

Molecular characterisation of norovirus contamination in wastewater and oysters



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Declaration

I, Paulina Rajko-Nenow, declare that the results presented are to the best of my knowledge correct. This thesis represents my own original work, except where specifically stated in the text and acknowledgements. The contribution of other authors are acknowledged. Any quotation or paraphrase from the published or unpublished work of another person has been duly acknowledged in this work which I present for examination. This work has not been previously accepted for a higher degree either at NUI Galway or elsewhere.

Date:..... **Signed:**.....

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List of abbreviations

BMS	<i>Bivalve molluscan shellfish</i>
CaCV	<i>Canine calicivirus</i>
CSO	<i>Combined sewer overflow</i>
CEN	<i>Comité Européen de Normalisation</i>
DT	<i>Digestive tissue</i>
ELISA	<i>Enzyme-linked immunosorbent assay</i>
EM	<i>Electron Microscopy</i>
EFSA	<i>European Food Safety Authority</i>
EU	<i>European Union</i>
EC RNA	<i>External control RNA</i>
FBVE	<i>Foodborne Viruses European</i>
FCV	<i>Feline calicivirus</i>
FSAI	<i>Food Safety Authority of Ireland</i>
FRNA	<i>F-specific RNA (bacteriophage)</i>
HPSC	<i>Health protection surveillance centre</i>
HAV	<i>Hepatitis A virus</i>
HEV	<i>Hepatitis E virus</i>
HBGA	<i>Histological Blood Group Antigen</i>
ICTV	<i>International Committee on Taxonomy of Viruses</i>
ISO	<i>International Organisation for Standardisation</i>
LOQ	<i>Limit of Quantification</i>
ID₅₀	<i>Median infectious dose</i>
MuNoV	<i>Murine norovirus</i>
MPN	<i>Most Probable Number</i>
NoV	<i>Norovirus</i>
NV	<i>Norwalk virus</i>
NVRL	<i>National Virus Reference Laboratory</i>
NRL	<i>National Reference Laboratory</i>
ORF(s)	<i>Open reading frame(s)</i>
PCR	<i>Polymerase Chain Reaction</i>
p.e.	<i>Population Equivalent</i>
C_q	<i>Quantification cycle</i>
R₀	<i>Reproduction number</i>
RdRp	<i>RNA-dependent RNA polymerase</i>
RT-PCR	<i>Reverse Transcription Polymerase Chain Reaction</i>
RT-qPCR	<i>Reverse Transcription Quantitative Polymerase Chain Reaction</i>
Se	<i>Sector</i>
SFPA	<i>Sea Fisheries Protection Authority</i>

SRSVs	<i>Small round structured viruses</i>
(t)LOD	<i>(theoretical) Limit of detection</i>
VLPs	<i>Virus Like particles</i>
WWT	<i>Wastewater treatment</i>
WWTP	<i>Wastewater treatment plant</i>

Abstract

Bivalve molluscan shellfish (BMS), such as oysters, when grown in waters impacted by human wastewater may bioaccumulate human enteric viruses such as Norovirus (NoV). NoV is the leading agent of foodborne viral gastroenteritis worldwide. A wide variety of different genotypes of NoV are believed to circulate in the community. This research project involved the quantification and identification of NoV genotypes in wastewater, oysters and in NoV outbreak related faecal samples. The overall aim of the project was to establish a link between NoV genotypes circulating in the community (through analysis of municipal wastewater) and their uptake in oysters. This link was further related to the risk to consumers by investigating NoV concentrations and genotypes in oysters and faecal samples related to two outbreaks of gastroenteritis.

During the initial part of this study, NoV concentrations and genotypes were determined in oyster samples collected monthly over a 2-year period from a commercial harvest area. Despite ongoing *E. coli* analysis indicating a category B classification under EU regulations, the area was closed for harvesting because of previous implication in a number of NoV related outbreaks. Total NoV (GI and GII) concentrations in oysters ranged from <LOQ to 20,080 genome copies g⁻¹ of digestive tissue (DT) and displayed a strong seasonal trend, greater concentrations occurring from October to March and correlated with the known increased seasonal prevalence of NoV illness in the population. Multiple NoV genotypes were identified in oysters from the harvest area; NoV GI.4, GI.3, GI.2, GII.4, GII.b, GII.2, GII.12, and GII.e. This study demonstrated that *E. coli* monitoring alone is not sufficient to assess the NoV contamination of shellfish harvesting area. Moreover, NoV genotypes changed over the study period, and unexpectedly high concentrations of NoV GI were present. The application of the nested RT-PCR targeting the part of the RNA-dependent RNA polymerase (RdRp) was suitable for genotyping, but its usefulness was insufficient to distinguish between all GII.4 variants.

To determine the pathway of NoV strains present in the community and those subsequently accumulated in oysters, weekly samples of influent, secondary treated effluent, and oysters adjacent to a wastewater treatment (WWT) outfall were collected

during the peak period of NoV community infections during 2010. A nested RT-PCR assay for NoV was used for phylogenetic analysis of NoV strains based on the N/S capsid domain of the genome. Over a 13 week study period, a total of 931 laboratory-confirmed cases of NoV GII infection were recorded in comparison to 16 cases of NoV GI. Despite the few cases of NoV GI reported, concentrations of NoV GI were similar to NoV GII detected in influent and effluent wastewater, and subsequently in oysters. The dominant strain implicated in NoV outbreaks in Ireland at this time was NoV GII.4 variant 2010. However, in addition to detecting NoV GII.4 variant 2010, multiple genotypes of NoV GI (GI.1, GI.4, GI.5, GI.6, and GI.7), NoV GII (GII.3, GII.4, GII.6, GII.7, GII.12, GII.13, and GII.17), and four putative NoV recombinant strains were identified in the wastewater and oyster samples. Different NoV genotype profiles were observed in influent, effluent and oysters possibly indicating differing survival characteristics or selective bioaccumulation by oysters of different NoV strains. This study demonstrated that a wide variety of NoV GI and GII strains is present in effluent wastewaters and may be potentially accumulated by oysters, highlighting that the role of oysters as a vector of multiple NoV GI and GII strains, including recombinants. In addition, it was demonstrated that the nested RT-PCR targeting the N/S domain of NoV genome allowed for better discrimination between strains, including GII.4 variants, and the recombinant identification than the nested RT-PCR targeting the part of RdRp gene.

Finally, the RT-qPCR and genotyping procedures previously implemented into the laboratory were used to investigate two separate oyster-related NoV outbreaks that occurred in Ireland in 2010 and 2012. In both outbreaks, NoV concentration in oysters exceeded 1000 genome copies g⁻¹ DT. In addition, highly similar or identical NoV sequences were detected in the faeces of individuals with gastroenteritis and in oysters that were served in restaurants and directly associated with illness. In faecal samples, GII.13 was the only genotype implicated in outbreak 1, detected using direct sequencing, whereas multiple genotypes were detected in outbreak 2, following the application of cloning procedures. This study demonstrated that various genotypes are present in oyster samples containing high NoV concentrations, and therefore, cloning

of faecal samples is vital to fully characterise the causative NoV genotype in outbreak investigations.

Overall the findings from this thesis demonstrate that NoV genotypes detected in oysters can change over time, and in general are reflective of strains circulating in community. NoV genotype profiles detected in the influent, effluent and oyster samples varied indicating differing survival characteristics through the treatment process or preferential accumulation of some genotypes in oysters. Phylogenetic analysis of NoV sequences can be used as a supportive tool in tracking the origin of NoV oyster-related outbreaks. However, cloning of nested PCR products is recommended prior to sequencing of the NoV-positive faecal samples since multiple NoV GI and GII genotypes can be identified in the faeces of individuals. Oysters containing NoV concentrations in excess of 1000 genome copies g⁻¹ DT were responsible for both gastroenteritis outbreaks investigated in this study. Concentrations of NoV in the closed harvest area frequently exceeded these values and would probably have made people ill if consumed. Therefore, NoV monitoring using RT-qPCR can be used to monitor NoV contamination within shellfish harvesting areas at risk of human wastewater pollution and be used for risk management purposes.

Chapter 1 Introduction

1.1 Bivalve molluscan shellfish

1.1.1 Classification and nomenclature of bivalve molluscan shellfish

The Bivalvia class belongs to the Mollusca phylum, one of the largest and most diverse groups in the animal kingdom. Bivalvia contains approximately 9 200 species and is the second-largest class of the phylum (Bouchet *et al.*, 2010). Bivalve molluscan shellfish (BMS) species commercially farmed in Europe include the native or flat oyster (*Ostrea edulis*), pacific oyster (*Crassostrea gigas*), common blue mussel (*Mytilus edulis*), Mediterranean blue mussel (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*), king scallops (*Pecten maximus*) and queen scallops (*Chlamys opercularis*), and clams including the hard shell clam (*Mercenaria mercenaria*), the razor shell clam (*Ensis spp.*) and the native clam (*Tapes decussatus*) (Lees, 2000). BMS have a shell composed of two equal, or almost equal, halves jointed together by an elastic ligament. BMS are classified based on the shell form such as colour, shape and marking. With the exception of scallops, BMS are sedentary animals that bury themselves in seafloor sediment (cockles) or remain anchored to one spot (mussels).

1.1.2 History of bivalve molluscan shellfish in Ireland

The history of seafood in Ireland dates to *ca* 3000 B.C. when the first inhabitants arrived in the north part of the island and travelled along the coastline to the south (Wilkins, 2004). Around the Irish coast, archaeological evidence of prehistoric settlements can be found in a form of large mounds of domestic remains containing shells and bones known as middens. At Dunloughan Bay in County Galway Ireland, shell middens were found to contain rich deposits of oysters, cockles, limpets, winkles, dog whelks and thus provide information on the diet of Bronze Age people (Wilkins, 2004). Until the nineteenth century, shellfish such as common or blue mussels were mainly gathered by the poor people and were considered as poor man's food ('*bia bocht*' in Gaeilge) (Mac Con Iomaire, 2006). BSM consumption became more-widespread amongst urban inhabitants at the time of industrial revolution with oysters being the first 'fast food'. In the 1950s, seafood festivals were considered an excellent way to increase tourism and yield revenue for local

communities and are commonly held to the present day (Mac Con Iomaire, 2006). In the early 1970s, the production of the Pacific oyster *Crassostrea gigas* was introduced into Ireland as this species was easier and faster to cultivate than the native oyster *Ostrea edulis*.

1.1.3 Bivalve molluscan shellfish production

World aquaculture production was over 59 million tonnes in 2010 with the contribution of China accounting for 61.4 percent (FAO, 2012). World BMS production has increased substantially in the last fifty years, from ~1 million tonnes in 1950 to about 14.6 million in 2010 tones. In 2009, the European aquaculture production was 2 483 717 tonnes, BMS accounted for 25% of it (FAO, 2012). In Europe the most important BMS markets are France, Italy and Spain. The United Kingdom and France produce mainly oysters, while Denmark, Ireland, the Netherlands and Spain produce mainly mussels.

In Ireland, modernisation of the aquaculture sector began in the 1970's after entry into the European Economic Community. Irish aquaculture is divided into the finfish sector and shellfish sector. In 2007, the total production value of the aquaculture production was €105.7 million (48.350 tonnes), of which 44.73% constituted the shellfish production sector. The overall value of the shellfish production increased threefold in the last ten years in Ireland (Figure 1.1) (Browne *et al.*, 2008). Mussels, pacific oysters, native oysters, clams and scallops are the main shellfish species being produced in the country presently (Browne *et al.*, 2008). Between 2005 and 2007, France was the key shellfish export market for Ireland, followed by Spain, Italy and the United Kingdom.

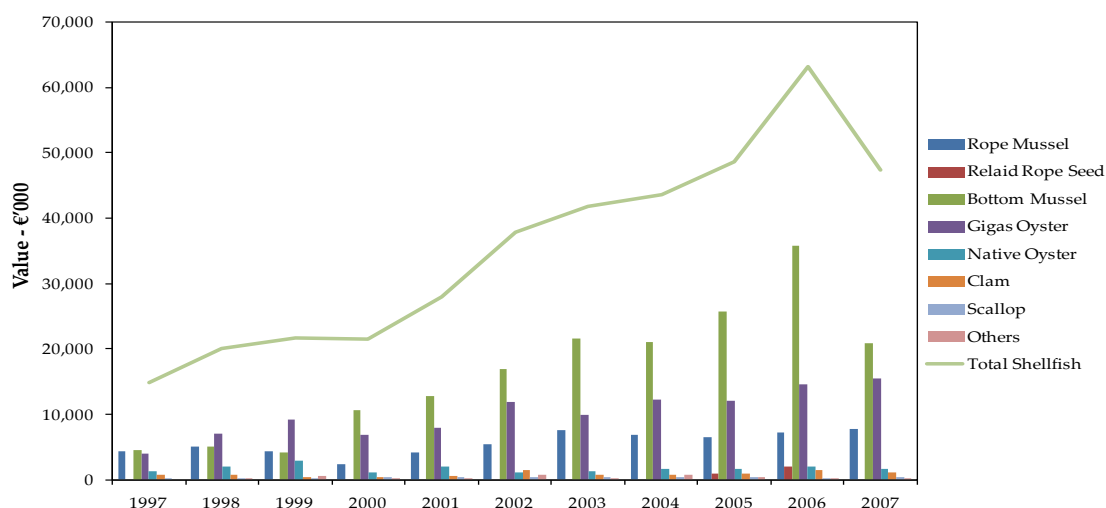


Figure 1.1 Shellfish production in Ireland.

Production value (value - €'000) of shellfish aquaculture in Ireland between 1997 and 2007 (Browne et al., 2008).

1.1.4 Bivalve molluscan shellfish and filter feeding

Most BMS are filter feeders that obtain their food by filtering small particles from the surrounding water. Large volumes of water are pumped across the gills by ciliary action. BMS are highly effective at filtering particles from water and as a result they can reduce turbidity, nitrogen, nutrients and organic matter, and can play a role in improving the water clarity and light transmission in aquatic environments which can be beneficial for other inhabitants such as seagrasses (Shumway *et al.*, 2003). Through filter feeding, shellfish improve water quality and as such bivalve shellfish aquaculture is considered to be a sustainable industry (Shumway *et al.*, 2003). As shellfish grow best in nutrient-rich water, they tend to thrive in areas such as estuaries or inshore sheltered coastal shallows. These inshore, shallow waters are often contaminated by human wastewaters that may contain pathogenic microorganisms. During the filter-feeding process, these microorganisms may become highly concentrated in the digestive tissues of BMS (Lees, 2000). Laboratory studies have demonstrated that oysters can bioaccumulate F⁺ coliphage, an enteric viral surrogate, up to concentrations 99 times greater compared with the surrounding water (Burkhardt and Calci, 2000).

1.2 Microbiological hazards associated with bivalve molluscan shellfish

As pathogens can be accumulated by BMS, they pose a recognised health risk when such contaminated shellfish are consumed. Moreover, as certain shellfish species are traditionally eaten raw or lightly cooked, the inactivation of pathogens during the cooking process is eliminated. Outbreaks of shellfish-associated diseases have been documented worldwide (Potasman *et al.*, 2002); The BMS species most frequently reported as being responsible for outbreaks of illness are oysters, followed by clams (Bellou *et al.*, 2013). BMS may transmit a variety of pathogenic microorganisms, including viruses, bacteria and parasites.

1.2.1 Bacterial disease associated with bivalve molluscan shellfish

The earliest reports of bacterial disease associated with the consumption of raw shellfish were documented at the end of the 19th century. Typhoid fever was the most common illness that was transmitted by sewage polluted oysters and caused numerous outbreaks in Europe, the United States and other countries (Allen, 1899). In the mid-1920s, several large outbreaks of typhoid fever occurred responsible for more than 1500 cases and 150 deaths reported in the USA (Rippey, 1994). The last shellfish-vectored case of typhoid fever was reported in the USA in 1954. The decrease and eventual elimination of typhoid cases associated with shellfish consumption coincided with general improvements in sanitation and the reduction in the significance of this pathogen in the population. Overall bacterial pathogens now represent a small percent of shellfish-associated outbreaks (4.0%) and cases (3.8%) reported between 1905 and 1990 (Rippey, 1994). This is due to general improvements in wastewater treatment technologies and the effectiveness of regulatory hygiene controls on shellfish production. Other bacteria such as *Vibrio* spp., *Salmonella* spp., *Shigella* spp., and *Listeria* spp. have also been associated with disease after the consumption of raw shellfish (Potasman *et al.*, 2002).

Vibrio spp. are native to both marine and estuarine environments, and their occurrence in shellfish are typically unrelated to faecal pollution. An exception is toxigenic *V. cholerae* O1, which is excreted in a large numbers by infected

individuals and can survive in wastewater (DePaola *et al.*, 2010). *Vibrio* spp. are halophilic, non-sporeforming bacteria that grow in warmer periods of the year. They are fast growing bacteria in nature and can multiply readily in oysters postharvest when not properly refrigerated. *Vibrio* spp. produce a wide range of clinical symptoms: *Vibrio vulnificus* can result in septicemia with a high mortality rate, others species are associated with gastroenteritis of varying severity (Table 1.1) (Rippey, 1994). *Vibrio vulnificus* and *parahaemolyticus* have been recently detected in oysters at retail during market survey in the USA; oysters sampled from the Gulf Coast had the highest numbers of both *Vibrio* spp. compared to other regions in USA during 2007 (DePaola *et al.*, 2010).

Oyster-related *Vibrio vulnificus* sepsis and death in shellfish consumers was first documented in 1975 (Blake *et al.*, 1979). Predisposing factors for the development of severe disease are chronic liver disease, diabetes mellitus, hemochromatosis, and other immunosuppressive disorders. The disease is characterised by a 24-h incubation period, followed by signs of sepsis, including fever, chills, hypotension, nausea, vomiting, and diarrhoea (Potasman *et al.*, 2002) (Table 1.1).

Between 1995-2000, 61 cases of illness were reported in the USA due to toxigenic *Vibrio cholerae* O1 of which 23% were associated with the consumption of undercooked seafood originating from the Gulf Coast (Steinberg *et al.*, 2001). Both O1 and other serotypes have been isolated from individuals with relatively severe gastroenteritis. Since the 1990s, non-O1 serotypes have been reported more frequently and the illness caused by them is generally less severe than caused by toxigenic O1 serotypes.

Shigella spp. are Gram-negative bacilli. Clinical manifestations of *Shigella* infection range from watery, loose stools to more severe symptoms such as fever, abdominal pain, tenesmus, and bloody diarrhea (Iwamoto *et al.*, 2010). An oyster-related outbreak of *Shigella sonnei* infection among 24 individuals occurred in Texas and was associated with the sewage dump overboard from the oyster harvest boat (Reeve *et al.*, 1989). In the USA alone, 118 cases of BMS-associated shigellosis were reported between 1973 and 2006 (Iwamoto *et al.*, 2010).

Table 1.1 Bacterial causes of shellfish-associated illness.

Bacteria	Incubation period	Duration	Illness/ symptoms	Source of contamination
<i>Salmonella typhi</i>	1-3 weeks	up to 4 weeks	Typhoid fever/malaise, headache, fever, cough, nausea, vomiting,	Human faeces/ sewage
<i>Salmonella Paratyphi</i>	1-10 days	2-3 weeks	constipation, abdominal pain, chills, bloody stools	
<i>Salmonella enterica (various serogroups)</i>	6-72 hours,	4-7 days	Stomach pain, headache, fever, nausea, vomiting, diarrhoea, chills	Human faeces/ animal bird faeces
<i>Vibrio vulnificus</i>	16 hours	2-3 days	Septicaemia/Malaise, chills, fever, prostration, cutaneous lesions, fatalities occur	Marine environment
<i>Vibrio parahaemolyticus</i>	2-48 hours	2-14 days	Abdominal pain, diarrhoea, nausea, vomiting, chills, headache	Marine environment
<i>Vibrio cholerae O1 and O139 serotypes</i>	1-5 days	2-5 days	Profuse, watery diarrhoea (rice-water stools), vomiting, abdominal pain, dehydration	Human faeces/ sewage
<i>Vibrio cholerae non-O1/non-O139</i>	2-3 days	up to 7 days	Watery diarrhoea (varies from loose stools to cholera-like diarrhoea)	Marine environment
<i>Shigella spp</i>	24-72 hours	5-7 days	Abdominal pain, diarrhoea, bloody & mucoid stools, fever	Human faeces/ sewage
<i>Campylobacter</i>	2-7 days	3-6 days	Diarrhoea (often bloody), severe abdominal pain, fever, anorexia, malaise, headache, vomiting	Animal/bird faeces/ slurry

Adapted from (Lee *et al.*, 2008)

1.2.2 Protozoan pathogens

The transmission of protozoan parasites via seafood is very rarely reported; however, protozoan-contaminated water is a frequent cause of outbreaks (Yoder *et al.*, 2008). The protozoan parasites such as *Cryptosporidium* spp., *Giardia duodenalis* and *Toxoplasma gondii* have the potential to be accumulated by shellfish from the surrounding waters (Robertson, 2007) (Table 1.2). To date, one oyster-associated outbreak of giardiasis has been reported (Iwamoto *et al.*, 2010). There is some evidence suggesting that both *Toxoplasma* and *Cryptosporidium* retain their infectivity for prolonged periods in shellfish (Robertson, 2007). However, the actual public health risk posed by protozoan parasites via shellfish ingestion is unclear due to lack of epidemiological data.

Table 1.2 Protozoan parasites detected in shellfish tissue with the possible potential to cause shellfish-related outbreaks.

Protozoan	Incubation period	Duration	Illness/ symptoms	Transmissible stage (size range)
<i>Cryptosporidium</i> spp	2-10 days	Up to 2 weeks	Profuse and watery diarrhoea, weight loss, nausea; low-grade fever	Oocyst (4-6 μ m)
<i>Giardia duodenalis</i>	1-45 days	1-2 weeks	Diarrhoea, malabsorption	Cyst (8-12 μ m)
<i>Toxoplasma gondii</i>	5-23 days	2-4 weeks	Lymphadenopathy, fever, congenital infections	Oocyst (10-12 μ m)

Adapted from (Robertson, 2007).

1.2.3 Human pathogenic viruses

While many different viruses can be detected in shellfish (Table 1.3), epidemiological evidence suggests that human enteric viruses such as norovirus (NoV) and hepatitis A virus (HAV) are the most common pathogens transmitted by

shellfish. The first linkage of shellfish-associated viral gastroenteritis was made in the winter of 1976-1977 in the UK when cooked cockles were epidemiologically linked to 33 separate outbreaks, effecting ~800 people (Appleton and Pereira, 1977). NoV is the leading cause of shellfish-borne outbreaks, and is responsible for 83.7% of outbreaks reported in scientific literature; this is followed by HAV which is responsible for 12.8% of outbreaks (Bellou *et al.*, 2013). The majority of NoV shellfish-associated outbreaks have been reported to occur in East Asia, followed by Europe, Oceania, America and Australia (Bellou *et al.*, 2013). In 1955, the first outbreak of infectious HAV was reported from Sweden when 629 cases were associated with the consumption of raw oysters (Roos, 1956). Since then many outbreaks of infectious hepatitis associated with BMS consumption have been reported worldwide (Richards, 1987 and Rippey, 1994). The largest outbreak of viral food poisoning reported was attributed to HAV in China in 1988 when more than 292,000 cases, including nine deaths were traced to the consumption of uncooked clams (Halliday *et al.*, 1991 and Tang *et al.*, 1991). The majority of HAV shellfish-associated outbreaks (83%) have been reported in Europe (Bellou *et al.*, 2013), where individuals lack immunity.

Infectious hepatitis can be also caused by hepatitis E virus (HEV). Infectious hepatitis caused by HEV is an acute, self-limiting liver disease that shares many features with HAV infection, but with some differences-particularly the high mortality rate amongst HEV-infected pregnant women. Thus far, just one outbreak of HEV related infectious hepatitis associated with shellfish has been recorded. During this outbreak illness was confirmed in 33 passengers who returned to the United Kingdom from a world cruise in 2008 (Said *et al.*, 2009). In Scotland, HEV was detected in 92% and 55% of the mussel samples collected from the west and east coast, respectively (Crossan *et al.*, 2012). These findings suggest that shellfish are a potential source of HEV infection in Europe.

Multiple human enteric viruses such as aichi virus, NoV, astrovirus, enterovirus and rotavirus were detected in both faecal and oyster samples following an outbreak of gastroenteritis when 205 cases were linked to the consumption of contaminated oysters (Le Guyader *et al.*, 2008). However, shellfish-associated

outbreaks attributed to rotaviruses, astroviruses, enteroviruses, and adenoviruses have been rarely reported worldwide (Bellou *et al.*, 2013). This may be attributed to: pre-existing immunity of the person consuming shellfish, low levels of viruses accumulated in shellfish to establish infection or underreporting of infections due to mild symptoms (Bosch, 2007). Many other viruses such as coxsackievirus or echovirus have been detected in shellfish tissues (Lees, 2000), but have not been associated with shellfish-borne illness.

Table 1.3 Viruses implicated in shellfish-borne outbreaks.

Virus	Incubation period	Duration	Illness/ symptoms	References
Norovirus	24-28 hours	1-3 days	Gastroenteritis/ Non-bloody diarrhoea, nausea, vomiting, abdominal pain, abdominal cramps, fever	(Hansman <i>et al.</i> , 2010)
Hepatitis A virus	10 to 50 days	10 to 30 days	Infectious hepatitis/ Fever, malaise, lassitude, anorexia, nausea, abdominal pain, jaundice	(Lee <i>et al.</i> , 2008)
Hepatitis E virus ¹	15-60 days	10 to 25 days	Infectious hepatitis/ Fever, malaise, lassitude, anorexia, nausea, abdominal pain, jaundice	(Panda <i>et al.</i> , 2007)
Sapovirus ¹	5 to 100 hours	12- 46 hours	Gastroenteritis/ Non-bloody diarrhoea, nausea, vomiting, stomach cramps	(Noel <i>et al.</i> , 1997)
Astrovirus ¹	1 to 2 days	48 to 72 hours	Gastroenteritis/ diarrhoea	(Lee <i>et al.</i> , 2008)

¹Only 1 or 2 shellfish-related outbreaks have been reported. Adapted from (Bellou *et al.*, 2013).

1.2.4 Current Food Hygiene regulation

In order to protect consumer health from contaminated shellfish and in an effort to maintain a successful industry, regulations have been introduced to help control the risk of illness. In the European Union (Anonymous, 2004), official controls focus on

the classification of the relaying and production areas for BMS. Under European Council Regulations EC 854/2004 the competent authority in each Member State is required to classify live BMS production areas according to the degree of faecal contamination based on the concentration of *E. coli* present in the shellfish flesh and intravalvular liquid. The reference method for this analysis is the five-tube, three dilution Most Probable Number (MPN) test specified in ISO 16649-3. However, alternative methods may also be used if it is validated against the reference method in accordance with the criteria in EN/ISO 16140.

Bivalve molluscs for sale onto the market must only be harvested from defined classified areas (Table 1.4). Only bivalve molluscs that meet the category A requirement and are also free from *Salmonella* spp. and marine biotoxins can go direct for human consumption. BMS product harvested from Class B areas must not exceed, in 90% of the samples, 4,600 *E. coli* per 100 g of flesh and intravalvular liquid. Class C areas require further purification of animals over a long period or heat treatment before being placed on the market (Table 1.4).

Table 1.4 Criteria for the classification of bivalve mollusc harvesting areas.

Category	Microbiological Standard per 100 g flesh and intravalvular fluid	Treatment required
Class A	<230 <i>E. coli</i>	May go direct for human consumption.
Class B	<4,600 <i>E. coli</i>	Must be depurated, heat treated or relayed to meet class A requirements.
Class C	<46,000 <i>E. coli</i>	Relay for 2 months to meet class A or B requirements-may also undergo heat-treatment.
Prohibited	>46,000 <i>E. coli</i>	Harvesting prohibited.

In Ireland, the Sea Fisheries Protection Authority (SFPA) is the Competent Authority for the Classification of Live Bivalve Mollusc Production Areas. The Marine Institute is the National Reference Laboratory (NRL) for monitoring the bacteriological and viral contamination of shellfish and advises SFPA on technical aspects relating to the classification of shellfish production areas. The SFPA and the

NRL work closely with the Food Safety Authority of Ireland (FSAI) to ensure that Irish shellfish are produced in accordance with the current regulations. The production areas sampled in the national monitoring programme are principally oyster and mussel cultivation areas, but some clam species are also included. Non BMS species including Sea Urchins which are Echinoderms are also classified.

