypes.

The prevalence of the GII.4 noroviruses and in particular the Lordsdale virus within the UK led to the decision of using Lordsdale virus VLPs in the proposed immunisation regime. Previous work demonstrated that a single immunising antigen gave rise to monoclonal antibodies directed towards the single antigen.

Thus the aim of the work discussed in this chapter was to incorporate GII and GV capsid proteins as immunising antigens to generate hybridomas that secreted monoclonal antibodies with potential cross-reacting properties that in the best case might be shared across all five genogroups. A combination of both GII and GV norovirus capsid proteins was considered to give a better opportunity of isolating broadly cross-reacting monoclonal antibodies as the mouse immune system would be exposed to the capsid protein of both GII and GV viruses. Thus the regions of identical amino acids are more likely to be the focus of any newly generated antibodies. The use of a single immunising antigen may limit the potential of cross-reactive antibodies to be isolated. The ability to detect GI noroviruses

would be a bonus as it is hypothesised that cross-reacting monoclonal antibodies to GII and GV may also bind with GI viruses. Previous work by C.Batten (PhD Thesis) had shown that monoclonal antibodies towards GI and GII viruses can be isolated. Monoclonal antibodies which bind human and murine norovirus capsid protein are critical in the development of a sensitive, rapid and simple assay capable of detecting noroviruses in a variety of samples, as well as the capability of detecting MNV seeded into shellfish tissue.

At the present time there is no monoclonal antibody available which covers both human and murine noroviruses. The isolation of such an antibody would provide a valuable tool into the development and optimisation of an immunoassay capable of detecting a broad range of noroviruses. This assay and downstream applications will aid investigations into the probable infectivity potential of these viruses, assuming that both murine and human noroviruses have a similar infectivity profile in shellfish tissue and other food substances. The low number of infectious virions required to cause an infection means that any assay developed needs a high level of sensitivity.

To achieve the successful isolation of cross-reactive monoclonal antibodies it was vital to implement an appropriate immunisation regime. GII.4 norovirus Lordsdale was chosen as the initial immunogen due to the high prevalence of this genotype as the causative agent in norovirus outbreaks. Three immunisations of Lordsdale VLPs will be followed by a single boost immunisation containing MNV (GV) VLPs. To ensure successful stimulation of the immune system, conformation of the production of Lordsdale antibodies is required; this will be achieved through antibody analysis by immunoblot and direct ELISA.

Once isolation of purified hybridomas is achieved, characterisation of the secreted monoclonal antibodies will be performed. Through a series of direct ELISAs against human VLPs a profile of the antibodies reactivity can be generated. It is also important to

determine the location of the amino acid epitope which the isolated monoclonal antibodies recognise; this can be initially achieved by immunoblot analysis.

The overall aim of this work is to obtain monoclonal antibodies which have a fully characterised reactivity profile and are hoped to be able to detect a broad range of noroviruses.

5.2 Characterisation of Lordsdale and Murine norovirus VLPs

1 – 50µg of purified antigen is required per immunisation to produce the desired immunological response (C.Batten, PhD thesis). Lordsdale virus VLPs had previously been expressed using the baculovirus expression system in our laboratory and were available at the outset of this project (S. Shipway, PhD thesis, 1999). Lordsdale virus VLPs produced through the baculovirus system were purified on a CsCl gradient and visualised by EM to ensure the presence of VLPs (S. Shipway, PhD thesis, 1999).

To ensure the desired quantity of Lordsdale virus antigen was used in the immunisation, the protein concentration of the VLP preparation was determined using the BCA protein assay kit (2.8.1 [Pierce]) to be 2074µg/ml. 50µg of immunising antigen was used in each immunisation per animal (2.10.3). MNV VLPs analysed previously (3.2) were used as boost antigen. The MNV VLPs were not included at the start of the immunisation regime to ensure that the mice produced a strong and sustained response against Lordsdale virus.

Lordsdale and MNV VLPs were analysed by SDS-PAGE (2.8.2) to ensure purity before incorporation into the immunisation regime. A single clear band was seen for LV VLPs at 58kDa, corresponding to the capsid protein of Lordsdale virus. An immunoblot of the MNV VLP is provided in section 3.2, whilst the preparation is not as pure as the LV VLP preparation it is sufficient for boost immunisations.

5.3 Immunisation Regime

Two Balb/C mice (Female, date of birth 13/1/2012, HO. Batch No. 1/10/67) were used for hybridoma production. A certified technician performed all procedures in accordance with Home Office guidelines. Prior to immunisation, tail bleeds of 200µl were taken from each mouse and prepared as previously described (2.10.4). Pre-immunisation serum was tested in a direct ELISA (2.8.8) against Lordsdale VLPs, MNV VLPs and Mouse Hepatitis Virus antigen. Both mice were screened against MHV due to the previous MHV infection in a separate batch of mice, described in Appendix 1. All values obtained were less than quadruple the negative control, 0.3 $OD_{450nm,}$ which were considered as negative for all antigens. Serum was also analysed for pre-existing cross-reactivity by immunofluorescence against MNV infected and uninfected Raw 264.7 cells (2.10.8), no detection of MNV or uninfected Raw 264.7 cells was visualised (data not shown, due to lack of detection).

Both mice were immunised following the standard regime shown in Table 12 and were used for hybridoma production as described previously (2.10.4).

The immunisation and selection regime was performed as described by Kohler and Milstein (132) and Oliver *et al* (187) as described in Chapter 3, with the previously stated modifications. As this immunisation regime was successful in producing monoclonal antibodies towards to desired antigen it was decided to use the same protocol, altering only the choice of antigens administered at each immunisation.

Table 12. Immunisation regime for the production of cross-reactive monoclonal antibodies.

Day	Details
0	Pre immunisation Bleeds
1	Subcutaneous Immunisation: 50µg LV VLPs + 50% Freund's Complete Adjuvant
11	Subcutaneous Immunisation: 50µg LV VLPs + 50% Freund's Incomplete Adjuvant
21	Subcutaneous Immunisation: 50µg LV VLPs + 50% Freund's Incomplete Adjuvant
35	Test Bleeds Subcutaneous Immunisation: Boost with 50µg MNV VLPs
39	Hybridoma Production

Hyper-immune sera taken on day 35, detected Lordsdale virus VLPs in an immunoblot whilst MNV VLPs were not detected (2.8.2). Immunoblots against Lordsdale virus antigen with pre and hyper immune sera for both mice are shown in Figure 20, the experiments were performed in duplicate however only one strip is shown. A band can be visualised at 58kDa, the size of Lordsdale capsid protein.

A direct ELISA (2.8.8) provided further confirmation of a successful immunisation schedule with Lordsdale VLPs hyper-immune sera detected Lordsdale virus VLPs at 1:51,200 dilution of the hyper immune sera; however it was unable to detect MNV VLPs as shown in Table 13. The values obtained were judged as a sufficient immune response to proceed with hybridoma production. The lack of response against MNV VLPs was expected as these mice were housed in a facility which was routinely tested for the presence of MNV and MHV therefore were not been previously exposed to MNV and had not yet received the boost immunisation of MNV VLPs.

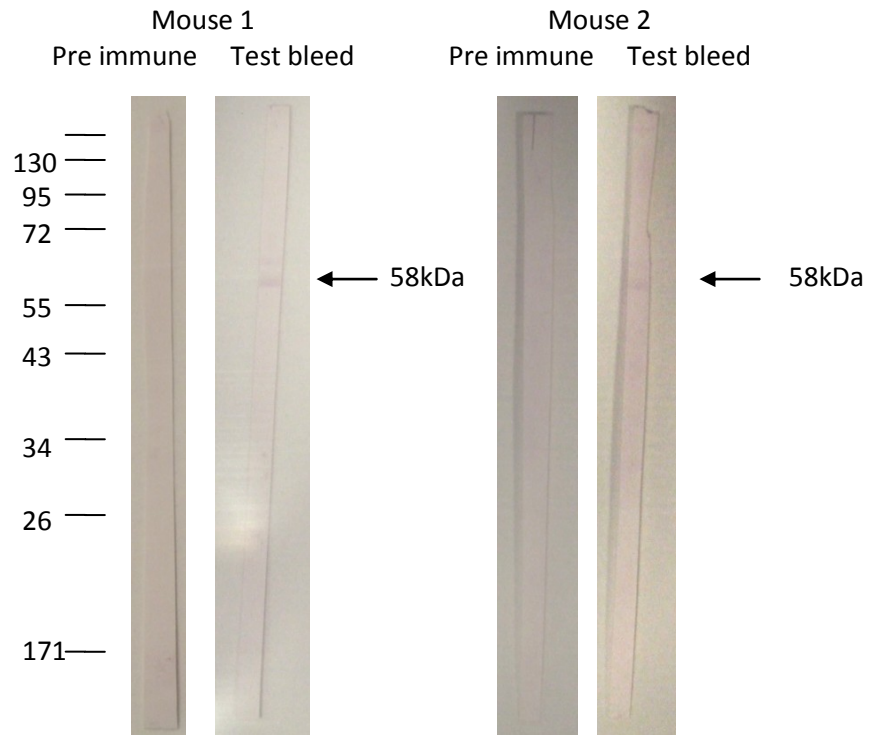


Figure 20. Immunoblot of pre-immune and hyper-immune sera against Lordsdale VLPs for mice 1 and 2. The molecular weights (kDa) are provided alongside.

Table 13. Direct ELISA OD values for mouse 1 and 2, (M1 and M2) pre-and hyper-immune sera against Lordsdale virus VLPs and MNV VLPs. Positive values are shown in bold.

	Dilution Factor										
	Sera	**1:100**	**1:200**	**1:400**	**1:800**	**1:1600**	**1:3200**	**1:6400**	**1:12800**	**1:25600**	**1:51200**
Pre-immune	*M1 LV*	0.075	0.076	0.089	0.086	0.074	0.074	0.072	0.076	0.079	0.074
	M2 LV	0.075	0.071	0.087	0.097	0.091	0.086	0.075	0.075	0.09	0.087
	M1 MNV	0.103	0.096	0.102	0.099	0.097	0.087	0.087	0.096	0.091	0.085
	M2 MNV	0.098	0.101	0.092	0.092	0.088	0.089	0.078	0.097	0.081	0.08
Hyper-immune	*M1 LV*	**1.42**	**1.269**	**1.477**	**2.021**	**1.672**	**1.905**	**1.684**	**1.72**	**1.589**	**1.514**
	M2 LV	**1.295**	**1.26**	**1.307**	**1.611**	**1.161**	**1.811**	**1.766**	**1.996**	**1.93**	**1.616**
	M1 MNV	0.283	0.216	0.207	0.096	0.157	0.164	0.161	0.146	0.148	0.151
	M2 MNV	0.281	0.204	0.176	0.188	0.176	0.171	0.179	0.187	0.2	0.212

Boost immunisations were administered to both mice with 50µg MNV VLPs. The inclusion of MNV VLPs was hypothesised to increase the chances of isolating a broadly cross-reactive monoclonal antibody which detected an epitope present across the capsid protein of genotypes of noroviruses. By subjecting the mouse immune system to two different genogroups of norovirus the regions of identical amino acids in the capsid protein are more likely to be the focus of any newly generated antibodies. The use of a single immunising antigen may limit the potential of cross-reactive antibodies to be isolated; this had been demonstrated through the work performed in Chapter 3. At the current time there are no reported antibodies which cover both the human and murine noroviruses, therefore it is unknown whether it is possible to successfully produce and isolate antibodies with these properties. Previous work by C.Batten (PhD Thesis) had shown that monoclonal antibodies with cross-reactive properties towards GI and GII viruses can be isolated; demonstrating the isolation of cross-reactive antibodies is possible amongst the human noroviruses.

5.4 Purification of isolated hybridomas

A fusion of viable spleen cells to SP2 cells (2.10.1.1) was performed as described at a 1:1 ratio (2.10.4) for both mice, four days after the boost immunisation. Once the hybridomas had grown to yield visible colonies in 96 well plates for 14 days, the hybridoma supernatant from each well was screened against Lordsdale VLPs and MNV infected Raw 264.7 cell lysate (2.10.6) in a direct ELISA (2.8.8). Wells were considered positive if they had a reading >0.3 OD_{450nm}, this was an arbitrary value to ensure a manageable number of hybridomas were purified. Hybridomas in 'positive' wells were picked into fresh wells on 96 plates containing DMEM-HAT medium (2.10.1.3) and purified by two rounds of terminal dilution (2.10.5).

Forty-two individual hybridomas were generated from the fusion, which produced OD values >0.3 against either Lordsdale VLPs or MNV infected Raw 264.7 cell lysate. Thirty- one

hybridomas subsequently produced OD values >0.3 against uninfected Raw 264.7 and SF9 cell lysates (2.10.8) in a direct ELISA (2.8.8) and were discarded. The discarded thirty-one hybridomas produced monoclonal antibodies which were not guaranteed to detect solely MNV or Lordsdale virus. The capability of these thirty-one hybridomas to detect a protein common to both Raw 264.7 and SF9 cell lines indicated their unsuitability for incorporation into detection assays. A positive result in the norovirus detection assay with the antibodies would not conclusively indicate the presence of norovirus capsid protein. Therefore to ensure that all results are reliable these cellular protein detecting antibodies were discarded.

Eleven hybridomas detecting either Lordsdale virus or MNV antigens but not Raw 264.7 or SF9 cell lysates were purified, expanded and stored at -135°C in DMEM supplemented with 10% DMSO.

Table 14. Timetable and summary of work performed in the generation of eleven monoclonal antibodies, intended to be cross-reactive to human and murine noroviruses.

Days post fusion	Work undertaken	Antigens tested (VLPs or cell lysate)
8	Screening of fast growing hybridomas by direct ELISA	LV, MNV and Raw 264.7
8	Positive hybridomas picked into a fresh 96 well plate	
10	Screening of 12 master trays by direct ELISA	LV VLPs and MNV
10	Positive hybridomas picked into a fresh 96 well plate	
14	Hybridomas (day 8 and 10) screened by direct ELISA.	LV, MNV, SF9 and Raw 264.7
14	Positive hybridomas picked into a fresh 96 well plate and double diluted	
15	Slow growing hybridomas picked on day 10 screened by direct ELISA	LV, MNV, SF9 and Raw 264.7
15	Positive hybridomas picked into a fresh 96 well plate and double diluted	
16	Hybridomas from master trays stored at -135°C in DMEM with 10% DMSO	
22-68	Isolated hybridomas purified by terminal dilution	LV, MNV, SF9 and Raw 264.7
55 - 83	Expansion of purified hybridomas and storage at -135°C in DMEM with 10% DMSO	

5.5 Characterisation of GII detecting antibodies

Eleven hybridomas generated from two fusions were analysed by direct ELISA against a panel of seven human norovirus VLPs (5 x GII and 2 x GI) available in our laboratory (Table 5) and MNV VLPs. The human VLPs were a kind gift from Dr. K.Green and cover a broad range of norovirus genotypes within genogroups (GI and GII). A broad sample group enables the antibody reactivity profile to be accurately determined.

A direct ELISA is an effective method for determining if an antibody is capable of detecting and binding human norovirus capsid proteins. If the antibodies are capable of detecting human noroviruses an OD value above the positive cut-off is obtained. The results of the ELISAs are shown in Table 15. OD values considered positive are shown in bold for each monoclonal antibody with an OD_{450nm} above 0.2, double the negative control CM64 of 0.093. Each hybridoma isolated was given a CM/hybridoma number, enabling the profile of each monoclonal antibody to be accurately recorded.

Table 15. Direct ELISA results of eleven monoclonal antibodies with a panel of seven human norovirus VLPs and MNV VLPs. Positive values are shown in bold with OD_{450nm} > 0.2.

Hybridoma Number	MNV VLPs (GV)	LV VLPs (GII.4)	SV VLPs (GI.2)	Toronto VLPs (GII.2)	Hawaii VLPs (GII.1)	Maryland VLPs (GII.4)	Snow Mountain VLPs (GII.2)	Desert Shield VLPs (GI.3)
153	- (0.132)	**+ (3.176)**	- (0.133)	- (0.120)	- (0.128)	**+ (2.182)**	- (0.120)	- (0.096)
154	- (0.108)	**+ (3.182)**	- (0.104)	- (0.105)	- (0.114)	**+ (1.892)**	- (0.121)	- (0.087)
155	- (0.119)	**+ (3.245)**	- (0.149)	- (0.114)	- (0.121)	**+ (3.208)**	- (0.179)	- (0.103)
156	- (0.109)	**+ (3.191)**	- (0.122)	- (0.108)	- (0.106)	**+ (3.203)**	- (0.107)	- (0.107)
157	- (0.114)	**+ (3.227)**	- (0.120)	- (0.103)	- (0.105)	**+ (3.176)**	- (0.110)	- (0.111)
158	- (0.106)	**+ (3.123)**	- (0.111)	- (0.105)	- (0.097)	**+ (3.144)**	- (0.096)	- (0.100)
159	- (0.123)	**+ (3.134)**	- (0.109)	- (0.101)	- (0.096)	**+ (3.147)**	- (0.105)	- (0.126)
160	- (0.078)	**+ (1.679)**	**+ (1.275)**	- (0.054)	- (0.093)	- (0.101)	- (0.046)	**+ (0.321)**
162	- (0.145)	**+ (3.133)**	- (0.102)	- (0.094)	- (0.087)	**+ (3.148)**	- (0.121)	**+ (0.412)**
163	- (0.108)	**+ (3.125)**	- (0.110)	- (0.094)	- (0.081)	**+ (3.148)**	- (0.099)	- (0.097)
166	- (0.068)	**+ (0.262)**	- (0.047)	- (0.051)	- (0.054)	- (0.071)	- (0.087)	- (0.046)

The eleven monoclonal antibodies detected Lordsdale virus VLPs (immunising antigen); however, none could bind MNV VLPs (boosting antigen). During the screening process, it transpired that all monoclonal antibodies which could bind MNV also detected uninfected Raw 264.7 cells. This was not the desired or expected outcome and led to no monoclonal antibodies which could detect MNV. The antibodies secreted by these hybridomas were detecting an epitope present in Raw 264.7 mouse macrophages, as well as MNV. Secreted antibodies could not be guaranteed to be detecting solely MNV and were therefore discarded.

Nine of the eleven monoclonal antibodies which detected Lordsdale virus could also detect Maryland VLPs. Maryland virus (MD-145) is a GII.4 norovirus, located in the same cluster as Lordsdale, Bristol and Camberwell viruses. This strain of virus was isolated from an outbreak of gastroenteritis at a Maryland nursing home in 1987 and was shown to have only one amino acid difference to the capsid protein of Camberwell virus (GII.4) which has a high homology to Lordsdale virus (72). Figure 21 displays the differences between Maryland virus (MD-145) and Camberwell virus, both GII.4. It is therefore highly likely that the nine monoclonal antibodies are detecting the same epitope in both Lordsdale and Maryland capsid proteins.

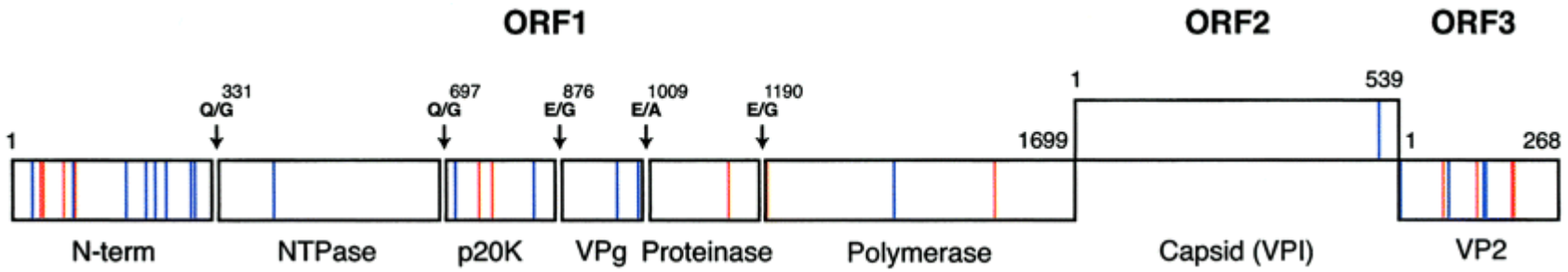


Figure 21. Figure taken from K.Green *et al* (72), demonstrating differences in amino acids between the Maryland virus (MD-145) and Camberwell virus, both GII.4. Blue and red bars indicate the conservative and non-conservative amino acid substitutions, respectively. As described previously there is only one amino acid which differs between these viruses in the capsid protein.