The implementation of various national monitoring programmes in developed countries has been effective at almost completely eliminating bacterial illness associated with BMS consumption (Lees, 2000). Despite the bacteriological monitoring of shellfish harvesting that is carried out, outbreaks of viral illness associated with BMS consumption continue to occur.

1.3 Norovirus

1.3.1 Taxonomic classification of *Caliciviridae* family

The family *Caliciviridae* was defined in 1979 by the International Committee on Taxonomy of Viruses (ICTV) (Matthews, 1979). The name of the family comes from the Latin word Calyx, which means cup or chalice and is derived from the typical cup-shaped depression on the virion surface (known as the “Star of David” morphology) when viewed using electron microscopy. Other common features of this family include; the formation of a capsid by a number of copies of a major structural protein and the lack of a methylated 5' end on the viral genome (Black *et al.*, 1978, Burroughs and Brown, 1978, and Green *et al.*, 2000). The genomes of caliciviruses are made up of single-stranded polyadenylated RNA, which can be organised into two or three open reading frames (ORFs) (Clarke and Lambden, 1997). This differentiates them from the closely related family of *Picornaviridae*, which utilise one long ORF to synthesise their viral proteins (Meyers *et al.*, 1991).

Currently five genera of the family *Caliciviridae*; Lagovirus, Nebovirus, NoV, Sapovirus, and Vesivirus have been recognised (King *et al.*, 2011) (Table 1.5). The type species of the Lagovirus genus is rabbit haemorrhagic disease virus. This virus is extremely virulent and was responsible for killing 14 million domestic rabbits in China over a nine month period, when it first emerged in 1984 (Cooke, 2002 and Forrester *et al.*, 2006). Feline calicivirus and vesicular exanthema of swine virus are only two officially recognised species of Vesivirus and cause severe respiratory illness, pneumonia and also limping in cats (Clarke and Lambden, 1997) and vesicular lesions in swine similar to those of foot-and-mouth disease (Clarke and Lambden, 1997), respectively. Nebovirus has recently been accepted as a new genus by the ICTV. The genus include viruses that cause diarrhoea and intestinal lesions in the small intestines of calves (Smiley *et al.*, 2002), but differs significantly from bovine NoVs in its genome organisation (Oliver *et al.*, 2006). NoV and Sapovirus mainly cause gastroenteritis infections in humans and were named after the geographical location, i.e. Norwalk in USA and Sapporo in Japan, where the type species was first isolated. Currently, molecular evidence exists to support the

presence of at least four additional genera of caliciviruses (Farkas *et al.*, 2008, L’Homme *et al.*, 2009, and Wolf *et al.*, 2012).

Table 1.5 Genera and species within the *Caliciviridae* family.

Genus	Type Species	Illness/ symptoms
Lagovirus	European brown hare syndrome virus	Acute and severe contagious, necrotising viral hepatitis that occurs in European brown hares and mountain hares; lead to dysfunction of various organs
	Rabbit hemorrhagic disease virus	Rabbit hemorrhagic disease; severe disseminated necrotic hepatitis, multifocal petechial haemorrhages in the liver and other organs with high mortality rate in rabbits
Nebovirus	Newbury-1 virus	Gastroenteritis in bovines
Norovirus	Norwalk virus	Gastroenteritis in humans and animals; bovine, porcine, murine, feline and canine species
Sapovirus	Sapporo virus	Gastroenteritis in humans and animal such as porcine and musteline species
Vesivirus	Feline calicivirus	Mild oral and upper respiratory infections/fever, conjunctivitis, nasal discharge, sneezing, and ulceration of the mouth in cats
	Vesicular exanthema of swine virus	Fever, vesicles on the epithelium of the snout, lips, nostrils, tongue, feet and mammary glands, disease affects vesicles swine and marine mammals

1.3.2 Norovirus discovery and genus classification

In 1972, an etiological agent of nonbacterial gastroenteritis (a 27-nm particle) was recognised using immune electron microscopy in faecal samples derived from children infected during an epidemic outbreak in Norwalk, Ohio, USA, and subsequently named as Norwalk virus (NV) (Kapikian *et al.*, 1972). NV became the prototype strain for a group of morphologically similar viruses, called “small round structured viruses” (SRSVs) that were commonly associated with epidemic gastroenteritis (Green *et al.*, 2000). In the early 1990s, the full-length genome sequences of NV and other related SRSVs were published (Jiang *et al.*, 1993 and Lambden *et al.*, 1993) and demonstrated the sequence similarity of these viruses to other members of the *Caliciviridae* family.

NoV exhibits considerable genetic diversity and can be divided into at least five genogroups, designated GI-GV, based on amino acid identity of the major capsid protein (VP1) (Zheng *et al.*, 2006). NoV strains that infect humans have been identified in GI, GII, and GIV, whereas the strains infecting bovine and murine species belong to GIII and GV, respectively (Figure 1.2). Each genogroup is further divided into genotypes, with at least eight genotypes belonging to GI and 21 genotypes belonging to GII (Hall and Aron, 2011). Interspecies transmission of NoV has not been reported so far, but the potential risk for zoonotic transmission cannot be precluded as NoVs closely related to human NoVs have been detected in pigs: GII.18 (Mattison *et al.*, 2007) and dogs: GIV.2 (Martella *et al.*, 2008).

Figure 1.2 Classification of noroviruses into 5 genogroups (GI-V) and 35 genotypes based on sequence diversity in the major capsid protein VP1.

**The scale bar of 0.1 reflects the number of amino acid substitution per site (Hall and Aron, 2011).*

Zheng *et al* demonstrated that new genotypes can be assigned when the amino acid sequence of the major capsid protein (VP1) differ by more than 14.1% and a minimum of 15% pairwise difference is achieved between the next-nearest genotype (Zheng *et al.*, 2006). For NoV GII.4 variants, the amino acid divergence of 5% has been reported between the VP1 sequence, and up to 2.8% within variants (Zheng *et al.*, 2010). However, due to the common occurrence of NoV recombinants, a dual nomenclature using both ORF1 and VP1 sequences has been suggested recently by Noronet network (Kroneman *et al.*, 2013).

1.3.3 Norovirus genomic organization and non-structural proteins

The NoV genome consists of ~7.7 kb positive-sense single-stranded RNA that is organized into three ORFs (Figure 1.3). The viral genome is covalently attached to a viral protein (VPg) at 5' end and contains a poly-A tail at the 3' end (Hardy, 2005). ORF1 (~5 kb) encodes a ~200 kDa non-structural polyprotein that is cleaved by a

viral 3C-like protease (3C) to yield six individual mature proteins that are crucial for norovirus replication. ORF2 (~1.8 kb) and ORF3 (~0.6 kb) encode the 57 kDa major structural capsid protein (VP1) and the 22 kDa minor basic structural protein (VP2), respectively (Donaldson *et al.*, 2008).

Figure 1.3 Norovirus genome organisation and atomic structure of the capsid.

The RNA genome of Norwalk virus is organised into three open reading frames (ORFs). The P2 region of the P domain (blue) is exposed on the surface of the capsid and contain the binding site for histo-blood group antigen (HBGAs) (magenta).

Adapted from (Bok and Green, 2012).

The processed non-structural proteins include: (i) p48, an N-terminal protein of unknown function (~48 kDa); (ii) NTP, nucleoside triphosphatase (~40 kDa) that is similar to the poliovirus 2C protein, but binds and hydrolyzes all NTPs; (iii) p22, a 3A-like protein of unknown function (~22kDa); (iv) VPg, a viral protein genome-linked that most likely play a role in replication and translation of viral RNA (~16 kDa) (Daughenbaugh *et al.*, 2003 and Herbert *et al.*, 1997); (v) 3C, a 3C-like protease that process the ORF1 polyprotein at cleavage sites: QG, EG, or EA and is similar to the picornavirus 3C protease (~19 kDa) (Donaldson *et al.*, 2008); and (vi) RdRp, RNA-dependent RNA polymerase is essential for *in vitro* viral replication and

contains the conserved GDD motif that has been found in many positive-strand RNA viruses (~57 kDa) (Fukushi *et al.*, 2004).

1.3.4 Norovirus structural proteins

In the early nineties, it was demonstrated that the NV capsid proteins (VP1) spontaneously self-assembled to form non-infectious virus like particles (VLPs) when expressed using a baculovirus replicon system (Jiang *et al.*, 1992). The x-ray crystal structure determined at 3.4 Å resolution for VLPs confirmed that the NV capsid is composed of 180 copies of a single protein (VP1) that is organised into 90 dimers in a T=3 icosahedral symmetry (Prasad *et al.*, 1999) (Prasad, 1994). Each NV VLP is composed of: (i) thirty C/C dimers, which are located at icosahedral 2-fold axes and are important for building of interior capsid shell and (ii) sixty A/B dimers, which surround icosahedral 5-fold axes of symmetry and play a role in receptor recognition as they extend away from the capsid surface (Donaldson *et al.*, 2008) (Prasad *et al.*, 2000). A single VP1 protein (monomer) is organised into two domains (Figure 1.3):

- **The shell (S) domain** contains an N-terminal arm and an eight-stranded antiparallel β sandwich fold that is commonly found in viral capsid proteins and is involved in formation of the icosahedral shell (Prasad *et al.*, 1999).

- **The protruding (P) domain** is composed of P1 subdomain and P2 subdomain, which is a large insertion in the P1 domain. The P1 subdomain is connected to the S domain by a flexible hinge, whereas the P2 subdomain is the most surface exposed region of the capsid protein (Donaldson *et al.*, 2008).

Structural variation is the lowest within the S domain, intermediate within the P1 subdomain and the highest within the P2 subdomain (Chakravarty *et al.*, 2005) and reflects the different functions of these components in the VP1 capsid protein. The hypervariable P2 subdomain contains the carbohydrate receptor-binding pockets (Cao *et al.*, 2007 and Tan *et al.*, 2003). The VLPs structures of GI.1 and GII.4

in the complex with A-type HBGA revealed a different ligand interaction sites within the P2 subdomain (Donaldson *et al.*, 2008). This suggests that VLPs of different NoV genogroups can interact with the same HBGAs through distinctive receptor-binding interface.

The minor structural protein (VP2) is incorporated into the capsid in one or two copies in infectious virions (Hardy, 2005). The VP2 protein interacts with an isoleucine 52 of VP1 located within a highly conserved motif IDPWI in the S domain and the mutation of this particular residue precluded VP2 from incorporating into the VLPs (Vongpunsawad *et al.*, 2013). The function of VP2 has not been revealed yet, but it could possibly play a role in supporting capsid assembly and packaging the viral genome (Hardy, 2005 and Vongpunsawad *et al.*, 2013).

1.3.5 Recombination

The high genetic variability observed in RNA viruses can be attributed to three evolutionary forces such as point mutation, reassortment (exclusively undergone by segmented RNA genomes) and recombination. However, the high genetic diversity of the *Caliciviridae* family has been attributed to point mutation and recombination. The high rate of nucleotide substitutions, due to short replication times, high multiplicity and a lack of proofreading activity of RdRp, allows RNA viruses to continuously generate mutant genomes (Holmes, 2010). Recombination involves the exchange of genetic information between non segmented RNA genomes, but genetic divergence is restricted by the necessity to maintain a functional viral RNA genome. Recombination in viruses is an important mechanism of evolution as newly-emerged progeny virus may have different pathogenesis and virulence from the parental viruses. Mathijs *et al.* demonstrated that co-infection of a mouse cell line with two different parental murine norovirus (MuNoV) isolates led to the formation of recombinant progeny that exhibited distant biological properties from the parental viruses (Mathijs *et al.*, 2010).

Snow Mountain virus was first documented naturally-occurring NoV recombinant. Hardy *et al* (1997) showed that the nucleotide sequence of the capsid region of Snow Mountain recombinant (GII.c/GII.2) had 94% identity to the capsid

region of Melksham virus (GII.2), but only 79% identity in the RdRp region. The most recent estimate, at least 22 NoV recombinant types have been described within GI (1), GII (16), GIII (2), GIV (1) and GV (2) (Hansman *et al.*, 2010) along with inter-genogroup recombinant GI.3/GII.4 (Nayak *et al.*, 2008) (Table 1.6).

Table 1.6 Norovirus recombinants.

Norovirus genogroup	Prototype strain	RdRp genotype	Capsid genotype	Average breakpoint ¹
GI	WUGI/01/JP	GI.2	GI.6	5
GII	Picton/03/AU	GII.b	GII.1	-26
	Snow Mountain/76/US	GII.c	GII.2	-55
	E3/97/Crete	GII.4	GII.2	-25
	Pont de Roide 673/04/Fr	GII.b	GII.2	-21
	SydneyC14/02/AU	GII.b	GII.3	+23
	Sydney2212/98AU	GII.a	GII.3	-59
	Chiba1/04/JP	GII.4	GII.3	-7
	771/05/IRL	GII.4/GII.d	GII.4	-595-212
	Nyiregyhaza/1077/02HUN	GII.b	GII.4	Not available
	S63/99/Fr	GII.2	GII.5	+35
	Hokkaido133/03/JP	GII.d	GII.5	-18
	Kunming/04/CH	GII.6	GII.7	-54
	Mc37/01/Th	GII.4	GII.10	+4
	SaitamaU1/02/JP	GII.4	GII.12	-38
	Minato14/99/JP	GII.6	GII.15	-53
	VannesL23/99/US	GII.5	GII.15	-31
GIII	B-1SVD/03/US	GIII.2	GIII.1	-20
	CV521/02/US	GIII.1	GIII.2	-10
GIV	S28/06/DE	ND	ND	+17
	MNV-4/05/US	ND	ND	+55
GI/GII	L8775/06/ID	GI.3	GII.4	+22

¹Numbering is in relation to start of ORF2;

ND, genotype not defined.

Adapted from (Hansman *et al.*, 2010).

Recombination ‘hotspots’ are regions in a genome that exhibit elevated rates of recombination in comparison to other parts of genome. A breakpoint is defined as location where the recombination event occurs in a sequence (Eden *et al.*, 2013). In most cases, the NoV recombination breakpoint is located at the RdRp/capsid

junction suggesting that the start of the capsid gene constitutes a recombination hotspot for NoV (Hansman *et al.*, 2010). Recombination hotspots have been identified within the picornaviruses and coronaviruses and recombination events have been seen to occur *in vitro* throughout the entire genome, except for the capsid regions encoding for VP1 and VP3 in picornaviruses (Lai, 1992). In addition, a more complex pattern of NoV recombination involving the intra-genotypic exchange of the capsid P2 domain between variants of GII.4 has been reported recently (Lam *et al.*, 2012).

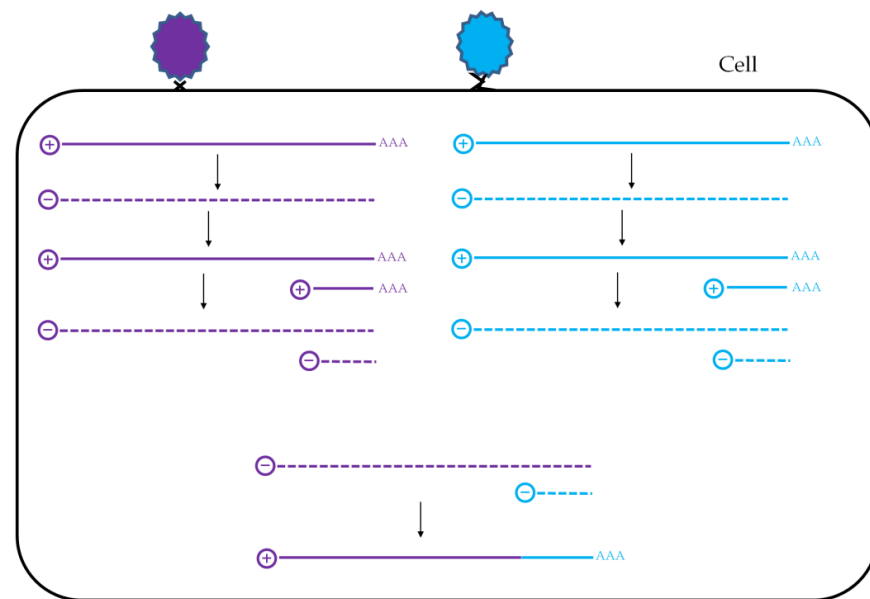


Figure 1.4 *Recombination mechanisms proposed for norovirus.*

*Recombination occurs when the RNA dependant RNA polymerase switches templates from the full-length negative strand from one parental virus to an available negative subgenomic RNA from another parental virus. Adapted from (Bull *et al.*, 2005).*

A copy choice model for NoV recombination has been proposed based upon RNA replication and the synthesis of subgenomic RNA (sgRNA) by internal initiation (Bull *et al.*, 2005). This model consists of four subsequent steps (Figure 1.4). First, negative full-length genome intermediates (dashed lines) are transcribed by the RdRp. Second, positive stranded genomic RNA and sgRNA (straight lines) are generated after RdRp binds to RNA promoter sequences located at the start of ORF1 and ORF2. Third, negative full-length genome RNA and sgRNA species are synthesised from positive RNA. Finally, recombination occurs by template

switching of the RdRp during positive strand synthesis. In this model, the viral RNA complex pauses, and subsequently switches from one template to another due to the occurrence of complex secondary structure regions that forms a binding site for the RdRp (Nagy *et al.*, 1999).

1.4 Norovirus epidemiology

1.4.1 Transmission routes of infection

NoV transmission occurs through the faecal-oral route either via the consumption of contaminated food and water or through direct person-to-person contact. According to the Foodborne Viruses European (FBVE) network, person to person transmission was responsible for 88% of 5035 gastroenteritis outbreaks caused by NoV over a 5-year period. Foodborne and waterborne transmission were responsible for 10% and 2% of the outbreaks respectively (Kroneman *et al.*, 2008). NoV was responsible for the majority (57.3%) of enteric outbreaks (n=75) that occurred in long-term care facilities between 1997 and 2007; the majority of these outbreaks were attributed to transmission by person-to-person contact (Greig and Lee, 2009). *Barker et al* using a human challenge study demonstrated that human NoVs could be consistently transferred via contaminated fingers to surfaces such as toilet tops, door handles, and telephone receivers (Barker *et al.*, 2004). Contamination of surfaces in hospital wards was shown to decrease with the implementation of strict hygiene rules for staff and visitors (Gallimore *et al.*, 2008).

Food can be contaminated by contact with faecal material that is derived from either an environmental source or an infected food handler. BMS are frequently identified as a vector responsible for NoV associated foodborne outbreaks. On occasion these can be large scale and involve international outbreaks (Guyader *et al.*, 2006 and Westrell *et al.*, 2010). Salads and soft fruits such as berries can be contaminated with NoV by polluted irrigation or washing water and have been responsible for large-scale international outbreaks of illness (Maunula *et al.*, 2004, Mäde *et al.*, 2013, and Showell *et al.*, 2007). Outbreaks associated with NoV contamination of drinking water have also been reported (Arvelo *et al.*, 2012, Breitenmoser *et al.*, 2011, and Di Bartolo *et al.*, 2011). Food handlers suffering from NoV gastroenteritis can contaminate food at any point from harvest to serving (de Wit *et al.*, 2007 and McIntyre *et al.*, 2012). Furthermore, the excretion of NoV from asymptomatic food handlers can also be an infection source during an outbreak (Barrabeig *et al.*, 2010 and Yu *et al.*, 2011). Bidawid *et al.*, (2000) demonstrated that

hand washing can significantly reduce the probability that virus (HAV) will be transferred from contaminated fingers to food items (lettuce).

1.4.2 Illness

NoV illness is usually described as mild and self-limiting. The incubation time is generally between 24 and 28 hours; symptoms include non-bloody diarrhoea (in 87% of patients), vomiting (74%), fever (32%), nausea (48%), abdominal pain (51%) and abdominal cramps (44%), and generally resolve in 1-3 days as shown in a cohort study (Rockx *et al.*, 2002). Vomiting, nausea and fever primarily may occur on the first day of illness. Of all the symptoms, diarrhoea lasts the longest, with an average duration of 4 days; however, in some individuals diarrhoea can persist for up to 4 weeks after onset. Vomiting occurred more often in children aged >1 years, whereas diarrhoea was more frequent in younger children <1 year (Rockx *et al.*, 2002) (Lopman *et al.*, 2004). Although NoV infects persons of all age groups, it has been reported to be more prevalent in children (age, 0.5-17 years) and elderly patients (age ≥65 years) (Rockx *et al.*, 2002). In the elderly, young children and the immunocompromised, symptoms during NoV infection can be more severe and have some serious consequences, such as prolonged illness and even death (Mattner *et al.*, 2006 and Trivedi *et al.*, n.d.). Nelson *et al.*, (2012) demonstrated that some patients have a disturbed microbiota in the intestinal tract (elevated Proteobacteria), following NoV infection, and then may be at increased risk for long-term health complications including post-infection irritable bowel syndrome.

1.4.3 Shedding

NoV is present in high concentration in faeces; 5×10^8 to 1.6×10^{12} genome copies per gram of faeces were detected after experimental infection with NoV GI.1 Norwalk virus (Atmar *et al.*, 2008). Comparable concentrations of NoV GII.4 were reported in the faeces of symptomatic individuals; however, higher NoV concentrations were associated with older aged patients and prolonged diarrhoea (Lee *et al.*, 2007) (Lai *et al.*, 2013). After resolution of disease symptoms, NoV shedding can be detected up to 3 weeks post infection in healthy adults, but it can be particularly long (up to 100

days) in children <1 year (Kirkwood and Streitberg, 2008 and Rockx *et al.*, 2002). In transplant recipients or patients with hematologic and oncologic disorders, NoV infection can lead to chronic disease characterised by prolonged illness and the presence of NoV at high concentrations in faeces for up to several years (Carlsson *et al.*, 2009 and Ludwig *et al.*, 2008). It is unknown if these prolonged shedders remain infectious for others. In England, asymptomatic NoV infections were identified in 12% of 2205 healthy individuals with the highest prevalence of asymptomatic infections in children aged <5 years, and during the winter season (Phillips *et al.*, 2010).

1.4.4 Infectivity

The prototype NV when used in human volunteer studies was established to be highly infectious. The probability of infection from a single NV particle was estimated at ~0.5 which is the highest that has been reported so far for any known virus. Increasing NV doses led to an increasing probability of becoming ill amongst volunteers and the median infectious dose (ID₅₀) was estimated at 18 disaggregated virus particles or 2.6 aggregated virus particles (Teunis *et al.*, 2008). For oyster-related outbreaks, the ID₅₀ estimated for NoV GI and GII ranged between 1.6 and 7.51 genome copies per oyster consumed (Thebault *et al.*, 2013). During an outbreak, the reproduction number (R₀) of NoV was estimated at 14.05 in the absence of hygiene measures; roughly every primary case infected 14 secondary cases. In the same study, the implementation of hygiene measures had an effect on the reduction of R₀ to 2.13 (Heijne *et al.*, 2009). During the 2009 pandemic of influenza A (H1N1), the R₀ was estimated at a lower value which ranged between 1.0 and 2.0 in different outbreaks studies (Glass *et al.*, 2012).

1.4.5 Human susceptibility and immunity

Although NoV is highly infectious, a number of human volunteers remained uninfected after challenge with high doses of NV (Johnson *et al.*, 1990 and Matsui and Greenberg, 2000). Non-secretor (Se⁻) individuals are genetically resistant to NV infection as they do not express the H-type-1 oligosaccharide on the surface of

epithelial cells and in mucosal secretions that act as a ligand required for Norwalk virus binding (Lindesmith *et al.*, 2003). A number of secretor individuals (Se⁺) were also protected from NV infection, suggesting the presence of acquired immunity characterized by rapid production of NV-specific mucosal IgA (Lindesmith *et al.*, 2003). Human challenge study have shown that protective immunity may be absent or short lived (a few months) after infection with NV (Johnson *et al.*, 1990). Long-term immunity against NoV has not been established (Parrino *et al.*, 1977). Furthermore, immunity to one strain may not confer cross-genogroup protection against infection with another strain (Wyatt *et al.*, 1974) or even variants within a genotype (Donaldson *et al.*, 2010). Therefore, one susceptible individual may suffer repeated NoV infection throughout their lifetime.

1.4.5.1 Receptor binding

Histo-blood group antigens (HBGAs) are complex carbohydrates found on the surfaces of erythrocytes and the mucosal epithelium of respiratory, genitourinary and digestive tracts (Ravn and Dabelsteen, 2000). These carbohydrates determine human blood types and are commonly present in biological fluids, such as saliva, intestinal contents, milk and blood. The expression of HBGAs is regulated by the fucosyltransferases genes FUT1, FUT2, and FUT3, which are polymorphic in the human population. The synthesis of HBGAs begins with the addition of single monosaccharides to a disaccharide precursor by specific enzymes. There are four major types of disaccharide precursors: type 1 (Gal β 1-3GlcNAc β), type 2 (Gal β 1-4GlcNAc β), type 3 (Gal β 1-3GalNAc α) and type 4 (Gal β 1-3GalNAc β). In the type 1 pathway, the α -1,3-fucosyltransferases (encoded by FUT3 gene) adds a fucose residue to the Gal β 1-3GlcNAc β precursor at an α -1,3 linkage, leading to synthesis of trisaccharides of a Lewis A (Le^a) antigen, known as the non-secretor phenotype. In the secretor phenotypes, the α -1,2-fucosyltransferase (FUT2 gene) adds a fucose to the type 1 precursor at α -1,2 linkage, leading to creation of the H type 1 antigen. Further action on the H type 1 antigen by FUT3 or the A and B enzymes lead to biosynthesis of tetrasaccharides Le^b, A type 1, and B type 1, respectively. Antigen A

and B type 1 may be further transformed into the pentasaccharide A Le^b and B Le^b, respectively (Figure 1.5).

Figure 1.5 HBGA biosynthesis pathway based on the type 1 disaccharide precursor.

The entire pathways can occur on a type 2 precursor. The corresponding products Le^a and Le^b in type 1 are Le^x and Le^y for type 2, respectively. Abbreviations: Enzyme A, N-acetylgalactosamine transferase; enzyme B, galactosyltransferase; Fuc, L-fucose; FUT2, α -1,2-fucosyltransferase; FUT3, α -1,3/4-fucosyltransferase; Gal, D-galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine (Tan and Jiang, 2005).

Different NoV strains have variable HBGA-binding patterns. The hypervariable P2 that protrudes furthest from the capsid shell as well as some surface-exposed P1 residues are essential for specific ligand–receptor interactions. Two binding profiles have been distinguished for NoV: those that bind A or B and H antigens and those that bind Le or H antigens. However, studies with VLPs have shown that some strains such as GII.4 Farmington Hill are capable of binding antigens belonging to both groups (Donaldson et al 2010). Specific binding patterns are not genotype or genogroup exclusive, and it has been demonstrated

that a single amino acid replacement can change the binding pattern of the GII.4 VLPs. A comparison of various GII.4 VLPs (equivalent to NoV GII.4 strains from 1987 to 2006) showed that the evolution of this genotype relies on both antibody escape and receptor switching to persist in the human population. Strains of NoV GII.4 are predominant worldwide due to their ability to bind A, B, and O secretors, which represents 80% of the human population (Lindesmith et al 2003).

1.5 Molecular epidemiology of Norovirus

1.5.1 Distinct properties of NoV GII.4 genotype

Different NoV genotypes and genogroups have distinct properties regarding transmission, prevalence, and host genetic factor. The FBVE network published data on NoV outbreaks in Europe over 5-year period (Kroneman *et al.*, 2008) revealing that NoV GII.4 genotype caused the majority of person-to-person outbreaks, whereas a relatively high number of foodborne outbreaks were caused by non GII strains (Figure 1.6). In healthcare settings, NoV GII.4 was responsible of 80% of all outbreaks; however, in day care centres and schools NoV GI genotypes were found more frequently (Kroneman *et al.*, 2008). In a systematic review, it was found that waterborne outbreaks were significantly more likely to be caused by NoV GI genotypes (Matthews *et al.*, 2012). It has been proposed that NoV GI genotypes are more resistant to environmental factors than NoV GII (Nordgren *et al.*, 2009), therefore their prolonged survival in the water environment may facilitate waterborne transmission.

Figure 1.6 Distribution of transmission pathways between GII.4, GII non 4 and non GII NoV genotypes.

Modified from (Kroneman et al., 2008).