Monoclonal antibodies CM160 and CM162 had the broadest cross-reactivity of the eleven isolated antibodies. CM160 recognised an epitope in GI noroviruses Southampton and Desert Shield alongside Lordsdale virus and CM162 detected Lordsdale, Maryland and Desert Shield VLPs in a direct ELISA. Despite the range of noroviruses detected by these antibodies, this was not as extensive as hoped for at the beginning of this experimental procedure.

Only two of the eleven monoclonal antibodies could detect GI noroviruses. It is unusual that both antibodies could not detect similar GII viruses and only GI alongside Lordsdale. Results suggest that there must be an identical or similar conformational or linear epitope present in these capsid proteins.

Isotyping of the monoclonal antibodies was performed using the Mouse Typer Sub-Isotyping kit (2.8.11). Isotyping provides information on which type of heavy chain present in the antibody which determines the class and subclass of the immunoglobulin molecule. The Isotyping kit discriminates between the four mouse IgG subclasses IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 as well as IgA and IgM and the two immunoglobulin light chains lambda (λ) and kappa (κ). The summary of the monoclonal antibody Isotypes is provided in Table 16.

After isotyping the eleven isolated antibodies and taking into account the original well the hybridomas were picked from to avoid expanding identical clones, seven individual hybridomas were purified. If hybridomas originated from the same well on the master 96 well plates after the fusion and they had the same isotype, they were assumed to be identical. It is likely that these colonies were located close to or next to each other so that the same cells were picked into separate wells and after purification by terminal dilution the same hybridomas had been isolated. The seven separate hybridomas may detect different epitopes in the capsid protein and as such may detect a different range of antigens.

Table 16. Isotype and light chain of the eleven monoclonal antibodies isolated

Hybridoma Number	Isotype	Light Chain
153	IgG_{2b}	κ
154	IgG_{2b}	κ
155	IgG_{2b}	K
156	IgA	K
157	IgG_{2b}	K
158	IgG_{1}	K
159	IgG_{1}	K
160	IgG_{2b}	K
162	IgG_{1}	K
163	IgG_{2a}	K
166	IgG_{2a}	K

Reactivity of the eleven monoclonal antibodies with Lordsdale VLPs and MNV infected Raw 264.7 cell lysate was assessed by immunoblot (2.8.6.1**Error! Reference source not found.**). None of the monoclonal antibodies showed reactivity against either antigen; therefore it can be assumed that the epitopes detected by the antibodies are a conformational dependent epitope, due to the denaturing conditions of the SDS-PAGE on the capsid protein. If the epitope detected by the monoclonal antibody is conformational dependent, the denatured protein will not have the intact 3D protein folding enabling the antibody to bind.

A conformational dependent epitope limits the incorporation of these antibodies into diagnostic detection assays, as viral particles may be present in environmental and faecal samples both degraded and intact. A potential linear epitope detecting antibody enables the development of a sensitive assay, detecting protein in both states. Due to the lack of isolation of antibodies which displayed the required characteristics, further experimental plans could not take place. It was intended that purified antibodies could form the basis of a norovirus capture assay to assess the possible risk of infection to humans, however for this to be a success a linear epitope detecting antibody was required.

5.6 Discussion

Lordsdale and Murine norovirus VLPs were used to immunise two Balb/c mice, with the aim of generating broadly cross-reactive monoclonal antibodies towards a range of human noroviruses. From immunisation of two mice with Lordsdale and MNV VLPs, eleven monoclonal antibodies were isolated that could detect Lordsdale VLPs in a direct ELISA; two of these antibodies also demonstrated broader cross-reactive properties against human noroviruses. The availability of a range of five human norovirus VLPs, as well as MNV VLPs aided the screening of hybridomas and thus the selection of authentic monoclonal antibodies. The screening of antibodies with Lordsdale and MNV VLPs, as well

as uninfected cell lines Raw 264.7 and SF9 allowed the selection of hybridomas secreting antibodies to the desired capsid protein and elimination of non-norovirus detecting monoclonal antibodies.

Inclusion of MNV VLPs in the boost immunisation was hypothesised to enable B cells to be produced by the mice which secreted antibodies recognising an epitope in the capsid protein of both human and murine noroviruses. This was an ambitious goal, as it was not a guaranteed outcome that cross-reactive antibodies could be produced and isolated. One possible explanation may be that the inclusion of MNV VLPs in only the boost immunisation may not have provided adequate time for the mice to generate a strong and sustained immune response with the generation of the desired B cells. The planned immunisation regime was intended to produce monoclonal antibodies which had cross-reactive properties towards Lordsdale virus and MNV as well as other GI or GII viruses. Monoclonal antibodies specific for MNV were expected to be isolated, even if no cross-reacting antibodies were produced.

Results suggest that it is difficult to produce broadly cross-reactive monoclonal antibodies against GII and GV noroviruses and it is unlikely that a possible common epitope which monoclonal antibodies could detect in the capsid protein of all noroviruses could be isolated from the immunisation of just two mice with this experimental strategy. Furthermore, it is not known if it is possible to produce a cross reactive monoclonal antibody across all five genogroups. Currently one of the broadest cross-reactive monoclonal antibodies (MAb 14-1) previously reported can detect human noroviruses GI.1, 4 and 8 and GII. 1-7 (215). This antibody was reported in 2007, before this project was undertaken in 2009. Despite the existence of this antibody, it did not cover murine noroviruses (GV) as well as both human genogroups (GI and GII).

Decisions were made to incorporate two different genogroups of norovirus into the regime. To use any isolated monoclonal antibodies in a diagnostic assay, they must be able to detect human noroviruses. GII.4 noroviruses are the major strain involved in outbreaks of noroviruses in the community, therefore by including one of these viruses in the regime it was hoped that antibodies would detect an epitope in the capsid protein of a range of GII.4 viruses.

Alongside the requirement to detect human noroviruses, it was hoped that murine noroviruses would also be detected. At the current time, only murine noroviruses can be reliably propagated in *in vitro* cell culture. For this reason if an antibody was able to detect both human and murine noroviruses it could be incorporated in an array of immunoassay and expand the current understanding of these viruses. A comparison between OD values obtained in an ELISA could be directly compared to the infection potential of the sample when propagated in cell culture.

The immunisation schedule used in this body of work had been previously used in our laboratory generate antibodies which could detect an epitope present across the GI viruses (15). The use of this schedule was intended to produce broadly cross-reactive monoclonal antibodies across the norovirus genogroups. GII.4 norovirus Lordsdale was chosen as the initial immunogen due to the high prevalence of this genotype in norovirus outbreaks. Using Lordsdale VLPs as the initial antigen was intended to ensure that the mice produced a sustained and intense response. In addition, the inclusion of MNV VLPs in the boost immunisation was intended to expose the immune system to a further norovirus antigen to potentially generate antibodies towards a conserved epitope between both viruses. It was hypothesised that the conserved epitope between the GII.4 and GV viruses would also be present across the other genogroups, GI and GII.

By calculating the number of potential epitope sites it may be possible to determine approximately how many immunisations are required to generate such an antibody. If the probability is small, it would be necessary to conduct a large scale immunisation program or a change of the immunising antigen would be required.

The eleven monoclonal antibodies obtained from the fusion were screened by direct ELISA to a variety of norovirus capsid proteins to look for evidence of cross-reactivity. Nine monoclonal antibodies obtained appear to be highly specific, capable of detecting solely the immunising antigen (Lordsdale, GII.4) or an antigenetically related capsid protein (Maryland, GII.4) in a direct ELISA. For a diagnostic ELISA to be able to detect all genogroups of norovirus in clinical samples monoclonal antibodies detecting an epitope in the capsid protein, present in all noroviruses needs to be isolated. Despite boost immunisations of MNV VLPs administered to both mice, no antibodies could be isolated which detected this antigen, which did not also display cross-reactivity with uninfected Raw 264.7 or SF9 cell lysates.

The epitope recognised by the isolated eleven antibodies is within the Lordsdale capsid protein. The Lordsdale VLP preparation is composed of solely capsid protein, therefore it is highly unlikely that monoclonal antibodies detect norovirus non-structural proteins. Immunoblot analysis of the monoclonal antibodies against LV and MNV VLPs was performed to confirm the cross-reactivity pattern. None of the antibodies reacted to the antigens tested, suggesting that the epitope recognised by these antibodies was conformational dependent. Recognition of a conformational epitope prevents the incorporation of these antibodies into an antigen capture ELISA for the detection of native norovirus in clinical samples, which contain both viable and degraded capsid protein. Conformational epitopes depend upon the 3D protein folding of the desired epitope to remain intact to ensure detection. For virions which have degraded, the capsid protein will

have fragmented and is no longer guaranteed to still contain the required conformation of proteins to allow antibody binding.

The results of this fusion suggest that it is possible to obtain broadly cross-reacting monoclonal antibodies by immunising with VLPs and screening with capsid fusion proteins. However, these antibodies did not display the range of cross-reactivity desired across GI, GII and GV noroviruses using this choice of immunogens and immunisation regime.

The inability to isolate a monoclonal antibody which detected a linear epitope in norovirus capsid protein was a disappointment. While conformational dependent epitopes can be detected in intact viral particles, there is no guarantee that these will still be intact if the particle degrades. In diagnostic detection assays to determine the presence of norovirus in both faecal and shellfish samples, both intact and degraded virions can be present. To assess the true extent of an infection it is vital to detect all fragments of particles. Degraded virions were once viable and potentially capable of infection, to ensure detection of proteins from these virions in a diagnostic assay a linear epitope detecting monoclonal antibody is required. Linear epitope detecting antibodies have an increased potential in being able to detect both viable and degraded norovirus virions. The ability to detect both viable and degraded capsid protein provides the opportunity to assess the potential infectivity of noroviruses in particular matrices by detecting all norovirus which was initially present, this is vital for the prevention of further outbreaks

Improvements could have been made to the immunisation regime by using both immunogens, Lordsdale and MNV, simultaneously in the initial immunisations which could increase the possibility of obtaining cross-reactive monoclonal antibodies. Future experiments would include incorporating a broader range of antigens into a longer immunisation schedule which may increase the possibility of cross-reactive monoclonal antibodies being isolated. Further assessment of the amino acid sequence similarity of the

human and murine noroviruses would also be beneficial. Potential avenues of research include identifying the sequences of identical amino acids between the different genogroups. From these sequences peptides could be generated and utilised in future immunisation schedules, to generate cross-reactive antibodies. It was not possible to perform these experiments in the time frame of this research.

Despite intentions of the monoclonal antibody production resulting in antibodies which detect an epitope common to both human and murine noroviruses, this did not occur. It is important to note that it was not guaranteed that antibodies with these properties would be generated. The mouse immune system is a natural system, which will vary between mice. The immunisation of two mice with the same immunogen and regime will result in antibodies which detect different epitopes. An epitope may be located in any region of the capsid which is surface exposed. The norovirus capsid is composed of 90 dimers; within this protein it is the P1 and P2 domains which are on the exposed surface.

While antibodies which detect an epitope in the P domain are ideal, by immunising with norovirus VLPs both intact and fragmented sections of the capsid protein are presented to the immune system. This can result in antibodies binding to regions of the S domain, which are not exposed in intact virions, as experienced in the work described here.

Ideally, the use of only intact virions as the immunogen would dramatically increase the possibility of isolating antibodies binding an epitope in the P2 domain. Unfortunately at this current time, there is no way to generate only intact capsid. However even this technique does not guarantee that the epitope will be linear rather than conformational. It may be possible in future experiments to use peptides composed of short sections of sequence in the P2 domain which are common across GI, GII and GV viruses for use in the immunisation. By selecting the desired conserved amino acid sequence, monoclonal

antibodies may be selected which have the desired properties; this method would decrease the overall number of monoclonal antibodies due to the strict screening constraints.

The eleven antibodies have only been used as part of the direct ELISA technique, an increase in sensitivity could be achieved by incorporating these antibodies into a DELFIA based system. Due to time constraints these antibodies could not be incorporated into the development of a detection assay for noroviruses in shellfish. Therefore an existing GII.4 monoclonal antibody will be used in developing a sensitive assay.

Chapter 6 Development of diagnostic tests for the detection of GII noroviruses in oyster tissue

6.1 Introduction

Foodborne illnesses involving seafood have become a global issue. With more than 63.5 million tons of seafood caught and consumed globally each year the potential for widespread outbreaks of infectious illnesses are vast (154). In America 10-19% of the 76 million foodborne illnesses reported each year involved seafood, equating to one illness per 250,000 servings (212,249). Shellfish have been shown to account for 64% of seafood related infectious outbreaks worldwide (249). Shellfish are particularly susceptible to viral contamination and are a transmission route for human infection due to the frequency of being eaten raw or lightly cooked. The lack of cooking increases the potential for illness caused by viruses or organisms which could have been inactivated by exposure to high temperatures (25).

Oysters are bivalve shellfish which grow predominantly in shallow, coastal waters. These molluscs feed by filtering litres of seawater through their gills daily. It is this method by which they feed that makes them susceptible to accumulating noroviruses within their digestive tissue. When bivalve shellfish pump water over their gills, suspended particles are captured and passed through the digestive tract, which digests the captured food particles. Norovirus virions are concentrated in the hepatopancreas, whilst other particles move on through the midgut before being expelled. The mechanism of norovirus accumulation in oyster digestive tissues has not been characterised in detail. Virus particles may accumulate in oysters by mechanical entrapment, direct chemical bonding, Van der Waals bonding, H^+ ion bonding or other ionic bonding (42,138,235).

Oysters concentrate faecal coliform bacteria from the surrounding water in their environment by more than four-fold, indicating the pool of infectious diseases these organisms can harbour (25). Numerous viruses enter coastal waters through direct discharge of domestic sewage into the ocean (212), although only noroviruses and hepatitis A viruses are associated with infectious outbreaks from the consumption of shellfish (83). The accumulation of noroviruses within the tissue does not affect the shellfish and they do not display any signs of infection.

Bivalve molluscs grow naturally on the sea bed in coastal locations and are dredged by fishing vessels. Sewage from both sewage outlets and discharged from boats represent a significant public health risk. Contribution of these sources is difficult to determine due to the intermittent nature of the discharge. Across the UK there are approximately 5500 sewage treatment plants, however only around 130 of these have tertiary treatments which disinfect the material before discharge. These 130 sewage works discharge directly into regions which are harvested for oysters and other shellfish, disinfectant treatment is employed to prevent the release of viruses and bacteria into these areas. It is important to note that if the sewage system is exposed to unexpected environmental stresses, such as floods, the storm drainage network is designed to release directly into the sea. However this does expose the shellfish growing beds to potential sources of bacterial and viral contamination. Noroviruses are stable in the environment and have been detected in both waste water treatment plant effluent and surface waters (169,240,242).

The persistence of noroviruses in shellfish tissue has become an important public health concern. The current method to produce virus-free oysters involves placing the shellfish in clean water tanks for a minimum period of 48 hours, a process called depuration or relaying. This method is only employed in shellfish which originate from class B growing beds; shellfish from class A beds can be sold straight into the food chain and therefore are

not required to undergo this process. Depuration has been shown to be inefficient (158,172) as, after bioaccumulation, only 7% of Norwalk virus is depurated compared to a 95% reduction in bacterial levels (210). The size of the oyster will affect its bioaccumulation activity, with larger specimens being able to pump larger quantities of water although not all oysters function with equal effectiveness (174).

The bioaccumulation of noroviruses in shellfish tissue is dependent on many factors such as water temperature, mucus production, glycogen content of connective tissue and gonadal development (25). The seasonal change of water temperature causes physiological changes within the oysters which affect their ability to accumulate virus particles, as well as the survival of noroviruses in the surrounding waters. Specifically, viral survival in estuarine waters has also been shown to be affected by temperature and sunlight exposure (173). Both these factors (low temperature and limited sunlight) are most prevalent during the colder months and have been suggested to lead to the seasonal outbreaks of noroviruses in the human population during colder weather (25,25,164). A recent study has suggested that GII.4 noroviruses are slower to accumulate in oyster digestive tissue over a 24 hour period than GI viruses (163). After one hour of bioaccumulation the GII.4 viruses were predominately in the gills and took 24 hours to accumulate in the digestive tract. In comparison to GI.1 noroviruses which were more efficiently accumulated directly in the digestive tissue after 1 hour of bioaccumulation. This genotype also displayed more efficient accumulation during the colder months (163,164). Despite an increase in efficiency both genogroups were shown to be mostly in the digestive tissue after 24 hours. It is unlikely that oysters would be harvested within 24 hours of exposure; however, this demonstrates that different binding ligands must be used in the tissue for the two genogroups. Oysters are a slow growing organism; they will typically grow about two inches in length during each growing season (April to September). To reach a size that is suitable for harvesting it will take up to four years. An oyster could be exposed to norovirus

at any point during this period; noroviruses can persist in water of temperatures 4, 25, and −20°C for up to 2 months, complete degradation of the norovirus genome was observed after 100 days (183).

Recombinant norovirus VLPs have been found to bind specifically to digestive ducts in oysters via carbohydrate structures which have a terminal N-acetylgalactosamine residue in the α-linkage. This α-linkage also overlaps the same binding site which is used for norovirus VLP attachment to human digestive cells (141). The binding site has been mapped to the viral capsid P2 domain. A study by Tian *et al* has demonstrated that A-like Histoblood group antigens (HBGAs) were present in 21 oyster samples tested (236) and HBGAs have previously been recognised as receptors for noroviruses in humans (106). The ability of noroviruses to bind to digestive tissue in both shellfish and humans at the same binding site has been suggested to point to a co-evolution mechanism (141).

It has been shown that despite the high levels of infection and therefore high level of viruses in sewage, GII.4 viruses are not predominant in oyster-related outbreaks (58,119,135). This may be due to the higher efficiency and speed of GI noroviruses to bioaccumulate in the oyster digestive tissue in comparison to GII viruses. It has been suggested that binding of GII.4 viruses to the gills may slow the passage of viral particles to the mouth and subsequently the digestive tissues, however, these viruses do reach these tissues after a 24 hour period (163,164).

Noroviruses are highly infectious, taking only a few virions (<6) to infect a human, so it is important to detect even low levels of viral contamination in shellfish to prevent outbreaks (159). Due to a lack of a cell culture method, noroviruses can currently only be detected by molecular approaches involving an initial viral concentration step. Due to the current lack of standardised detection methods internationally, there has been a trend for in-house and commercial methods being developed.

The most widely used detection method is real-time reverse transcription PCR which has been developed to cover the genetic diversity of the human noroviruses, although most assays are for either genogroup I or II (60,118,157,238). At the present time there no legal requirements for testing shellfish for the presence of norovirus and with no published reference method, there are a large number of in house methods all of which have different protocols. The Centre for Environment, Fisheries & Aquaculture Science (Cefas) have recently described a qRT-PCR norovirus testing method which is derived from the proposed standard for quantifying norovirus in foodstuffs, currently being developed by a joint European Committee for Standardisation (CEN) and International Standards Organisation (ISO) group. This technique provides no indication of viral infectivity as both intact and degraded RNA is quantified, but is a step forward in standardising detection methods. Quantitative RT-PCR (qRT-PCR) is limited by its time consuming nature, the need for high technical expertise, expensive machinery, consumables and the extensive labour required. qRT-PCR is also dependent on removing inhibitors during the viral concentration step.