Reports of NoV gastroenteritis outbreaks follow a clear winter seasonality in northern hemisphere; however an off-season peak was reported across Europe in spring 2002 (Kroneman *et al.*, 2008, Lopman *et al.*, 2009, and Mounts *et al.*, 2000). Winter and healthcare-related outbreaks are more likely to be caused by NoV GII, especially genotype 4 (Kroneman *et al.*, 2008 and Matthews *et al.*, 2012). In general, NoV GII.4 is associated with lower attack rates in foodborne outbreaks when compared with other genotypes (Noda *et al.*, 2008). In contrast to person-to-person outbreaks, shellfish-related outbreaks are associated with the detection of multiple NoV GI and GII genotypes in both shellfish and faecal samples (Le Guyader *et al.*, 2012 and Matthews *et al.*, 2012). Based on NoV sequencing data collected over a few years from various outbreaks, it was demonstrated that NoV genotype profiles can be used to differentiate transmission modes (Verhoef *et al.*, 2010). Currently, online tool exists and provides an estimate of the probability that an outbreak was caused by foodborne transmission (Verhoef *et al.*, 2009 and Verhoef *et al.*, 2011).

NoV GII.4 has caused 60-80% of all reported outbreaks in the last ten years in the United States, Europe, and Oceania (Kroneman *et al.*, 2008) (Siebenga *et al.*, 2009). The pandemic outbreaks observed since the mid-90s have been due to the emergence and spread of GII.4 variants (Lindesmith *et al.*, 2008). To date, it has been confirmed that four variants of NoV GII.4 such as the Grimsby 1996, the Farmington Hills 2002, the Hunter 2004 and the Den Haag 2006b have had a global distribution (Figure 1.7) (Noel *et al.*, 1999 and Siebenga *et al.*, 2009). Although some of GII.4 variants such as 2003 Asia or Yerseke 2006a caused epidemics, they were geographically limited (Siebenga *et al.*, 2009). At the beginning of 2010, the GII.4 Den Haag 2006b was displaced by a new variant of GII.4 New Orleans 2009 in the USA (Vega, 2011) and Europe (Mathijs *et al.*, 2011, Rajko-Nenow *et al.*, 2013, and Van Beek *et al.*, 2012). Such displacement is a feature of NoV GII.4 epidemiology (Hansman *et al.*, 2010) however it is not yet clear why NoV GII.4 variants are so successful in causing the pandemics. Lindesmith *et al.* suggested that the surface-exposed carbohydrate ligand binding domain in the NoV capsid is under heavy immune selection and most likely evolves by antigenetic drift to avoid short-term herd immunity (Lindesmith *et al.*, 2008).

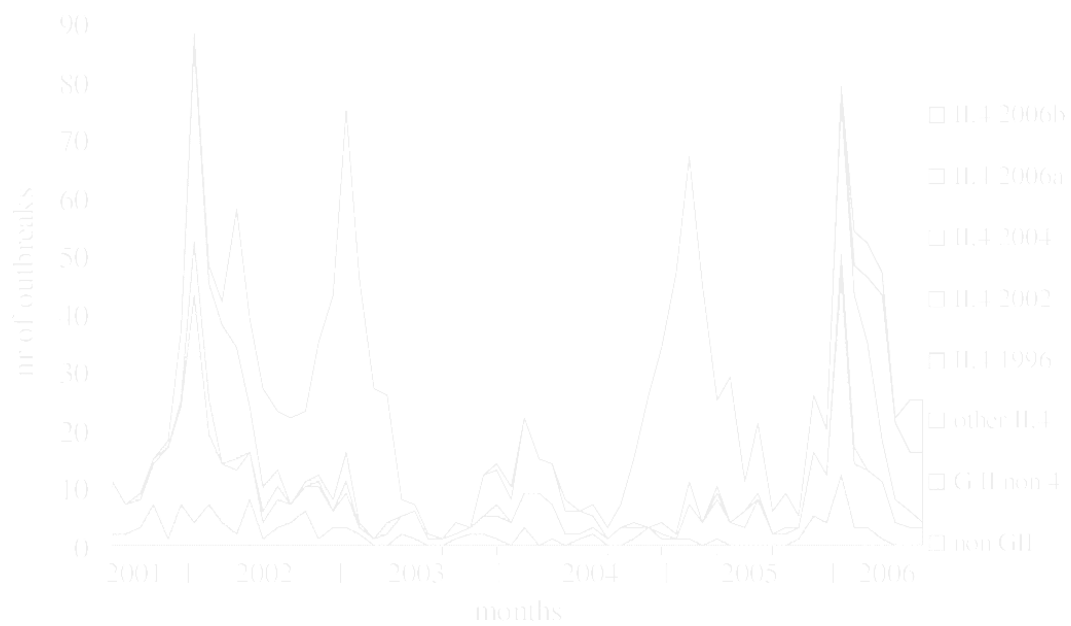


Figure 1.7 NoV genotypes and variants implicated in outbreaks in Europe 2001-2007.

Adopted from (Kroneman et al., 2008).

1.5.2 Reporting of NoV infections in Ireland

The national surveillance of NoV in Ireland is shared between the Health Protection Surveillance Centre (HPSC) that collects the epidemiological data on all reported outbreaks and the National Virus Reference Laboratory (NVRL) that provides a molecular diagnostic service. Faecal samples from patients of all ages are to be examined by the NVRL if an outbreak meets the following amended Kaplan criteria: (i) vomiting occurring in more than half of all patients, (ii) the average duration of illness is 12-60 h, (iii) an incubation period after exposure of 15-48 h, (iv) both healthcare providers and patients affected, and (v) the absence of bacterial pathogens (NDSC, 2003).

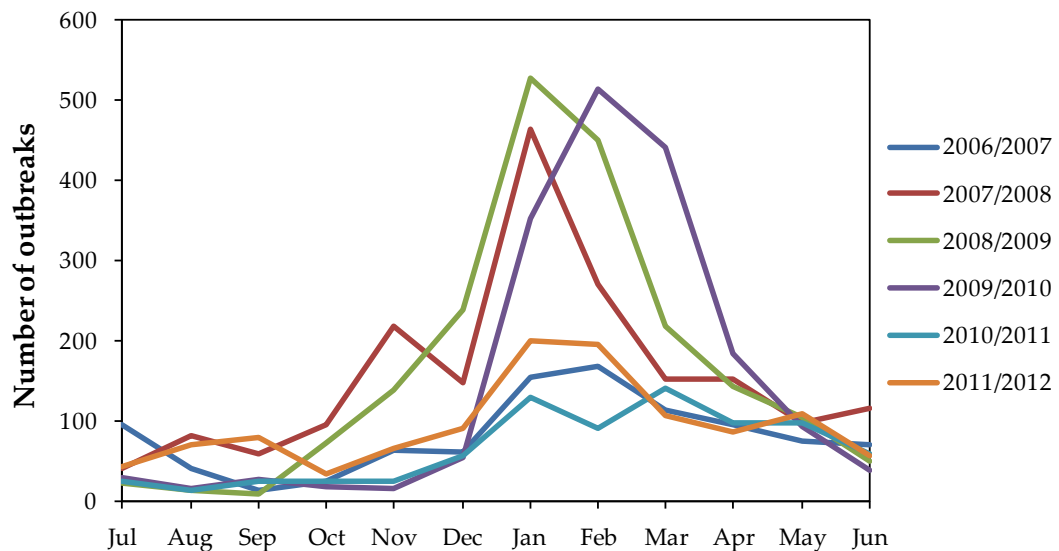


Figure 1.8 Seasonal distribution of confirmed outbreaks of norovirus, 2006-2012.

Based on data from (Cloak et al., 2012).

The vast majority of recorded NoV gastroenteritis cases is reported from healthcare settings and only limited data are available concerning food-related outbreaks. A variety of NoV GI and GII genotypes have been detected in influent wastewater suggesting that multiple genotypes are circulating in Irish population at any one time (Rajko-Nenow *et al.*, 2013). It has been demonstrated that a variety NoV genotypes (GII.2, GII.4, GII.6, GII.b, and GII.13) were responsible for acute gastroenteritis in Irish children ≤ 5 years, whereas only a single genotype of GII.4 was found in adults (Waters *et al.*, 2008). To date, the highest number of NoV outbreaks was reported in 2010 ($n=1789$) (Figure 1.8) and coincided with the emergence of NoV GII.4 New Orleans 2009 (Rajko-Nenow *et al.*, 2013).

1.6 Norovirus contamination in the water environment

Human NoV, similar to other enteric viruses, can be shed in more than 10^9 particles per gram of faeces (Atmar *et al.*, 2008), and therefore is frequently detected in municipal wastewaters. As NoV is quite stable in the environment, it is not completely removed during the wastewater treatment process (Flannery *et al.*, 2012); in consequence it is commonly detected in treated wastewater effluent and subsequently in aquatic environments such as, river water, groundwater, and marine water (Flannery *et al.*, 2012, Koh *et al.*, 2011, Mans *et al.*, 2012, and Wyn-Jones *et al.*, 2011). NoV can be detected in wastewater during the entire year; however, the concentration of NoV GI and GII is significantly greater over winter months as a consequence of the high frequency of NoV infection in the community (Flannery *et al.*, 2012). Outbreaks of shellfish-associated gastroenteritis have been attributed to the overflow of untreated sewage during high rainfall events contaminating harvesting areas (Doyle *et al.*, 2004). Recently, it has been shown that combined sewer overflow (CSO) effluents contain a higher percentage of infectious viruses than UV treated effluents, and thus CSO discharges may be an important cause of infectious viruses entering shellfish harvest areas (Flannery *et al.*, 2013a).

1.6.1 Norovirus surrogates

No cell culture system exists for human NoV thus its survival kinetics can only be estimated using molecular methods. Although the RT-PCR methods are sensitive and specific, they do not provide information on virus infectivity (Nuanualsuwan *et al.*, 2002 and Pecson *et al.*, 2011). Therefore, viral surrogates such as poliovirus, feline calicivirus (FCV), MuNoV, hepatitis A (HAV) and F-specific RNA (FRNA) bacteriophage are widely used to predict the survival and inactivation of human NoV. Using molecular methods, Duizer *et al.* showed that human NoV is more resistant to heat, high and low pH, ultra-violet light and disinfectants than canine calicivirus (CaCV) and the respiratory FCV (Duizer *et al.*, 2004). As FCV has been shown to be sensitive to heat and pH, it is a poor surrogate for human NoV (Cannon *et al.*, 2006 and Duizer *et al.*, 2004), whereas MuNoV has shown to be stable

in the environment across a wide pH range (pH 2 to 10) (Cannon *et al.*, 2006). Most recently, it has been demonstrated that real-time RT-qPCR significantly overestimates the survival of FRNA bacteriophage GA in seawater when compared to a plaque assay and is therefore unsuitable for determining the inactivation rates of viruses (Flannery *et al.*, 2013b).

1.6.2 Survival of enteric viruses

Several factors play a role in the survival of human enteric viruses in the marine environment. At freezing or nearly freezing temperatures, viruses may remain infectious for many months. Gantzer *et al* (1998) demonstrated that in seawater, it took 671 days to inactivate 90% of poliovirus and HAV at 4°C, but only 25 days at 25°C. Ultraviolet light can inactivate viruses through direct damage to the nucleic acids leading to the formation of pyrimidine dimers or other photo-products (Lytle and Sagripanti, 2005). Sinton *et al* (2002) demonstrated that bacteriophage inactivation rates in the presence of sunlight are 10 times greater than in the dark. NoV GI, NoV GII and bacteriophage were both reduced to a greater extent under summer conditions (235 Wm⁻², 17°C) than winter (56 Wm⁻², 10°C) (Flannery *et al.*, 2013b). The association of enteric viruses with suspended solids often prolongs their survival of viruses in water environment (Gerba, 2005). It has been shown that poliovirus persists longer in filtered seawater or artificial water than unfiltered natural seawater (Wetz *et al.*, 2004).

1.6.3 Bioaccumulation and depuration of NoV in oysters

As previously mentioned, oysters may become contaminated with human pathogens when they are grown in wastewater-contaminated waters and can transmit NoV to consumers when eaten raw illness. Over a two-year survey, NoV was detected in the 76.2% of monthly oyster samples collected from 39 designated harvesting areas in the United Kingdom. However, concentrations of NoV were detected below the limit of quantification (<100 genome copies g⁻¹) of the RT-qPCR assay in 65.1% of NoV GI and 42.8% of NoV GII positive samples, respectively (Lowther *et al.*, 2012). During this study, 30% of the oyster samples had the NoV

concentrations of >1,000 genome copies g⁻¹ during the winter months (Lowther *et al.*, 2012). In another study at an experimental shellfishery, mean concentrations of NoV GI and GII were 26,915 and 69,183 genome copies g⁻¹, respectively in oysters that were placed adjacent to a WWTP outfall (January-March) (Rajko-Nenow *et al.*, 2013).

Oysters may bioaccumulate NoV from their surrounding waters within a few hours following the discharge of untreated wastewater (Flannery *et al.*, 2013a). Faecal bacteria concentrations in contaminated BMS are reduced rapidly and can be almost totally eliminated within 2 days when placed in clean seawater (Love *et al.*, 2010). However, NoV has frequently been detected in BMS after depuration of 23 hours (20°C) (McLeod *et al.*, 2009), 29 days (20°C) (Nappier *et al.*, 2008) and 10 days (10°C) (Ueki *et al.*, 2007) in clean seawater.

It has been shown that NV binds to a specific carbohydrate receptor present in shellfish digestive tissue, which appears to facilitate virus persistence inside the shellfish and may explain the inefficiency of the depuration process in reducing NoV concentrations (Le Guyader *et al.*, 2006 and Tian *et al.*, 2006). NoV GI.1 recognise type A-like HBGA ligand present on cells of the oyster digestive tissue and the expression of this ligand varies during the year, whereas NoV GII.4 recognise a sialyated carbohydrate motif expressed in various oyster tissues (Maalouf *et al.*, 2011 and Maalouf *et al.*, 2010). It has been also suggested that NoV GI strains persist better in oyster tissues than NoV GII due to its specific binding to A-like HBGA ligand, and therefore are often detected in oyster-related outbreaks (Le Guyader *et al.*, 2008 and Le Guyader *et al.*, 2012).

1.7 Detection and molecular characterisation

1.7.1 Electron microscopy

Traditionally, NoV was detected in faecal specimens where the viral particles were visualised using electron microscopy (EM). However, direct detection of NoV by negative straining technique is only possible when at least 10^6 virus particles are present per gram of faeces (Richards *et al.*, 2003); therefore, its application has been limited to stool samples only. NoV does not have the distinct calyx surface morphology of other SRSVs, such as Sapovirus (Caul and Appleton, 1982) and is therefore more challenging to identify. As a result highly trained personnel are required (Lopman *et al.*, 2002) to reliably detect NoV particles by EM. In immuno-EM, faecal samples can be visualized after reaction with antibody derived from convalescent phase sera from infected individuals with gastroenteritis. However, as NoV is constantly evolving, an antibody-antigen reaction for all genotypes is not guaranteed (Atmar *et al.*, 2008).

1.7.2 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of NoV antigen in faecal samples and is based on the use of hyperimmune anisera raised against recombinant NoV capsid proteins. The production of monoclonal antibodies towards recombinant VLPs allows the construction of a multivalent antibody panel with wide-ranging reactivities capable of detecting a broad-range of both NoV GI and GII genotypes. There are a few commercially available ELISA kits and these can be carried out in less than two hours and are cost-effective to perform. The IDEIA™ Norovirus ELISA test utilises wells coated with NoV GI and GII specific monoclonal antibodies and was recently compared to two RT-PCR methods. The IDEIA™ Norovirus ELISA test demonstrated a lower sensitivity (78.9%) in comparison to two RT-PCR methods (>90%), but equally high specificity (100%). These ELISA methods can be useful for large-scale testing in hospital settings to rapidly screen for viral gastroenteritis outbreaks rather; however, ELISA-negative samples should be confirmed with the RT-PCR assay (Kele *et al.*, 2011).

ELISA methods are generally fast sensitive and cheap, and are therefore widely used for NoV detection in stool samples. However, concentration of NoV in environmental samples such as wastewater, seawater and oyster are too low to be detected using ELISA method. Therefore, molecular-based techniques have become the most widely utilised method of detection for NoV in many types of samples as they are highly specific and sensitive

1.7.3 RT-PCR

RT-PCR is the most widely used technique for the detection of NoV in clinical samples; in food samples such as BMS, salads and soft fruits; in water samples, including drinking water, river water, seawater, and wastewaters and in swab samples from contaminated surfaces (Flannery *et al.*, 2012, Larsson *et al.*, 2013, Mans *et al.*, 2012, Verhaelen *et al.*, 2012, and Wyn-Jones *et al.*, 2011). The detection of NoV in foodstuff and other environmental samples such as wastewater is particularly challenging due to (i) low concentrations of virus particles found in these samples (ii) PCR inhibitors present in sample matrix, and (iii) the high genetic diversity within the NoV genus.

Recently, the European Committee for Standardization (CEN) working group (TC275/WG6/TAG4) has developed a standardised method for determination of HAV and NoV in foodstuff using real-time quantitative RT-PCR (Lees, 2010). In general, low virus concentrations in environmental samples (such as soft fruit, salad vegetables, bottled water, BMS, and wastewater) can be detected by applying a virus concentration procedure prior to the detection method. Adsorption-elution methods, ultracentrifugation, protein K digestion and polyethylene glycol precipitation are frequently used concentration methods for the NoV detection from environmental samples. To remove PCR inhibitors such as polysaccharides and glycogen present in the shellfish matrix, viral RNA is extracted through lysis of capsid with guanidine thiocyanate and preferential adsorption of RNA onto silica (Schrader *et al.*, 2012). In the CEN method, the real-time RT-PCR primers amplify a short sequence (~100 bp), located at the ORF1/ORF2 junction, which is the most conserved part of the NoV genome.

1.7.4 Genotyping

Genotyping of NoV is challenging due to the genetic variability of the virus and the need for sufficient genetic material. Food and environmental samples frequently contain a low viral contamination and the amplification of long fragments of NoV genome from these matrices can be problematic while this is not the case for clinical samples that usually contains high quantities of virus (Baert *et al.*, 2011 and Stals *et al.*, 2012). The real-time RT-qPCR product is widely used for the NoV detection in all sample types. However, as the RT-qPCR assay targets the most conserved region of NoV genome and generates a relatively short product, it is not suitable for NoV genotyping. Therefore, a conventional RT-PCR targeting larger and more variable region of NoV genome is used to distinguish between different NoV genotypes.

Currently, five regions of NoV genome have commonly been targeted for genotyping purposes (A-E) (Figure 1.9). Region A and B are amplified by primers sets targeting the RNA polymerase gene, and regions C, D and E are amplified by primers targeting the major capsid protein VP1 gene. Region A and C are the most widely used regions for genotyping (Vinjé *et al.*, 2004 and Vinjé *et al.*, 2003). Simultaneous genotyping of regions A and C can be useful in identifying NoV recombinants. It has been demonstrated that genotyping based on the capsid gene (region C and D) allows for better differentiation between NoV strains than genotyping based on the polymerase gene. The best discrimination between NoV strains can be achieved if full length capsid sequencing is used. However, it has been shown that region D is suitable for identifying new GII.4 variants and it is widely used by diagnostic laboratories (Mattison *et al.*, 2009).

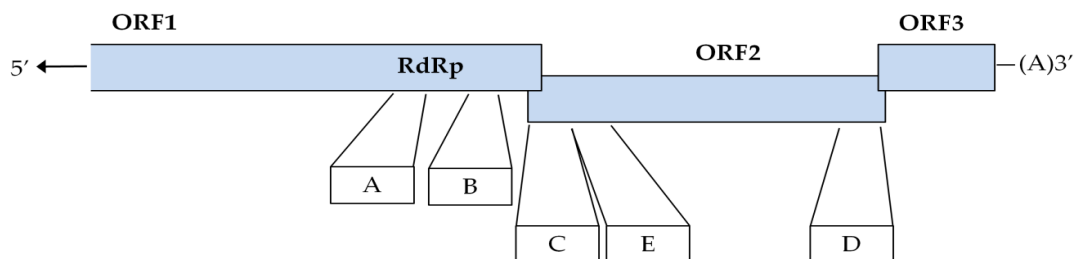


Figure 1.9 Region (A-E) of NoV genome commonly used for genotyping.

RdRp: RNA-dependent RNA polymerase (Mattison et al., 2009).

Recently, the norovirus working group (Noronet) demonstrated that the pairwise distance cutoff method (Zheng *et al.*, 2006) is no longer a suitable approach to classify NoV (Kroneman *et al.*, 2013). A separate nomenclature has been proposed for the polymerase region (ORF1) and the capsid region (VP1) because of the importance of the emergence of new recombinants (Kroneman *et al.*, 2013). The recent changes in the NoV nomenclature include: (i) renaming of the GI.e, GII.b and GII.d polymerase clusters (ORF1) into GI.P9, GII.P21 and GII.P22, respectively (ii) new GII.4 variants will be named according to year and location of the first full-length capsid sequence in public domain, instead of the first year in which it became epidemic: e.g. GII.4 2010 is now GII.4 New Orleans 2009, (iii) a new genotype has been added: GI.9 (prototype: HQ637267, Norovirus Hu/GI/Vancouver730/2004/CAN), (iv) polymerase based genotypes are now written with a 'P': GII.P4 (Kroneman *et al.*, 2013). NoV genotyping tool is an open-access typing-tool that allows users to upload NoV nucleotide sequences >100 bp and assign a genotype based on new NoV nomenclature of the partial polymerase (ORF1) and the partial capsid region (VP1) of NoV genome (Kroneman *et al.*, 2011).

The NoV recombination hotspot is located at the junction between ORF1 and ORF2 of the NoV genome. Therefore, the recombinant virus can be identified when both the polymerase and capsid regions are sequenced and then compared with the reference strains of NoV. In order to exclude multiple infections or contamination within a sample, a PCR amplicon covering the ORF1/ORF2 junction should be generated, sequenced and aligned with the sequences of the putative parental strains to confirm recombination event (Stals *et al.*, 2012).

1.8 Genetic information

Phylogenetic analysis is used to reconstruct the evolutionary relationship between nucleotide sequences. The evolutionary history inferred or estimated from phylogenetic analysis is usually graphically displayed as a tree or treelike diagram. The individual sequences are known as taxa, and the nucleotide bases of the sequence are referred to as characters. The genetic code defines a mapping between tri-nucleotide sequences (codon) and an amino acid and is universal for nearly all organisms with some exceptions such as the human mitochondria (Barrell *et al.*, 1979). The universal genetic code is usually represented as RNA code (Figure 1.10) as it is the direct template for protein synthesis. There are 61 'sense' codons and 3 'non-sense' codons or 'stop' codons that signals a termination of translation. An open reading frame (ORF) that is able to encode for a protein starts with AUG codon (methionine) and terminates with one of the 'stop' codons (UAA, UAG, UGA). The genetic code is degenerated, since all amino acids except methionine and tryptophan are encoded by more than one codon. The genomes of the majority of viruses have overlapping genes as a result they can encode two or more proteins from a single nucleotide sequence utilising multiple reading frames.

1.8.1 Mutations within the genetic material

The duplication of the genetic material is generally carried out with a high level of precision. However, point mutations can occur during the replication process when the DNA or RNA polymerase incorrectly incorporates a non-complementary nucleotide. Additionally, the structure of nucleic acid may be damaged by modification of the bases, which can be induced by exposure to oxygen free radicals, ultraviolet or ionizing radiation and various chemicals. If the point mutation occurs within the protein coding sequence of a gene, it can have an impact on the phenotype of an organism (Allison, 2007). The term point mutation also includes insertions or deletions of a single base pair (indels).

5'end	Second letter of the codon								3'end	
	U		C		A		G			
First letter of the codon	U	UUU	Phy	UCU	Ser	UAU	Tyr	UGU	Cys	U
		UUC	Phy	UCC	Ser	UAC	Tyr	UGC	Cys	C
		UUA	Leu	UCA	Ser	UAA	STOP	UGA	STOP	A
		UUG	Leu	UCG	Ser	UAG	STOP	UGG	Trp	G
	C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
		CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	C
		CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A
		CUG	Leu	CCG	Pro	CAG	Gln	AGG	Arg	G
	A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
		AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	C
		AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	A
		AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg	G
	G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
		GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	C
		GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A
		GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	G

Figure 1.10 The universal genetic code.

A point mutation involving a substitution of a purine for another purine (A, G) or a pyrimidine by another pyrimidine (C, T) is known as transitions. In contrast, transversions are interchanges of purine for pyrimidine bases, which therefore involve exchange of one-ring for two-ring structures or vice versa (Figure 1.11) (Salemi and Vandamme, 2009). Transversions are less common than transitions due to chemical and steric reasons. In the human genome, transitions appear twice as often as transversions (Allison, 2007). Single nucleotide substitutions may or may not have an effect on the phenotype; it depends on whether a significant change in an amino acid sequence of a protein is produced.

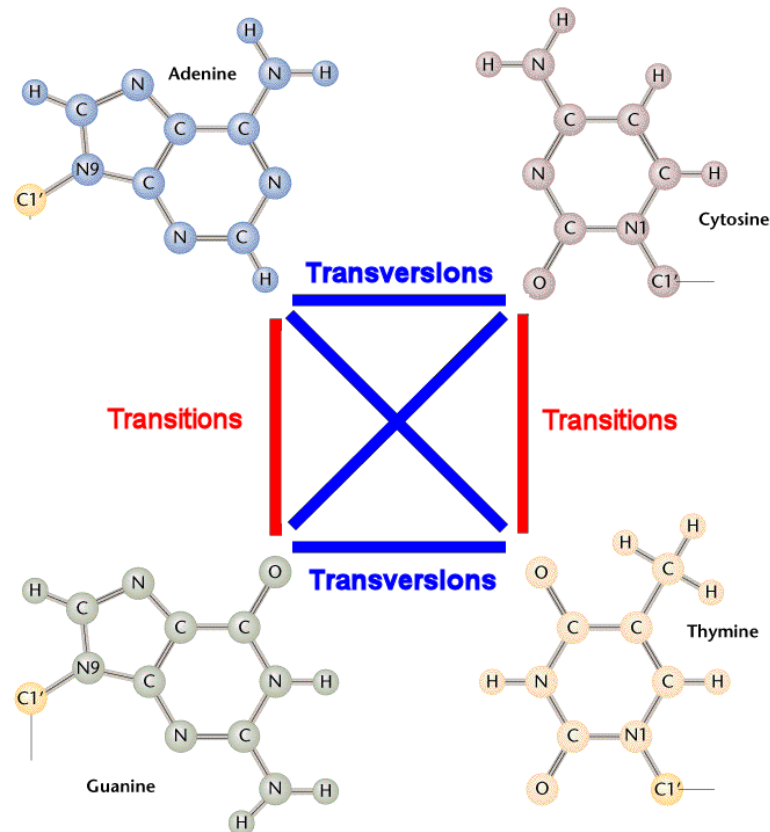


Figure 1.11 The schematic model of DNA substitution between the four organic bases (Carr, 2013).

A point mutation at the 3rd position of the codon usually does not lead to an amino acid substitution. In contrast, the first position is more conserved and point mutations usually result in an amino acid change (Salemi and Vandamme, 2009). Mutations that change the nucleotide sequence but do not alter the amino acid sequence are called silent or synonymous mutations (Figure 1.12). When a mutation results in the incorporation of a different amino acid, it is called a nonsynonymous mutation or missense mutation. The non-conservative missense mutation can have a functional consequence in the protein if this missense-mutation affects the catalytic site of an enzyme or drastically alters the configuration of the protein (McConkey, 2004). Nonsense mutations occur when the substitution converts a coding triplet into one of the 'stop' codons and can truncate the protein resulting in non-functional product.

A frameshift mutation is caused by either an insertion or a deletion of a number of nucleotides that is not evenly divisible by three. This can disrupt the

reading frame for mRNA translation leading to either a completely different translation from the original or it can lead to a termination signal. Indels include deletions, insertions, and a combination of both thereof and are less common in coding regions than in non-coding regions. A frameshift mutation, when occurring in coding regions, can lead to the acquisition of new gene function.