By contrast an Enzyme-Linked Immuno-Sorbent Assay (ELISA) based detection system does not require the removal of PCR inhibitors from the sample and offers a cheap, rapid, efficient and easy-to-interpret alternative to RT-PCR. The development of an improved detection assay had been identified by the Food Standards Agency as an area in need of further development.

In this chapter, the existing detection techniques, ELISA and qRT-PCR will be compared with Dissociation Enhanced Lanthanide Fluoro-Immuno-assay (DELFIA), developed in our laboratory and refined for detecting and typing human noroviruses. The aim is to determine how sensitive these procedures are when detecting noroviruses in oysters.

GII.4 norovirus clinical samples were used as a model human norovirus for assay evaluation as they are readily available in comparison to GI samples. GI outbreaks are a rare occurrence in the general population, compared to the frequent outbreaks of GII.4 noroviruses. In this study GII.4 stool samples were utilised; with the samples being seeded directly into the shellfish digestive tissue instead of being bio-acculmated in the digestive system, therefore bypassing the gills, which have been suggested to trap GII noroviruses. For this reason the use of GII viruses rather than GI clinical samples does not affect the proposed aim of work.

6.2 Characterisation of clinical sample 7

As human noroviruses cannot be cultured, viral particles must be obtained from clinical samples of norovirus infected patients. Anonymised faecal specimens were obtained prior to the start of the project, kindly supplied by Dr. Owen Caul, Bristol HPA. These samples were anonymised and had been discarded following routine diagnostic tests; therefore the samples did not need ethical approval and met HPA approved ethical criteria for assay evaluation and development. Each sample was screened by electron microscopy (EM) at Bristol HPA to confirm the presence of norovirus. Freezing can destroy the characteristic viral morphology that allows diagnosis by EM, so samples were stored at 4°C upon arrival.

From over 100 norovirus EM positive samples, twelve samples were selected because of their large volume (>20ml) to ensure a plentiful supply for analysis. The twelve selected samples were thoroughly characterised by sequencing of the capsid protein region, as well as by detection assays, ELISA, DELFIA and a GII specific qRT-PCR. After characterisation, ‘sample 7’ was chosen for further experimental analysis because this faecal sample had the greatest volume and an increase in OD values above the other samples in a direct ELISA assay.

To characterise sample 7, it was subjected to genotype assignment by sequence analysis. Nucleotide primers which are based on a single norovirus genotype sequence are unable to detect a broad range of genotypes within one stool sample. For this reason it was important to use degenerate primers which are able to bind to sequences across all norovirus genotypes.

Nucleotide primers used for sequencing of sample 7 were genogroup II specific and amplified a 223 base pair region of the capsid protein gene (ORF2), nucleotides 5362-5584 in the Lordsdale virus genome (79). It was been shown previously that ORF2, containing the sequence encoding the capsid protein and ORF3, have a high level of genetic variation (77,148). This variation also broadly separates genogroups I and II. CapIIa and CapIIb (79) primers were designed to provide capsid sequence data for further strain characterisation as well as being genogroup specific allowing rapid genetic grouping of various noroviruses within the clinical specimens. The region these primers bind to are located within the conserved shell domain region.

Reverse transcription – PCR (RT-PCR) was performed as described by S.Green *et al* (79) with sample 7 after RNA extraction from the original stool sample (2.7.6.3) and cDNA synthesis (2.7.5.1). Purified cDNA samples were sent for nucleotide sequencing at Geneservice (2.7.4) (Source Bioscience, Oxford).

Sequence data was viewed using SeqMan and multiple alignments were performed using MegAlign (both part of the DNA LaserGene software programme). Multiple alignments were carried out using CLUSTAL W (233) and sequence distances and phylogenetic trees were constructed within the MegAlign software programme.

Sequence data obtained for sample 7 is shown in Figure 22. Geneservice sequencing measure the peaks of fluorescence generated by the fluorescently labelled

dideoxynucelotides (ddNTPs) which emit light at different wavelengths for each of the four ddNTP chain terminators. In automated sequencing this can be performed in a single reaction.

The reference sequences used for alignment and sequence data comparison are a range of genogroup I and II noroviruses which are listed in Table 17, adapted from a table by K.Y Green *et al* (73). The reference sequences cover GII genotypes I – 7 to allow a broad range of sequences for correct genotyping, as sample 7 had been assigned to GII.4 by previous work (Sarah Garner). The reference sequences also included genogroup I sequences, ensuring accurate grouping of sample 7 into the relevant genogroup and genotype, if previous sequencing was not accurate.

Table 17. Human norovirus reference sequences used in multiple alignments, indicating the genogroup, genotype, virus name and accession number.

Genogroup/Genotype	Name	Accession Number
GI.2	Southampton	L07418
GI.3	Birmingham	AJ277612
GI.5	Musgrove	AJ277614
GII.I	Hawaii	U07611
GII.I	Girlington	AJ277606
GII.2	Snow Mountain	U70059
GII.2	Melksham	X81879
GII.3	Toronto	U02030
GII.3	Mexico	U22498
GII.3	Auckland	U46039
GII.4	Bristol	X76716
GII.4	Lordsdale	X86557
GII.4	Grimsby	AJ004864
GII.4	Camberwell	AF145896
GII.5	Hillingdon	AJ277607
GII.6	Seacroft	AJ277620
GII.7	Leeds	AJ277608
GII	Saitama/U3	AB039776

The alignment data shown in Figure 22 displays the nucleotide divergence of sample 7 from Lordsdale virus (GII.4, accession number X86557). Figure 22 indicates sample 7 has a large number of identical nucleotides to the Lordsdale virus sequence in this region with just four single nucleotide changes. Sample 7 had an identical nucleotide sequence to the Bristol virus sequence. Nucleotides which differ from the prototype Lordsdale virus are highlighted in red shown in Figure 22. The previously published human norovirus sequence data show a large and varied divergence from each other, as expected (70,70,77,78,128,148,149,155,213).

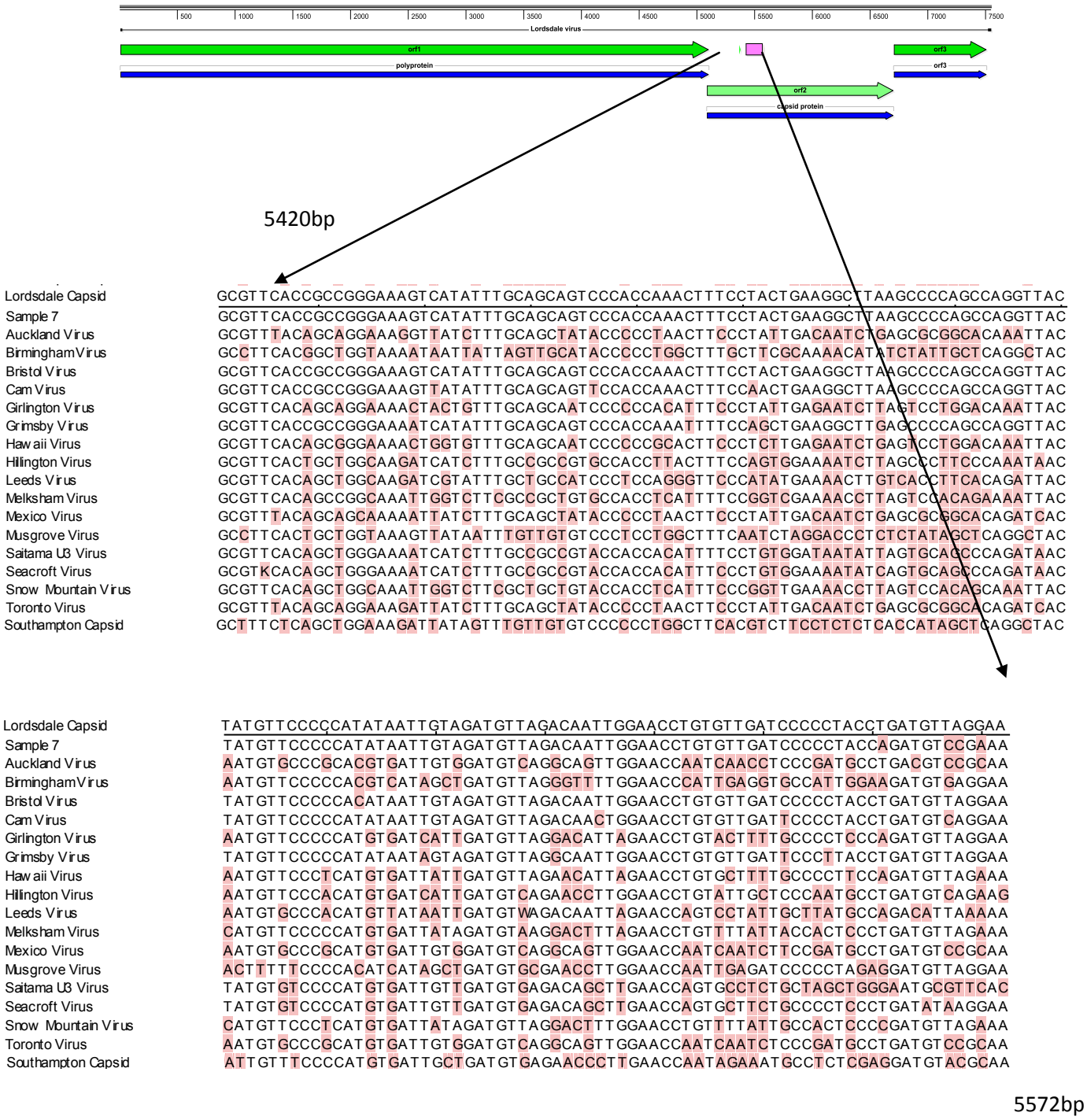


Figure 22. Sequence alignment data for sample 7 and 18 published norovirus sequences for the nucleotide region 5420-5572. Nucleotides divergent from Lordsdale are shown in red.

The amino acid sequence alignment data shown in Figure 23 indicates the number of mis-matches in a range of norovirus sequences including clinical sample 7. In Figure 23 the nucleotides which differ from the Lordsdale virus amino acid sequence are highlighted in red. There are no amino acid residues which differ from the Lordsdale, Bristol or Camberwell virus capsid in sample 7. The data shown in Figure 22 and Figure 23 show that this clinical sample contains human noroviruses highly likely to be GII.4.

Lordsdale virus genome

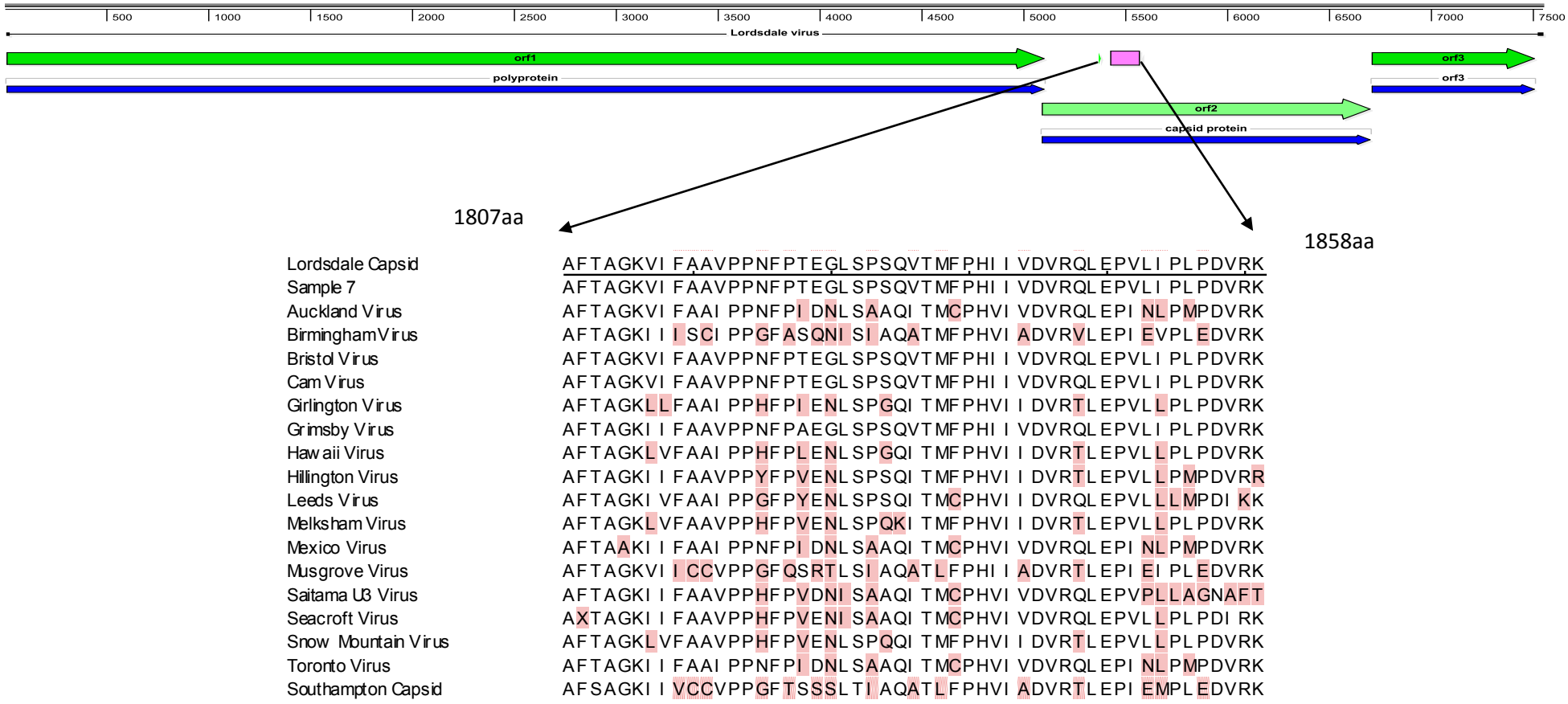


Figure 23. Alignment data for amino acid sequence of sample 7 against a panel of 18 human norovirus sequences for amino acid residues 1807 – 1858 in Lordsdale virus. This region is located in ORF2 as displayed above. Amino acids divergent from Lordsdale are shown in red.

Phylogenetic analysis of the sequenced 153 bp/51 amino acid region of sample (located at nucleotides 5420 to 5572 in the Lordsdale virus genome) was performed with a range of 18 previously reported norovirus sequences, as shown in Figure 24. Previous alignment of the sample 7 nucleotide and amino acid sequences suggested that this virus was related to the GII.4 noroviruses.

Despite the assumption that sample 7 is a GII.4, in other regions of the genome this sample may display sequence similarity to other noroviruses. As the initial purpose of this work was to select a sample for analysis that could be used in qRT-PCR and immunoassay techniques sample 7 fulfilled these criteria.

A number of recombinant human noroviruses have previously been described which have an RdRp from a separate cluster of noroviruses when compared to the capsid protein sequence, such as Snow Mountain and Saitama viruses. This may have occurred with sample 7, however would require further sequence analysis to confirm. For the proposed experimental strategy this was not required.

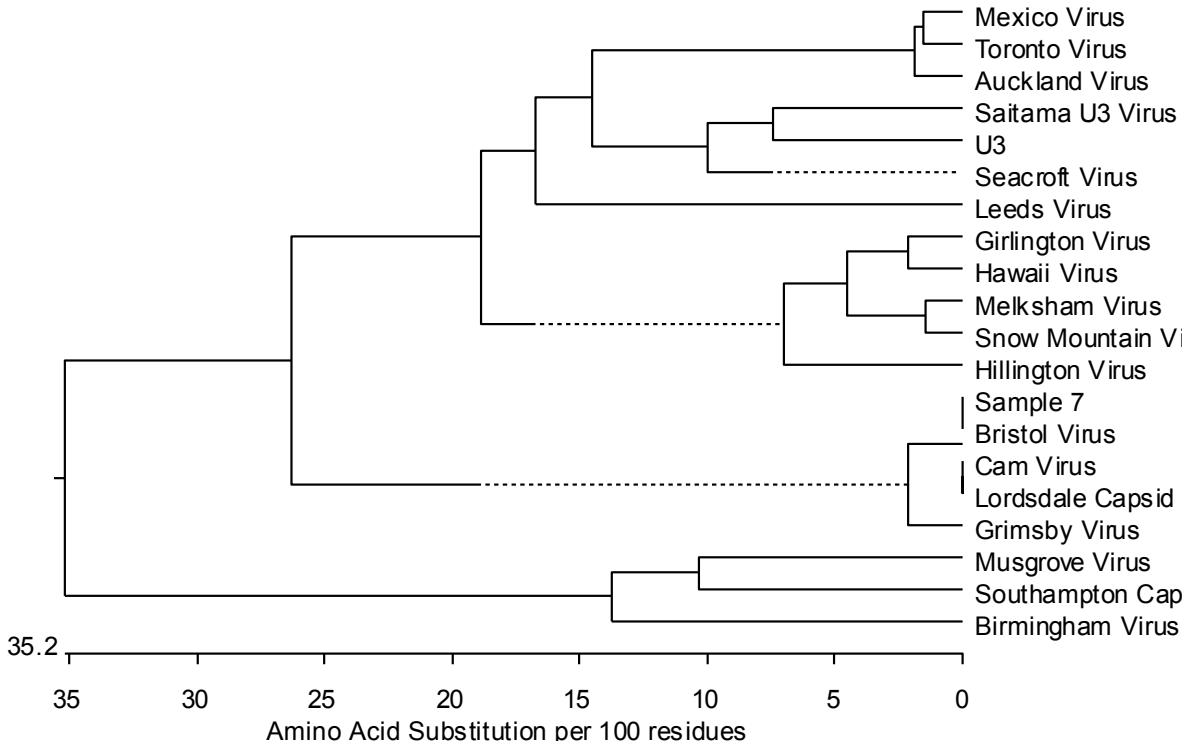


Figure 24 . Phylogenetic tree of the amino acid sequence data for sample 7 and 18 human norovirus sequences, amino acids 1807 to 1858 of the Lordsdale virus. The sequences are separated by evolutionary distance and amino acid substitutions.

The branch lengths of the phylogenetic tree are proportional to the evolutionary distance between sequences and the distance scale in nucleotide substitutions per position is shown in Figure 24.

Upon study of the alignment data and phylogenetic tree generated, sample 7 was shown to be closely related to the GII.4 noroviruses Bristol, Lordsdale, Camberwell and Grimsby. These viruses are members of the most prevalent genotype within the UK, GII.4 which have been reported to cause the majority of recorded norovirus outbreaks (20,219). As expected the GI noroviruses and other GII noroviruses are positioned in separate clusters to the GII.4 viruses.

6.2.1 Enzyme linked-Immuno-sorbent assay (ELISA)

A simple antigen capture ELISA had already been developed which was specific for GII.4 Lordsdale virus clinical samples and formed the basis for further development (247). It was ultimately intended to use a broadly cross-reactive monoclonal antibody for the detection of noroviruses in oysters. However, due to previously unsuccessful attempts to produce a monoclonal antibody which covered all human noroviruses, an existing GII.4 monoclonal antibody available in our laboratory was chosen for 'proof of principle' assay development. The Lordsdale virus-specific ELISA was used as a starting point and model system for developing an ELISA to apply to shellfish tissue. Sample 7 was tested in a modified Lordsdale virus specific ELISA to determine that the titres of virus were sufficient to be detectable and could be used in future experiments.

The capture antibody used in the GII.4 ELISA was polyclonal antiserum, raised to Lordsdale VLPs in rabbits. The detection antibody (22.2/CM7) was a monoclonal antibody generated from immunisation of mice with Lordsdale VLPs (S.Shipway, 1999, PhD Thesis).