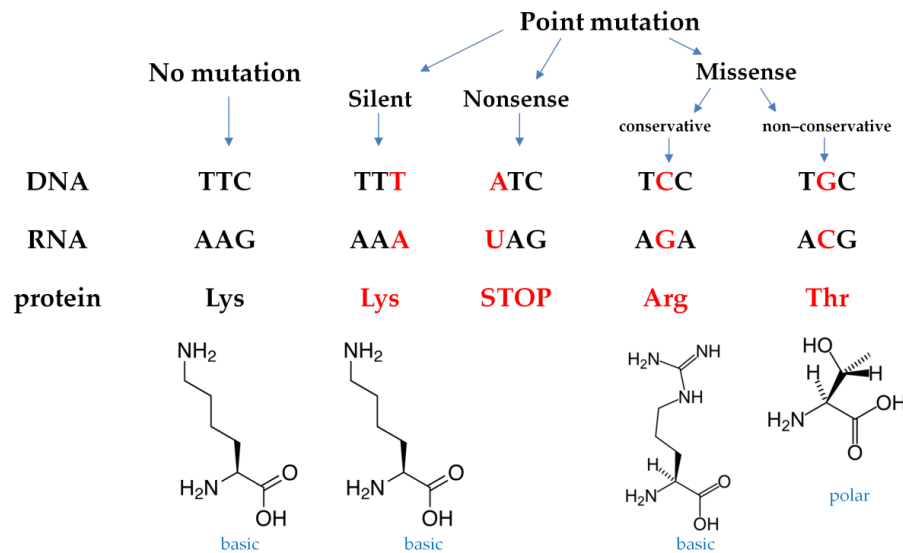


Figure 1.12 Influence of point mutation amino acids composition of protein.

Another type of mutation occurs when two separate strands of nucleic acid combine to form a new single strand which is called recombination. In viruses, recombination occurs when at least two related viral genomes co-infect the same host cell. In viruses with segmented genomes, the segments can be reshuffled during superinfection and progeny virus can receive two segments from different ancestor viruses. This process is called reassortment (Cann, 2001). In 2009, the reassortment of avian, human, and swine influenza viruses resulted in the emergence of pandemic influenza (H1N1) strain causing deaths in 91 people (Trifonov *et al.*, 2009).

1.8.2 Nucleic acid sequence databases

Before constructing a phylogenetic tree, it is important to find and retrieve nucleotide sequences that can be used to build the dataset. There are two basic types of search strategies for finding a set of related sequences, namely by keyword search

(e.g. sequences of known properties) and by similarity (e.g. looking for sets of homologous sequences).

The main search engines for keyword searching are Sequence Retrieval System (SRS) and Entrez. SRS is a powerful tool that was developed by T. Etzold at the EMBL-EBI (Hinxton, UK). Entrez, maintained at the NCBI, provides access to following databases simultaneously: Nucleotide (nucleic acid sequences data), Protein (protein sequences data), MEDLINE (database with abstracts from the medical literature), Structure (3-D structure data), Genome (genomic maps for each chromosome or genetic element), PopSet (multiple sequence alignments of DNA sequences of different populations or species), OMIM (human genes and genetic disorders) and Taxonomy (the names of all organisms in the genetic databases).

The most widely used technique for similarity searching is the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). The BLAST algorithm breaks the query sequences into a small subset of letters, known as query words. Each query word is then compared against the scoring matrix to determine the additional words (neighbourhood words) that match quite closely to the original query word. The neighbourhood words with a score higher than the neighbourhood score threshold are extended in both directions to construct a local alignment. For each local alignment, the cumulative score is calculated by taking into account both gaps and substitutions. The resulting alignments, called the high-scoring segment pairs (HSPs), are reported in the table with the cumulative score and E values. The E value represents the number of the HSPs that would be expected purely by chance, so the lower it is the greater the biological significance of the result.

In addition to general databases, the secondary databases (specialised databases) are used in searching in a well-defined set of sequences. In the specialised databases, sequence is frequently accompanied by a high quality annotation that allows for faster, more specific search (Salemi and Vandamme, 2009). The specialised databases are frequently used to enable rapid exchange of epidemiological information worldwide. Sequences of human viruses such as HIV, influenza, hepatitis A, B, and C and NoV can be submitted to or retrieved from specialised databases. Noronet is a specialised database for NoV that focuses on the

emergence and distribution of NoV GII.4 variants, preparation of a standardised NoV nomenclature and setting up typing library (RIVM, 2013).

1.8.3 Sequence alignment

After generating the sequence dataset, multiple alignment of homologues (derived from common ancestry) can be constructed. The basic dot-plot method can be used to visualise similarity in two large genomic fragments. In the dot matrix, the characters of sequences are represented by columns (sequence 1) and rows (sequence 2) and the dot is placed in the case of identical residues. If a region of high similarity appears, it will be shown by a diagonal line in the plot. Figure 1.13 shows the dot plot for the ORF2 of NoV GI and GII created in MegAlign (Lasergene, DNASTar).

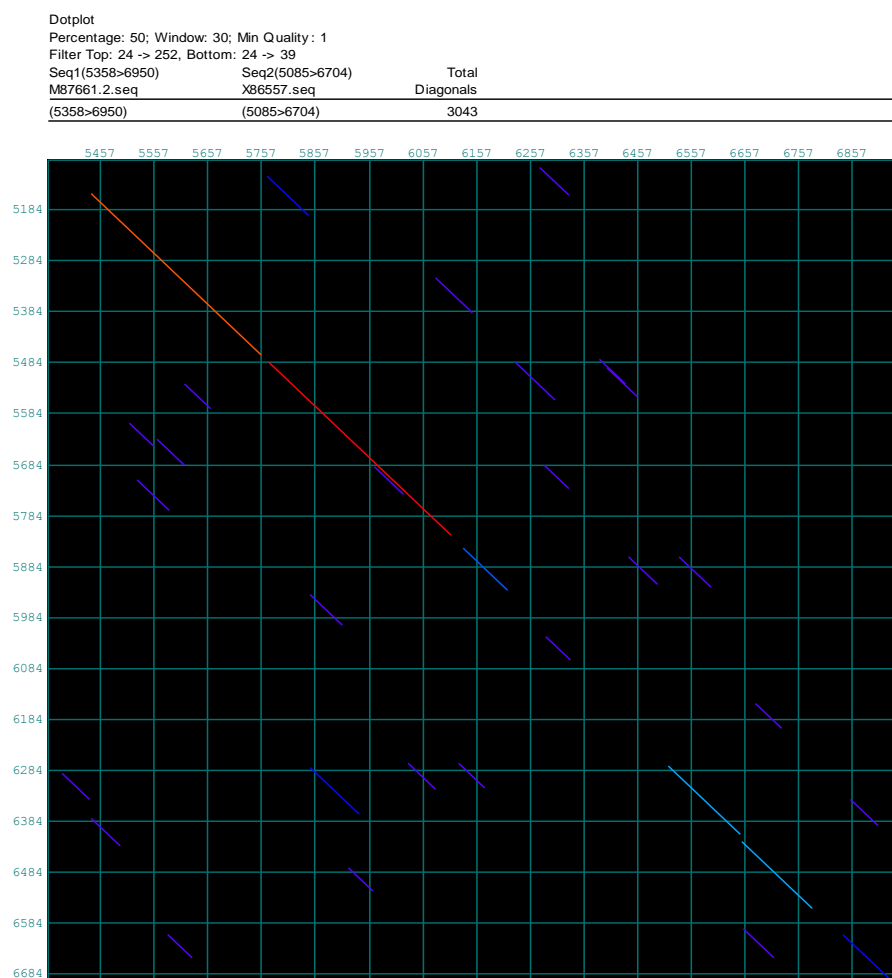


Figure 1.13 Dot-plot of two nucleotide sequences representing the ORF2 of NoV genome: Norwalk GI (M87661.2) and Lordsdale GII (X86557).

The colour of the line reflects the degree of similarity between two NoV sequences, taking into account both the percent match and the match length. Blue indicates the weakest matches and red the strongest.

Two sequences can be aligned by the dynamic programming method (Needleman and Wunsch, 1970, Gotoh, 1982), which guarantees to find the alignment with the highest score. Optimal alignment is calculated by scoring all possible pair of aligned residues, using a weight matrix, and penalising the gaps. The formula used for scoring the gaps is called the affine gap penalty and follows: $GP = G + Ln$. Where, G is a gap opening penalty, L is a gap extension penalty and n is the length of the gap (Baxevanis, 2005). Theoretically, the dynamic programming approach can be used for multiple alignments; however, it is time-consuming and impossible for more than four sequences.

The progressive alignment is a heuristic method for multiple sequence alignments which build an alignment up stepwise. Firstly, the most closely related sequences are aligned (pairwise alignment) and then progressively the more dissimilar ones are added. During this process, the distances are calculated and a guide tree is constructed using the neighbour-joining (NJ) clustering method (Saitou and Nei, 1987). The NJ tree then determines the order in which the alignment is progressively built up. Once the gaps are introduced between the two closest sequences in the alignment, they can only be enlarged, but not removed. The progressive alignment does not guarantee the optimal alignment, mistakes can occur during the early alignment which can not be corrected later. ClustalW (Thompson *et al.*, 1994) and ClustalX (Thompson *et al.*, 1997) programs carry out the alignment of sequences using the progressive alignment approach. Both can be downloaded freely from the EMBL/EBI server (<http://www.ebi.ac.uk/Tools/msa/>). Additionally, a Clustal algorithm can be run as a subroutine of the BioEdit and the multiple alignments can be improved by manual editing.

1.9 Models of evolution

DNA sequences that derive from a common ancestor change in the course of time as they evolve independently under the influence of different evolutionary forces. Evolutionary distance between these aligned sequences can be simply measured by counting the number of sites where they differ, which is known as the observed distance or p -distance. If 12 sites are dissimilar in two aligned sequence that are 100 base pair long, then $p=12\%$ or 0.12. However, the p -distance approach can underestimate the number of substitutions per site that have actually occurred. This is due to multiple substitution events at the same site (e.g. A to G to C) or a back mutation (e.g. A to C to A) that results in hiding some occurring changes. Figure 1.14 shows the relationship between expected genetic distance d (true genetic distance) and observed p -distance (observed distance). The linear correlation between p -distance and genetic distance d is observed for highly similar sequences and as evolutionary time goes by; the p -distance reaches saturation level and is no longer proportional to the genetic distance. To estimate the true genetic distance that can not be observed, mathematical methods are needed to infer it from the data (Salemi and Vandamme, 2009).

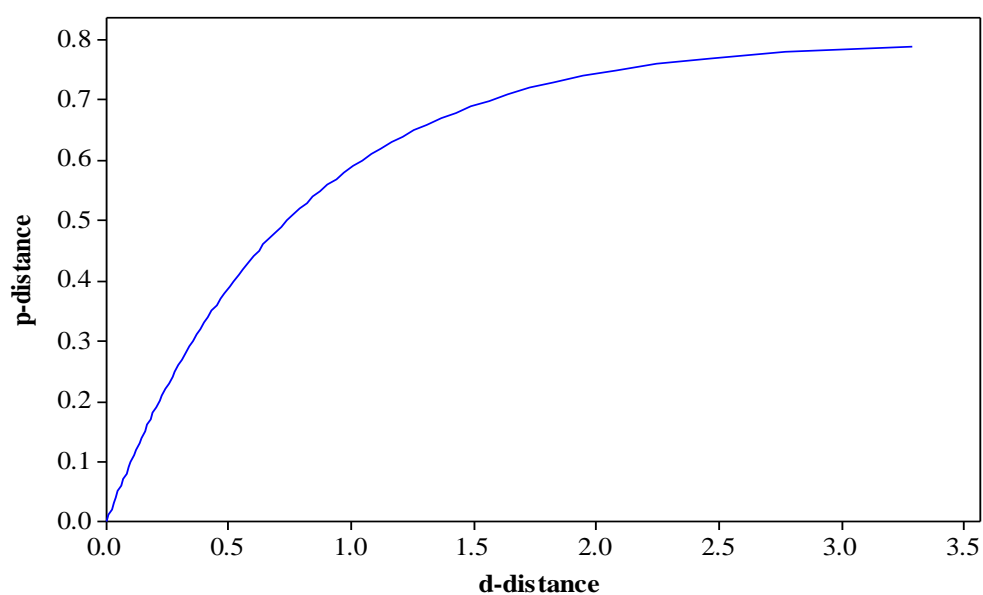


Figure 1.14 Relation between expected genetic distance d and observed p -distance.

1.9.1 Markov models of nucleotide substitution

The number of nucleotide substitutions in DNA sequences can be estimated by the Markov process model. This probabilistic model uses instantaneous rate matrix Q , shown on Figure 1.15, to specify the relative rates of changes of each nucleotide along the sequence. Markov models belong to time-homogeneous, time-continuous and stationary models that are based on the following assumptions:

- For any site in the sequence, the rate of change from base i to base j is independent from the base that occupied that site before i . 'the future does not depend on the past' (Markov property)
- The rates of substitution does not change over time (homogeneity)
- The relative frequencies of nucleotide bases are at equilibrium ($\pi_a, \pi_c, \pi_g, \pi_t$) (stationary)

$$Q = \begin{matrix} & \begin{matrix} \text{A} & \text{C} & \text{G} & \text{T} \end{matrix} \\ \begin{matrix} -\mu(a\pi_c + b\pi_g + c\pi_t) \\ g\mu\pi_a \\ h\mu\pi_a \\ j\mu\pi_a \end{matrix} & \begin{matrix} a\mu\pi_c \\ -\mu(g\pi_a + d\pi_g + e\pi_t) \\ i\mu\pi_c \\ k\mu\pi_c \end{matrix} & \begin{matrix} b\mu\pi_g \\ d\mu\pi_g \\ -\mu(h\pi_a + j\pi_c + f\pi_t) \\ l\mu\pi_g \end{matrix} & \begin{matrix} c\mu\pi_t \\ e\mu\pi_t \\ f\mu\pi_t \\ -\mu(i\pi_a + k\pi_c + l\pi_g) \end{matrix} \end{matrix}$$

Figure 1.15 The Q matrix. The instantaneous substitution rate from nucleotide i to nucleotide j is represented by an exact position in the matrix.

The letter a, b, c...l describes the relative rate of each nucleotide substitution to any other (Figure 2.6) and μ describes nucleotide substitution per site per unit time. The nucleotide frequencies are presented by π_a, π_c, π_g , and π_t . Furthermore, time-reversible Markov models, which are the only models considered in this thesis, assume the equal rate of chance from nucleotide i to nucleotide j and inversely ($a=g, b=h, c=j, d=i, e=k, f=l$). Using the specified model of evolution and Q matrix, it is possible to compute the probability of the replacement of nucleotide i by nucleotide j after a certain time of evolution. When probabilities for all possible replacements are known, the expected genetic distances between two sequences can be calculated

according to the evolutionary models specified by the Q matrix (Salemi, Vandamme et al. 2009).

1.9.2 Nucleotide substitution models

The simplest JC69 substitution model (Jukes and Cantor, 1969) assumes that the equilibrium frequencies of the four bases are equal ($\pi_a = \pi_c = \pi_g = \pi_t = 0.25$) and that all kinds of substitutions are equally likely to occur ($a = b = c = d = e = f = 1$) i.e there is zero free parameters in the Q matrix. Some of the commonly used models of evolution are outlined in the Table 1.7 with the summary of parameters. The Q matrix can be specified up eight parameters some of which are provided below:

- base frequencies
 - equal if $\pi_a = \pi_c = \pi_g = \pi_t$,
 - unequal if $\pi_a + \pi_c + \pi_g + \pi_t = 1$, all the bases frequencies are estimated from the data;
- nucleotide substitution rates
 - all types of substitution are equally likely to occur if $a = b = c = d = e = f = 1$
 - if the nucleotide substitution rates are unequal, $a + b + c + d + e + f = 1$, total number of five free parameters exists, ($a = A$ to C , $b = A$ to G , $c = A$ to T , $d = C$ to G , $e = C$ to T , $f = G$ to T).
 - the nucleotide substitution can be divided into transversions ($T_v = \{a, c, d, f\}$) and transitions ($T_s = \{b, e\}$, where purine transitions $T_{sr} = \{b\}$ and pyrimidine transitions $T_{sy} = \{e\}$). These can be expressed by two independent rate parameters out of five:
 - the transition transversion ratio $\kappa = T_s/T_v$
 - the purine transitions pyrimidine transitions ratio $\gamma = T_{sr}/T_{sy}$

Table 1.7 Nucleotide substitution models.

Model	References	Parameters in the Q matrix		
		Free parameters	Nucleotide frequencies	Substitution rate
JC69	(Jukes and Cantor, 1969)	0	equal	$a=b=c=d=e=f$
K80	(Kimura, 1980)	1	equal	$a=c=d=f, b=e$ (ratio κ)
F81	(Felsenstein, 1981)	3	unequal	$a=b=c=d=e=f$
HKY85	(Hasegawa <i>et al.</i> , 1985)	4	unequal	$a=c=d=f, b=e$ (ratio κ)
F84	(Thorne <i>et al.</i> , 1992, Felsenstein, 1993)	4	unequal	$a=c=d=f, b=e$ (ratio κ)
TN93	(Tamura and Nei, 1993)	5	unequal	$a=c=d=f, b,e$ (ratio κ and ratio γ)
GTR	(Tavaré, 1986)	8	unequal	$a+b+c+d+e+f=1$

1.9.3 Rate heterogeneity among site – the gamma distribution

By analysing the real data (Fitch and Margoliash, 1967), it has been revealed that nucleotide substitution occurs at variable rates across majority of genes. Rate heterogeneity has an important impact on the inference of genetic distances when carrying out the phylogenetic analysis. Site-dependent rate mutation is commonly incorporated into models of nucleotide evolution by the use of a gamma (Γ) distribution (Figure 1.16). In this approach, the mean of the distribution is equal to 1 and the variance to $1/\alpha$. The shape parameter can be calculated and incorporated into the model of evolution. For $\alpha \leq 1$, the distribution is L-shaped with strong rate heterogeneity, meaning that most sites have very low substitution rate (invariable) apart from a few sites with high rates. For $\alpha > 1$, the distribution is bell-shaped with weak rate heterogeneity, meaning that the most sites have intermediate rates in spite of a few site that have very low or very high substitution rates. By adjusting the parameter α , the gamma model accommodates different levels of heterogeneity in a variety of data sets (Yang, 1996).

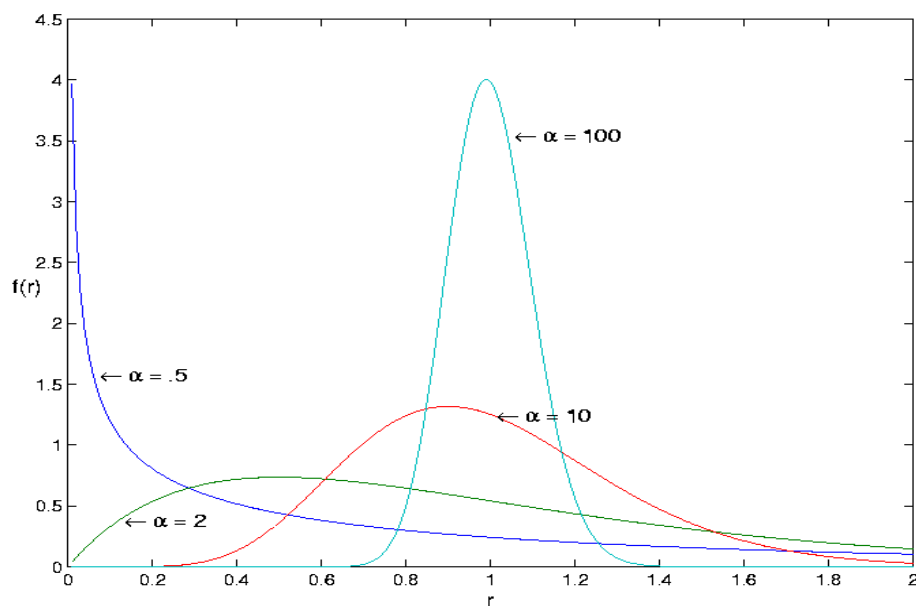


Figure 1.16 Density function, $f(r)$, of the gamma distribution of substitution rates at sites (r).

The α parameter determines the shape of the Γ distribution and the degree of rate heterogeneity (Gowri-Shankar, 2003).

1.10 A phylogenetic tree

The evolutionary relationships between different taxa can be illustrated by a diagram known as a phylogenetic tree (Figure 1.17) (due to its resemblance to a tree). Each phylogenetic tree can be made up of nodes and branches. A node is a point at which branches diverge from a line on the tree. External (terminal) nodes correspond to the comparable taxa (e.g. virus strains of a single species) from which the tree was derived and are also known as operational taxonomic units (OTUs). Branches join the external nodes indirectly via internal nodes (also referred to as hypothetical taxonomic units or HTUs), which correspond to the hypothetical progenitors of the OTUs.

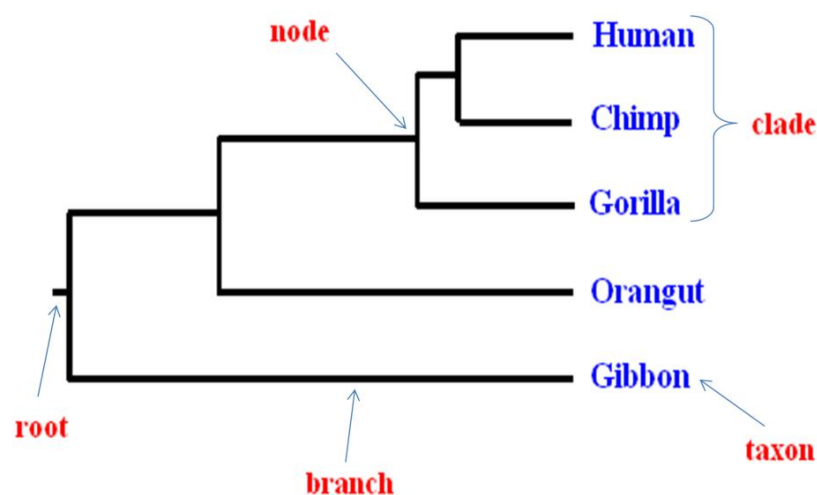


Figure 1.17 Components of a phylogenetic tree.

The nucleotide sequences of the TRIM5 α gene from different primate species were aligned by ClustalW method.

The various taxa that are connected by branches and diverge from the same node are called a clade or a monophyletic group. This group originates from a single common ancestor and has inherited a set of unique common traits/characters. Cladogram illustrates the relationship between taxa in which branch length does not have any meaning. While, phylogenetic tree (phylogram) represents the

inferred/estimated evolutionary relationship among taxa in which lengths of the branches correspond to the amount of character change (evolution) (Baldauf, 2003).

Phylogenetic trees can be either unrooted or rooted. Unrooted phylogenetic trees illustrate relationships between individual taxa only, without indicating which node represents the common ancestors for all OTUs. A rooted phylogenetic tree represents the divergence of a group of taxa from their most common ancestor, the root. The tree can be rooted when the OUT relatively distant from the rest of the data (known as an outgroup) is included into the data analysis. As an alternative, the midpoint rooting method can be used to set the root in the middle of the tree between the two most divergent OTUs (Baldauf, 2003).

1.10.1 Tree building methods

Methods used for calculating phylogenetic trees can be divided into distance-based methods and character-based methods. Distance matrix methods are based on an explicit model of substitution and progress in two stages. Firstly, the dissimilarities are computed for all pairs of sequences and these are then converted into evolutionary distances. The estimated values of the evolutionary distances depend on the evolutionary model that has been applied. Secondly, the matrix of distance values is used to generate a phylogenetic tree by using progressing clustering or minimum evolution (Baxevanis, 2005). The main distance based methods are cluster analysis (e.g the unweighted pair group method with arithmetic mean (UPGMA) or the neighbour joining (NJ)) and minimum evolution (ME). The UPGMA method uses unweighted distances and assumes a constant rate of evolution and all the taxa are equidistant from the root of the ultrametric tree (known as the molecular clock hypothesis). The NJ (Saitou and Nei, 1987) is commonly used to build the distance based tree due to its fast computational speed and relative success in finding a correct tree. In contrast to the UPGMA algorithm, the NJ does not assume molecular clock hypothesis and produces an unrooted tree. The optimality criterion in the ME method (Rzhetsky and Nei, 1992) relies on searching for an optimal tree with minimal sum of the branch lengths; however, this method can be applied to less than ten taxa.

Maximum parsimony (MP) is a character-based method which is not based on an explicit model of evolution. The MP method operates on a matrix of discrete character states, for example an alignment of DNA sequences in which states are the nucleotide bases. The principle of this method searches for a tree, or collection of trees, that require the minimum number of evolutionary steps to explain a given set of data. The most preferable is the simplest explanation of the character substitution in the set of sequences. When constructing a topology tree with the MP method, for a single sequence site, the minimum amount of substitutions required among the branches is inferred. The length of a full tree is computed as the sum of the scores for all the sites. A number of trees can be evaluated; however, the maximum parsimony tree is a tree with minimum number of required changes. The method is particularly sensitive to the 'long-branch attraction' and an inferred tree can differ from a true tree. Rapidly evolving taxa are often placed together on the same tree due to the high number of mutations even though they do not share a common ancestor.

Maximum likelihood (ML) is another character-based method that examines the different tree topologies. In the phylogenetic inference, the likelihood value is the probability of observing a set of aligned sequences under a proposed tree structure and specified model of evolution: $L = \Pr(\text{data} | \text{tree}, \text{model of evolution})$. The ML is derived for each base position in the alignment. The ML algorithms search for the optimal set of parameters (tree and model of evolution) that have the highest likelihood of producing the observed data. The likelihood of a number of tree topologies is calculated and a tree with the greatest score is considered the best one. The main advantage of ML methods is the statistical properties that allow different tree comparison and hypothesis testing. However, the calculation of the tree likelihood can be computationally intensive.

1.10.2 Evolutionary model selection

Phylogenetic reconstruction requires a number of assumptions about the process of DNA substitutions. These assumptions can be based on implicit models e.g. parsimony methods or explicit models e.g. distance and maximum likelihood

methods. To apply phylogenetic methods with confidence, one should select the evolutionary model that they rely on. The most appropriate model can be selected and justified by the use of statistically based methods. It is important to choose a model that is complex enough to explain the data, while bearing in mind that intricate models can be computationally intensive and might misestimate the parameters.

1.10.2.2 Likelihood Ratio Test

One measure of comparing the relative accuracy of two evolutionary models is the Likelihood Ratio Test (LRT). A more complex model is compared to a simpler model to check if it fits a particular dataset significantly better. The LRT statistic is calculated as follows:

where L_0 specifies the likelihood under the less complex model (the null hypothesis) and L_1 specifies the likelihood of the same data under the more complex model (the alternative hypothesis). When the null hypothesis is correct and compared models are nested, the LRT statistic is asymptotically chi-square distributed (χ^2) with q degrees of freedom, where q is the difference in the number of free parameters between two models. The two models are considered as nested when one is a special case of the other such as the Jukes-Cantor model (JC69) (the base frequencies are equal to 0.25) and the Felsenstein model (K81) (the base frequencies do not have to be equal). The LRT can be easily calculated for nested models only and can be carried out in a hierarchical manner where the schematic pathway begins with the simplest and progresses to the more complex models. The null model is rejected when the p value of the LRT is less than 0.01 based on a χ^2 distribution. A mixed χ^2 distribution can be used in some cases, when one of the parameters is fixed at its boundary (Salemi, Vandamme et al. 2009).

1.10.2.3 Akaike Information Criterion

Another approach for selecting the most appropriate model of evolution for a given data set is the Akaike Information Criterion (AIC) (Akaike 1974) that allows comparison of the competing models at the same time. The AIC is computed as:

where L_i is the maximum likelihood for the model i and K_i is the number of free parameters in the model i . To choose the best-fitting model, the AIC calculates the maximum likelihood of proposed models and penalizes each of them with respect to a number of parameters. Parameter-rich models obtain a larger penalty (K) than a simple one, keeping as much complexity in the model as needed. The model with the smallest AIC value is preferred. The main benefit of using the AIC is its ability to compare both non-nested and nested models simultaneously.

1.10.2.4 JModelTest

JModelTest (Posada, 2008) is an independent program used for the statistical selection of the best-fit models of nucleotide substitution. It utilises one of five different selection strategies, including hierarchical likelihood ratio test and the Akaike information criterion. It accepts the aligned sequences files in NEXUS format. JModelTest output file provides a block of commands in NEXUS format, which can be executed in PAUP* to specify the parameters of selected model.

1.10.3 Search for the tree that fits the data

The number of possible trees increases exponentially with the number of taxa included in analysis (Felsenstein, 1978). For small data sets (<10 taxa) exhaustive search may be used to find an optimal tree. It relies on evaluating all possible strictly bifurcating trees (each ancestral lineage gives rise to exactly two descendent lineages) to find global optimum topology (McCormack and Clewley, 2002). Heuristic search can be applied to large datasets; however, as it is hill-climbing algorithm it does not guarantee to find the global optimum tree (entrapment in local

optimum). Many branch-swapping methods are employed to evaluate trees. One of the branch-swapping methods available in PAUP* is tree bisection and reconnection (TBR) strategy (Lewis, 1998) that involves cutting one branch of a tree and then reconnecting two subtrees by creating a new branch that joins a branch on one subtree to a branch on the other (Salemi and Vandamme, 2009)

1.10.4 Bootstrapping

Bootstrap is a commonly used tree evaluation method that measures the reliability of an inferred phylogenetic tree. The main advantage of the bootstrap sampling technique is that it works with many phylogenetic methods e.g. distance, parsimony or maximum likelihood. This method performs in a two-step manner. Firstly, a column of characters is randomly sampled from an original set of sequences and placed in a new alignment. Next, another column is selected at random from the original set and added to the new alignment. This continues until the newly created data set has the same number of total characters as the original data set. When taking a random sample from the original alignment, each column of characters can be chosen with the same probability as any of the other columns (sampling with replacement). The process of creating the new data sets can be repeated hundreds of times. It is recommended to resample from 200 to 2000 times (Hedges, 1992 and Zharkikh and Li, 1992). In the second step, a tree is constructed for each newly created data set. The frequency with which a given branch of optimal tree is found in the replicate trees is the bootstrap proportion. This proportion can be used as a measure of the reliability of individual branches in the optimal tree. The bootstrap values are superimposed on the original tree and it has been shown by (Hillis and Bull, 1993) that bootstrap values greater than 70% usually correspond to the 'real' clade with very high probability.

1.11 Detecting recombination

Similarity plot analysis is used for detecting recombination events and compares the recombinant sequence with a number of reference sequences. The measure recorded is the similarity value between the query sequence (potential recombinant) and each of the reference sequences. Figure 1.18 shows the similarity plot for the recombinant sequence R. The similarity plot shows that R is the most similar to sequence A in the 5' end of the sequence and the most similar to sequence B in the 3' end of the sequence. Sequences C and D are not related to the recombinant query. Bootscanning is another method used for recombinant event detection. In this method, an alignment of reference sequences and a potential recombinant are sequentially divided into overlapping sub-regions (i.e. windows) for which a bootstrap value is computed. The bootstrap value is then plotted in a scatter plot using alignment coordinates on the x-axis and the bootstrap value on the y-axis. The advantage of bootscanning is that often a much greater switch is observed in comparison to that seen for the similarity value. This allows much more precise mapping of recombination points (Lole *et al.*, 1999 and Salemi and Vandamme, 2009).

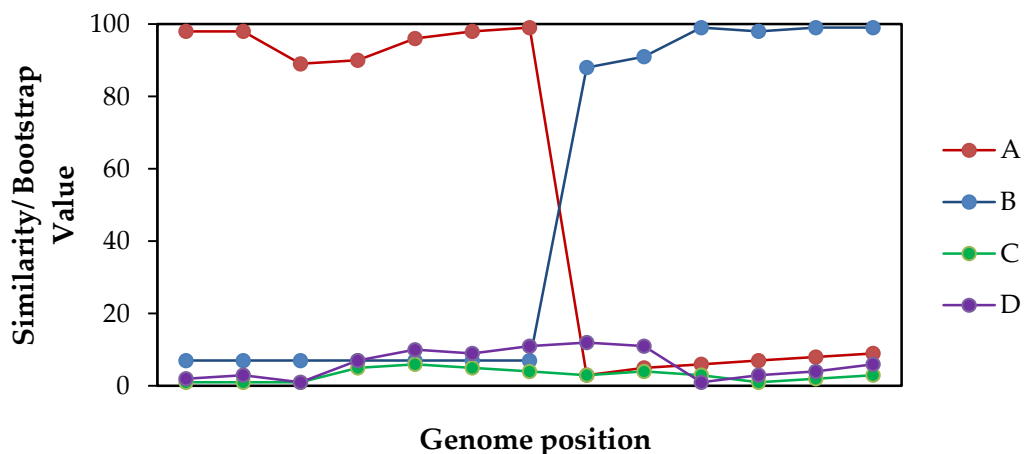


Figure 1.18 Similarity plot of recombinant strain R.

R is a recombinant of strains A and B.

1.12 References

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1.13 Scope of the thesis

The main objective of this research project was to investigate the diversity of NoV genotypes in oysters and relate these to NoV genotypes circulating in the population by analysing wastewater. A secondary objective was to determine the NoV genotype profiles in influent and effluent wastewater and in oyster samples to establish whether survival through the WWT and uptake by oysters vary for different NoV strains. A final objective was to determine NoV contamination (concentrations and genotypes) in oysters directly associated with gastroenteritis outbreaks in Ireland.

Chapter 2 outlines the methodology used during the course of this research project.

Chapter 3 involved a two-year investigation of a shellfish harvest area that has been closed due to previously being implicated in NoV gastroenteritis outbreaks. NoV GI and GII concentrations in oysters were quantified using RT-qPCR and characterised using nested RT-PCR, followed by cloning and sequencing. This established the NoV concentrations and genotype profiles present in the harvest area.

Chapter 4 Investigated the diversity of NoV GI and GII genotypes present in influent and effluent wastewaters during a period of high rates of NoV infections in the community in an attempt to establish a NoV contamination pathway for oysters. The work highlighted the highly diverse nature of the NoV genotypes present and the potential for the environmental spread of multiple NoV genotypes.

In **Chapter 5**, two separate oyster-related NoV outbreaks in Ireland were investigated. This study demonstrates that a range of NoV genotypes can be detected in highly contaminated oysters; however, not all appeared to be successful in causing NoV infections.

Chapter 6 outlines the principle conclusions from this research project along with future recommendations.

The author's publications and presentations are listed in **Appendix A**.

Chapter 2 Materials and Methods

2.1 Standard laboratory protocol

Good laboratory practice was adhered to at all times. To reduce the risk of potential contamination, all master mixes and solutions for molecular use were prepared in the separate laboratory (Clean room). The shellfish proteinase K extract and wastewater concentrate were prepared in the Bacteriology laboratory and RNA extraction was carried out in laminar airflow cabinet located in the separate room (RNA extraction lab). Gloves were worn at all times and nucleic acid free pipette tips were used to set up all reactions to prevent contamination. Real-time PCR standards were prepared, diluted and added to the 96-well plate in the Media Preparation laboratory using a specially assigned set of pipettes. Finally, to prevent carry-over of amplified DNA sequences, PCR reactions were set up in a separate area (PCR cabinet) to that used for cloning in the PCR room. To avoid cross contamination, each laboratory was distinguished by unique coloured rocks and lab coats.

2.1.1 ISO 17025 Laboratory accreditation

The National Reference Laboratory (NRL) for monitoring the bacteriological and viral contamination of bivalve shellfish is accredited by the Irish National Accreditation Board (INAB) to ISO 17025. ISO 17025 specifies all of requirements that testing laboratories must demonstrate to prove that they are technically competent to generate valid results. As a part of INAB accreditation the quality system and test methods are audited by independent technical experts to ensure compliance with the standard. The test method "Detection of Norovirus genogroups I and II bivalve shellfish by real-time reverse transcription polymerase chain reaction (RT-PCR)" was accredited by INAB in 2010. The NRL participates in proficiency testing organised by European Union Reference Laboratory (CEFAS) to compare performance of the RT-qPCR assay (ISO/TS 15216-1).

2.2 Reagent preparation

2.2.1 Solutions and buffers

1 M HCL

To make up 1 litre; 82 ml of 37% HCl (Sigma-Aldrich, UK) was added to a 1 l Schott bottle and then the volume was adjusted to 1 l with distilled water.

2.5 M MgCl₂

To make up 1 litre; 238.02 g MgCl₂ (Sigma-Aldrich, UK) was gradually added to a 1 l Schott bottle contains 0.5 l of distilled water. When all the solids had dissolved, the volume was adjusted to 1 l with distilled water. The solution was sterilised by autoclaving at 121°C for 15 min.

0.14 M NaCl

To make up 1 litre; 8.18 g NaCl (Sigma-Aldrich, UK) was added to a 1 l Schott bottle contains 0.5 l of distilled water. When all the solids had dissolved, the volume was adjusted to 1 l with distilled water and solution was sterilised by autoclaving at 121°C for 15 min.

50 mM glycine- NaOH (pH 9.5) with 1% beef extract buffer

To make up 1 litre; 3.75 glycine (Sigma-Aldrich, UK) and 10.0 g beef extract (Sigma-Aldrich, UK) were added to 900 ml distilled water in a 1 l Schott bottle. Then, the pH was adjusted to 9.5 with 1 M NaOH, the volume adjusted to 1 l with distilled water and the buffer was sterilised by autoclaving at 121°C for 15 min.

1 M NaOH

To make up 1 litre; 40g NaOH (Sigma-Aldrich, UK) was dissolved in 500 ml distilled water in a 1 l Schott bottle When all the solids had dissolved, the volume was adjusted to 1 l with distilled water and the solution was sterilised by autoclaving at 121°C for 15 min.

Proteinase K (100 µg/ml)

To make up 200 ml, 20 mg of proteinase K (30 units/mg, Sigma-Aldrich, UK) was added to 200 ml of molecular biology grade (MBG) water. It was shaken to dissolve and stored in working aliquots of 2 ml in 15 ml tubes at -20°C for maximum of 6 months.

TBE buffer (Tris-Borate-EDTA buffer)

To make up 2 l, 200 ml of 10 × concentrate of Tris-Borate-EDTA buffer (Sigma-Aldrich, UK) was added to 1800 ml of distilled water and mix by inversion.

2.2.2 Media for cloning**LB broth**

To make up 1 litre; 10 g of Tryptone (Oxoid), 5g of yeast extract (Oxoid) and 5 g of NaCl (pH 7.0) (Oxoid) was dissolved in 500 ml distilled water. Then, the volume was adjusted to 1 l with distilled water and the media was sterilised by autoclaving at 121°C for 15 min.

LB plates/ampicillin

To make up 1 litre; 15 g of Nutrient agar (Oxoid) was added to 1 l of LB broth, and then sterilised by autoclaving at 121°C for 15 min. The medium was allowed to cool (50°C) before the addition of 1 ml of ampicillin (100 mg/ml, Sigma-Aldrich, UK) to a final concentration of 50 µg/ml ampicillin. The media was poured into 85 mm petri dishes and stored at 4°C for a maximum of 30 days.

IPTG stock solution (100 mM)

MBG water (Sigma-Aldrich, UK) was added to 1.2 g of isopropyl-β-D-thiogalactopyranoside (IPTG, Promega, UK) to a final volume of 50 ml. The IPTG solution was filter sterilised and stored at 4°C.

X-Gal stock solution (50 mg/ml)

100 mg of 5-bromo-4-chloro-3-indolyl- β -d-galactoside (Promega, UK) was dissolved in 2 ml N,N'-dimethyl-formamide (Sigma-Aldrich, UK). The solution was covered in aluminium foil and stored at -20°C.

2.3 Polymerase chain reaction

2.3.1 Real-time reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was performed using RNA Ultrasense™ one-step quantitative RT-PCR system (Invitrogen, Carlsbad, CA) on an AB7500 real-time PCR instrument (Applied Biosystems, Foster City, CA). Five µl of sample RNA was added into 20 µl of the appropriate reaction mix (for the detection of NoV GI, NoV GII and Mengo virus) containing final concentrations of 1 × reaction mix, 12.5 pmol forward primer, 22.5 pmol reverse primer, 6.25 pmol probe, 1 × Rox and 1.25 µl of enzyme mix was added to the designated wells of the 96-well plate. The primers and probes used are listed in Table 2.1. The plate was sealed and then centrifuged at 1000 rpm for 60 s. The reaction plate was incubated for 60 min at 55°C, 5 min at 95°C with 45 cycles of 15 s at 95°C, 1 min at 60°C, and 1 min at 65°C. Fluorescent data were collected by instrument at in the extension stage (1 min at 65°C).

Table 2.1 Primers and probes used in the RT-qPCR.

Target	Primer/probe	Sequence (5' – 3')	Reference
NoV GI	QNIF4 (Forward)	CGCTGGATGCGNTTCCAT	(Silva <i>et al.</i> , 2007)
	NV1LCR (Reverse)	CCTTAGACGCCATCATCATTTAC	(Svraka <i>et al.</i> , 2007)
	¹ NVGG1p (Probe)	TGGACAGGAGAYCGCRATCT	(Svraka <i>et al.</i> , 2007)
NoV GII	QNIF2 (Forward)	ATGTTTCAGRTGGATGAGRTTCTCWGA	(Loisy <i>et al.</i> , 2005)
	COG2R (Reverse)	TCGACGCCATCTTCATTCACA	(Kageyama <i>et al.</i> , 2003)
	¹ QNIFS (Probe)	AGCACGTGGGAGGGCGATCG	(Loisy <i>et al.</i> , 2005)
Mengo virus	Mengo 110 (Forward)	GCGGGTCCTGCCGAAAGT	(Pinto <i>et al.</i> , 2009)
	Mengo 209 (Reverse)	GAAGTAACATATAGACAGACGCACAC	(Pinto <i>et al.</i> , 2009)
	² Mengo 147 (Probe)	ATCACATTACTGGCCGAAGC	(Pinto <i>et al.</i> , 2009)

¹Probe labelled 5' 6-carboxyfluorescein (FAM), 3' 6-carboxy-tetramethylrhodamine (TAMRA)

²Probe labelled 5' 6-carboxyfluorescein (FAM), 3' MGB (minor groove binder).

2.3.2 Nested RT-PCR

2.3.2.5 Targeting the part of RNA-dependent RNA polymerase region

The part of the RNA-dependent RNA polymerase was amplified using the nested RT-PCR under condition specified in Chapter 3. First round of PCR products were amplified using G1, G2 and SM31 primers in one reaction tube. For each sample, the second round PCR products were generated in two separate reaction tubes for NoV GI (Ando/E3) and NoV GII (NI/E3); resulting in similar length nested PCR for both genogroups products (112 bp for GI, and 113 bp for GII). Sequences of the nested PCR primers can be found in Table 2.2.

Table 2.2 Primers used in the nested RT-PCR targeting part of ORF1 of NoV genome.

Primer	NoV genogroup	Sequence (5' – 3')	Reference
G1 (Forward)	GI	TCNGAAATGGATGTTGG	(Green <i>et al.</i> , 1998)
G2(Forward)	GI	AGCCNTNGAAATNATGGT	(Green <i>et al.</i> , 1998)
SM31(Reverse)	GI and GII	CGATTTTCATCATCACCATA	(Green <i>et al.</i> , 1998)
Ando (Forward)	GI	GTGAACAGYATAAAYCANTGG	(Maguire <i>et al.</i> , 1999)
NI (Forward)	GII	GAATTCATCGCCCACTGGCT	(Green <i>et al.</i> , 1998)
E3 (Reverse)	GI and GII	ATTCATCATCACCATA	(Green <i>et al.</i> , 1998)

2.3.2.6 Targeting N/S domain of capsid region

The hemi-nested RT-PCR targeting the N/S domain of the NoV genome was carried out under conditions specified in Chapter 4 and 5. Primers sequence used in the hemi-nested PCR can be found in Table 2.3.

Table 2.3 Primers used in the nested RT-PCR targeting the ORF1/ORF2 junction of NoV genome.

Primer	NoV genogroup	Sequence (5' – 3')	Reference
COG1F (Forward)	GI, 1 st round	CGYTGGATGCGNTTYCATGA	(Kageyama <i>et al.</i> , 2003)
GISKR (Reverse)	GI, 1 st round	CCAACCCARCCATTRTACA	(Kojima <i>et al.</i> , 2002)
COG2F (Forward)	GII, 1 st round	CARGARBCNATGTTYAGRTGGATGAG	(Kageyama <i>et al.</i> , 2003)
GIISKR (Reverse)	GII, 1 st round	CCRCCNGCATRHCCRTTTRTACAT	(Kojima <i>et al.</i> , 2002)
GISKF (Forward)	GI, 2 nd round	CTGCCCCGAATTYGTAATGA	(Kojima <i>et al.</i> , 2002)
GISKR (Reverse)	GI, 2 nd round	CCAACCCARCCATTRTACA	(Kojima <i>et al.</i> , 2002)
GIISKF (Forward)	GII, 2 nd round	CNTGGGAGGGCGATCGCAA	(Kojima <i>et al.</i> , 2002)
GIISKR (Reverse)	GII, 2 nd round	CCRCCNGCATRHCCRTTTRTACAT	(Kojima <i>et al.</i> , 2002)

2.3.3 Cloning and sequencing primers

Plasmid specific PCR primers were used following cloning to determine if the inset was in the correct size, orientation, and in frame (Table 2.4).

Table 2.4 Primers used in the colony PCR and chain termination PCR.

Primer	Sequence (5' – 3')	Application
pTAg (Forward)	GCTATGACCATGATTACGCCAA	Colony PCR, sequencing of pGEM vectors
pTAg (Reverse)	TGTAAAACGACGGCCAGTGAA	
M13 (Forward)	GTAAAACGACGGCCAG	Colony PCR of pCR4-TOPO vector
M13 (Reverse)	CAGGAAACAGCTATGAC	
T3 (Forward)	ATTAACCCTCACTAAAGGGA	Sequencing of pCR4-TOPO vector
T7 (Reverse)	TAATACGACTCACTATAGGG	

2.4 Purification of the nested RT-PCR products

2.4.1 Chroma Spin Purification

All the nested RT-PCR positive samples were purified before cloning using CHROMA SPIN™ columns (Unitech, Dublin, Ireland). The contents of the Chroma Spin tubes were inverted several times to resuspend the gel matrix, ensuring no air bubbles were present. The tubes were placed in the collecting tube. The tubes were centrifuged at $700 \times g$ for 5 min to compact the silica gel. The collecting tube was discarded. Approximately 40 μ l of the nested RT-PCR product was loaded into the centre of the column and centrifuged at $700 \times g$ for 5 min using a new collecting tube.

2.5 Cloning of PCR product into pGEM-T Easy vector

2.5.1 pGEM-T Easy plasmid

The pGEM-T Easy vector is supplied linearised with a single 3' thymidine (T) overhangs at the insertion site. This improves the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by the *Taq* polymerase (Figure 2.1).

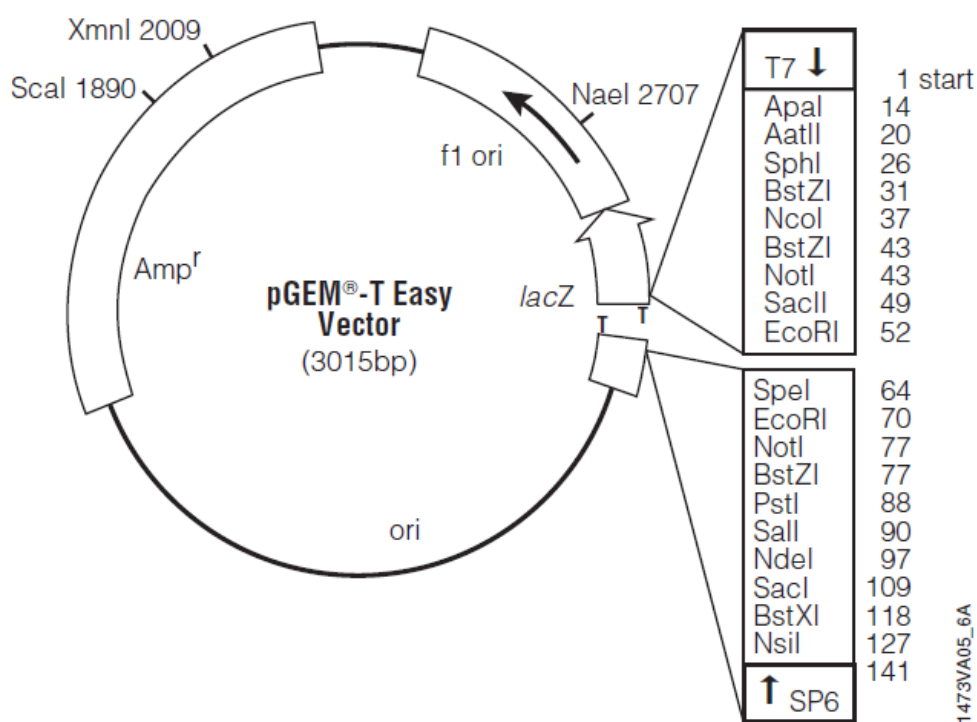


Figure 2.1 pGEM®- T Easy Vector.

2.5.2 Ligation

Purified PCR products were ligated into the pGEM®-T Easy Vector (Promega, UK). Tubes containing the pGEM®-T Easy Vector and Control Insert DNA were centrifuged to collect contents at the bottom of the tubes and 2 × Rapid Ligation Buffer was mixed by vortex. Ligation reaction was set up as outline in Table 2.5. The reaction was mixed by pipetting, and incubated for 1 hour at room temperature.

Table 2.5 Ligation into the pGEM®-T Easy Vector.

Component	Per sample (μl)	Positive control (μl)	Background (μl)
2 \times Rapid Ligation Buffer	5	5	5
pGEM®-T Easy Vector (50ng)	1	1	1
PCR product	3	0	0
Control Insert DNA	0	2	0
T4 DNA ligase (3 Weissunits/ μl)	1	1	1
MBG water	0	1	3
Total volume	10	10	10

2.5.3 Transformation of JM109 competent cells

High efficiency competent cells JM109 (1×10^8 cfu/ μg , Promega, UK) were placed on ice to thaw. The tubes containing the ligation reaction were briefly centrifuged and 2 μl of each ligation reaction was added to a sterile 1.5 ml microcentrifuge tube on ice. A volume of 50 μl of competent cells was carefully transferred into each tube and gently mixed and placed on ice for 20 min before being heat shocked at 42°C for 45 s. The tubes were immediately returned to ice for 2 min. Room temperature LB medium (950 μl) was added to the tubes containing transformed cells. The tubes were then shaken (~150 rpm) for 1.5 hour at 37°C. One hundred μl of 100 mM IPTG and 20 μl of 50 mg/ml X-Gal were spread on LB/ampicillin plates and placed in the incubator at 37°C for 30 min to allow for absorption of IPTG. Approximately 100 μl of each transformation culture was spread onto duplicate LB/ampicillin/IPTG/X-Gal plates. The plates were inverted and incubated overnight at 37°C. The plates were examined the next day for growth of white colonies.

2.5.4 Screening of colonies using colony PCR

To determine whether colonies contained the insert of interest, 5 white colonies were screened using colony PCR. A colony PCR master mix was prepared as outlined in Table 2.6, and 50 μl aliquots were prepared in a 0.5 ml thin walled PCR reaction tubes. The edge of a white colony was touched with a 1 μl loop and

streaked onto one section of the grid plate (LB/ampicillin). The PCR colony screening plate was incubated overnight at 37°C.

Table 2.6 Colony PCR master mix.

Component	Final concentration	Volume (µl)
10 × PCR buffer	1 ×	5
25 mM MgCl ₂	1.25 mM	2.5
25 mM dNTPs	0.2 mM each dNTP	0.4
50 µM pTAg (Forward)	1 µM	1
50 µM pTAg (Reverse)	1 µM	1
Taq polymerase	2.5 U	0.5
MBG water		39.6
Total volume		50

A colony PCR mix was inoculated with the colony by putting the loop into the reaction tube and mixing vigorously. PCR was run under following conditions; denaturation at 96°C for 10 min, 30 amplification cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 1 min; and an extension of 72°C for 10 min. Correct size PCR products were identified by agarose gel electrophoresis (2%, TBE buffer) using 100 bp ladder (Invitrogen). Bands with the correct size were seen as a 311/312 bp for NoV (200 bp from the vector plus insert size). Positive control (control insert DNA) supplied with the pGEM-T Easy Systems was recognised as a 742 bp band.

2.5.5 Purification of colony PCR product (ExoSAP-IT)

ExoSAP-IT was removed from -20°C and placed on ice throughout the procedure. A colony PCR reaction product (10 µl) was mixed with 4 µl of ExoSAP-IT and then incubated at 37°C for 15 min to degrade remaining primers and nucleotides. To inactivate ExoSAP-IT, the tubes were incubated on a heating block at 80°C for 15 min. Purified PCR product was stored in -20°C prior further analysis.

2.6 Cloning of PCR product into pCR[®]4-TOPO plasmid

2.6.1 pCR[®]4-TOPO plasmid

This plasmid vector (pCR[®]4-TOPO) is supplied linearised with a single 3' thymidine (T) overhangs for TA cloning and therefore enables direct cloning of PCR products via their poly-A tail (Figure 2.2)

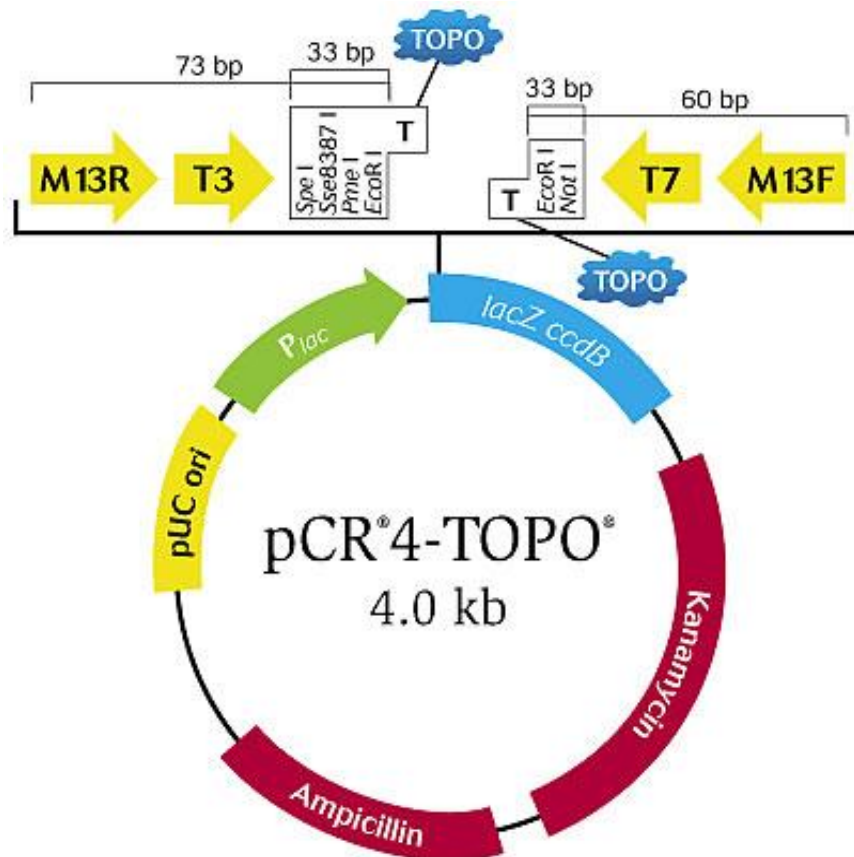


Figure 2.2 TA pCR 4 – TOPO cloning vector.

2.6.2 Ligation of the nested PCR product

The purified products were ligated into a pCR[®]4-TOPO[®] (Invitrogen, UK). A 6 μ l reaction mix containing 4 μ l of PCR product, 1 μ l of salt solution and 1 μ l of TOPO vector was made for each sample. The reaction was mixed gently, incubated for 20 min at room temperature, and then placed in ice.

2.6.3 Transformation into One Shot TOP10

One Shot TOP10 Chemically Competent *E. coli* cells (1×10^9 cfu/ μg , Invitrogen) were placed on ice to thaw. Two μl of the TOPO Cloning reaction was added into a vial of One Shot Chemically Competent *E. coli*, mix gently and incubated for 30 min on ice, before being heat-shock at 42°C for 30 s. The tubes were then placed on ice for 2 min following the addition of 250 μl of S.O.C. Medium (Invitrogen). The tubes were then shaken horizontally for 1 hour on a shaking platform at 225 rpm at 37°C incubator. Approximately 100 μl of transformed cells were plated onto duplicate LB/ampicillin plates and incubated overnight at 37°C .

2.6.4 Screening of colonies using colony PCR

To determine whether colonies contained the insert of interest, 5-10 colonies were screened using colony PCR. A colony PCR master mix was prepared as outlined in Table 2.7, and 50 μl aliquots were prepared in a 0.5 ml thin walled PCR reaction tubes. The edge of a white colony was touched with 1 μl loop and streaked onto one section of the grid plate (LB/ampicillin). The PCR colony screening plate was incubated overnight at 37°C .