22.2/CM7 can detect Lordsdale virus VLPs at a 1:10,000 dilution (0.2µg/ml) in a direct ELISA and detect Lordsdale virus-like viruses as efficiently as chicken IgY (S.Shipway, 1999, PhD Thesis). 22.2/CM7 did not recognise Toronto virus VLPs or two stool samples containing rotavirus in a direct and capture ELISA respectively. 22.2/CM7 immunoprecipitated full length Lordsdale virus capsid protein which had been expressed in the rabbit reticulate lysate coupled transcription/translation system. This monoclonal antibody did not immunoprecipitate either Lordsdale virus capsid protein expressed as N-terminal or C-terminal fragments and therefore it was determined that the antibody detected a conformational epitope in the full length Lordsdale virus capsid protein (S.Shipway, PhD thesis).

An optical density (OD_{450}) value above 0.2 was taken as a positive cut-off in the GII.4 antigen capture ELISA. This cut-off was double the OD_{450nm} value of the negative control used, 0.098. The negative control was a pre-immunisation serum taken prior to immunisation and used as the capture antibody; this serum should not contain any antibodies directed towards Lordsdale virus VLPs. Lordsdale virus VLP antigen was used as the positive control. Lordsdale VLPs were previously generated in our laboratory and gave an OD_{450} value of 1.2, at 0.2µg/ml (1:10,000 dilution), when used in conjunction with detection antibody 22.2/CM7. 100µl of faecal sample 7 produced an OD_{450} value of 0.822 (n=3) in the standard ELISA.

Sample 7 was considered to contain Lordsdale virus after analysis of sequence data produced from extracted RNA and amplification of the CapIIa and CapIIb primer region and values obtained from the ELISA. The titres of virus present in sample 7 were high enough to be reliably detectable in a capture ELISA.

6.2.2 Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA)

The DELFIA has a number of features which are designed to make it a superior alternative to the conventional ELISA. The DELFIA aims to quantify the level of antibody binding by measuring the light emitted when the fluorescent label decays after being excited by a light source at a shorter wavelength. DELFIA does not require time-dependent signal detection like the ELISA or the addition of a stop solution. Instead of relying on an enzymatic reaction, the oxidisation of TMB using the HRP enzyme, the DELFIA utilises the dissociation of the fluorescent label at a low pH and can also incorporate multiple fluorescent labels in a single well; these features produce a highly sensitive and flexible assay. Fluorescence is the amount of light emitted at a longer wavelength by the sample after it has absorbed light at a shorter wavelength. Fluorescence is a two wavelength analysis which reduces the possibility that two unrelated samples will have an identical excitation and emission spectra.

The DELFIA used in this project uses the same polyclonal antiserum and detection antibody in the previously described Lordsdale virus specific antigen capture ELISA. DELFIA uses a Europium (Eu)-labelled species-specific antibody conjugate and an enhancement solution to cause dissociation of the Eu lanthanide label. The fluorescence produced when Eu dissociates at a low pH, is measured at wavelength of 615nm after excitation at a lower wavelength using a time-resolved fluorescence reader (TRF). This fluorescence is stable for several hours, when protected from sunlight and evaporation; all measurements were taken within 20 minutes of development.

The positive control used was Lordsdale virus VLPs with detection antibody 22.2/CM7, a reading of 278k was obtained, at 0.2µg/ml. The negative control was pre-immunisation serum, used as the capture antibody; a reading of 1270 TFR was obtained at 615nm. A

value above 3000 TFR was taken as the positive cut-off, approximately double the negative control.

Sample 7 produced an average TFR value of 82353 (n=3) in the Lordsdale virus specific DELFIA. Fluorescence is 'unit less', similar to absorbance units in spectrophotometric analysis.

The DELFIA data indicated the quantity of viral capsid protein present in sample 7 was sufficient to be reliably detectable and therefore used in future experiments.

6.2.3 Development of DELFIA and ELISA for the detection of GII.4 viruses

To determine the limit of sensitivity of both detection assays (ELISA and DELFIA) for sample 7, a dilution series of the clinical sample was performed and results compared to this preliminary data.

In the antigen capture ELISA a reading above OD_{450} 0.2 indicated a positive result (double the negative control value of pre – immunization serum, 0.098). For the DELFIA a reading above 2000 TFR indicated a positive result approximately double the negative control, 1169. Each assay was performed in triplicate. The data in Table 18 and Table 19 show the values obtained in the ELISA and DEFLIA detection assays for sample 7.

The average and standard deviation of these values was calculated and shown alongside the raw data obtained. Inter-assay variation between each assay was also calculated for each dilution of sample 7, as the repeats of the experiment were performed on separate plates and different days. Inter-assay variation is measured as a percentage of the coefficient of variation (CV) as shown in the equation below. CV is a ratio of the standard deviation (σ) to the mean (μ).

$$\%CV = \frac{\sigma}{\mu} \times 100$$

The percentage CV gives an indication of the reproducibility of the assay. There is a high percentage of variation, 32-99%, between dilution repeats in the DELFIA, while, ELISA showed less variation with 3-62% between dilution repeats. The high percentage CV may be due to several factors, such as the natural variability of stool sample aliquots, the distribution of capture antibody across the wells and the efficiency of the conjugate and substrate step. Each stool sample may contain a variable number of viral capsid proteins causing uneven distribution, these samples are not purified and therefore there is no uniformity across the samples. Human error during the pipetting of the double dilution series may have resulted in uneven distribution of capsid protein across the wells.

Figure 25 displays the average values for each dilution in both the ELISA and DELFIA. The DELFIA error bars appear much smaller when compared to the ELISA data; however the former is on a logarithmic scale, due to the large numbers obtained. The initial volume of stool sample is 100µl in the 1:1 ratio. The ELISA is shown to drop under the cut-off at approximately a 1:48 dilution, approximately 2µl of stool sample detected. In comparison the DELFIA could still detect GII.4 capsid protein at a 1:192 dilution, approximately 0.5µl of stool sample, a fourfold increase in sensitivity.

Despite lower reproducibility for the DELFIA shown by the percentage inter assay variation; this assay was able to detect Lordsdale norovirus in all three replicates at a 1:64 dilution, which is still a twofold increase in sensitivity over the ELISA, in which all three replicates produced positive values at a 1:32 dilution.

Error bars in Figure 25 indicate the highest and lowest values obtained for each dilution in each assay.

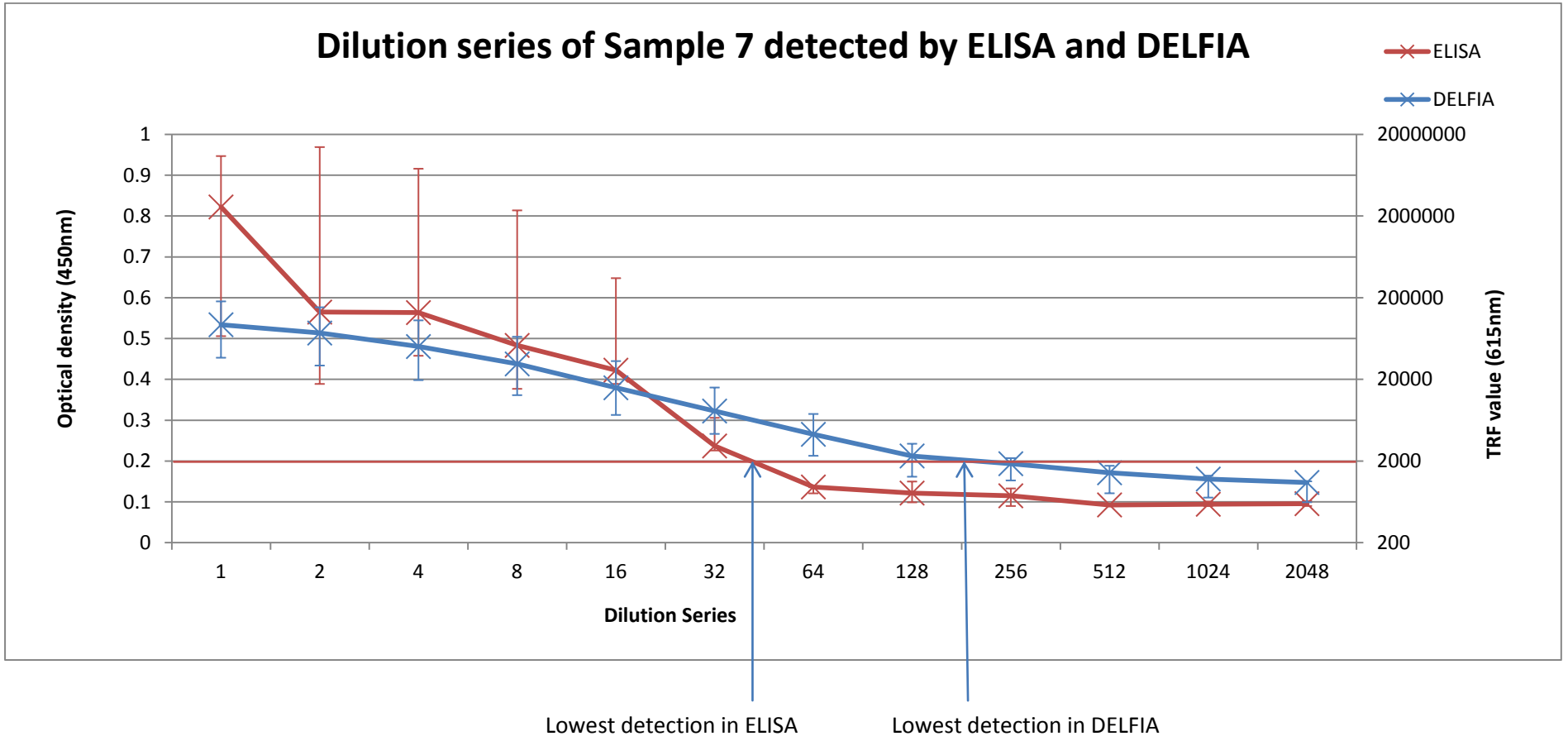
	Repeat Number					
Ratio	**1**	**2**	**3**	**Average (n=3)**	**Standard Deviation**	**Inter assay variation (%)**
1:1	**1.01**	**0.50**	**0.94**	0.82	0.27	33.56
1:2	**0.33**	**0.38**	**0.96**	0.56	0.35	62.09
1:4	**0.31**	**0.45**	**0.91**	0.56	0.31	55.55
1:8	**0.25**	**0.37**	**0.81**	0.48	0.29	60.49
1:16	**0.23**	**0.38**	**0.64**	0.42	0.21	49.80
1:32	0.17	**0.22**	**0.30**	0.23	0.06	27.09
1:64	0.15	0.12	0.13	0.13	0.01	11.71
1:128	0.11	0.15	0.09	0.12	0.02	21.34
1:256	0.12	0.13	0.09	0.11	0.02	19.42
1:512	0.09	0.09	0.09	0.09	0.003	3.29
1:1024	0.09	0.10	0.09	0.09	0.005	6.21
1:2048	0.09	0.10	0.09	0.09	0.006	6.30

Table 18. Assay data obtained from the GII.4 antigen capture ELISA, with a dilution series of sample 7 performed in triplicate. Positive values are shown in bold.

	Repeat Number					
Ratio	**1**	**2**	**3**	**Average (n=3)**	**Standard Deviation**	**Inter assay variation (%)**
1:1	**62916**	**36885**	**180632**	**93477**	76591	81.93
1:2	**39515**	**29593**	**152763**	**73957**	68428	92.52
1:4	**26188**	**19603**	**105769**	**50520**	47960	94.93
1:8	**13405**	**12835**	**66610**	**30950**	30883	99.78
1:16	**6528**	**7350**	**33531**	**15803**	15358	97.18
1:32	**4388**	**4306**	**15917**	**8203**	6680	81.42
1:64	**2878**	**2324**	**7550**	**4250**	2870	67.53
1:128	**2387**	1288	**3261**	**2312**	988	42.76
1:256	**2244**	1159	**2174**	1859	607	32.66
1:512	1755	807	1753	1438	546	38.01
1:1024	1578	715	1327	1206	443	36.78
1:2048	1528	627	1131	1095	451	41.22

Table 19. Assay data obtained from the GII.4 antigen capture DELFIA, with a dilution series of sample 7 performed in triplicate. Positive values are shown in bold.

Figure 25. Comparison of ELISA (red) and DELFIA (blue) assays for the detection of sample 7 in a twofold dilution series. The positive cut-off is indicated by the red line.



6.2.4 Quantitative Reverse-Transcription-PCR (qRT-PCR)

Quantitative RT-PCR is a highly sensitive detection method which has been modified and developed to detect noroviruses in faecal samples; it incorporates both the synthesis of cDNA from RNA and the subsequent qPCR to measure the quantity of RNA genomes per ml. The quantity of RNA is measured utilising a DNA probe, RING2-TP. This DNA probe is a small nucleotide fragment, which has a fluorophore (FAM) reporter at one end and a quencher (TAMRA) at the opposite end. The reporter and the quencher are closely located which prevents detection of the fluorescence prior to PCR amplification. As the probe is broken down by the 5' to 3' exonuclease activity of the polymerase this causes the fluorophore and quencher to separate allowing the emission of fluorescence which can be quantified. As the reaction progresses the level of fluorescence will increase with each cycle. qRT-PCR requires primer sequences located over a conserved area of the genome, which has proved a challenging undertaking due to the great sequence diversity of the norovirus genome particularly in the capsid protein sequence. In this project GII specific primers described by Kageyama *et al* (118) were used.

The quantity of RNA genomes in sample 7 could be determined using qRT-PCR and compared to the sensitivity of the Lordsdale virus specific antigen capture ELISA and DELFIA in detection of GII noroviruses in stool samples. qRT-PCR is currently the gold standard assay for the detection of noroviruses with a high sensitivity and ease of genogrouping the samples.

Viral RNA was extracted and purified (2.7.6.3) from the clinical sample 7 and eluted in 30µl UHQ H_2O. 5µl of viral RNA was added to the qRT-PCR reaction (2.7.5.2). The one-step qRT-PCR prevents cDNA contamination as cDNA synthesis and subsequent PCR are performed within the same tube.

Primers used in the qRT-PCR experiments were described by Kageyama *et al*, detecting a sequence located across the ORF1 and ORF2 junction, a highly conserved region of the genome (118). This region is located at nucleotides 5003-5100 in the Lordsdale virus genome (GII.4); with the primers amplifying a 98bp region.

To determine the number of genomic copies of RNA (per ml) in sample 7, a standard curve with 10-fold serial dilutions of a known quantity of the full length Lordsdale virus *in vitro* RNA (GII.4) was generated (10^{-1}-10^{-12} copies). The full length Lordsdale virus sequence had previously been inserted into the pSP73 vector (with the Xba-Xho cassette removed); this plasmid contains a T7 promoter upstream of the Lordsdale virus genome sequence. T7 RNA polymerase is a DNA-dependent RNA polymerase, producing 5' – 3' synthesis of RNA on either single or double stranded DNA downstream from the promoter.

The circular Lordsdale virus DNA plasmid was linearised using restriction enzyme HpaI and run on an agarose gel (2.5.4) to ensure complete linearization prior to transcription of RNA. Linear DNA was transcribed to RNA using the T7 RNA polymerase kit (2.7.6.1) and treated with DNase to remove template DNA.

Transcribed RNA was visualised on a denaturing agarose gel (2.5.5) stained with ethidium bromide to ensure no RNA degradation was present, as shown in Figure 26 alongside a single stranded RNA ladder.

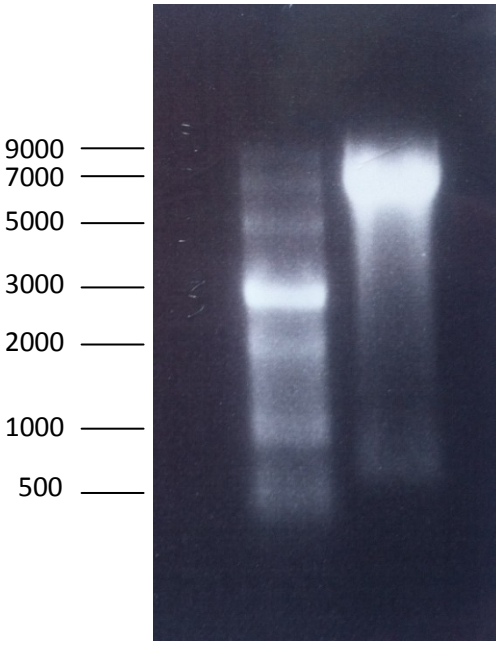


Figure 26. 0.9% denaturing agarose gel of 1.3µg Lordsdale RNA alongside a single stranded RNA ladder (NEB), with the size in bases indicated.

Figure 26 indicates that there was some degradation of the RNA present; however it was decided to proceed with qPCR experiments with this reference standard. A strong band at just over 7000bp indicates successful production of full-length Lordsdale virus genomic RNA. This RNA was diluted in UHQ H_2O to obtain a concentration of 1ng/ml, determined by using a Nanodrop^{TM}. The 1ng/ml material was used to generate a 10 fold dilution series 10^{-1} to 10^{-12} used as the standard curve in qRT-PCR experiments.

Control experiments were performed with the Lordsdale virus plasmid RNA to ensure that amplification visualised in the qPCR was produced from RNA and not remaining DNA after DNase treatment. qPCR reactions were performed with samples of Lordsdale virus RNA and DNA both with and without reverse transcriptase and negative control H_2O. This provides an indication of the amplification produced solely by any remaining DNA. The Lordsdale virus DNA displayed amplification both with and without reverse transcriptase as expected. Lordsdale virus RNA only had amplification with reverse transcription, demonstrating that no DNA was remaining from RNA production.

The threshold in each qRT-PCR experiment was located to ensure positive readings are taken above the background fluorescence level. In qRT-PCR a positive result was determined by the accumulation of a fluorescence signal above the threshold after each cycle. The Ct value is calculated by the number of cycles required for the fluorescence level to cross the threshold. Rn is the fluorescence of the reporter dye divided by the fluorescence of the passive reference dye, calculated by the Applied Biosystems 7500 software. ΔRn is the baseline corrected normalised reporter this is calculated by, ΔRn (cycle) = Rn (cycle) – baseline.

Several factors can affect the C_t value obtained; the use of different qRT-PCR master mix components, the quantity of ROX™ passive reference dye present and the efficiency of the PCR reaction. All possible steps were undertaken to avoid these factors altering the C_t values, such as using the same master mix, reference dye quantities and components for each reaction.

All amplification data were collected and analysed using sequence detector software 7500 version 2.0.5 (Applied Biosystems). The data generated from the 10 fold dilution series were used to plot a standard curve from which the quantity of RNA in the unknown samples can be determined.

The quantity of RNA in sample 7 was calculated to be 2.89×10^7 RNA copies per ml in the original stool sample.

Human stool samples can contain high concentrations of norovirus, up to 10^{11} viral particles per g (8). At the present time there is no threshold infectivity limit for norovirus detected by qRT-PCR. Previous studies reported that a 10^{-6} dilution was the highest dilution of a stool containing norovirus in which this assay could routinely detect viral RNA in both semi-nested and real-time GII methods (10). Jothikumar *et al* developed a qRT-PCR assay for the detection of norovirus RNA in stool samples and assessed its sensitivity using a 10 fold dilution series of norovirus GII. It was determined that the assay could detect <10 copies of viral genome per reaction (117). A 'positive result' i.e. detection of norovirus RNA was determined as samples with a Ct value below 45 in at least two replicates and no evidence of amplification of the negative control. The lowest level of total norovirus (GI & GII) reported by Cefas, using the recently described CEN qRT-PCR method in a sample definitively associated with an outbreak is 152 copies/g (161).

qRT-PCR analysis of sample 7 had indicated that this clinical sample contained approximately 2.87 x 10^7 RNA copies/ml when compared to the standard dilution series of Lordsdale RNA. As stated qRT-PCR can reliably detect as low as 10 norovirus RNA copies per reaction from stool samples. For this reason it was deemed that the 2.87 x 10^7 RNA copies/ml, equal to 2.87 x 10^6 RNA copies in the proposed 100µl seeding volume would be sufficient for detection.