Table 2.7 Colony PCR master mix.

Component	Final concentration	Volume (μl)
10 \times PCR buffer	1 \times	5
25 mM MgCl_2	1.25 mM	2.5
25 mM dNTPs	0.2 mM	0.4
50 μM M13 Forward	1 μM	1
50 μM M13 Reverse	1 μM	1
Taq polymerase	2.5 U	0.5
MBG water		39.6
Total volume		50

The colony PCR mix was inoculated with the colony by putting the loop into the reaction tube and mixing vigorously. PCR was run under following conditions; denaturation at 96°C for 10 min, 30 amplification cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min; and an extension of 72°C for 10 min. Correct size PCR

products were identified by agarose gel electrophoresis (2%, TBE buffer) using 100 bp ladder (Invitrogen). Bands with the correct size insert were seen as a 462 and 476bp for NoV GI and GII, respectively.

2.6.5 QIAquick purification

Purification of colony PCR products containing the correct size of inset was carried out using QIAquick PCR Purification (Qiagen) prior to sequencing. Briefly, 200 μ l of Buffer PB was added to 40 μ l of the PCR product and mix thoroughly with a pipette. Then, sample was loaded into the centre of the QIAquick column and centrifuged at $17,900 \times g$ for 60 s. Flow-through was discarded and 0.75 ml of Buffer PE was added into the column and centrifuged at $17,900 \times g$ for 60 s. Flow-through was discarded; the QIAquick column was placed back in the same tube and centrifuged again. The QIAquick column was placed in a clean 1.5 ml tube and 30 μ l Buffer TE was added to the centre of the QIAquick membrane. The column was let stand for 1 min and, then centrifuged at $17,900 \times g$ for 60 s.

2.7 DNA sequencing

The chain termination method was used to obtain DNA sequences (Sanger and Coulson, 1975). This method requires modified 2',3'-dideoxynucleotides triphosphates (ddNTPs) that differs from deoxynucleotides by having a hydrogen atom at 3' carbon rather than an OH group. These modified molecules can not form a phosphodiester bond with the next deoxynucleotide and terminate DNA chain elongation; as a result different size DNA fragments are generated. Afterwards, all obtained fragment are separated according to size, and fluorescently labelled ddNTPs are read by automated sequencing machines.

2.7.1 Cycle sequencing

A cycle sequencing reaction mix for the desired number of reactions was prepared as outlined in Table 2.8 using the ABI PRISM BigDye Terminator v 3.1 Ready Reaction kit (Applied Biosystems, Foster City, CA). The sequencing PCR conditions were 25 cycles of, 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. Samples were held at 4°C until ready for cycle sequencing clean-up.

Table 2.8 Cycle sequencing reaction mix.

Component	Final concentration	Volume (μ l)
BigDye Terminator v 3.1 Ready Reaction mix	1 \times	4
BigDye Terminator v 3.1 Sequencing Buffer	1 \times	2
Forward or reverse primer (10 μ M)	4 pmol	0.4
Template		1
MBG water		12.6
Total volume		20

2.7.2 Purification of cycle sequencing PCR products

The cycle sequencing PCR products were purified to remove unincorporated dye terminators and salt using DyeEx 2.0 Spin Column (Qiagen). The DyeEx 2.0 Spin Column was vortexed to resuspended the resin, then centrifuged at 750 \times g for 3 min. The spin column was transferred into a clean centrifuge tube and then 20 μ l of the

cycle sequencing PCR reaction was loaded directly onto the centre of slanted gel-bed surface. Samples were centrifuged at $750 \times g$ for 3 min. The spin column was discarded and the tubes were dried in a block heater at 80°C for approximately 25 min. The pellet was resuspended in $25 \mu\text{l}$ of Hi-Di Formamide, and then denaturated at 95°C for 2 min. Samples were vortexed, cooled on ice and transferred into the correct position on an autosampler's tray.

2.7.3 DNA sequencing

Sequencing was performed on an automated Sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems). The dye-labelled DNA fragments migrated through the POP6 polymer (on 61 cm capillaries) in an electrical field and were separated according to the size. The sample injection time was set for a 10 s. All resulting chromatograms were analysed using Sequencing Analysis 5.2.0 software (Applied Biosystems). Quality of sequence data was assessed using KB Basecaller programme (Applied Biosystems), which assigned a quality value (QV) prediction for each base (Figure 2.3). QV of 20 corresponds to 1% probability of error in base call. The higher QV value the lower probability of error in base calling. The bases with $\text{QV} < 20$ were re-checked for a background noise, signal intensity and mis-called nucleotides and the errors were corrected manually if possible.

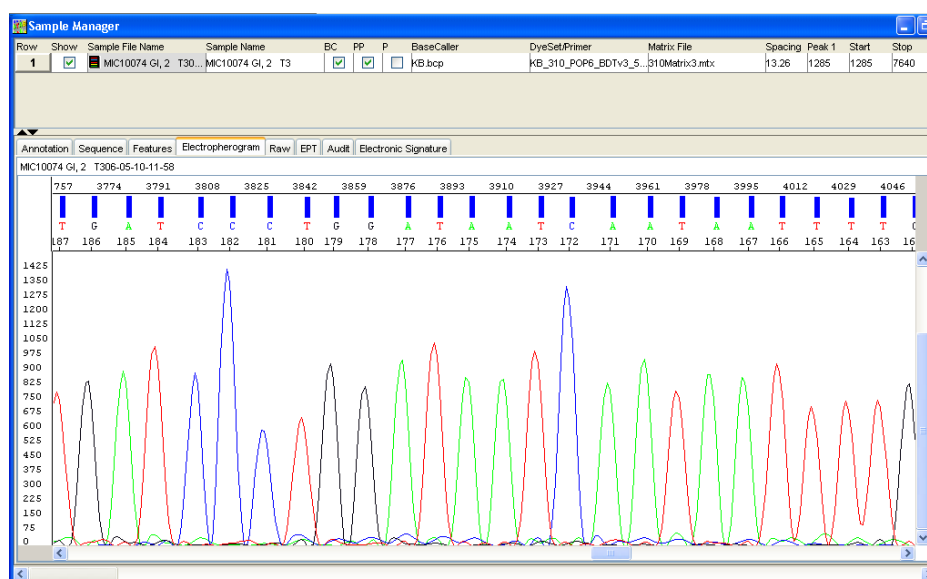


Figure 2.3 Chromatogram visualised using Sequencing Analysis 5.2.0.

2.8 Preparation of double-stranded DNA (dsDNA) control stocks

2.8.1 Control plasmids

NoV GI and GII control plasmids were separately constructed by ligation of the real-time PCR target DNA sequence into the pGEM-3Zf (+) vector at a *Sam*I restriction site (Figure 2.4) such that the target sequence was downstream of a promoter sequence for the T7 RNA polymerase. NoV GI and GII plasmids were developed by Dr. F.S Le Guyader, IFREMER and distributed by the European Union Reference Laboratory (EURL), CEFAS, UK.

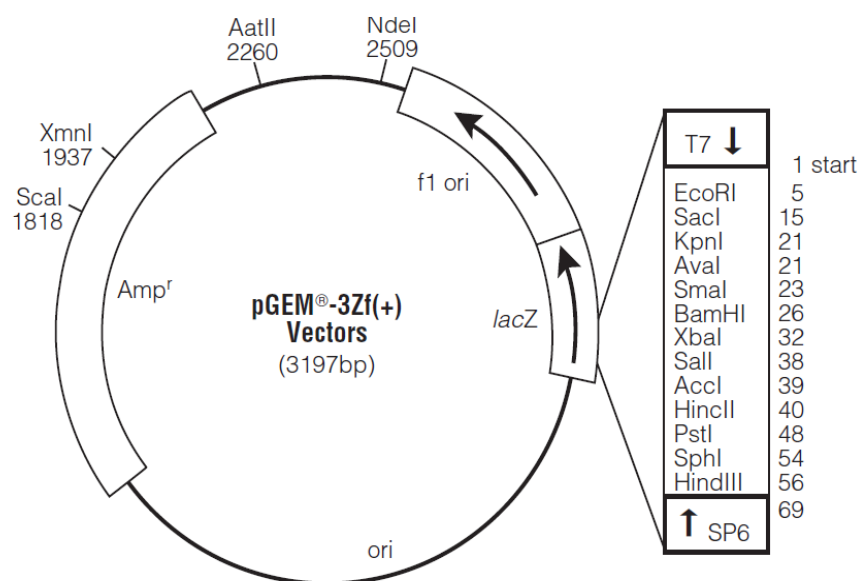


Figure 2.4 pGEM®-3Zf(+) vector circle map and sequence reference points (Promega).

2.8.2 Transformation and screening for NoV GI and GII insert

One μ l of supplied control plasmid (containing NoV GI or NoV GII target sequence) was added to 50 μ l of JM109 competent cells (Promega, UK), which were transformed followed the protocol previously described in section 2.4.3. Then, a colony PCR was performed as described before in section 2.4.4. NoV GI and GII DNA fragments were identified by 2% agarose gel electrophoresis using 25 bp ladder (Invitrogen) and were seen as a 243 bp (NoV GI insert 90 bp + 153 vector) and a 248 bp (NoV GII insert 95 bp + 153 vector) bands, respectively.

2.8.3 Purification of plasmid DNA

LB broth supplemented with ampicillin (final concentration 100 µg/ml, Sigma-Aldrich, UK) was inoculated with a single colony containing the plasmid of interest. Four 50 ml tubes containing 5 ml of culture were incubated at 37°C with shaking at 110 rpm. The plasmid DNA was purified from the cultures (4 × 3 ml) using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, UK).

Briefly, four tubes were centrifuged at 12,000 × g for 1 min and pellet cells were resuspended in 200 µl Resuspension Solution by pipetting up and down. Then, 200 µl of the Lysis Solution was added and mixed by gentle inversion. The cell debris was precipitated by the addition of Neutralization/Binding Solution (350 µl) and tubes were mixed by inversion. The samples were centrifuged at 12,000 × g for 10 min and supernatant (cleared lysate) was transferred into a clean 2.0 ml tube. To prepare a GenElute Miniprep Binding Column, 500 µl of the Column Preparation Solution was added to each miniprep column and centrifuged at 12,000 × g for 1 min. The flow-through liquid was discarded. The cleared lysate was transferred to the GenElute Miniprep Binding Column and centrifuged at 12,000 × g for 1 min. The flow-through liquid was discarded. To wash the column, 750 µl Wash Solution was added to the column and centrifuged at 12,000 × g for 1 min. The flow-through liquid was discarded and the column was centrifuged again at maximum speed for 2 min. The column was transferred to a new collection tube and eluted in 50 µl MBG water.

Then, four elutes were pooled and the plasmid DNA (150 µl) was further purified using the QIAquick PCR purification kit (Qiagen) as previously described in section 2.6.5. Briefly, 750 µl of Buffer PB was added to 150 µl of the PCR product and mix thoroughly with a pipette according to manufacturer's protocol and eluted in 30 µl MBG water.

2.8.4 Quantification of plasmid dsDNA

The purity and concentration of the NoV GI and GII plasmids were determined using the Nanodrop (concentration >200 ng/µl and A_{260}/A_{280} 1.7–2.0 were accepted). The mass of an individual plasmid molecule was calculated by multiplying the plasmid length in bp by 607.4 (the molecular weight of an average base pair) and

dividing by the Avogadro constant (6.02×10^{23}). The number of bp in the NoV GI and GII plasmids are 3287 and 3292, respectively. Therefore, the mass of the NoV GI plasmid is 3.32×10^{-18} g and the NoV GII plasmid is 3.32×10^{-18} g. The concentration of each plasmid was divided by the mass in g of a single plasmid molecule to calculate the concentration of plasmid DNA in copies μl^{-1} . Working stocks of 10^5 copies μl^{-1} were prepared for NoV GI and GII plasmid and stored in -20°C .

2.9 Preparation of external control RNA (EC RNA) stocks

2.9.1 Linearization of plasmid dsDNA

To enable linearization of the purified plasmid, each plasmid (NoV GI and NoV GII) underwent digestion using XbaI restriction enzyme (Promega, UK) (Table 2.9). Four individual reactions for both NoV GI and GII were prepared and incubated at 37°C for 3 hours. Successful linearization was determined using gel electrophoresis (0.8%, TBE buffer) and by comparing the linearised plasmid to non-linearised plasmid. The linearised plasmid was purified using QIAquick PCR purification kit (section 2.6.5) and eluted in 50 µl of MBG water.

Table 2.9 Linearisation of plasmid DNA.

Component	Final concentration	Volume (µl)
Plasmid DNA (2 µg)		1-10
XbaI (12 U/ µl)	0.6 U/ µl	1
10 × Reaction buffer D	1 ×	2
BSA (10 µg/ µl)	0.1 µg/ µl	0.2
MBG water		Up to 20
Total volume		20

2.9.2 In vitro RNA transcription

One µg of purified linearised plasmid DNA was added to an *in vitro* RNA transcription reaction mix (Riboprobe® In Vitro Transcription Reagents, Promega, UK) (Table 2.10) and incubated at 37°C for 2 hours.

Table 2.10 In vitro RNA transcription protocol.

Component	Final concentration	Volume (µl)
5 × transcription buffer	1 ×	20
100 mM DTT	10 mM DTT	10
RNasin		2.5
2.5 mM rNTP mix		20
Linearised template (1 µg)		1-20
SP6 RNA polymerase		3
MBG water		Up to 100
Total volume		100

Then, 5 μl of RNase-free DNase was added to the reaction and incubated at 37°C for 15 min.

2.9.3 RNeasy mini purification

The RNA transcripts were purified using the RNeasy mini kit according to the manufacturer's instructions (Qiagen). Briefly, 350 μl of Buffer RTL was added into 100 μl of the sample and mixed thoroughly. Ethanol (250 μl , 100%) was added and the 700 μl solution was pipetted immediately into an appropriately labelled RNase spin column and centrifuged for 15 s at 8000 \times g. The flow-trough was discarded and the column was washed with Buffer RW1 (350 μl). DNase I incubation mix (80 μl) was added directly to the RNeasy spin column membrane and incubated at room temperature for 15 min. Buffer RW1 (350 μl) was added to the column, then the column was centrifuged at 8000 \times g for 15 s and the flow-trough was discarded. Buffer RPE (500 μl) was added to the RNeasy spin column, centrifuged for 15 s at 8000 \times g and the flow-trough was discarded. Buffer RPE (500 μl) was added again to the RNeasy spin column and centrifuged for 2 min at 8000 \times g to ensure that no ethanol is carried over during RNA elution. The flow-trough was discarded and the column was placed into a new 2 ml collection tube. MBG water (100 μl) was pipetted directly onto the centre of the silica-gel membrane and the RNA was eluted by centrifugation at full speed for 1 min.

2.9.4 DNA contamination check

NoV GI and GII target specific real-time PCR master mix was prepared as described before in section 2.2.1, divided into two and the reverse transcriptase (RT) enzyme was inactivated in one portion by heating at 95°C for 5 min. NoV GI and GII dsDNA standard curves were prepared and added to designated wells containing both the RT active and RT inactive master mix. One μl of 1×10^7 transcript μl^{-1} NoV GI or GII was added to 5 μl of MBG water and tested using (i) RT active PCR master mix and (ii) RT inactive PCR master mix. The concentration of EC RNA [copies μl^{-1}] was calculated using RT inactive (C_{INACT}) and RT active (C_{ACT}) PCR master mix. If the

detectable levels of EC RNA stock ($C_{\text{DEACT}}/C_{\text{ACT}} \times 100\%$) were greater than 0.1% then the stock was accepted for use.

2.9.5 Quantification of EC RNA

For both NoV GI and GII, the concentration of *in vitro* transcribed RNA was measured using the Nanodrop ($A_{260}/A_{280} \sim 2.0$). The mass of an individual RNA molecule was calculated by multiplying the RNA length in ribonucleotides by 320.5 (the molecular weight of an average ribonucleotide) and dividing by the Avogadro constant (6.02×10^{23}). The number of ribonucleotides in the NoV GI and GII transcripts is 126 and 131, respectively. To calculate the concentration of EC RNA in transcript copies μl^{-1} , the concentration of EC RNA in $\text{ng } \mu\text{l}^{-1}$ was divided by the mass in grams of a single EC RNA molecule. Working stocks of 10^7 copies μl^{-1} were prepared for NoV GI and GII transcripts and stored in -20°C .

2.10 Phylogenetic analysis

Two fragments of NoV genome (A and C) were sequenced and used in phylogenetic manipulations during this study (Table 2.11).

Table 2.11 Norovirus genome domains used in phylogenetic analysis.

Fragment (primers)	Length of fragment	Genome position	Region/ ORF	Application
NoV GI (Ando/E3)	112 bp	4768 to 4880	Region A/ ORF1	Phylogenetic analysis
NoV GII (NI/E3)	113 bp	4495 to 4598**	Region A/ ORF1	Phylogenetic analysis
NoV GI (GISKF/GISKR)	330 bp	5342 to 5671*	Region C / junction ORF1/ORF2	Phylogenetic analysis and recombination
NoV GII (GIISKF/GIISKR)	344 bp	5046 to 5389**	Region C / junction ORF1/ORF2	Phylogenetic analysis and recombination

*with respect to Norwalk virus (M87661),

**with respect to Lordsdale virus (X86557)

2.10.1 Outgroup selection

GIV.2 Norovirus lion/GGIV.2/Pistoia/387/06/ITA (EF450827) and Norovirus Bo/Newbury2/1976/UK (AF097917) sequences were used as an outgroup to root a phylogenetic tree in chapter 4 and 5, respectively. Tree consisting of NoV GII.4 variants only, used Human calicivirus NLV/Oberhausen 455/01/DE (AF539440) virus as an outgroup. In chapter 6, NoV GII.4 Bristol (X76716) and NoV GI.1 Norwalk virus (M87661) were chosen as an outgroup for NoV GI and NoV GII trees, respectively.

2.10.2 Reference strains

Reference strain were retrieved from GenBank website (<http://www.ncbi.nlm.nih.gov/GenBank/>) and incorporated into analysis. For region A, the NoV reference strains included in the phylogenetic analysis are listed in Table 2.12. The NoV ORF1 and ORF2 nomenclature was adopted from the Noronet website. The NoV reference strains used for the analysis of region C are included in Table 2.13, whereas the reference strains for NoV GII.4 variants can be found in Table 2.14.

Table 2.12 ORF1 reference strains for phylogenetic analysis.

ORF1	Prototype strain	Accession Number	ORF2 type	Comment
I.1	Norwalk/1968/US	M87661	I.1	ORF2 type I.1
I.2	Southampton/1991/UK	L07418	I.2	
I.3	NLV/VA98115/1998	AY038598	I.3	
I.4	Chiba virus	AB042808	I.4	
I.6	Hesse 3/1997/DE	AF093797	I.6	
I.a	Desert Shield virus DSV395	U04469	I.3	ORF2 type Kageyama 2004 I.12
1.b	WUG1	AB081723	I.6	ORF2 type Kageyama 2004 I.8
I.c	SzUG1	AB039774	I.5	ORF2 type Kageyama 2004 I.9
I.d	Vesoul576/2003/France	EF529738	I.3	ORF2 type Kageyama 2004 I.11
I.e	Chatellerault709/2004/France	EF529737	I.3	ORF2 type Kageyama 2004 I.13
I.f	GI/Otofuke/1979/JP	AB187514	I.3	ORF2 type Kageyama 2004 I.14
II.1	Hawaii/1971/US	U07611	II.1	
II.2	Melksham/1994/UK	X81879	II.2	
II.2	Hu/MK04/2004/JP	DQ456824	II.2	
II.3	Toronto/1991/CA	U02030	II.3	
II.4	Bristol/1993/UK	X76716	II.4	
II.4	Hu/GII.4/Dongen46/2006/NL	EF126961	II.4	II.4 variant 2004
II.5	NLV/MOH/99	AF397156	II.5	

II.6	Saitama U16	AB039778	II.6	
II.7	Saitama U4	AB039777	II.7	associated with ORF2 II.7, II.6, II.9, II.14
II.11	Sw918/1997/JP	AB074893	II.11	
II.12	Hu/5017.34/2003/JPN	EU187437	II.3	associated with ORF2 II.4 2003 variant, II.2, II.3, II.5, II.10, II.12, II.13
II.12	Hu/Hiroshima/60-1015/2005/JP	AB354299	II.2	
II.15	Hiroshima/66-1110/2006/JP	AB360387	II.15	
II.16	Hiroshima/60-1015/2005/JP	AY772730	II.16	associated with ORF2 II.16, II.17
II.17	Briancon870/2004/France	EF529741	II.17	
II.18	Sw/OH-QW101/2003/US	AY823304	II.18	
II.19	Sw/OH-QW170/2003/US	AY823306	II.19	
II.a	Human calicivirus strain Arg320	AF190817	II.3	
II.b	Pont de Roide 673/2004/France	AY682549	II.2	associated with ORF2 II.1, II.2, II.3
II.c	Snow Mountain virus	AY134748	II.2	
II.d1	Hokkaido/133/2003/JP	AB212306	II.5	associated with ORF2 II.2 and II.5
II.d1	Hu/OsakaNI/2004/JP	DQ366347	II.2	
II.d2	Human norovirus Saitama	AB231337	II.3	associated with ORF2 II.3
II.e	Hu/OC07138/07/JP	AB434770	II.4	II.4 2007 variant
II.f	Hu/NLV/S63/1999/France	AY682550	II.5	associated with ORF2 II.5
II.g	Goulburn Valley G5175 B/1983/AUS	DQ379714	II.13	associated with ORF2 II.1, II.12, II.13
II.h	OC97007/97/JP	AB089882	II.2	associated with ORF2 II.2
II.j	E3/1997/Crete	AY682552	II.2	associated with ORF2 II.2 and II.4
II.k	OC96065/1996/JP	AF315813		no known associated ORF2
II.NA1	Leverkusen267/2005/DE	EU424333	II.NA1	
II.NA2	Hu/NoV/GII/YURI/JP	AB083780	II.NA2	

Table 2.13 ORF2 reference strains for phylogenetic analysis.

ORF2	Prototype strain	Accession Number	Comment
I.1	Norwalk/1968/US	M87661	
I.1	SRSV-KY-89/89/J	L23828	
I.2	Southampton/1991/UK	L07418	
I.2	HU/NLV/Whiterose/96/UK RNA	AJ277610	
I.3	Desert Shield 395/1990/SA	U04469	
I.3	NLV/VA98115/1998	AY038598	
I.4	Chiba 407/1987/JP	AB042808	
I.4	Hu/NLV/Queen's Arms/Leeds/92/UK	AJ313030	
I.5	Musgrove/1989/UK	AJ277614	
I.5	SzUG1	AB039774	
I.6	Hesse 3/1997/DE	AF093797	
I.6	HU/NLV/Sindlesham/95/UK	AJ277615	
I.7	Winchester/1994/UK	AJ277609	
I.7	NLV/IF2036/2003/Iraq	AY675555	
I.8	Boxer/2001/US	AF538679	
II.1	Hawaii/1971/US	U07611	
II.1		AJ277606	
II.2	Melksham/1994/UK	X81879	
II.2	HU/NLV/Girlington/93/UK	AY134748	
II.3	Toronto/1991/CA	U02030	
II.4	Bristol/1993/UK	X76716	
II.5	Hillingdon/1990/UK	AJ277607	
II.5	NLV/MOH/99	AF397156	
II.6	Seacroft/1990/UK	AJ277620	
II.6	Saitama U16	AB039778	
II.7	Leeds/1990/UK	AJ277608	
II.7	NLV/Gwynedd/273/1994/US	AF414409	
II.8	Amsterdam/1998/NL	AF195848	

ORF2	Prototype strain	Accession Number	Comment
II.8	Saitama U25	AB039780	
II.9	VA97207/1997US	AY038599	
II.9	Hu/GII/Goulburn Valley G5175 C/1983/AU	DQ379715	
II.10	Erfurt 546/2000/DE	AF427118	
II.10	Mc37	AY237415	
II.11	Sw918/1997/JP	AB126320	Swine norovirus
II.11	Sw/NLV/Sw918/1997/JP	AB074893	Swine norovirus
II.12	Wortley/1990/UK	AJ277618	
II.12	Chitta virus	AB032758	
II.13	Fayetteville/1998/US	AY113106	
II.13	Kashiwa47	AB078334	
II.14	M7/1999/US	AY130761	
II.14	Hu/Maizuru/010426/2001/JP/3385	EF547404	
II.15	J23/1999/US	AY130762	
II.15	NLV/Mex7076/1999	AF542090	
II.16	Tiffin/1999/US	AY502010	
II.16	Hu/NLV/GII/Neustrelitz260/2000/DE	AY772730	
II.17	CS-E1/2002/US	AY502009	
II.17	Hu/NoV/Katrina-17/2005/US	DQ438972	
II.18	Sw/OH-QW101/2003/US	AY823304	Swine norovirus
II.18	Swine/GII/OH-QW125/03/US	AY823305	Swine norovirus
II.19	Sw/OH-QW170/2003/US	AY823306	
II.NA1	Luckenwalde591/2002/DE	EU373815	
II.NA2	Hu/NoV/GII/YURI/JP	AB083780	
II.NA3	IF1998/2003/Iraq	AY675554	
II.NA4	Chiba/040502/2004/JP	AJ844470	

Table 2.14 NoV GII.4 variants reference for ORF1 and OFR2.

GII.4 variant ORF1	GII.4 variant ORF2	Accession number (ORF1)	Accession number (ORF2)	Comment
Bristol	Bristol	X76716		
Bristol	Bristol		X86557	
Camberwell	Camberwell	AF145896		
Camberwell	Camberwell		AY030098	
1996	1996	DQ078829		
1996	1996		AF080558	
2001	2001	AB294779		
2001	2001		AB303929	
2002	2002	AY502023		
2002	2002	AY485642		
2002CN	2002CN		DQ364459	
2002CN	2002CN	EU310927		
	2003		AB220921	Recombinant with ORF1 type II.12
	2003		DQ369797	Recombinant with ORF1 type II.12
2004	2004	AB294785		
2004	2004		AY883096	
2004	2004	EF126961		
2004	2004		DQ078794	
2006a	2006a	EF126963		
2006a	2006a	EF126964		
2006b	2006b	EF126965		
2006b	2006b	EF126966		
2006b	2006b		EF684915	
	2007		AB434770	Recombinant with ORF1 type II.e
2008	2008		AB445395	
2010			GU445325	New Oreland 2009

2.11 Multiple alignment

Nucleotide sequences were aligned using ClustalW algorithm of the MegAlign (DNAsstar, Inc., Madison, WI, USA) software and compared with reference strains. If multiple alignment required editing then BioEdit software version 7.0.5.3 was used (Figure 2.5). Outgroup sequence was selected as a first sequence in the alignment. Corrected aligned data set was saved as a FASTA file. To convert aligned FASTA sequence file into NEXUS format, Readseq programme (<http://www.ebi.ac.uk/Tools/sfc/readseq/>) was used.

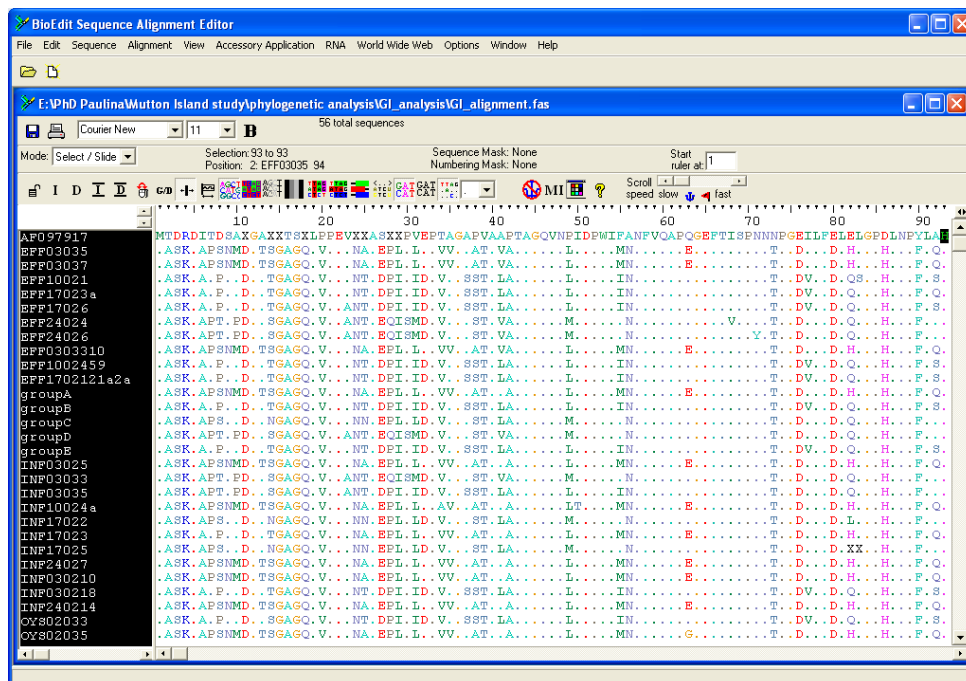


Figure 2.5 Amino acid sequence aligned using BioEdit Sequence Alignment Editor.