6.3 Detection of human noroviruses in oyster tissue

Under EU regulations (No. 853/2004, 854/2004, 2073/2005, 2074/2005), oysters are required to be monitored and classified appropriately. EU method ISO TS 16649-3 is required for official control testing and monitoring of *E.Coli,* indicating the level of faecal pollution that the oysters were exposed to in the harvesting area.

However, these regulations are focused predominately towards reducing bacterial levels in shellfish and have failed to prevent outbreaks associated with viruses (26). To detect noroviruses in shellfish and prevent infection, it is vital to develop rapid and sensitive assays. Currently, there is no legal requirement for testing shellfish for norovirus in EU regulations and no standardised detection assay. There has been a push across the EU to produce a standard qRT-PCR method for the detection of noroviruses; the technical specification of this method was published in 2012. The release of this method as a validated international standard will be released by 2017. It is expected that EU regulations will adopt this method as a virus standard and incorporate it into EU legislation. The relationship between the number of infectious virus particles and the quantity of genome copies detected in qRT-PCR is unknown and it is unclear what risk low level positive oysters pose to the general population.

It is known that current treatment methods to reduce viral levels such as depuration are ineffective. Oysters which had been artificially contaminated with GII.6 samples for 72hrs

followed by depuration for 10 days were tested using qRT-PCR described by Kageyama *et al*, after 72hrs of exposure an average of 1.7 x 10^3 RNA copies per g of digestive tissue were detected and 1.5 x 10^3 and 1.8 x 10^3 copies/g were detected after the 3 and 10 days depuration respectively. These experiments indicated that there is no decrease in the levels of norovirus RNA after depuration; however, it does not indicate the levels of infectious norovirus present in the tissue (118,241). Alternative treatments such as heat treatment and high pressure may be more effective but produce changes to the oyster tissue which are unappealing to the consumer (129,146). The recommended method to prevent norovirus contamination in oysters is to ensure that the oysters are grown in uncontaminated waters and not exposed to faecal matter. In naturally contaminated oysters the Kageyama *et al* qRT-PCR detected an estimated 3.3 x 10^6 to 1.5 x 10^8 RNA copies per oyster (61,118). However, at present the quantity of norovirus in oysters which results in illness has not been defined.

There are a number of common procedures used to extract enteric viruses and viral RNA from environmental samples, acid absorption-elution (110,220), direct glycine buffer elution (143), virus precipitation using Cat-Floc (201), polyethylene glycol (110), solvent extraction using chloroform (178) and chloroform/butanol (7).

Many studies have aimed to compare the extraction procedures in order to develop a sensitive detection method. Baert *et al* (10) artificially seeded mussels with a 10-fold serial dilution of a GII.4 sample, no RNA could be detected at the low inoculum levels using direct RNA extraction, whilst the alkaline virus elution concentration method was able to detect inoculum at a 10^{-4} dilution with a semi-nested GII assay. It was estimated that around 100 copies of norovirus were detected from seeded mussels, indicating around 100 fold loss of virus had occurred (10).

Column-based RNA purification methods, such as the Qiagen RNeasy kit have been shown to recover more RNA than silica based methods. Gentry *et al* (117) evaluated both RNA extraction protocols and demonstrated that the Qiagen RNeasy kit was able to recover 80% of RNA copies from seeded oyster compared to 0.175% from the silica based methods. In dilution assays using the Qiagen RNeasy kit, RNA could be detected at a 1 x 10^{-3} dilution, which was estimated to be 8 RNA copies. Other studies have also investigated the sensitivity of qRT-PCR, to detect norovirus RNA in naturally contaminated oysters, determining that for GII viruses, the values obtained were near or at the limit of sensitivity (117).

These studies indicate the importance of using an extraction procedure which maximises the recovery of viral particles, in turn ensuring that a true understanding of the quantity of noroviruses present is obtained. Within the extraction procedures the most crucial step is the viral recovery, therefore it is vital to select an appropriate method. However at the current time there is no optimal method for norovirus recovery from shellfish for commercial testing. The digestive diverticulum is used as the starting material for viral extraction as it has been shown to be the target of contamination within the tissue (203).

In this project organic flocculation and polyethylene glycol (PEG) precipitation was used for extraction and concentration of norovirus proteins from oyster tissue, as described by Atmar *et al* with slight variation (6,7). PEG is used as it allows the precipitation of noroviruses at neutral pH and at high ionic concentrations without precipitation of other organic material.

In the original study, Atmar *et al* artificially seeded oysters with 100µl of 10 fold dilutions of a 50% suspension of stool sample; the limit of detection was determined to be 10^{-2} dilution of seeded stool sample by RT-PCR. This is theoretically 0.5µl of stool sample detected in the whole oyster tissue. The study assumed that the original stool sample contained 10^{5}-10^{6}

particles per ml, as no viral particles could be viewed using EM, which requires a minimum of 10^6 viral particles. This would assume that the extraction and detection methods are able to detect 50 – 500 viral particles (6). Polyethylene glycol (PEG) is often used for this purpose, as it easily allows the precipitation of these viruses at neutral pH and at high ionic concentrations without precipitation of other organic material

The method described by Atmar *et al* (6,7) has been used extensively for the detection of noroviruses from oysters (17,136,140,157,186). As described by Le Guyader *et al* (140) this method can be improved by using a commercially available RNA extraction kit for the detection of norovirus RNA in a qRT-PCR. In the work described here, TRIzol was utilised alongside the Purelink RNA extraction kit (Invitrogen, 2.7.6.3) to ensure successful RNA isolation.

Alternative methods, such as ultracentrifugation based approaches to pellet the noroviruses have been described however these require expensive equipment which was unavailable.

It has been described previously that detecting noroviruses in naturally contaminated shellfish is hindered by the low levels of virus present and the presence of inhibiting substances which prevent detection (140).To address this issue, oysters were initially tested both unseeded (100µl PBS) and seeded with 100µl clinical sample 7, to determine the sensitivity of methods, DELFIA, ELISA and qRT-PCR, as described in the preliminary experiments with sample 7. By seeding the oysters with sample 7 it is hypothesised that the level of norovirus will be increased sufficiently to be detected reliably in each assay.

6.3.1 Extraction of norovirus viral particles from oysters

Pacific rock oysters (*C.gigas*) were used in all experiments involving oyster tissue. *C.gigas* oysters are resilient to extreme weather conditions and spawn more frequently than the

native oyster (*ostrea edulis*). The rock oyster is available all year round, whereas the native oyster is harvested from September through to the end of April. This all year availability led to *C.gigas* oysters being the predominant oyster consumed in the UK. The pacific oyster is an estuarine species and attaches to rocky surfaces in shallow or sheltered waters. This makes them particularly vulnerable to faecal contamination from sewage outlets and run off from adjacent farmland.

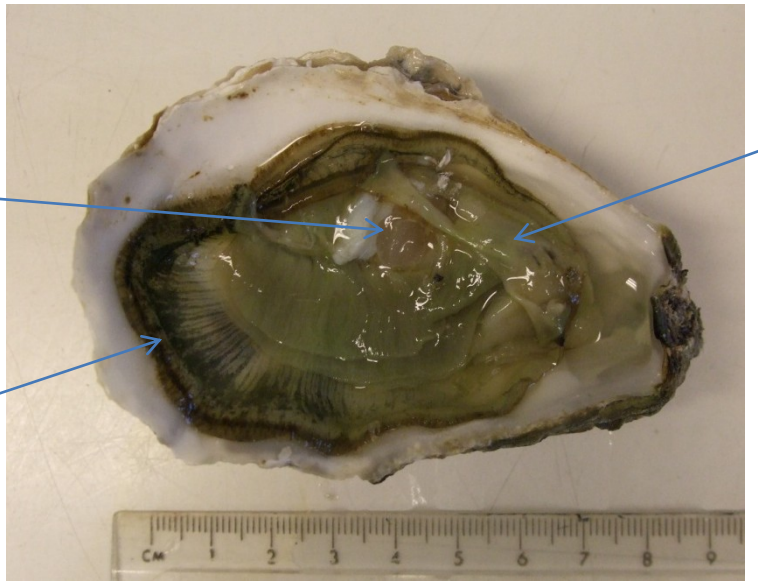
The all year round availability and high level of consumption of the Pacific rock oysters (*C.gigas*) in the UK led to these oysters being used in experiments to compare the sensitivity of norovirus detection methods.

From the oysters, tissue was removed and the hepatopancreas was dissected out and homogenised (2.11.1). The average oyster tissue weighed between 7 – 13g and dissected digestive tissue between 0.2 – 1g. Digestive tissues comprised of between 2.8 – 8% of the total weight of the oyster, excluding the shell.

Figure 27 shows images of a pacific rock oyster (*Crassostrea gigas*), intact (a), with the upper shell removed to display the oyster tissue (b) and the dissected digestive tissue (c). The organs are covered by a sheet of connective tissue, the mantle; this was removed in image b) to display the location of the internal organs.

a)

b)



c)

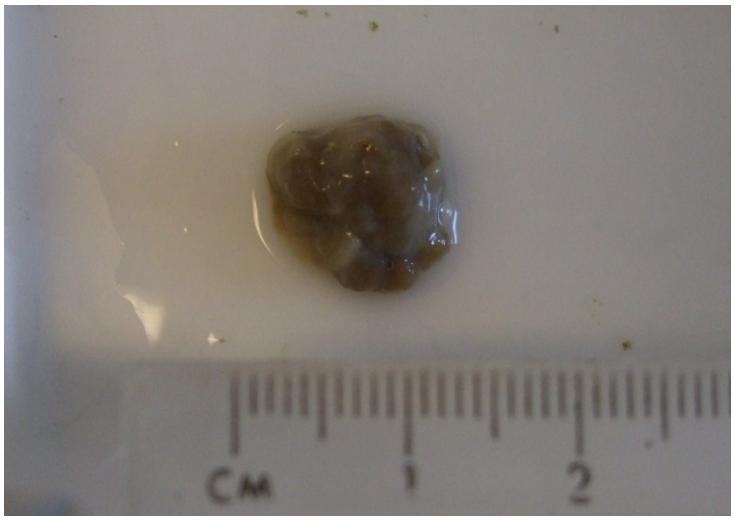


Figure 27. a) Pacific rock oyster (*C.gigas*) **b**) Upper shell removed, displaying the internal organs **c**) dissected digestive tissue

6.3.2 Development of ELISA, DELFIA and qRT-PCR for the detection of GII noroviruses in oysters

A preliminary set of 18 oysters, three separate seeded and three separate unseeded oysters were analysed by each method, ELISA, DELFIA and qRT-PCR to determine if the GII.4 stool sample 7 could be reliably detected above the background in each detection assay. It was unknown if the oysters would contain inhibitory substances which would affect the detection of either norovirus RNA or protein. It was thought that the unseeded oysters will provide a nil result, demonstrating there are no pre-existing noroviruses in the tissue. A 100µl aliquot of sample 7 was added to each oyster sample to be tested as a seeded sample. A 100µl volume of stool sample contained approximately 2.87 x 10^6 RNA copies; this was calculated from previous qRT-PCR data of sample 7 quantified using a 10 fold dilution series of *in vitro* Lordsdale RNA. Previous studies have demonstrated that the chloroform/butanol extraction procedure enables between 50 – 500 virions to be detected (6); therefore the 100µl sample should contain a sufficient quantity of norovirus virions to be reliably detected, it was felt above this quantity there would be no increase in detection rates by the chosen assays. It was important to ensure there were an excess number of virions present, as some virions would be lost during the extraction and purification procedures and therefore not detected.

Each oyster was shucked and dissected (2.11.1) and either norovirus protein or RNA was extracted as described previously (2.11.1.1 and 2.11.1.2). In protein extractions, the pellet was suspended in 400µl UHQ H_2O, of which 100µl was used in the ELISA or DELFIA detection assays.

The data obtained for the preliminary ELISA and DELFIA to detect a GII.4 sample in oyster digestive tissue is shown in Table 20 and Table 21 respectively. The GII.4 antigen capture ELISA and DELFIA were performed as described previously (2.8.9 and 2.8.10). A negative

control of PBS (as antigen) was used in each assay, alongside the unseeded oyster sample. The positive cut-off value was an OD value >0.24 in the ELISA and a TRF value >2000 in the DELFIA; both double the negative control.

As shown in Table 20, none of the values obtained in the ELISA for either seeded or unseeded oysters were above the positive cut-off, however in Table 21 it can be seen that in the DELFIA both the unseeded and seeded oyster values are above the positive cut-off. These data show that the DELFIA was able to detect GII.4 norovirus in all six tested oysters and show that the ELISA is not as sensitive as the DELFIA. It is important to recognise that both assays tested different oysters, which may result in a number containing pre-existing norovirus whilst others did not.

Table 20. ELISA preliminary data for six oysters, three seeded with 100µl GII.4 sample 7 and three with 100µl PBS. OD values at 450nm are given.

Unseeded Oyster			Seeded Oyster			
1	**2**	**3**	**4**	**5**	**6**	**Negative**
0.072	0.098	0.076	0.091	0.153	0.147	0.123

Average unseeded oyster values: 0.082

Average seeded oyster values: 0.130

Table 21. DELFIA preliminary data for six oysters, three seeded with 100µl GII.4 sample and three with 100µl PBS. TRF values at 615nm are given.

Unseeded Oyster			Seeded Oyster			
7	**8**	**9**	**10**	**11**	**12**	**Negative**
5215	**6916**	**7005**	**6593**	**8549**	**8029**	1027

Average unseeded oyster values: **6378**

Average seeded oyster values: **7723**

Six separate oysters were subsequently tested in a GII qRT-PCR to calculate the number of RNA genomes per ml which could be detected. Three oysters seeded with 100µl sample 7 and three unseeded oysters with 100µl PBS were extracted using the TRIzol extraction procedure and purified using the Pure Link kit (2.7.6.3), in an elution volume of 30µl. The concentration of RNA in each sample was obtained using the Nanodrop™ and shown in Table 22. The concentration of total RNA recovered is reported, the Nanodrop is not selective for norovirus RNA, therefore despite the high RNA concentration, only a small proportion may be norovirus.

The RNA genomes per ml can be calculated using qRT-PCR with primers which are specific for GII norovirus RNA. This method provides an accurate value of the quantity of norovirus RNA present in the oyster digestive tissue.

5µl of the extracted RNA was tested using a GII specific qRT-PCR (2.7.5.2) as described by Kageyama *et al* (118). All three oysters seeded with sample 7 were positive for GII norovirus RNA, with quantities of 1.7×10^7, 1×10^7 and 1.7×10^7 RNA genomes per ml. Initially 2.87×10^6 norovirus RNA copies were seeded into the oysters (100µl of sample 7), after extraction and purification, the sample was eluted in 30µl, which should contain the same number of RNA copies as the original sample (assuming there was no loss during the procedure). Of the 30µl sample, 5µl was tested in the GII qRT-PCR which was expected to contain approximately 4.7×10^5 RNA copies. In the three seeded oysters analysed by the same procedure it was determined that oysters 1 and 3 contained approximately 8.5×10^4 RNA copies and oyster 2, 5×10^4 RNA copies in the 5µl sample tested. The number of RNA copies was approximately 5.5 fold less in the seeded oyster extractions than expected from the theoretical values. The theoretical values do not take into account any loss of virions during the extraction and purification process which will result in a reduction in the number of RNA copies detected. Despite the same extraction and purification procedure being

performed with each oyster, there was a variation in the number of GII RNA copies which were detected. Oyster 2 had 1.7 fold less RNA copies than oysters 1 and 3, which had an identical number of RNA copies, this difference may be due to inefficiencies in the procedure or in the natural variation of the oysters. It was unknown if these oysters contained pre-existing norovirus RNA which may have also affected the values obtained. In comparison only one of the three unseeded oysters was negative for norovirus RNA. The two unseeded oysters in which norovirus RNA could be detected, had slightly lower concentrations of RNA per ml with 8×10^5 and 4.5×10^5 RNA genomes per ml, when compared to the three seeded oysters. Oyster 3 was negative and produced no amplification plot in the qRT-PCR; therefore no norovirus GII RNA was present. This oyster was not tested for GI norovirus RNA; therefore it may contain other noroviruses which were not detected by the GII qRT-PCR. This result indicates that the extraction procedure is not contaminating the samples with norovirus and that the positives recorded are true positives.

Table 22. Concentration of recovered total RNA from three unseeded and three seeded Oysters in ng/µl, as determined using the Nanodrop™. RNA genomes per ml, was calculated in a GII specific qRT-PCR.

	Oyster No.	RNA concentration ng/ul	RNA genomes per ml
Unseeded	1	4984	8×10^5
	2	5045	4.5×10^5
	3	3497	0
Seeded	4	4003	1.7×10^7
	5	3976	1×10^7
	6	3924	1.7×10^7

The preliminary investigations, using ELISA, DELFIA and qRT-PCR determined that oysters containing norovirus, either containing pre-existing contamination or seeded with 100µl sample 7 (2.87 x 10^6 RNA copies in the 100µl sample and 4.7 x 10^5 RNA copies in 5µl tested by qRT-PCR) could be detected by both DELFIA and qRT-PCR. Both methods have an increased sensitivity over the ELISA.

It was unexpected that the majority of oysters tested contained pre-existing norovirus contamination. However a recent study by Cefas showed that a significant percentage (76%) of oysters tested from UK oyster growing beds contained norovirus. The virus was detected at low levels in more than half of the positive samples (52%). The study took place between 2009 and 2011; more than 800 samples were obtained from 39 oyster harvesting areas across the UK (FSA, press release, November 2011).

At the start of the experimental design it was expected that a few oysters may contain norovirus protein or RNA, however, after preliminary testing of nine unprocessed oysters, three were negative in the ELISA, two out of three returned positive in the qRT-PCR and all three were positive in the DELFIA. The negative results for the oysters in the ELISA may be due to the lack of sensitivity of this method, relying on the efficiency of the HRP enzyme. Between the DELFIA and qRT-PCR only one of the six oysters was negative for norovirus protein and RNA respectively. DELFIA and qRT-PCR detection techniques could detect faecal samples containing norovirus seeded into the oyster in all samples tested and the values obtained were slightly above the unseeded oyster values in both assays. It is important to note that due to no *in vitro* culture methods being available for human noroviruses it is unknown whether these viral particles are infectious or are a mixture of degraded and inactive protein and RNA.

The detection antibody in the DELFIA detects a conformational epitope in the capsid protein. The capsid protein may have degraded and no longer be infectious and the

detection antibody would still detect the protein if the conformational epitope was still intact. The primers used in the qRT-PCR recognise a very small region in the capsid protein sequence (200bp) of the GII genome (which is approximately 7.4kb in length). RNA may have degraded and be non-infectious while qRT-PCR is still able to detect this small region and produce an amplification plot.

All eighteen oysters (nine unprocessed and nine seeded oysters) tested were from two separately caught batches but from the same oyster farm. Therefore it maybe that this particular growing area is exposed to sewage contaminated water (either sewage outlets or boat discharge), an infectious handler or agricultural run-off.

Due to the unexpectedly high prevalence of pre-existing norovirus contamination in the oyster digestive tissue, the experimental design was altered. Accumulation of norovirus in the digestive tissue could be seen as beneficial to this project, as the original aim of seeding the oyster tissue with a GII.4 stool sample was to replicate naturally contaminated oysters and then detect the sample in a GII assay. Discovery of pre-existing norovirus in the ideal location meant that further tests were performed to determine if a quantity of stool sample (100µl, containing approximately 2.87×10^6 GII norovirus RNA copies) could be detected above the pre-existing norovirus contamination present in the digestive tissue.