2.11.1 Selecting model of evolution and tree building

Sequence data in NEXUS format were executed in the jModelTest version 0.1.1 program to carry out statistical selection of best-fit models of nucleotide substitution. Model was selected based on hierarchical likelihood ratio test or Akaike criteria strategy. PAUP* commands containing the necessary information with the parameters for the model of substitution were copied into commands in

PAUP*. Maximum likelihood trees were constructed with PAUP* version 4.0 based on selected model of evolution. The reliability of the generated tree was estimated by bootstrap analysis of 1,000 replicates using the neighbor-joining method. The constructed phylogenetic trees were viewed using TreeView version 1.6.6. The maximum likelihood method could not be used to construct a phylogenetic tree for short sequences (<100 bp, region A); therefore the neighbor-joining method was applied.

2.12 Characterisation of recombinants

Detection of potential NoV GI and GII recombinant sequences was carried out using the Recombination Detection Program, version 4.14 (RDP4, Figure 2.6), comprising the following programs: RDP, GENECONV, BOOTSCAN, MaxChi, CHIMAERA, SISCAN, and 3SEQ. The default settings for each of the programs in RDP4 were used, except for BOOTSCAN and SISCAN, where a window size of 40 and a step size of 20 were used. The potential NoV recombinants were considered putative recombinant sequences if they were detected by at least 3 different programs.

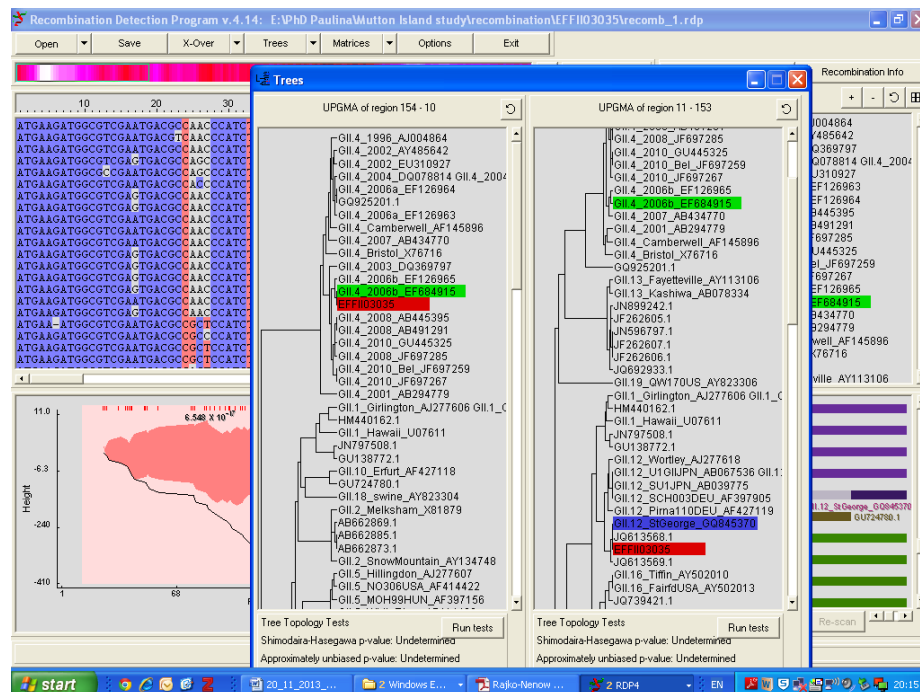


Figure 2.6 Detection of potential recombinant sequences using the Recombination Detection Program.

2.13 References

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**Chapter 3 Characterisation of
norovirus contamination in an Irish
shellfishery using real-time RT-
qPCR and sequencing analysis**

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Abstract

Norovirus (NoV) is the single most important agent of foodborne viral gastroenteritis worldwide. Bivalve shellfish, such as oysters, grown in areas contaminated with human faecal waste may become contaminated with human pathogens including NoV. A study was undertaken to investigate NoV contamination in oysters (*Crassostrea gigas*) from a shellfishery over a 24 month period from October 2007 to September 2009. Oyster samples were collected monthly from a commercial shellfish harvest area classified as category B under EU regulations, but that had had been closed for commercial harvesting due to its previous association with NoV outbreaks. Real-time reverse transcription quantitative PCR (RT-qPCR) was used to determine the concentration of human NoV genogroups I and II (GI and GII) in monthly samples. Total NoV (GI and GII) concentrations in NoV positive oysters ranged from 97 to 20,080 genome copies g⁻¹ of digestive tissue and displayed a strong seasonal trend with greater concentrations occurring during the winter months. While NoV GII concentrations detected in oysters during both years were similar, NoV GI concentrations were significantly greater in oysters during the winter of 2008/09 than during the winter of 2007/08. To examine the NoV genotypes present in oyster samples, sequence analysis of nested RT-PCR products was undertaken. Although NoV GII.4 is responsible for the vast majority of reports of outbreaks in the community, multiple NoV genotypes were identified in oysters during this study: GI.4, GI.3, GI.2, GII.4, GII.b, GII.2, GII.12, and GII.e. NoV GI.4 was the most frequently detected genotype throughout the study period and was detected in 88.9% of positive samples, this was followed by GII.4 (43.7%) and GII.b (37.5%). This data demonstrates the diversity of NoV genotypes that can be present in sewage contaminated shellfish and that a disproportionate number of non-NoV GII.4 genotypes can be found in environmental samples compared to the number of recorded human infections associated with non-NoV GII.4 genotypes.

3.1 Introduction

Bivalve molluscs, such as oysters, are filterfeeding shellfish that can bioaccumulate human pathogenic microorganisms when grown in waters impacted by sewage. Faecally contaminated shellfish represent a risk to human health, especially when consumed raw or lightly cooked (Halliday *et al.*, 1991 and Richards, 1985). Norovirus (NoV) is recognised as the most frequent cause of shellfish-associated illness worldwide and has been linked to numerous outbreaks of acute gastroenteritis in consumers (Lees, 2000). Current control measures rely on the use of bacterial indicator organisms such as *Escherichia coli* (*E. coli*) to assess the faecal contamination of shellfish (Butt *et al.*, 2004 and Lees, 2000). Despite these controls, oysters that are fully compliant with existing bacterial standards continue to be associated with NoV outbreaks in Europe (Baker *et al.*, 2010, Doré *et al.*, 2010 and Le Guyader *et al.*, 2010) and elsewhere (David *et al.*, 2007 and Huppertz *et al.*, 2008).

In general, NoV gastroenteritis is considered a mild and self-limiting illness, involving diarrhoea and vomiting, with symptoms lasting for 2–3 days (Kaplan *et al.*, 1982). NoV is spread by the faecal–oral route and outbreaks are most often reported in healthcare settings, where they contribute to significant financial costs (Lopman *et al.*, 2004). Although NoV infections in the community occur throughout the year, the annual peak of NoV infection during the winter months can be associated with dry and cold environmental conditions (Lopman *et al.*, 2009).

NoV is one of five genera recognised within the family *Caliciviridae*, and human NoV contains a positive sense single-stranded RNA genome that is organised into three open reading frames (ORFs). ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase; ORF2 encodes the major capsid protein; and ORF3 encodes a minor capsid protein (Jiang *et al.*, 1993). The NoV genome can undergo recombination, which occurs relatively frequently at the ORF1/ORF2 junction (Bull *et al.*, 2005). NoV exhibits considerable genetic diversity and has been classified into five distinct genogroups (GI–GV) based on phylogenetic analysis of the capsid protein (Zheng *et al.*, 2006). Of the five genogroups NoV GI, NoV GII, and less frequently NoV GIV infect humans and each genogroup is further

subdivided into numerous genotypes. NoV GII and, in particular, variants of the NoV GII genotype 4 (GII.4), are most often associated with outbreaks in healthcare and other enclosed settings where person-to-person transmission occurs (Kroneman *et al.*, 2008). NoV GI genotypes have, however, been reported in one study in Sweden as being more frequently implicated in water-related (drinking and recreational) outbreaks than NoV GII (Lysen *et al.*, 2009). Shellfish related outbreaks are often associated with multiple NoV genotypes (GI and GII) that are detected both in contaminated shellfish and faeces of infected individuals (Le Guyader *et al.*, 2006).

The detection of NoV in environmental samples relies on molecular techniques that have been developed to overcome a number of issues, such as low target virus concentrations, the presence of inhibitory substances in sample matrices and the diversity of the NoV genome (Le Guyader *et al.*, 2009). Broadly reactive and sensitive real-time quantitative reverse transcription PCR (RT-qPCR) assays that target the conserved ORF1/ORF2 junction of the NoV genome, have been designed for the detection and quantification of NoV GI and GII in shellfish (da Silva *et al.*, 2007, Kageyama *et al.*, 2003, Loisy *et al.*, 2005 and Svraha *et al.*, 2007). RT-qPCR procedures have been used successfully in a number of studies to assess the concentrations of NoV in shellfish (Flannery *et al.*, 2012, Kageyama *et al.*, 2003, Loisy *et al.*, 2005 and Lowther *et al.*, 2008). More recently, a standardised method based on RT-qPCR detection of NoV and Hepatitis A in food has been developed by the European Committee for Standardisation (CEN) working group (TC 275/WG6/TAG 4) – detection of viruses in food (Lees and TAG4, 2010) and provides a tool to quantify NoV concentration in shellfish. While this method does not distinguish between infectious and non-infectious virus particles it has been used to demonstrate that the risk of illness associated with oysters increases with increasing concentrations of NoV genome copies present (Lowther *et al.*, 2012). RT-qPCR, therefore, can be a useful tool for assessing the extent of NoV contamination in shellfish which may indicate the potential risk associated with the consumption of such contaminated shellfish. In this study, we used RT-qPCR to investigate the concentrations of NoV in oysters, over a two year period, from a shellfish harvest

area that was closed for commercial harvesting because it had been previously associated with outbreaks of NoV illness. We also undertook phylogenetic analysis to establish the profile of NoV genotypes present in the oysters over this time.

3.2 Material and methods

3.2.1 Study site selection

An oyster harvest area, which was closed for harvesting due to previous incidents of NoV contamination leading to illness outbreaks, was selected for this study. The harvest area may be impacted by a number of wastewater treatment plant discharges (WWTP), including from one that provides UV disinfection in addition to secondary treatment and serves a population equivalent (p.e.) of 10,000, located approximately 1 km from the harvest area; and another providing secondary treatment only (p.e. of 420,000), located approximately 10 km from the site.

The selected sampling site was monitored for *E. coli* levels by the Sea Fisheries Protection Authority (SFPA) as part of the national classification of shellfish harvest area. During the study period the site was consistent with a category B site. Between October 2007 and September 2009, *E. coli* results for the majority of the samples were $< 230 E. coli 100 g^{-1}$ (category A – upper limit) and none of the obtained results was greater than $4600 E. coli 100 g^{-1}$ (category B – upper limit).

3.2.2 Shellfish sampling and processing

Oyster (*Crassostrea gigas*) samples were collected on a monthly basis ($n = 23$) over a 2-year period, from October 2007 to September 2009, from a single site within the closed harvest area. Sampling was carried out by an SFPA officer and approximately 18 oysters were transported to the Marine Institute laboratory within 48 h under chilled conditions ($< 15^{\circ}\text{C}$). Ten alive oysters were cleaned by rinsing under running tap water and opened using a flame sterilised shucking knife. The digestive tissues (DT) of 10 oysters were transferred to a sterile Petri dish, weighed and chopped. Per 1 gram of DT, twenty five microlitres of Mengo virus strain MC₀ was added as an internal positive control (IPC) virus controlling for the virus extraction efficiency (Costafreda *et al.*, 2006) followed by 1 ml of proteinase K solution ($100 \mu\text{g ml}^{-1}$; Sigma-Aldrich, Dorset, UK). The sample was then incubated at 37°C , with shaking, for 60 min followed by incubation at 60°C for 15 min. The sample was centrifuged at $3000 \times g$ for 5 min and the shellfish proteinase K extract

(supernatant) was stored for a period of less than 1 month, at -80°C , prior to RNA extraction.

3.2.3 RNA extraction

Viral RNA was extracted from 500 μl of shellfish proteinase K extract using the NucliSENS® miniMAG® extraction platform and NucliSENS® magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. A negative RNA extraction control (molecular biology grade water) was included alongside each batch of shellfish sample. Prior to nested RT-PCR, each shellfish sample was extracted at four different dilutions of shellfish proteinase K extract: neat, 1:1.25, 1:2 and 1:5 (dilutions prepared in PBS, Oxoid, Basingstoke, England). Viral RNA was eluted into 100 μl of elution buffer and was stored at -80°C until further analysis.

3.2.4 RT-qPCR assay

3.2.4.7 Preparation of standards and controls

Plasmids pGEM-3Zf(+) carrying the GI and GII target sequences containing restriction site (BamHI) to check for contamination (supplied by Dr. Françoise S. LeGuyader, Ifremer, Nantes, France) were transformed into competent cells and transformant clones were screened. Plasmid dsDNA was isolated from bacterial cultures using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Dorset, UK), and then purified using the QIAquick PCR Purification Kit (Qiagen, West Sussex, UK). The purified plasmids were quantified by spectrophotometry at 260 nm using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE). For both NoV GI and NoV GII, single-use aliquots containing dsDNA of 10^5 genome copies μl^{-1} were prepared for use as quantification standards. In addition, the purified dsDNA plasmid was linearised, and transcribed in vitro using the Riboprobe Transcription System (Promega, UK) for preparation of controls for RT-qPCR inhibition. After DNase treatment, NoV GI and NoV GII external control (EC) RNA were purified and quantified by spectrophotometry at 260 nm. The dsDNA standards and EC RNA controls were stored at -20°C .

3.2.4.8 Quantification of NoV in shellfish samples

RT-qPCR was performed using RNA Ultrasense™ one-step quantitative RT-PCR system (Invitrogen, Carlsbad, CA) on an AB7500 real-time PCR instrument (Applied Biosystems, Foster City, CA). For NoV GI and NoV GII analyses of shellfish samples, 20 µl of the appropriate reaction mix containing final concentrations of 1 × reaction mix, 12.5 pmol forward primer, 22.5 pmol reverse primer, 6.25 pmol probe, 1 × Rox and 1.25 µl of enzyme mix was added to the designated wells of the 96-well plate. This was followed by duplicate 5 µl aliquots of sample RNA or extraction control. Previously described primers and probes were used for separate analysis of NoV GI and GII. For NoV GI forward primer QNIF4 (da Silva *et al.*, 2007), reverse primer NV1LCR, and probe NVGG1p (Svraka *et al.*, 2007) and for NoV GII forward primer QNIF2 (Loisy *et al.*, 2005), reverse primer COG2R (Kageyama *et al.*, 2003), and probe QNIFS (Loisy *et al.*, 2005) were used for the RT-qPCR assays. The reaction plate was incubated for 60 min at 55°C, 5 min at 95°C with 45 cycles of 15 s at 95°C, 1 min at 60°C, and 1 min at 65°C. No template controls using molecular biology grade water were included for the NoV GI and the NoV GII assays.

To determine the concentrations of NoV (genome copies g⁻¹ DT), a dilution series (10⁵ to 10¹ copies µl⁻¹) of the NoV GI and NoV GII DNA plasmids was included in duplicate on each RT-qPCR run. The number of NoV copies in each positive sample was determined by comparison of the quantification cycle (*C_q*) value obtained to the standard curves. The final concentration was then adjusted, based on dilution factors, and expressed as detectable virus genome copies g⁻¹ DT. The limit of detection (LOD) for NoV GI and NoV GII was 20 detectable genome copies g⁻¹ DT for shellfish.

To test for the presence of RT-PCR inhibitors, 5 µl of sample RNA was added to a further two wells to which 1 µl of EC RNA (10⁷ genome copies µl⁻¹) was added. A log dilution series of the NoV GI and GII EC RNA ranging from 10⁷ to 10⁴ copies µl⁻¹ was included on each RT-qPCR run. The mean *C_q* value obtained for samples that included the EC RNA was used to calculate the quantity of EC RNA detected in the sample which was then used to estimate PCR amplification efficiency which was expressed as a percentage. Samples with an amplification

efficiency of less than 25% were not accepted and in such cases the sample's RNA was reanalysed at a 1:10 dilution.

For extraction efficiency (determined using the IPC; Mengo virus), forward (Mengo209) and reverse (Mengo110) primers and probe (Mengo147) were used as described previously by Pintó et al. (2009). Twenty microlitres of one-step reaction mix was added to the adjacent wells of the 96-well plate followed by duplicate of 5 µl aliquots of sample or extraction control RNA. The reaction mix was prepared with the same one-step RT-qPCR system containing the same concentrations of reaction mix, primers, probe, Rox and enzyme mix as was used for NoV analysis. The *C_q* value of the sample was compared to a standard curve obtained by preparing log dilutions from the same batch of Mengo virus as was used to seed samples for analysis. This was expressed as percentage extraction efficiency and samples with an extraction efficiency of less than 1% were not accepted and in such cases RNA extraction was repeated.

3.2.5 Nested RT-PCR and cloning

Reverse transcription (RT) was performed by adding 5 µl of extracted RNA to 3.9 µl molecular biology grade water containing 500 ng of random hexamers (Promega, UK). The mixture was heated at 70°C for 5 min, chilled on ice, and then added to 6.1 µl of reaction mix containing, as final concentrations, 10 mM Tris-HCl (pH = 8.3), 50 mM KCl, 5 mM of MgCl₂, 1 mM of dNTPs, 20 U of RNasin and 50 U of Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). The RT protocol involved incubation for 10 min at 23°C, followed by 60 min at 37°C and inactivation at 95°C for 5 min. Fifteen microliters of cDNA was added to 35 µl of the PCR mixture, yielding a total of 50 µl of a reaction mixture consisting of 10 mM Tris-HCl (pH = 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 20 pmol of each primer, and 1 U AmpliTaq polymerase. The primers (G1, G2, and SM31) used in the first round of PCR were those described by Green et al. (1998). One microliter of the first round PCR product was added to 49 µl of the PCR mastermix containing 10 mM Tris-HCl (pH = 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1 U AmpliTaq polymerase, and 20 pmol of each primer (Ando, E3

for NoV GI and NI and E3 for NoV GII). In the nested PCR, previously described primers were used: Ando (Maguire *et al.*, 1999), E3, and NI (Green *et al.*, 1998). The nested PCR was carried out in separate tubes for NoV GI and NoV GII. The thermocycling format used in both rounds of PCR was as follows: an initial denaturation at 94 °C for 2 min; 30 amplification cycles with denaturation at 95°C for 1 min, annealing at 40 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The amplification products were examined by gel electrophoresis (2% agarose) and molecular weights were determined by comparison with 25 bp DNA ladder (Invitrogen, Carlsbad, CA). The nested PCR products (113 bp for NoV GI and 112b p for NoV GII) were purified using CHROMA SPIN™ columns (Unitech, Dublin, Ireland) and then cloned into the pGEM®-T Easy Vector System (Promega, UK). Transformation of bacterial cells and growth on agar plates was performed according to the manufacturer's instructions. Five white colonies per plate were analysed for the presence of the correct insertion size by PCR with a reaction mixture consisting of 10 mM Tris-HCl (pH = 8.3), 50 mM KCl, 1.25 mM MgCl₂, 0.2 mM dNTPs, 2.5 U AmpliTaq polymerase, and 50 pmol of each primer (pTAg forward and reverse). PCR consisted of a denaturation step at 96 °C for 10 min; 30 amplification cycles at 95 °C for 1 min, 37 °C for 1 min and 72 °C for 1 min; and an extension step at 72 °C for 10 min. When the target band was observed (311 bp and 312 bp for NoV GI and NoV GII respectively) using gel electrophoresis (2% agarose), the PCR products were purified with ExoSAP-IT® (USB Corporation, Cleveland, OH) and sequenced in both directions with the pTAg primers.

3.2.6 DNA sequencing and phylogenetic analysis

The ABI PRISM BigDye Terminator v 3.1 Ready Reaction kit (Applied Biosystems, Foster City, CA) was used according to the manufacturer's protocol and the PCR products were purified by DyeEx 2.0 Spin Kit (Qiagen, West Sussex, UK) to remove incorporated dye terminators. Nucleotide sequences of the partial polymerase region were aligned using ClustalW algorithm of the MegAlign (DNASTar, Inc., Madison, WI, USA) software and compared with reference strains retrieved from

GenBank. The genotype nomenclature of the reference strains has been adopted from the norovirus genotyping tool (National Institute of Public health and the Environment, The Netherlands, <http://www.rivm.nl/mpf/norovirus/typingtool>). All identical sequences were grouped together and relabelled with an alphabetic letter (groupA–groupJ). Phylogenetic analysis was carried out using HKY85 as a model for nucleotide substitution and a phylogenetic tree was constructed by the neighbour-joining method using PAUP* version 4.0 (Swofford, 2003). The reliability of the generated tree was estimated by bootstrap analysis of 1000 resamplings using PAUP* and the tree was drawn using FigTree version 1.3.1.

3.2.7 Reference strains

The reference strains of NoVs included in the phylogenetic analysis were obtained from GenBank and are as follows (accession numbers in brackets); GIV.2 norovirus lion (EF450827), GI.I SRSVKY89 (L23828), GI.2 Southampton (L07418), GI.b WUG1 (AB081723), GI.4 Baltimore (AF414404), GI.4 Chiba (AB0428008), GI.6 Hesse (AF093797), GI.c SZUG1 (AB039774), GI.3 NLV (AY038598), GI.d Vesoul (EF529738), GI.a Desert Shield (U04469), GI.f Otofuke (AB187514), GI.e Chatellerault (EF529737), GII.k OC96065 (AF315813), GII.18 Swine (AY823304), GII.d Hokkaido (AB212306), GII.15 Hiroshima (AB360387), GII.6 Saitama (AB039778), GII.2 Melksham (X81879), GII.f Human (AY682550), GII.1 Hawaii (U07611), GII.e HU2007JP (AB434770), GII.h OC97007 (AB089882), GII.5 MOH (AF397156), GII.c Snow Mountain (AY134748), GII.17 Briancon870 (EF529741), GII.3 Toronto (U02030), GII.b Pont de Roide (AY68259), GII.j E3 (AY682552), GII.g Goulburn Valley (DQ379714), GII.a Arg320 (AF190817), GII.12 HUMAN5017JPN (EU187437), GII.4 Bristol (X76716), GII.4 2006b (EF126966), GII.4 2006a (EF187497), and GII.4 2004 (AB294785), and GII.4 2008 (AB491291).

3.2.8 Statistical analysis

Samples that were determined negative by RT-qPCR for a particular norovirus genogroup were scored 10 genome copies g^{-1} DT for that genogroup (half the LOD). The Anderson–Darling test for normal distribution was applied to each data set and

the NoV concentrations in oysters were logarithmically (base 10) transformed to achieve a normal distribution. Minitab statistical software version 16 (Minitab Inc., State College, PA) was used for the data analysis.

3.3 Results

3.3.1 NoV concentrations in oysters

NoV (GI or GII) was detected in 95.6% ($n = 22$) of the oyster samples analysed ($n = 23$) over the study period with total NoV (GI and GII) concentrations in positive oysters ranging from 97 to 20,080 genome copies g^{-1} DT. NoV concentrations in oysters demonstrated a strong seasonal trend. The mean concentration of total NoV in oysters during both winter periods (October–March) was 4365 genome copies g^{-1} DT (range 363 to 20,080) compared with the mean concentration of 193 genome copies g^{-1} (range $< LOD$ to 4060) in oysters analysed during the two summer periods (April to September). This difference was found to be statistically significant using a two tail unpaired t-test ($p < 0.001$). The mean concentration of total NoV was 960 genome copies g^{-1} DT higher in the second year (October 2008/September 2009) of the study than in the first year (October 2007/September 2008).

For the entire study, the mean concentrations of NoV GI and NoV GII detected in oysters were 1568 and 1837 genome copies g^{-1} DT respectively (Figure 3.1). The NoV GI concentrations detected in oysters differed significantly between the first and second years ($p = 0.046$). This difference was pronounced during the winter months and in the winter of 2008/09. The mean NoV GI concentration in oysters was 5713 (range from 1020 to 15,100) genome copies g^{-1} DT compared to just 211 genome copies g^{-1} DT in the winter of 2007/08. This difference was highly significant ($p < 0.001$). No difference in NoV GI concentrations was detected between the two years during the summer period with the mean concentrations of $< LOD$ and 70 genome copies g^{-1} DT present in oysters during the summer of 2007 and 2008, respectively. In the winter of 2007/08, the mean concentration of NoV GII was 4337 (range 353 to 6910) compared with 1765 (range 938 to 4980) genome copies g^{-1} DT in the winter of 2008/09, but this difference was not statistically different. Mean concentrations of NoV GII detected in the summer of 2008 and 2009 were 912 and 179 genome copies g^{-1} DT, respectively, however these concentrations were not significantly different. Data from the Health

Protection Surveillance Centre (HPSC) for the corresponding geographic region to the investigated area showed a peak in general community reports (not specifically food-related) of NoV gastroenteritis cases (n = 71) occurred in January 2008. The greatest concentrations of NoV in oysters were detected in the following month (February 2008). Conversely in the second year, the greatest concentrations of NoV in oysters were detected in November 2008 and preceded the peak number of NoV cases (n = 99) in the community that occurred in February 2009.

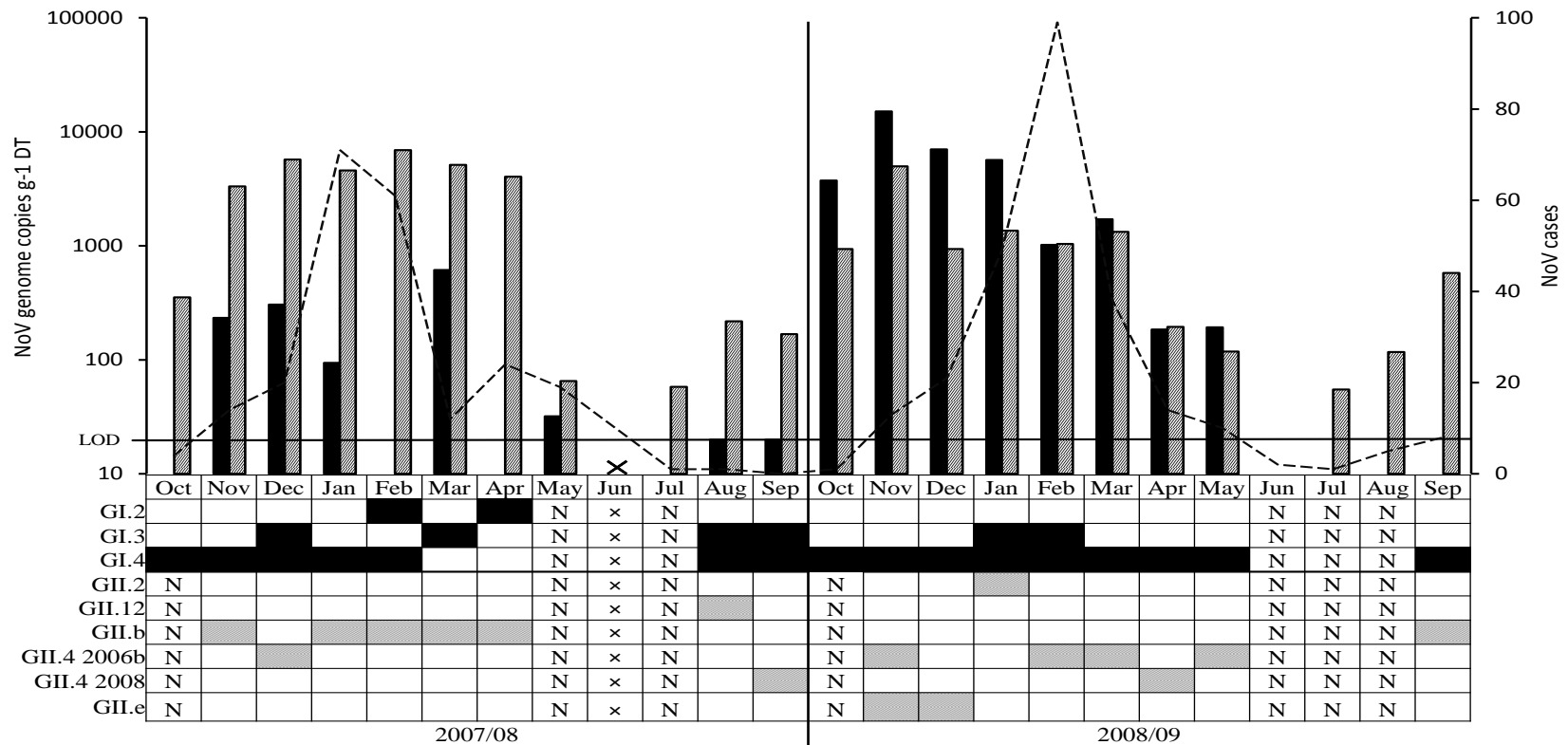


Figure 3.1 Monthly clinical cases of NoV gastroenteritis and concentrations and genotypes of NoV detected in oysters.