A control experiment was performed using a twofold dilution series of sample 7 (0 -300µl) through the intended protein extraction procedure, chloroform/butanol as described previously (2.11.1.1) and analysed by DELFIA. These results were compared directly to a twofold dilution series of sample 7 not put through the extraction procedure, as such these would be unmodified samples. This experiment ensured that the 100µl stool sample (approximately 2.87×10^6 GII norovirus RNA copies) seeded into oyster digestive tissue could be extracted and detected in the DELFIA over and above any pre-existing norovirus contamination present.

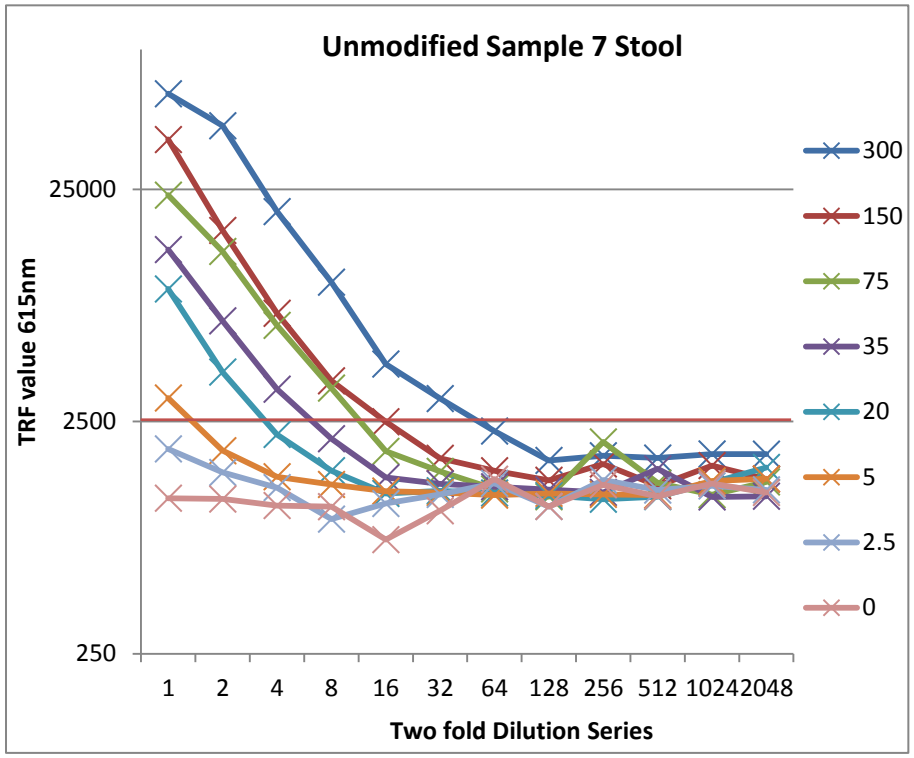
The TRF value obtained from the negative control UHQ H_2O, 0µl stool (1284) was doubled to give a positive cut-off value of TRF 2500. Each stool sample not extracted through the chloroform/butanol extraction procedure was diluted to give a final volume of 400µl, identical to the final volume of those samples which were processed through the chloroform/butanol procedure.

As an example, 75µl of unmodified sample 7 diluted to a final volume of 400µl in PBS, is equal to a 1 in 2.6 dilution of the sample 7. Therefore in the 100µl sample tested in the DELFIA there was an equivalent of 18µl of unmodified stool sample 7. Within the 18µl there are approximately 5.1×10^5 GII norovirus RNA copies, as calculated from the qRT-PCR values for sample 7.

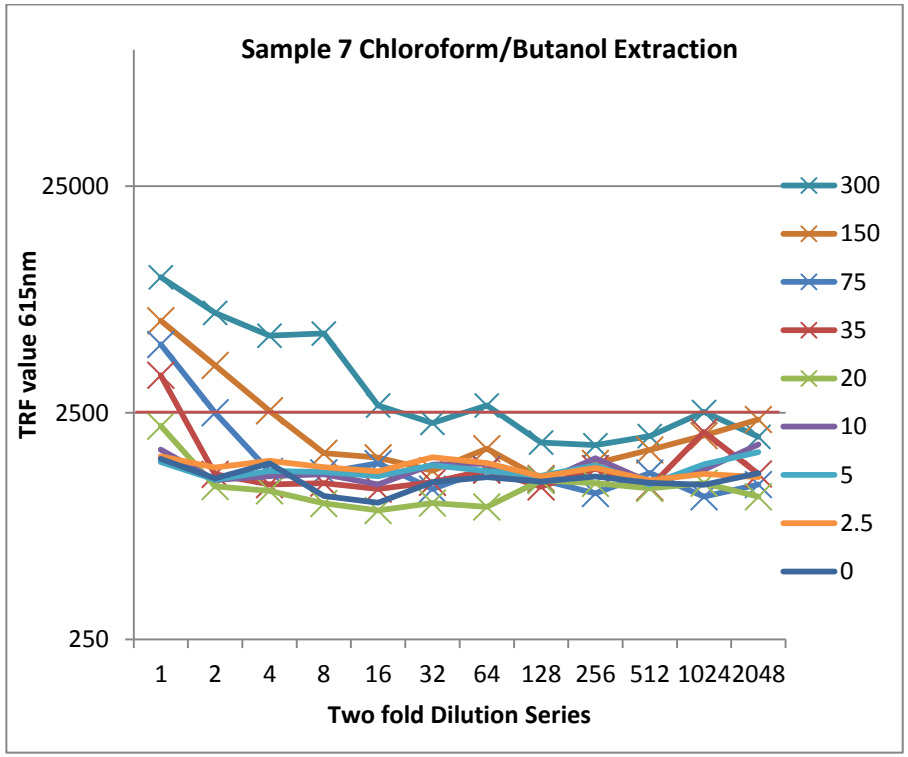
Figure 28 shows the values obtained from the two fold dilution series for sample 7 protein extracted with and without the chloroform/butanol procedure.

Figure 28. Average DELFIA values of a twofold dilution series of sample 7**, a)** Sample 7 diluted stool sample equivalent to the extracted sample pellet **b)**. Sample 7 protein extracted through the chloroform/butanol procedure. The positive cut-off is indicated by the red line.

a)



b)



After extraction by chloroform/butanol, sample 7 norovirus capsid protein could be detected in the processed 35µl stool sample, extracted into a total volume of 400µl. The initial 35µl stool sample is equivalent to 9µl stool sample when the dilution factor is taken into account in the final volume tested of 100µl (equivalent to 2.96 x 10^5 RNA genomes per ml, calculated from the original qRT-PCR data obtained for sample 7). The chloroform/butanol processed stool sample 7 could also be detected at a 1:2 dilution for 75µl of sample 7 (equivalent to 9µl stool sample in the final analysis sample volume of 100µl). In comparison the unmodified 5µl sample, which had not undergone the extraction procedure, in which there is 1.25µl of stool sample in the 100µl analysis sample volume, could still be detected undiluted. These experiments indicate that the chloroform/butanol procedure did result in significant loss of stool sample (7 fold loss), as demonstrated by the decrease in TRF values obtained.

Each dilution was performed in duplicate; the average of these values is shown in Figure 28.

6.4 Comparison of ELISA, DELFIA and qRT-PCR assays in the detection of GII noroviruses in naturally contaminated oysters

Due to the high level of stool sample loss during the extraction procedure and the indication in the preliminary results that the majority of the oysters tested contain norovirus protein and RNA it was decided that only unseeded oysters would be tested. Naturally contaminated oysters concentrate the virus via ligands present in the digestive tissue, making results obtained more relevant to commercial and environmental testing. The protein extractions from natural oyster tissue were analysed in an antigen capture GII.4 ELISA and DELFIA (2.8.9 and 2.8.10). A two-fold dilution series was performed to determine the limit of detection in each assay. The quantity of RNA molecules per ml in the last positive detected dilution in the assays can be determined using the values obtained in

a GII qRT-PCR of the same sample. A positive result in the qRT-PCR does not guarantee a positive result in the DELFIA or ELISA. The qRT-PCR is a GII assay whilst the DELFIA and ELISA are specific against Lordsdale virus (GII.4).

6.4.1 GII norovirus detection in naturally contaminated oysters by ELISA

Ten oysters were obtained from a local supplier, shucked and the digestive tissue dissected (2.11.1). From the digestive tissue of each oyster, RNA was extracted using the TRIzol extraction procedure, from approximately ¼ of the sample (2.11.1.2) and proteins were extracted and purified using the chloroform/butanol procedure from the remaining approximate ¾ of the sample (2.11.1.1). The resulting pellet from the protein extraction was suspended in 400µl UHQ H_2O, with 100µl used as the neat sample in both ELISA and DELFIA experiments. Extracted and purified RNA was eluted in 30µl UHQ H_2O, of which 5µl was used in each qRT-PCR reaction. Protein extractions were tested by DELFIA and ELISA (2.8.9 and 2.8.10) and purified RNA was analysed using qRT-PCR (2.7.5.2). The experimental strategy outlined above is shown in Figure 29.

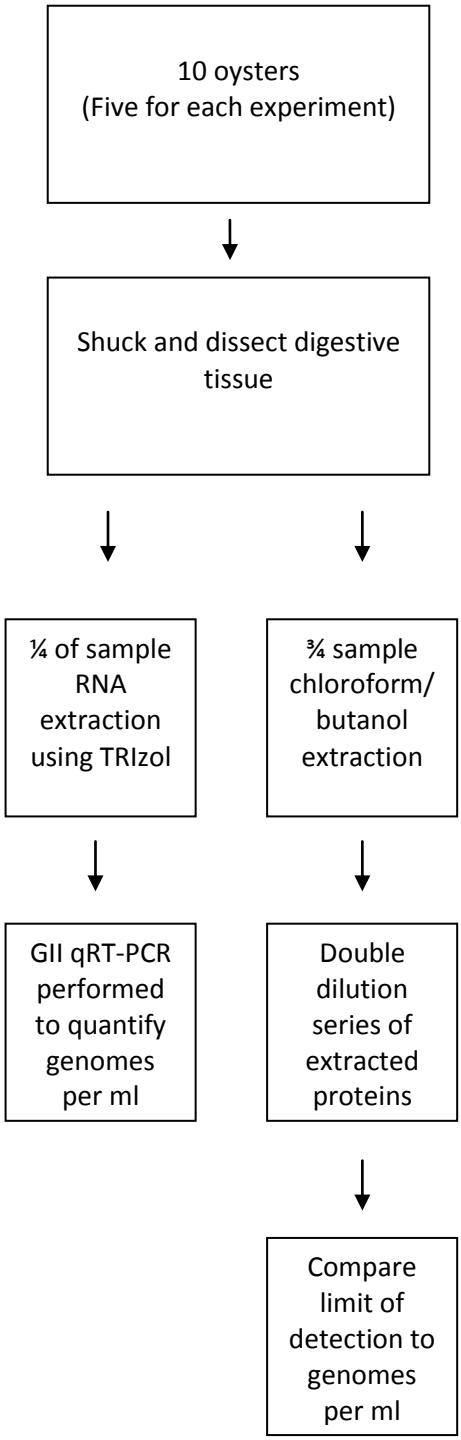


Figure 29. Experimental strategy designed to analysis the norovirus RNA and capsid protein content of naturally contaminated oysters.

Preliminary results previously obtained showed that the ELISA was not able to detect Lordsdale virus in oyster extracts which had been seeded with a stool sample containing 2.87×10^6 GII RNA genomes. Naturally contaminated oysters would contain a lower number of virions and are therefore unlikely to be detected by the ELISA. The ten oyster virus extracts were analysed by ELISA to test this hypothesis. The results are shown in Table 23, a positive cut-off value of OD 0.260 was used; double the negative control, UHQ H_2O (0.131).

Table 23. Average GII.4 ELISA data for ten oysters, positive results are shown in bold. The experiment was performed in duplicate.

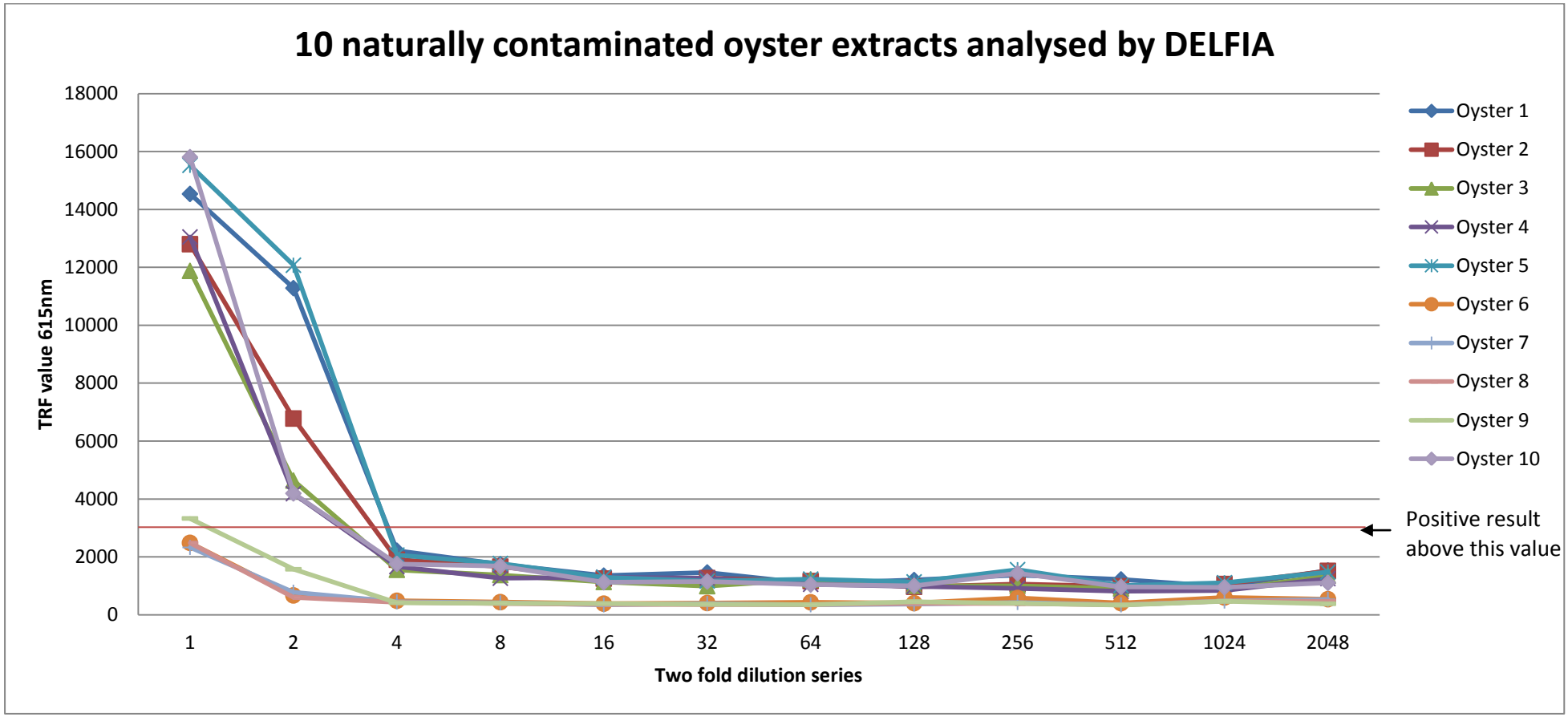
Oyster Number	OD_{450}	ELISA Designation
1	0.103	-
2	0.101	-
3	0.103	-
4	0.087	-
5	0.106	-
6	0.215	-
7	0.228	-
8	0.201	-
9	0.147	-
10	0.203	-
Sample 7	**2.110**	**+**
UHQ H_2O	0.131	-

The OD_{450nm} values for all ten oyster extracts were below the 0.26 cut-off, indicating that the viral contamination was too low to be detected by the ELISA for GII.4 noroviruses.

6.4.2 GII.4 norovirus detection in naturally contaminated oysters by DELFIA

The same ten oyster samples were subsequently analysed in a twofold titration in the DELFIA. Each sample was tested in duplicate and values were determined as positive above a threshold of 3000 TRF, double the negative control UHQ H_2O (1415 TRF). The average of the duplicate values are shown in Figure 30, the red line shows the positive cut-off. As shown in previous data, the DELFIA provides an increase in detection sensitivity when compared to the ELISA. An increase in sensitivity is due to the use of lanthanide chelates with the addition of an enhancement solution causing the instant release a fluorescent molecule which is not dependent on an enzymatic reaction.

Figure 30. Average values obtained for the dilution series of ten oyster samples, analysed by DELFIA.



Six oysters (1, 2, 3, 4, 5, and 10) gave high readings in the undiluted (neat) sample, oyster 9 was just above the positive threshold in the neat sample and the remaining three oysters were all negative in the DELFIA. A 1:2 dilution of the oyster extract still produced values above the positive threshold for six of the oysters (1, 2, 3, 4, 5, and 10); however no capsid antigen could be detected in any of the samples at a 1:4 dilution. In contrast the ELISA could not detect virus in any of the undiluted oyster extract samples.

Six of the oyster extract samples gave clear 'positive' results in the DELFIA assay, establishing that this assay, utilising a GII.4 specific monoclonal antibody has the required sensitivity for the detection of noroviruses in shellfish.

6.4.3 GII norovirus detection in naturally contaminated oysters by Quantitative Reverse-Transcription-PCR (qRT-PCR)

Further analysis of the oyster samples used in both the ELISA and DELFIA was performed using a GII qRT-PCR, as described in the preliminary experiments. Simultaneous extraction of RNA was performed on ¼ of the oyster digestive tissue, as the remaining ¾ was used in the viral protein extraction procedure. Extracted and purified RNA was eluted in 30µl UHQ H_2O (2.11.1.2), 5µl was used in each qRT-PCR experiment (2.7.5.2). Tenfold dilutions of a known concentration of *in vitro* produced Lordsdale virus RNA was used to produce a standard dilution series to aid the quantification of the unknown samples. The results are shown in Table 24, the Ct values and RNA genomes per ml are given for each oyster.

Oyster	Ct value	RNA genomes per ml
1	28	3.4×10^5
2	30	5.4×10^4
3	29	1.6×10^5
4	29	1.4×10^5
5	30	5×10^4
6	32	1×10^5
7	32	1.6×10^5
8	31	1.8×10^5
9	30	5.3×10^5
10	31	3×10^5
Average	30.2	2.02×10^5
UHQ	0	0

Table 24. GII qRT-PCR data from 10 oysters, indicating Ct values and RNA genomes per ml of sample.

6.4.4 Analysis of the sensitivity of the DELFIA compared to qRT-PCR in genomes per ml

The data obtained from the qRT-PCR, by which the RNA genomes per ml was calculated can be applied to the DELFIA TRF values obtained to calculate the approximate number of virions which may be present in each dilution sample. RNA genomes per ml detected in each dilution provides an indication of the sensitivity of the DELFIA, however it assumes that each RNA genome detected in the qRT-PCR originates from an intact virion, which is detected in the DELFIA.

The viral pellet obtained from the chloroform/butanol extraction procedure was suspended in 400µl, of this 100µl was used as the neat sample in the DELFIA, then diluted in a twofold dilution series. The qRT-PCR calculates the number of RNA genomes per ml of sample; therefore this value was divided by 10 to obtain the number of RNA genomes in the 100µl undiluted sample. Calculations were performed for all ten oysters which were analysed in the DELFIA, as shown in Table 25.

Table 25. Potential GII RNA genomes present in each sample tested in the DELFIA across a twofold dilution series. Dilution wells with a positive result in the DELFIA are shown in bold.

Oyster/Dilution	1	2	4	8	16	32	64	128	256	512	1024	2048
1	**34000**	**17000**	8500	4250	2125	1062	531	265	132	66	33	16
2	**5400**	**2700**	1350	675	337	168	84	42	21	10	5	2
3	**16000**	**8000**	4000	2000	1000	500	250	125	62.5	31	15	7
4	**14000**	**7000**	3500	1750	875	437	218	109	54	27	13	6
5	**5000**	**2500**	1250	625	312	156	78	39	19	9	4	2
6	10200	5100	2550	1275	637	318	159	79	39	19	9	4
7	16000	8000	4000	2000	1000	500	250	125	62	31	15	7
8	18000	9000	4500	2250	1125	562	281	140	70	35	17	8
9	**53000**	26500	13250	6625	3312	1656	828	414	207	103	51	25
10	**30500**	**15250**	7625	3812	1906	953	476	238	119	59	29	14

The theoretical number of norovirus RNA genomes which a DELFIA would be able to detect ranged from 34,000 to 2500. While the DELFIA does not detect RNA, the number of RNA copies which could be present in the sample was calculated from the qRT-PCR data obtained from the oyster samples. The qRT-PCR data was used to determine how many RNA genomes could be detected in a certain volume of oyster sample, however this does rely on several assumptions. It assumes that there was no sample loss during the experimental procedure and that the DELFIA was able to detect all available intact capsid proteins which contain RNA genomes. It can be seen in Table 25 that if the number of RNA genomes is outside of this range they could not be detected in a GII.4 DELFIA. As the qRT-PCR used in these experiments detected only GII norovirus RNA and the DELFIA detected specifically GII.4, the lack of detection of some samples which contain high numbers of RNA genomes may be due to the oysters containing other GII noroviruses as well as GII.4.

These experiments also demonstrated that there were oysters tested which were positive in the DELFIA when the sample was undiluted and at a 1:2 dilution but were not detected when the same sample was diluted 1:4. When the number of RNA genomes was applied using the qRT-PCR data to these dilutions the quantity of RNA is higher than in some of the other oyster samples which were positive in the DELFIA at the same dilution. Oysters with a high number of GII RNA genomes, but were negative in the GII.4 DELFIA may contain multiple noroviruses with differing genotypes, which has been reported by previous studies (58,135). Oysters with multiple GII noroviruses contamination will contribute to the qRT-PCR values but will not be detected in the GII.4 DELFIA. These results also assume that for each RNA genome detected there is an intact capsid protein detected in the DELFIA; however neither of these assays can differentiate between infectious and non-infectious particles and may be detecting degraded particles. This will cause an overestimation of the true number of infectious norovirus virions present in the oyster tissue.

6.5 Discussion

An investigation into the prevalence of norovirus in oysters requires the development of a highly sensitive assay. This study aims to compare the sensitivities of three methods focussed on the detection of GII noroviruses. These methods can then be used to study GI outbreaks with the incorporation of a GI monoclonal antibody as these viruses had been previously reported to be the dominant source of shellfish related outbreaks of norovirus.

It was hoped that monoclonal antibodies isolated from the hybridomas produced in Chapter 5 would display cross-reactive properties which could be used to develop an immunoassay for the rapid and sensitive detection of all human noroviruses in oysters. Unfortunately this was not possible, so a previously developed GII.4 ELISA was used as a model system for the detection of GII.4 noroviruses in oysters.

Methods currently employed to identify viral contamination in bivalve shellfish involve RT-PCR, however these methods are expensive, require highly skilled technical staff and dedicated equipment. It is therefore vital to undertake work aimed at developing an ELISA or DELFIA based method which is rapid, simple to perform and provides a high level of sensitivity in any diagnostic laboratory.

An experimental strategy was developed to optimise a model method which could be used in the detection of human noroviruses. Initial experiments involved fully characterising a clinical sample from over 100 EM norovirus positive samples for future seeding experiments. Characterisation included sequencing of the ORF2 region and determining that the titres of virus present in the clinical sample were sufficient to be detected in a GII.4 specific antigen capture ELISA and DELFIA. Characterisation also involved quantifying the number of RNA genomes per ml by GII specific qRT-PCR.

It was vital to fully characterise clinical sample 7 to ensure that results obtained from the oyster experiments were valid and could be compared to results gained prior to seeding. The full reactivity profile of the sample and definitively quantified the number of RNA genomes present was determined.

Initial experiments aimed at comparing the sensitivity of ELISA and DELFIA techniques at detecting GII.4 sample 7 indicated that the DELFIA assay was at least twofold more sensitive than the ELISA assay. 2.89×10^7 RNA copies per ml of GII RNA were present in sample 7, determined by qRT-PCR.

These data suggest that the DELFIA, which utilises fluorescence as the detection method, has higher detection sensitivity than the ELISA. The europium labelled conjugate antibody enables detection of very low levels of antigen, as well as the instant dissociation of the lanthanide fluorescence with the addition of an enhancement solution results in a long-lasting and stable signal which is not susceptible to degradation of enzyme conjugate activity.

To investigate if clinical sample 7 could be detected once extracted from oyster tissue, preliminary experiments with detection assays ELISA, DELFIA or qRT-PCR were performed. The hypothesis was that the majority of the oysters tested would be negative for pre-existing norovirus contamination as they were destined for human consumption. However it became clear during the experimental process that oysters containing norovirus were more common than previously expected. The numbers of norovirus virions present in the oysters were too low to be detected in the ELISA even once seeded with clinical sample 7, whilst the six oysters tested in the DELFIA all gave values above the positive cut-off for the presence Lordsdale virus, both before and after seeding. Six further oysters were analysed for GII norovirus RNA by qRT-PCR, in which all three oysters seeded with sample 7 had detectable levels of GII RNA and in two of the three unseeded oysters GII RNA could also be

detected. The levels of pre-existing GII norovirus were unexpected. The Food Standards Agency and Cefas recently undertook a study of UK oyster growing beds which reported that up to 76% of oysters in the UK contain norovirus RNA (FSA, press release, November 2011).

It was important to ensure before commencing the development of assays for the detection of noroviruses in oysters, that the proposed clinical sample to be seeded into the oyster could be detected over and above any potential background norovirus contamination. The protein extraction procedure chosen to achieve the isolation of norovirus capsid protein from oyster tissue was organic flocculation and PEG precipitation, as described by Atmar *et al* with slight variation (6,7).

A comparison of TRF values obtained from unmodified clinical samples and clinical samples which had been processed through the chloroform/butanol extraction procedure in the DELFIA was performed. Analysis of the resulting values revealed that a seven fold loss of antigen was occurring after extraction by chloroform/butanol. The preliminary experiments had also hinted at this, as the seeded samples did not show a significant difference from the values obtained for the unseeded samples.

The discovery that the majority of the oysters analysed contained pre-existing GII norovirus contamination, meant the experimental strategy had to be changed. However the natural accumulation of norovirus was occurring in the correct location, the digestive tissue and assumed to be binding to the reported norovirus binding ligands, HBGAs. This could be used to advantage in the analysis, with results obtained being more applicable to environmental testing in commercial settings.

The decision to evaluate the ELISA, DELFIA and qRT-PCR methods with oysters which had the potential to be naturally contaminated with norovirus was taken, after the likelihood of

obtaining negative oysters was not guaranteed. The ability to evaluate these methods on potentially naturally contaminated oysters increases the relevance of the findings to be incorporated into regular screening of oyster beds, and other food or environmental samples.

Ten oysters, obtained from a local fishmonger and destined for human consumption were analysed by ELISA and DELFIA in a twofold dilution series. In the case of the ELISA, no norovirus GII.4 capsid protein could be detected, while GII.4 capsid protein could be detected at a 1 in 2 dilution of the extracted protein sample in six of the oysters analysed by the similar method DELFIA. These results show that the DELFIA is capable of detecting norovirus in naturally contaminated oysters, while the ELISA lacks the level of sensitivity required. The protein extraction method used in these experiments, organic flocculation and PEG precipitation used for extraction and concentration of norovirus proteins had been previously described by Atmar *et al* with slight variation for the purification of norovirus capsid protein from oyster tissue (6,7).

Development of adequate methods for the detection of norovirus contamination in the oysters was further evaluated using GII qRT-PCR which was able to detect norovirus RNA in the same ten oyster samples as tested by DELFIA and ELISA. The Ct values (obtained from the Applied Biosystems software) were used to calculate the approximate number of RNA genomes which could be present in each dilution of the oyster sample analysed in the DELFIA. The detected quantity of RNA ranged from 34,000 to 2500 RNA genomes. The DELFIA does not detect RNA, instead providing data on the level of GII.4 capsid protein detected. This data was used in conjunction with the qRT-PCR values obtained for the oyster samples to calculate the number of RNA copies which could be present in the sample tested.

The quantity of RNA genomes in the positive dilution samples by DELFIA is approximate and assumes that there was no sample loss during the experimental procedure and that the DELFIA was able to detect all available capsid protein, which contains the RNA genome. The relationship between GII.4 capsid protein detection by DELFIA and GII RNA genomes by qRT-PCR provide simply an indication of relative sensitivity between these two assays. The actual number of virions the DELFIA can detect is hard to definitively quantify. The qRT-PCR used in these experiments detected only GII norovirus RNA and the DELFIA detected specifically GII.4, the lack of detection of some samples which contain high numbers of RNA genomes may be due to the oysters containing other GII noroviruses as well as GII.4. The quantity of norovirus virions in naturally contaminated oysters are low when compared to the quantities seen in human stool samples (186).

qRT-PCR is able to detect as low as 8 RNA genomes in a sample, in these results the DELFIA was only capable of detecting a theoretical minimum of 2500 RNA genomes. As only >6 virions are required to cause an infection, qRT-PCR remains the gold standard for detecting noroviruses in shellfish and is the basis for the newly described CEN standardised methods, however the DELFIA does offer a cost-effective alternative for routine screening at a lower sensitivity. Oysters tend to be harvested on a small scale; the introduction of an on-site norovirus qRT-PCR detection assay would be difficult for small companies to implement as it requires a high output and running costs. The DELFIA offers a superior sensitivity when compared to the ELISA, requiring less sample volume and a lower antibody concentration, which would translate into significant cost savings for the user. The lower cost per test of the DELFIA compared to the qRT-PCR, also makes it an ideal assay for on-site norovirus testing in non-scientific laboratories.

However it remains impossible to determine the number of infectious norovirus virions contained within an oyster with either method. This inability will remain until an *in vitro*

cell culture method can be developed for these viruses. Currently the molecular techniques of qRT-PCR and DELFIA offer the best alternative for molecular detection.

There are however, flaws in the experimental design. All ten oysters were obtained from the same local supplier in quick succession and therefore potentially originate from the same oyster growing bed. It may be that it is just this oyster bed which is contaminated with GII norovirus. The number of sewage treatment plants which discharge directly into oyster growing beds (approximately 130) in the United Kingdom is an area for concern, despite additional disinfectant treatments which are employed to prevent norovirus contamination. In future experiments screening oysters from various oyster beds located across the country at different times throughout the seasons could be examined and compared for levels of norovirus contamination. All ten oysters analysed in this study were positive for GII RNA in the qRT-PCR, while only seven had values above the positive cut-off in the DELFIA. The DELFIA used in this study detected only Lordsdale GII.4 capsid protein, while qRT-PCR was broadly cross-reactive for all norovirus GII RNA. Therefore the oysters analysed may have contained a number of different GII noroviruses, inflating the quantity of RNA reported in comparison to the GII.4 DELFIA. Further sequencing of the RNA isolated from the oyster samples would provide a definitive assessment of the genogroup and genotypes of noroviruses contained within the oyster tissue. Sequencing would allude to the sensitivity of the DELFIA at detecting all GII noroviruses within oysters, or if there are a high proportion of GII viruses which this detection assay is not reporting.

Neither DELFIA nor qRT-PCR could discriminate between infectious and non-infectious particles, so the risk of infection from these quantities of norovirus is unclear. The ten oysters used in these experiments were obtained from the same supplier in the same batch, however the exact location of the growing beds in relation to any sewage outlets or if the beds were in an area of high boat traffic is unknown. If the experiments performed in

this study were repeated it would be necessary to include a number of oysters which had been through the depuration system, alongside samples which had already been proven negative for norovirus contamination. These data would provide a complete data set on the sensitivity of these assays at detecting low levels of viral contamination. While it is not essential to determine the genotype of a norovirus contamination, this data would provide an insight into the point source of contamination when compared to other samples. The availability of the GII.4 Lordsdale DELFIA which has the ability to detect norovirus in naturally contaminated oysters will allow further investigations into the prevalence of contamination. The rapid and affordable DELFIA should increase the ability for any diagnostic laboratory to screen oysters for noroviruses routinely.

qRT-PCR does not indicate the genotype of virus, only that the amplified genomic RNA is GII. To develop a more sensitive assay capable of genotyping, the primers used would need to be more specific; however this would require an increase in reactions to be performed to enable screening for a larger number of genotypes. A different set of primers would also be required for each genotype. qRT-PCR is an expensive method to set-up and perform as it requires a high level of technical expertise to be performed in routine diagnostic laboratories. An alternative is to develop a broader qRT-PCR assay which can cover multiple genogroups which would require degenerate primers covering a large number of variable sequences. The DELFIA technique is able to determine the genotype of a virus using a strain specific monoclonal antibody, allowing basic typing of viruses; however this would increase the number of experiments required. This allows the point source of infection to be isolated and may help in tracing and ultimately preventing further outbreaks of infection.

Automation systems have been described for both DEFLIA and qRT-PCR on 96 well plates, which are available commercially. AutoDELFIA (Perkin Elmer) is an automatic immunoassay system designed for routine diagnostics or screening laboratories. This system performs all

sample and reagent handling including measurement of fluorescence automatically. The wide availability of automated qRT-PCR systems has led to many commercially available systems. These systems perform all steps necessary to set up 96 quantitative PCR reactions including transferring the master mix and RNA template into the PCR amplification plate and performing the reaction. Due to the availability of these automated systems the increase in the number of assays required to screen for all norovirus genogroups and genotypes may not cause a significant increase in workload. However there would be a significant increase in cost compared to a single detection assay.

To detect multiple genogroup and genotypes of noroviruses in one DELFIA experiment a monoclonal antibody with broad reactivity would be required. Shiota *et al* have described the isolation of a monoclonal antibody (MAb14-1) which displays cross-reactive properties across GI.1, 4 and 8 as well as GII.1-7; this is currently one of the broadest reacting monoclonal antibodies (215). The development of a 'catch-all' monoclonal antibody is vital for the development of an assay which could be used in a diagnostic laboratory for detection of all norovirus outbreaks.

When detecting noroviruses in food, a quick, cheap and reliable test is required to process large number of samples. The ELISA has the lowest cost per test, it is simple to perform and can process large numbers of samples, however its inability to detect norovirus in naturally contaminated oysters remove it as the diagnostic assay of choice for norovirus detection. The DELFIA has the advantages of an ELISA, in that it is easy to perform, but gives increased sensitivity and a large scope for flexibility when compared to the ELISA. The DELFIA has an increased cost per test over the ELISA, but is significantly cheaper than qRT-PCR. The DELFIA was unable to detect three oysters which were positive for GII RNA in qRT-PCR, however it is unknown if these oysters did contain GII.4 noroviruses.

Precise genotyping is not required for general norovirus screening in oysters, as just the presence of norovirus is enough to raise alarm bells. Essential to the development of a conclusive norovirus detection system is the optimisation of a reliable cell culture method. This would remove the need for molecular detection techniques and ensure only infectious virions were measured.

The clear positive results in the DELFIA assay, diagnosis GII.4 norovirus contaminants of oysters on sale for human consumption firmly establishes the concept that an immunoassay using monoclonal antibodies can be developed and is sufficiently sensitive for the detection of noroviruses in shellfish.

Chapter 7 Discussion

Noroviruses are highly diverse and are considered the major cause of non-bacterial gastroenteritis worldwide (5). This highly infectious virus is easily transmitted amongst humans and at the present time there is no effective treatment or health intervention.

Norovirus research has been stalled by the lack of a reliable *in vitro* cell culture model and the low titres of virus present in clinical samples. The development of an assay which has a broad specificity for detecting noroviruses has been hindered by these same factors. This has led to a need for an assay which is able to detect all norovirus genogroups and genotypes in various matrices.

A vast range of food produce has been implicated in outbreaks of human norovirus. Norovirus is thought to contribute to an estimated 30 – 50% of all foodborne outbreaks of gastroenteritis (253). Studies have shown that norovirus could be detected in 76% of oyster samples which were taken from 39 production areas tested in the UK, all sites tested returned at least one positive result (162).

The contamination of bivalve shellfish with norovirus from human sewage is a well-recognised health risk (142). Assessment of potential norovirus contamination of bivalve shellfish has relied on the use of *E.Coli* as an indicator; however this method has been shown to be inadequate at predicting the presence of norovirus in the tissue (3). Nucleic acid amplification methods are currently the most common molecular approach employed for the detection of norovirus RNA (118,245). The work described here was aimed at developing and optimising molecular detection assays for noroviruses in shellfish tissue samples, which offered a sensitive and cost efficient alternative to qPCR.

The development of recombinant baculoviruses which express capsid protein and spontaneously assembles to form VLPs (113) has led to a number of advancements in the

study of noroviruses. The ease of large scale production of norovirus VLPs has led to the ability of developing polyclonal antiserum and monoclonal antibodies using VLPs as the immunising antigen for incorporation into diagnostic assays (39,87,247). The lack of antibodies with a broad specificity has prevented the development of assays which could be incorporated into routine diagnostic testing. The work in this thesis was aimed at developing antibodies with cross-reactive properties which could fill this gap.

Discovery of human norovirus surrogate, MNV (255) has enabled research into the replication strategy of these viruses as well as furthering understanding on the stability of these viruses in complex food matrices. Until development of a method to propagate human noroviruses in cell culture, there has been a heavy reliance on surrogates such as MNV.

This thesis describes the production and isolation of twelve monoclonal antibodies, following immunisation with MNV VLPs. The monoclonal antibodies were analysed against seven human norovirus VLPs, of which none displayed cross-reactive properties. Five of the twelve antibodies detected an epitope located in the MNV capsid protein, the remaining seven were assumed to be directed towards non-structural proteins. Analysis suggested that these five antibodies recognised a conformational dependent epitope in the capsid protein. Despite all five antibodies detecting MNV VLPs they could not be incorporated into a capture ELISA/qRT-PCR for the detection of native virus, providing evidence that the epitope was located in the shell domain. These results impacted on the intention to use these antibodies for the detection of MNV seeded into oysters to evaluate the relationship between ELISA and qRT-PCR values. Isolation of monoclonal antibodies which detect a conformational dependant epitope in the shell domain of MNV capsid protein will be important tool in future research into the characterisation of MNV. MNV

continues to provide invaluable data on human norovirus replication, however this is based heavily on assumptions that both viruses replicate in a similar manner.

Other norovirus surrogates may be more appropriate than murine norovirus in research into norovirus accumulation in oysters and the isolation of cross-reactive antibodies. It has been described that Tulane virus (TV), a monkey calicivirus which is genetically closely related to human noroviruses and recognises the same receptors may be a more suitable surrogate than MNV, despite being in a different genogroup. TV is more sensitive to pressure treatment when seeded into oyster tissue than MNV, but shows the same sensitivity to pH being more stable in acidic conditions than neutral pH (151). Inclusion of an alternative surrogate as the immunogen in the development of monoclonal antibodies with broadly cross-reactive properties is an area for potential research.

The norovirus capsid protein has two major domains, the shell (S) domain connected by a flexible hinge to a protruding (P) domain (198). The P domain is divided further into P1 and P2; it is within the P2 domain that the hyper-variable region is located. Recombinant viruses have been reported, when the RdRp from a cluster GII.4 and a capsid protein which originates from a separate cluster of noroviruses are combined (85,88,111,124,244). Recombination events and the mutation of regions within the sequence of the genome is a mechanism that noroviruses have evolved to evade detection in the host immune system (153,185).

This thesis describes the incorporation of a previously characterised monoclonal antibody epitope, LEDVRN into the P2 domain of the MNV capsid. This linear epitope is conserved amongst GI but not GII noroviruses. Chimeric MNV provided an alternative route to the production of monoclonal antibodies which detect a linear epitope. As noroviruses regularly mutate and recombinate; it was hoped that this novel insertion would not affect successful folding of the capsid protein. The LEDVRN epitope nucleotide sequence was

successfully incorporated into the MNV genome however no viable virus could be propagated.

Due to unsuccessful production of viable virus, it was not possible to develop an antigen capture ELISA. This method would have been used to compare values obtained from natural GI contamination in environmental samples and chimeric MNV, providing some assumptions to be drawn on the potential risk of human infection from the titres of virus present. Chimeric MNV could be propagated and the values obtained in the ELISA compared to the CPE visualised in chimeric MNV infected Raw 264.7 cells.

One study has reported amino acid changes in the P domain of noroviruses which were isolated from patients with chronic norovirus infection (185), demonstrating the ability of noroviruses to accept novel mutations and still result in viable virus. While the production of viable virus was not successful in this work, it has provided information that further optimisation is required to ensure any future mutational insertions produce protein-protein interactions which are tolerated to ensure successful capsid folding. In particular, attention must be paid to the affects of any change in amino acid charge and potential side chain interactions which a novel insertion could cause. This work has demonstrated that the LEDVRN amino acid sequence produces unfavourable interactions when the capsid protein folds to produce an intact virion. Additional investigation into where these unfavourable interactions lie would assist future successful mutational inserts.

Since 1995, new epidemic variants of GII.4 have emerged every two to three years, with population immunity and genetic drift as the driving forces behind the evolution (218). Due to the dominance of these viruses worldwide, surveillance networks such as Noronet and Calicinet have become an important means of tracking the emergence of novel strains. Historically, the GII strains have caused more severe illness than other versions of the virus, it is therefore important to ensure that diagnostic assays detect this genotype.

Eleven monoclonal antibodies were produced and isolated which detected Lordsdale and Maryland VLPs (labelled CM153-159, 162 and 163) . Interestingly CM160 and CM162 had broader reactivity than the other nine isolated antibodies, CM160 detected Southampton VLPs and both antibodies detected Desert Shield VLPs in a direct ELISA. Incorporation of both Lordsdale and MNV VLPs into the immunisation regime did not result in the expected isolation of antibodies with broad cross-reactive properties. Isolated antibodies were intended to be incorporated into diagnostic assays to enable assumptions to be drawn between MNV and human norovirus infectivity.

Further analysis demonstrated that the epitope detected by these antibodies was discontinuous and located in the S domain of Lordsdale virus. Location of the epitope sequence in the S domain indicated that it was unlikely that the antibodies would detect intact virions in an antigen capture ELISA. In diagnostic assays it is vital to bind both intact and degraded virions. While these antibodies could not be incorporated into a diagnostic assay, they are an important research tool in the study of Lordsdale virus epidemiology. To assess the true extent of an infection all fragments of particles must be detected since degraded particles were once intact and potentially capable of infection. Optimisation of the immunisation schedule and choice of immunogens is an important area for future work. The possible use of intact virions as the sole immunogens is one area for research to enhance the possibility of isolating antibodies which bind to a linear epitope.

The full distribution of norovirus in the environment is as yet unknown and recent research has suggested that oysters exposed to daily contamination (persistent sewage contamination) or a single accidental contamination event (accidental sewage discharge) resulted in a similar level of bioaccumulation (243). Without removing oyster consumption from the diet, thus removing the potential for infection it is of vital importance that sensitive detection assays continue to be developed and refined.

At the present time the majority of norovirus detection assays are based on molecular techniques, such as PCR until a cell culture model can be reliably reported. This work describes the development and optimisation of GII norovirus detection assays offering a cost-effective alternative to qPCR. At the present time the European Committee on Normalisation (CEN) is working to develop an ISO standard method for the detection of norovirus in food produce which is based on qPCR.

Norovirus RNA and capsid protein from oysters was detected by qRT-PCR and DELFIA respectively. Preliminary experiments performed indicated that the DELFIA was twofold more sensitive than ELISA when detecting the same GII.4 sample. As work proceeded it became clear that a higher proportion of oysters than expected had pre-existing norovirus contamination. Of the ten oysters' analysed containing pre-existing contamination, the ELISA did not detect GII.4 capsid protein, while the DELFIA could detect Lordsdale virus in seven of the oysters. qPCR proved the most sensitive assay with all ten oysters found to contain GII RNA.

The high number of oysters containing norovirus was not unexpected, it has been shown in studies that norovirus particles can survive in shellfish tissue for several months either by ionic binding or specific attachment (25,137). While qPCR remains the gold standard for detection and the basis of the newly described CEN standardised methods, the DELFIA could be employed as a sensitive alternative assay for small scale oyster production sites. This work demonstrates that the DELFIA has superior sensitivity compared to the ELISA for the detection of GII.4 noroviruses in oysters.

These techniques could also be employed to assess the risk posed to the human population by other bivalve shellfish species. It is hard to draw comparisons between the DELFIA and qRT-PCR results as the assays are detecting different genotypes of noroviruses and different components of the norovirus, capsid protein and genomic RNA respectively. The

DELFIA was developed to detect solely GII.4 while the qPCR can detect all GII viruses, potentially inflating the values obtained.

While optimisation of sensitive detection assays is of vital importance, none of the assays discussed can determine the number of infectious virions present in the matrices. Without this information, the potential risk of infection remains unclear. This work provides confirmation that the presence of norovirus in oysters is widespread but it is not clear what risk this poses to the human population. Long-term studies into the true extent of norovirus contamination in oysters, as well as determining where the cut-off lies for potential infection should be one of the main aims of future research.

Development of broadly reactive monoclonal antibodies is an ongoing process; this work defines antibodies which can detect either MNV or Lordsdale VLPs and bind epitopes located within the shell domain. None of these antibodies could be incorporated into an ELISA for the detection of intact virions in environmental samples. While chimeric MNV produced in this work could not provide an alternative route to obtaining an antibody which could fulfil this requirement, it does offer a potential alternative route with further research. The optimisation of an accurate and sensitive noroviruses detection assay is a vital area of research. These studies report the potential for incorporation of a GII.4 DELFIA into routine testing in oyster production areas, providing the opportunity for a greater number of shellfish produce to be analysed. While qPCR was shown to be a more sensitive assay, it requires a high level of expertise and financial input to perform, this work demonstrates that the DELFIA does provide a sensitive and cost effective alternative. Advances in this area await the ability to propagate human noroviruses in cell culture. This will allow in-depth studies into norovirus replication and infectivity.

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APPENDIX 1: **Production of Monoclonal antibodies against MNV and Lordsdale viruses**

Between the production of monoclonal antibodies described in Chapter 3 and the intention of performing a subsequent fusion, the animal facility at which the two remaining mice were located had an outbreak of Mouse Hepatitis Virus (MHV) and were thought to have contracted the virus.

MHV is a single-stranded, positive sense RNA virus and the genome ranges in size from 27 to 31 kb (99). There are many MHV strains in circulation; the strains are grouped according to their primary tissue types, polytrophic and enterotropic. The enterotropic strains replicate in the intestinal mucosa and rarely spread to other tissues.

Mouse hepatitis virus (MHV) is extremely contagious and easily spreads between mouse colonies, although it has a very low mortality rate in adult mice. Immuno-competent mice usually have subclinical infections, which are short in duration and are quick to clear. Studies investigating natural outbreaks are low, with most studies deliberately infecting mice with previously isolated MHV strains.

Mice which have been infected with MHV have been shown to modify unrelated T cell responses if the infection occurs at the time of immunisations (38). This infection can also cause immunodepression and immunostimulation depending on the time point at which the infection occurred (248). MHV is a member of coronaviruses and among MHV strains, it has been shown they have substantial variety of pathogenesis as MHV-JHM causes demyelinating disease in mice(228) while MHV-2 is highly hepatitogenic (97) . Effects of MHV on research has been studied for many years (14) and has been shown to effectively

alter the physiology of the mouse during an infection (99) and dysfunction of both T and B cells has also been described.

Despite the presence of MHV in the mice it was decided to proceed with the second fusion and screen purified hybridomas against MHV by PCR and ELISA to ensure all infected hybridomas are discarded. MHV is controlled in colonies by culling, cessation of breeding or by allowing the infection to burn out.

Experimental Strategy

Unfortunately no hybridomas with cross-reactive properties were isolated from the immunisation regime performed in Chapter 3, solely with MNV VLPs. The experimental strategy was altered to incorporate a boost immunisation of Lordsdale virus VLPs, this was hypothesised to enable to generation of cross reactive antibodies. The mice used in this strategy had previously been immunised with MNV VLPs as described in chapter 3. However the successful outcome of this plan depends upon the isolated antibodies not detecting MHV antigen.

The two mice (mice 1 and 2) had tail bleeds taken prior to the boost immunisation and analysed for reactivity against MNV infected Raw 264.7 cell lysate, the intended screening antigen by direct ELISA. A direct ELISA was performed (2.8.8) comparing the pre immunisation (day 0), tail bleeds (day 35) and tail bleed after MHV infection for each mouse. The results of the direct ELISA are shown in Figure 31 and Figure 32.

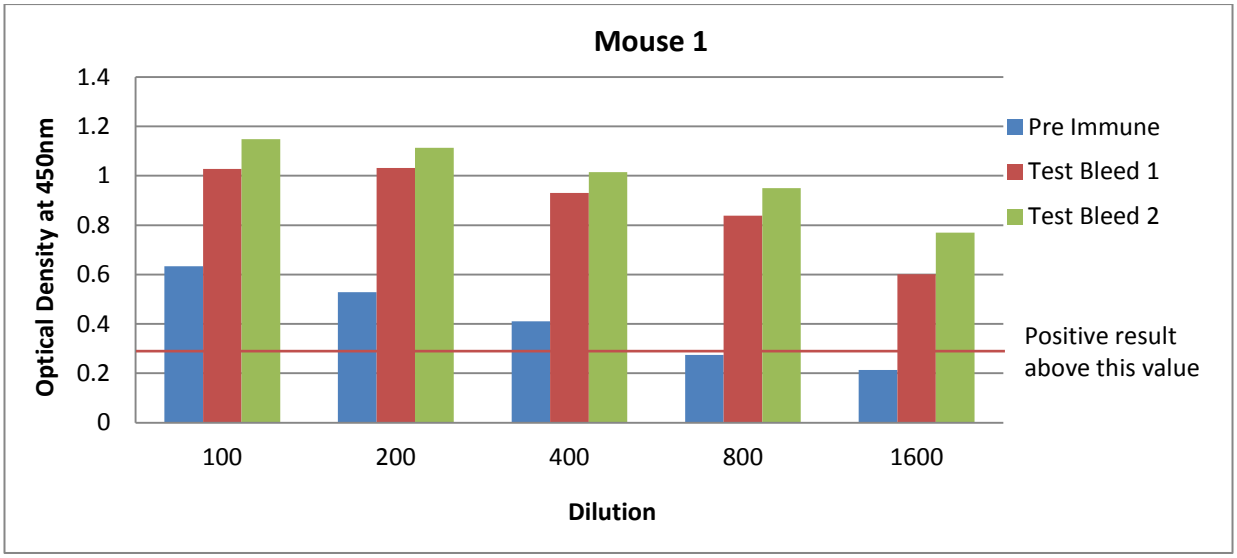

Figure 31. Mouse 1 pre-immune, test 1 and 2 serum against MNV VLPs in a direct ELISA at various dilutions

Figure 32. Mouse 2, pre-immune, test 1 and 2 serum against MNV VLPs in a direct ELISA at various dilutions.

Mice 1 and 2 produced similar values against MNV infected Raw 264.7 cell lysate to the previous direct ELISA values obtained from the test tail bleeds. After establishing that the mice had maintained an immune response against MNV by direct ELISA, after the previous immunisation regime, a boost immunisation with 50µg of Lordsdale VLPs was administered to both mice.

Mouse 1 was selected for hybridoma production, despite pre-existing antibodies detected by direct ELISA in both the pre and post immune bleeds against MNV. Mouse 1 was chosen as it produced higher optical density values against MNV infected Raw 264.7 cell lysate than mouse 2, as shown in Figure 31 and Figure 32. Mouse 1 was negative by immunoblot for pre-existing antibodies, as shown in Chapter 3. This was considered a sufficient immune response to continue with hybridoma production.

Hybridoma production

Three days after boosting with 50µg Lordsdale VLPs, mouse 1 was sacrificed and a fusion of B cells (spleen) and myeloma (SP2) cells (2.10.1.1) was performed as described (2.10.4) at a 1:1 ratio. Hybridomas were screened with MNV infected Raw 264.7 cell lysate in a direct ELISA (2.8.8), wells were considered positive if they had a reading above 0.3 optical density at 450nm. This cut off was to ensure that only a manageable number of hybridomas were selected for further cloning and characterisation. One tray from the master plates was also screened with Lordsdale VLPs (0.2µg/ml per well).

Hybridomas were picked and passed through three rounds of terminal dilution for purification prior to growing up individual hybridomas in bulk for antibody production. 24 individual hybridomas were generated from the hybridoma production, 23 had values which were above the positive cut-off against MNV infected Raw 264.7 cell lysate and 1 was positive for Lordsdale virus. The single hybridoma which was secreting antibodies

towards Lordsdale virus originated from the master tray which was screened against Lordsdale virus VLPs. The isolated hybridomas were also screened by direct ELISA against Raw 264.7 cell lysate to ensure no cellular cross-reactivity. 23 of the 24 hybridomas gave optical density values above the positive cut-off against uninfected Raw 264.7 cell lysate. With only the hybridoma which secreted antibodies towards Lordsdale virus showing no reactivity against MNV infected Raw 264.7 lysate or uninfected Raw 264.7 cell lysate.

Due to the MHV infection present in the mouse before being sacrificed, the resulting hybridomas were also screened against MHV antigen in a direct ELISA. The MHV direct ELISA was performed as previously described (2.8.8), with the wells coated in MHV antigen (50µl per well, Churchill Biotechnology, reconstituted in 5mls carbonate buffer). A positive result was taken as an optical density of 0.3 at 450nm or above. The results of this analysis are shown in Table 26.

Table 26. Hybridoma reactivity pattern against MNV infected Raw 264.7 cell lysate, uninfected Raw 264.7 cell lysate and MHV in a direct ELISA. Positive results are shown in bold.

	ELISA Designation		
Hybridoma Number	MNV lysate	Raw 264.7 lysate	MHV antigen
1	**+**	**+**	**+**
2	**+**	**+**	**+**
3	**+**	**+**	**+**
4	**+**	**+**	**+**
5	**+**	**+**	**+**
6	**+**	**+**	**+**
7	-	-	-
8	**+**	**+**	**+**
9	**+**	**+**	**+**
10	**+**	**+**	**+**
11	**+**	**+**	**+**
12	**+**	**+**	**+**
13	**+**	**+**	**+**
14	**+**	**+**	**+**
15	**+**	**+**	**+**
16	**+**	**+**	**+**
17	**+**	**+**	**+**
18	**+**	**+**	**+**
19	**+**	**+**	**+**
20	**+**	**+**	**+**
21	**+**	**+**	**+**
22	**+**	**+**	**+**
23	**+**	**+**	**+**
24	**+**	**+**	**+**

Due to reactivity of the hybridomas to MHV antigen in a direct ELISA it was also important to test them in a MHV PCR to determine if the hybridomas themselves contained MHV RNA. A MHV positive control was a kind gift from S.G. Siddell; this is a cloned cDNA copy of the MHV-JHM N protein gene cloned into pAT 153, 1mg/ml used at a 1:10,000 dilution in the PCR experiments.

To obtain RNA for the detection of MHV, hybridoma cells were harvested from 2 confluent wells in a 24 well tray and the RNA extracted as described previously (2.7.6.3) and stored at -80°C until required. Subsequently cDNA was synthesised from the isolated RNA as described (2.7.5.1) and stored at -20°C. cDNA obtained from the hybridomas was used for the detection of MHV by PCR (2.7). The primers used were described by Taylor *et al* (230) and the sequences of which are shown in Table 27. The MHV primers will produce a fragment of 225bp if MHV is present.

The hybridoma cDNA samples underwent PCR (2.7) and the products run on a 1.5% agarose gel (2.7.3), the results are shown in Figure 33.

Table 27. MHV Primer sequences described by Taylor *et al* (230), used for the detection on MHV cDNA in purified hybridoma cell lysate.

Primer Name	Sequence	Primer Length
MHV_F	**CAG-CCU-GCC-UCU-GCU-GUA-AAA-CC**	23bp
MHV_R	**GCC-UCC-AAA-GUU-CUG-AUU-AGG-GC**	23bp

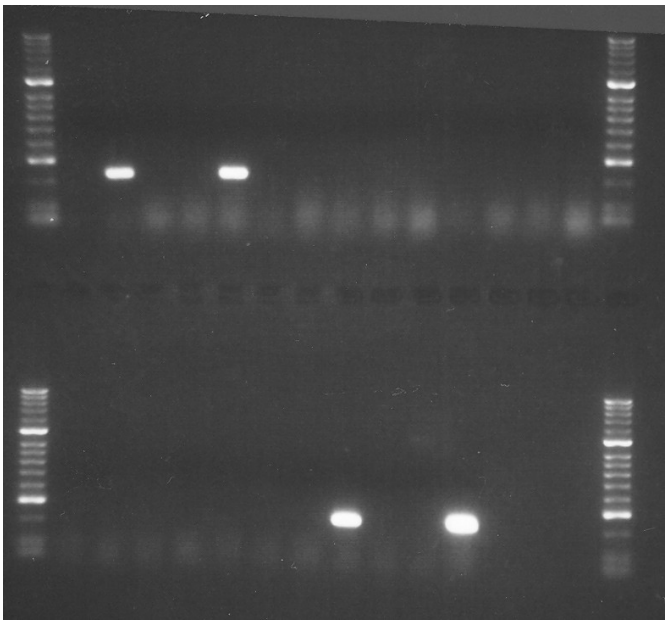

Figure 33. Products of the MHV PCR run on a 1.5% agarose gel. Lane 1 Hyper ladder II, Lane 2 negative control, H_2O, Lane 3 positive control, MHV-JHM. Followed by cDNA from hybridomas 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 24, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 13.

Hybridomas 3, 13 and 21 were positive for MHV cDNA, showing bands at 225bp the predicted fragment size and so were discarded. The remaining negative hybridomas were expanded and stored at -80°C.

Discussion

The MHV infection has severely hampered the potential of this second fusion, with 23 of the 24 hybridomas recovered testing positive to MHV antigen in a direct ELISA. This would indicate that these hybridomas are producing antibodies towards MHV, however after RNA was extracted and the synthesised cDNA was run in a MHV PCR, only three of the 24 hybridomas produced a fragment for MHV cDNA. 23 of the 24 hybridomas also tested positive in a direct ELISA against uninfected Raw 264.7 cell lysate. Only one hybridoma was secreting antibodies which detected Lordsdale virus but did not also detect MHV and uninfected Raw 264.7 cell lysate antigens.

Unfortunately due to an equipment fault these hybridomas were thawed to room temperature after they had been frozen at -80°C. These hybridomas were not transferred to liquid nitrogen storage (-125°C) storage due to the MHV contamination risk with other mouse cell lines that are used in the laboratory. An attempt was made to resurrect the hybridomas, however this failed.

APPENDIX 2: Single and Three Letter Amino Acid Code

Ala	(A)	Alanine
Arg	(R)	Arginine
Asp	(D)	Aspartic Acid
Asn	(N)	Asparagine
Cys	(C)	Cysteine
Gln	(Q)	Glutamine
Glu	(E)	Glutamate
Gly	(G)	Glycine
His	(H)	Histidine
Ile	(I)	Isoleucine
Leu	(L)	Leucine
Lys	(K)	Lysine
Met	(M)	Methionine
Phe	(F)	Phenylalanine
Pro	(P)	Proline
Ser	(S)	Serine
Thr	(T)	Threonine
Trp	(W)	Tryptophan
Tyr	(Y)	Tyrosine
Val	(V)	Valine