RT-qPCR results for NoV GI (black bars) and NoV GII (striped bars) are expressed in genome copies g^{-1} DT. Nested RT-PCR results for NoV GI (black blocks) and NoV GII (striped blocks) are shown in the grid below the graph. The LOD of the RT-qPCR is represented by solid line; (x) represents no sample and (N) represents negative samples from the nested RT-PCR. NoV clinical cases reported each month provided by HPSC are indicated by dotted line (Cloak et al., 2009).

3.3.2 Phylogenetic analysis of oyster samples

During the two-year sampling period, a variety of NoV genotypes were detected in oysters (Figure 3.2). Of the 23 samples analysed, 15 samples were positive for NoV GI by RT-qPCR compared with 18 samples by nested RT-PCR. Three NoV GI genotypes were identified from all positive samples by nested RT-PCR (Figure 3.2). NoV GI.4 was most frequently detected, present in 88.9% of the GI positive samples, followed by GI.3 (33.3%) and GI.2 (11.1%). NoV GI.2 was only identified in the first year of the study, whereas GI.3 and GI.4 were detected in both years.

Of the 23 samples analysed, 22 samples were positive using NoV GII RT-qPCR compared with 16 samples by nested RT-PCR. Five NoV GII genotypes were identified by nested RT-PCR (Figure 3.2). Genotype GII.4 was identified in 43.7% of GII positive samples, followed by GII.b (37.5%), GII.e (12.5%), GII.2 (6.2%) and GII.12 (6.2%). NoV GII.b was detected consistently from January to April 2008 and was the most prevalent genotype detected during the first year, whereas GII.4 was detected more frequently during the second year. NoV GII.2 and GII.12 were detected intermittently throughout the study period. Two variants of GII.4 were prevalent: 2006b and 2008. In November and December 2008 NoV GII.e genotype was detected and was found to have 100% identity to reference strain GII.e (AB434770).

Of all the samples analysed ($n = 23$) multiple NoV genotypes were found in 69.6% ($n = 16$) of the oyster samples. For instance, NoV GI.4, GII.4 2006b and GII.e were identified in oysters sampled in November 2008.

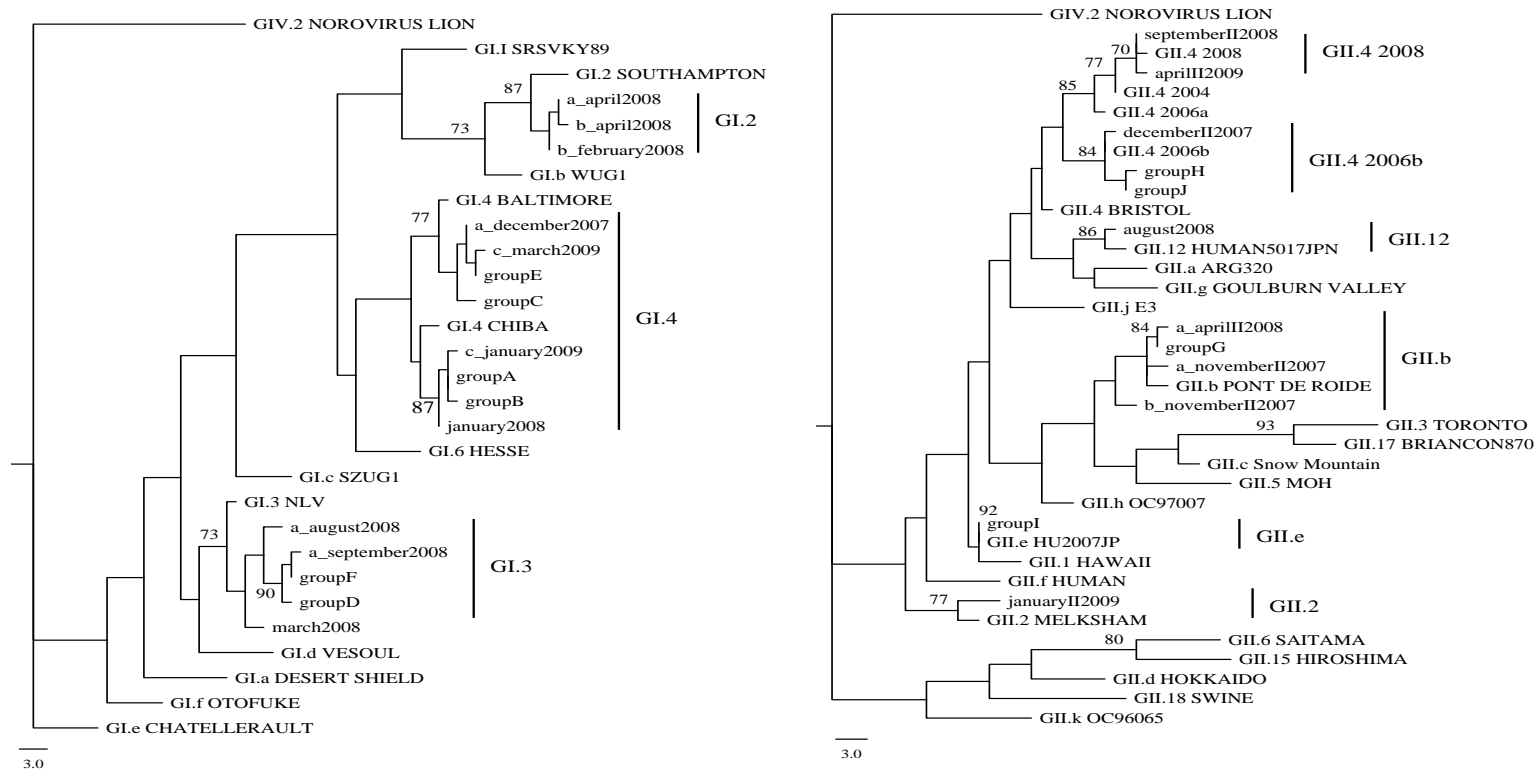


Figure 3.2 Phylogenetic trees for NoV sequences detected in oysters.

Phylogenetic trees for NoV GI (on the left) and NoV GII (on the right) include bootstrap scores for branches shown as a percentage of 1000 replicates. The scale at the bottom represents genetic distances in nucleotide substitutions per site. The name of the sequence preceded by a small letter was used to distinguish between multiple NoV sequences obtained from a single sample. All identical sequences were grouped together and relabeled with a capital letter (groupA–groupJ). The GenBank accession numbers of NoV reference strains are given in Section 4.2.8.

3.4 Discussion

We analysed oysters from a production area over a two year study period, which had previously been linked to outbreaks of NoV illness and was closed for harvesting. Despite this closure, ongoing *E. coli* monitoring of the harvest area indicated that it was compliant with a category B classification prescribed under EU regulation 854/2004 (Anonymous, 2004). Shellfish products harvested from category B areas may be placed on the market for human consumption following treatment such as depuration or relaying so as to meet category A requirements ($< 230 E. coli 100 g^{-1}$). Significant concentrations of NoV were detected in the majority of oyster samples tested over the two years of this study, particularly during the winter months. Total NoV concentrations greater than 100 genome copies g^{-1} DT were detected in 82.6% ($n = 19$) and concentrations greater than 1000 genome copies g^{-1} DT were detected in 52.2% ($n = 12$) of all the samples. While post harvest treatment, such as depuration, can efficiently reduce *E. coli* concentrations as currently practiced, it does not eliminate viruses and outbreaks of illness can still occur (Doré *et al.*, 2010 and Le Guyader *et al.*, 2006). It has been demonstrated that low concentrations of NoV present in oysters do not necessarily result in gastroenteritis when consumed. Lowther *et al.* (2012) suggest that oysters containing total NoV concentrations of < 100 genome copies g^{-1} DT represent a low health risk for consumers. Relatively high concentrations of NoV, > 1000 genome copies g^{-1} DT, may be responsible for a higher incidence of NoV illness (Doré *et al.*, 2010).

This study demonstrates that significant concentrations of NoV were present in oysters in the harvest area, despite compliance with category B health status. It is unlikely that post harvest treatment would reduce NoV contamination present in oysters during the winter months (mean concentration 6013 genome copies g^{-1} DT) to safe concentrations. In combination with the history of illness associated with oysters previously harvested from this area, the data demonstrates the inadequacy of the current reliance on *E. coli* monitoring alone to control risks associated with oyster consumption. Furthermore, it demonstrates the potential value of NoV

monitoring using RT-qPCR as an effective control to prevent oysters with significant concentrations of NoV from being placed on the market for consumption.

Total NoV contamination in oysters demonstrated a clear seasonal trend with elevated concentrations occurring during the winter months and is consistent with previous studies (Formiga-Cruz *et al.*, 2002, Le Guyader *et al.*, 2000 and Lowther *et al.*, 2008). The seasonal peak of NoV in oysters is probably due to the discharge of higher concentrations of NoV from WWTPs at this time due to peak community infections coupled with environmental factors such as lower temperature and decreased solar irradiation. It has been shown that enteric virus indicators can survive for prolonged periods of time at lower temperatures (Duizer *et al.*, 2004 and Sinton *et al.*, 1999) and they are more rapidly inactivated when exposed to elevated doses of sunlight (Sinton *et al.*, 1999). In addition, virus concentrations are reduced more rapidly at higher water temperatures (Doré *et al.*, 1998 and Doré *et al.*, 2000). In this study, total NoV concentrations detected in oysters were higher in the winter of 2008/09 than in the winter of 2007/08. According to the HPSC reports for the corresponding geographic region in Ireland to where the study was conducted, more NoV gastroenteritis cases were notified in the winter season 2008/09 than in the previous winter (Cloak *et al.*, 2009). In the second year of this study, peak NoV cases in the local community occurred after the increase in total NoV concentrations in oysters. This is consistent with another study that showed an increase in NoV concentrations in wastewaters prior to a peak in NoV cases in the community (da Silva *et al.*, 2007). In our study, it is possible that the shellfish harvest area is impacted by a number of WWTPs and increased NoV concentrations in oysters can precede the clinical notifications in the corresponding region of Ireland.

Interestingly, NoV GI concentrations in oysters differed significantly between years unlike NoV GII that was detected at comparable concentrations in both years of this study. NoV GI concentrations were greater than NoV GII concentrations in the second year and may be indicative of increased NoV GI infections occurring in the population at this time. A higher incidence of NoV gastroenteritis was reported in the winter of 2008/09 than 2007/08 in Ireland (Cloak *et al.*, 2009), England and Wales (HPA, 2012), and Belgium (Mathijs *et al.*, 2011). No clinical data is, however,

available showing a significant increase in NoV GI notifications for the winter period of 2008/09. Although NoV GII is responsible for the vast majority of reported clinical cases, environmental samples contain a greater variety of NoV genotypes. A number of studies have demonstrated that the prevalence and concentrations of both NoV genogroups may differ in wastewaters (da Silva *et al.*, 2007, Katayama *et al.*, 2008, Kitajima *et al.*, 2012 and La Rosa *et al.*, 2010). Other studies have demonstrated that they are present at similar concentrations to one another at the same time (Flannery *et al.*, 2012 and Lowther, 2011). It has been suggested that NoV GI is more resistant to the WWTP process than NoV GII (da Silva *et al.*, 2007), and also more stable in the water environment (Lysen *et al.*, 2009) and that these features may lead to increased concentrations of NoV GI in oysters. Recently, it has been demonstrated that NoV GI.1 binds to A-like carbohydrate structures in the digestive gland of oysters and that the expression of this ligand during the winter period can facilitate increased NoV GI accumulation (Maalouf *et al.*, 2010). This disproportionate concentration of NoV GI detected in oysters compared to clinical notifications may be as a result of increased expression of the ligand during the winter of 2007/2008. However, it has not been demonstrated whether expression of this ligand varies between the winter seasons.

Amongst the genotypes identified in oysters, NoV GI.4 was detected most frequently. This finding is consistent with previous studies that detected NoV GI.4 in influent and effluent wastewaters in Japan (Iwai *et al.*, 2009 and Kitajima *et al.*, 2012) and a river catchment in Spain (Pérez-Sautu *et al.*, 2012). It has also been reported that NoV GI.4 was the most frequently identified genotype in a large gastroenteritis outbreak of suspected waterborne transmission in Northern Italy (Di Bartoloa *et al.*, 2011) and in a large foodborne outbreak in which more than 200 people were affected by frozen raspberries in Finland (Maunula *et al.*, 2009). Verhoef *et al.* (2010) showed that NoV GI.4 along with GI.2 was more frequently detected in foodborne outbreaks than in those transmitted via person-to-person. NoV GI.4 may have some unique properties that allows for better persistence in the environment and possibly better accumulation in shellfish. NoV GI.2 and GI.3 were detected less frequently throughout the study than GI.4; however, they have been detected in

shellfish-related outbreaks worldwide (David *et al.*, 2007, Kageyama *et al.*, 2004, Le Guyader *et al.*, 2003 and Nakagawa-Okamoto *et al.*, 2009).

The second most frequently identified genotype in oysters was NoV GII.4, followed by NoV GII.b. In the first year of the study NoV GII.b was more prevalent in the oyster samples and was then replaced in the second year by NoV GII.4, especially the variant 2006b. NoV GII.b was first identified in a large multi-pathogen waterborne outbreak in France in 2000 (Gallay *et al.*, 2006) and since then has been recognised in many foodborne and person-to-person outbreaks across Europe (Bon *et al.*, 2005, Koopmans *et al.*, 2003 and Reuter *et al.*, 2005). NoV GII.b was circulating in the Irish population between 2006 and 2007 and was responsible for paediatric outbreaks reported at that time (Waters *et al.*, 2008). NoV GII.4 2006b variant was first identified in the summer of 2006 and became the dominant variant of GII.4 from October 2007 in Europe when the number of reported gastroenteritis outbreaks increased (Siebenga *et al.*, 2009). In Ireland, NoV GII.4 2006b can be linked to three clusters of NoV infections involving 62 cases reported following pilgrimages to Lourdes in late September 2008 (Verhoef *et al.*, 2008). From our study, NoV GII.4 2006b was the predominant variant of NoV GII.4 found in oysters and therefore probably the predominant variant circulating in the Irish population in this region during the winter of 2008/2009.

Phylogenetic analysis was carried out using the partial (< 100 bp) polymerase gene of the NoV genome. While this fragment of the NoV genome allows NoV genotypes to be categorised, its usefulness to distinguish between NoV GII.4 variants is limited, especially those that exhibit high nucleotide similarity in the polymerase region (GII.4 2004 and GII.4 2008). Alternative primers, targeting the capsid N/S domain of the NoV genome, may be useful in further elucidating the NoV genotype profiles in contaminated oyster samples (Nishida *et al.*, 2003). We undertook further sequencing analysis targeting the capsid N/S domain in one sample from November 2008 which revealed a link between the GII.e polymerase and the GII.4 2007 capsid genotype (GII.e/GII.4 2007) (data not presented). This GII.e/GII.4 2007 variant has been simultaneously identified in Belgium as causing outbreaks in November 2008, December 2008 and February 2009 (Mathijs *et al.*,

2011). As yet, data relating to the presence of the GII.e/GII.4 2007 variant in the Irish population is unavailable.

This study demonstrates that RT-qPCR monitoring provides a better assessment of NoV contamination risk within shellfish harvest area than current bacteriological monitoring alone. In a recent opinion by the European Food Safety Authority, it was recommended that risk managers should consider establishing an acceptable limit for NoV in oysters to be placed on the market (EFSA, 2012). Until such a limit is established RT-qPCR monitoring in oyster harvest areas could be conducted to establish a more comprehensive approach to risk management than currently offered from *E. coli* monitoring alone. In addition, this study demonstrated contamination of oysters with multiple genotypes of GI and GII NoV. Although no direct evidence is presented in this paper, it is possible that the disproportionate detection of NoV GI genotypes in oysters compared to the dominance of NoV GII.4 in clinical reports may be because of asymptomatic infections in the community, better environmental survival or preferential accumulation in oysters associated with NoV GI genotypes or a combination of these factors.

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**Chapter 4 Norovirus genotypes
present in oysters and in effluent
from a wastewater treatment plant
during the seasonal peak of
infections in Ireland in 2010.**

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Abstract

We determined norovirus (NoV) concentrations in effluent from a wastewater treatment plant and in oysters during the peak period of laboratory-confirmed cases of NoV infection in Ireland in 2010 (January to March). Weekly samples of influent, secondary treated effluent, and oysters were analyzed using real-time quantitative reverse transcription-PCR for NoV genogroup I (GI) and genogroup II (GII). The mean concentration of NoV GII (5.87×10^4 genome copies 100 ml^{-1}) in influent wastewater was significantly higher than the mean concentration of NoV GI (1.40×10^4 genome copies 100 ml^{-1}). The highest concentration of NoV GII (2.20×10^5 genome copies 100 ml^{-1}) was detected in influent wastewater during week 6. Over the study period, a total of 931 laboratory-confirmed cases of NoV GII infection were recorded, with the peak ($n = 171$) occurring in week 7. In comparison, 16 cases of NoV GI-associated illness were reported during the study period. In addition, the NoV capsid N/S domain was molecularly characterized for selected samples. Multiple genotypes of NoV GI (GI.1, GI.4, GI.5, GI.6, and GI.7) and GII (GII.3, GII.4, GII.6, GII.7, GII.12, GII.13, and GII.17), as well as 4 putative recombinant strains, were detected in the environmental samples. The NoV GII.4 variant 2010 was detected in wastewater and oyster samples and was the dominant strain detected in NoV outbreaks at that time. This study demonstrates the diversity of NoV genotypes present in wastewater during a period of high rates of NoV infection in the community and highlights the potential for the environmental spread of multiple NoV genotypes.

4.1 Introduction

Norovirus (NoV) is the leading cause of acute gastroenteritis outbreaks in Ireland (Cloak *et al.*, 2010) and is responsible for an estimated 90% of all epidemic non-bacterial outbreaks of gastroenteritis worldwide (Lindesmith *et al.*, 2003). NoV is highly infectious, with transmission occurring predominantly through person-to-person contact or by ingestion of faecally contaminated food or water (Matthews *et al.*, 2012). The majority of NoV outbreaks reported in Ireland occur in healthcare settings (Kelly *et al.*, 2008) and follow the pattern of winter seasonality demonstrated previously in the Northern Hemisphere (Mounts *et al.*, 2000).

NoV is a distinct genus of the family *Caliciviridae* that contains viruses with a positive-sense, polyadenylated RNA genome of approximately 7.5 kb. The genome of human NoV is organised into three open-reading frames (ORFs). A NoV recombination hot spot has been identified close to the ORF1/ORF2 junction (Bull *et al.*, 2005), and at least 22 NoV recombinants have been detected (Hansman *et al.*, 2010). The lack of a proofreading ability of the RNA dependent RNA polymerase (RdRp) contributes to a high error rate of NoV genome transcription, resulting in even greater genetic diversity (Barr and Fearn, 2010). Five NoV genogroups have been recognized (GI to GV) and may be subdivided into at least 29 genotypes (Zheng *et al.*, 2006). However, NoV GII genotype 4 (GII.4) accounts for the majority of acute gastroenteritis outbreaks characterized worldwide.

NoV is shed at high concentrations in the faeces of infected patients (10^8 to 10^{12} genomic copies per g), and may be shed for up to 8 weeks after symptoms have subsided (Atmar *et al.*, 2008). NoV particles may be detected in faeces of both symptomatic and asymptomatic individuals (Ayukekbong *et al.*, 2011 and Atmar *et al.*, 2008) and thus are commonly present in wastewater treatment plant (WWTP) effluents throughout the year (Flannery *et al.*, 2012 and Nordgren *et al.*, 2009). Shellfish harvest areas may be affected by the discharge of wastewater, and oysters can accumulate NoV from the surrounding waters through the process of filter feeding. Under European regulation (Anonymous, 2004), assessment of the sanitary quality of shellfish harvest areas relies on the use of the bacterial indicator organism

E. coli. However, *E. coli* has been shown to be an inadequate indicator of viral contamination (Flannery *et al.*, 2009), and numerous NoV outbreaks have been caused by shellfish compliant with the current regulations (Baker *et al.*, 2010, Doré *et al.*, 2010, and Le Guyader *et al.*, 2010). Furthermore, a characteristic of shellfish-related outbreaks is the presence of multiple NoV GI and GII genotypes in the faeces of infected patients (Le Guyader *et al.*, 2006 and Kageyama *et al.*, 2004), whereas outbreaks in closed or semiclosed settings are generally associated with a single GII genotype (Matthews *et al.*, 2012), most commonly GII.4 (Kroneman *et al.*, 2008).

In the present study, we investigated NoV concentrations in both influent and secondary treated effluent at a WWTP and in oysters placed adjacent to the WWTP discharge pipe over a 13 week period. In addition, the NoV concentrations/genogroups detected were subsequently compared with the number of laboratory-confirmed gastroenteritis cases caused by NoV GI and GII identified from outbreaks (community and food-related) investigated during that time. Finally, we characterised the NoV genotypes detected during a 5-week period, which had the highest NoV concentrations in municipal wastewater.

4.2 Materials and methods

4.2.1 Sample population

A WWTP providing secondary wastewater treatment and serving a population equivalent of 91,600 was selected for this study. The final effluent was discharged to coastal waters through a 420-m-long outfall pipe submerged at a depth of 10 m. One-litre, 24-hour composite samples of influent (n=13) and effluent (n=13) were taken on a weekly basis from January to March (week 1 to 13) of 2010 and analysed for NoV GI and NoV GII by real-time quantitative reverse transcription PCR (RT-qPCR). Oysters (*Crassostrea gigas*), previously demonstrated to be free from NoV contamination were suspended in mesh bags 1 m below the water surface and attached to a buoy anchored to the outfall pipe. Oyster samples (n=13), each consisting of a minimum of 10 animals, were collected 5 days after the wastewater samples, on a weekly basis (week 2 to 14). Oyster and wastewater samples were transported to the laboratory within 2 hours under chilled conditions (<15°C). The greatest concentrations of NoV in the influent and effluent wastewater samples were detected between weeks 5 and 9 of 2010, and these samples were selected for further molecular characterisation. Oyster samples corresponding to the selected wastewater samples (week 6 to 10) were also included in the sequencing analysis.

Stool samples (n=2734) received from symptomatic patients throughout Ireland between January and March 2010 were analysed for the presence of NoV RNA. The majority of the samples were received from healthcare settings. Stool samples received from representative outbreaks (n=8) that occurred between week 5 and 10 of 2010 were molecularly characterised. One NoV-positive stool sample was randomly selected per NoV outbreak.

4.2.2 Preparation of wastewater concentrate and shellfish proteinase K extract

A membrane-filter adsorption-elution method was used for the concentration of wastewater samples as previously described (Flannery *et al.*, 2012). Briefly, 40 ml of each wastewater sample was passed through a cellulose prefilter (Milipore, Billerica, MA) placed directly on a negatively charged filter (Millipore) with a 0.45- μm pore size, using a vacuum pump system. Twenty-five millilitres of 0.14 M NaCl

was passed through the filters that were then placed in 4 ml of 50 mM glycine-NaOH buffer (pH 9.5) with 1% beef extract and mixed for 20 min at 500 rpm. The virus eluate was transferred to an Amicon ultra-4 (50 kDa) (Millipore, Billerica, MA) centrifugal filter and centrifuged at $4000 \times g$ for 10 min. The retentate was resuspended in 550 μ l of molecular biology-grade water, and wastewater concentrate was stored at -20°C prior to RNA extraction.

Oyster samples were cleaned by rinsing under potable water and opened using a flame-sterilised shucking knife. For each sample, the digestive tissues (DT) of 10 oysters were dissected and chopped, and 2 g was transferred to a 50-ml tube containing 2 ml of $100 \mu\text{g ml}^{-1}$ proteinase K solution (Sigma-Aldrich) followed by the addition of 50 μ l of Mengo virus strain MC₀ as an internal positive control to control for extraction efficiency. This mixture was then incubated at 37°C with shaking for 60 min, followed by incubation at 60°C for 15 min. Following centrifugation at $3000 \times g$ for 5 min, the shellfish proteinase K extract (supernatant) was stored at -80°C prior to RNA extraction within 1 month.

4.2.3 RNA extraction procedure for shellfish and wastewater.

Viral RNA was extracted from 500 μ l of either shellfish proteinase K extract or wastewater concentrate using the NucliSENS® miniMAG® extraction platform and NucliSENS® magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France) according to manufacturer's instructions. A negative RNA extraction control (molecular biology grade water) was included with each batch of samples extracted. Viral RNA was eluted into 100 μ l of elution buffer and stored at -80°C . Additionally, each shellfish sample was extracted at three different dilutions of shellfish proteinase K extract: neat, 1:2, and 4:1 using Phosphate buffered saline (Oxoid, Basingstoke, UK), and eluted in 30 μ l of elution buffer prior to the nested RT-PCR.

4.2.4 NoV GI and GII quantification using one-step RT-qPCR

A previously described RT-qPCR assay was used to detect and quantify both NoV GI and GII in the influent, the effluent and oyster samples (Flannery *et al.*, 2012). For NoV GI analysis, previously described primers QNIF4 (da Silva *et al.*, 2007)

NV1LCR and probe NVGG1p (Svraka *et al.*, 2007) and for NoV GII analysis, primers QNIF2 (Loisy *et al.*, 2005), COG2R (Kageyama *et al.*, 2003) and probe QNIFS (Loisy *et al.*, 2005) were used. For internal positive control, primers Mengo209, Mengo110 and probe Mengo147 used were the same as those described by Pintò *et al.* (Pintó *et al.*, 2009).

Plasmids pGEM-3Zf(+) carrying the NoV GI and GII target sequences containing restriction site (BamHI) to check for contamination (supplied by Dr. Francoise S. Le Guyader, Ifremer, Nantes, France) were used to enable quantification of NoV RNA in copies per μl . A log dilution series of GI and GII DNA plasmids (range 1×10^1 to 1×10^5 copies μl^{-1}) were included in duplicate on each RT-qPCR run and the number of NoV RNA copies per μl was determined. The limit of detection (LOD) for NoV GI and GII was 20 genome copies g^{-1} of DT and 25 genome copies 100 ml^{-1} for shellfish and wastewater samples, respectively. All the samples were assessed for RT-PCR inhibition using external control RNA (Flannery *et al.*, 2012). Samples with an amplification efficiency of less than 25% were not accepted and in such cases the sample RNA was reanalysed at a 1:10 dilution. All of the samples were also assessed for extraction efficiency using Mengo virus as internal process control. Samples with of greater than 1% extraction efficiency were accepted for inclusion in this study.

4.2.5 NoV molecular characterisation and genotyping

A selection of influent and effluent wastewater, oyster and NoV outbreak samples received between weeks 5 and 10 of 2010, were further characterized and sequenced as follows. 8 μl of the RNA was treated with 1 U of RNase-Free DNase (Promega, UK) according to the manufacturer's protocol. Reverse transcription (RT) was performed in a 30 μl reaction volume containing 1 mM of dNTPs, 10 mM of dithiothreitol, 0.75 μg of random hexamers, 33 U RNase inhibitor, 300 U of SuperScript™ II Reverse Transcriptase (Invitrogen, UK) and 4.5 μl Superscript II buffer as described previously by (Nishida *et al.*, 2007). RT was carried out at 42°C for 75 min, followed by an inactivation step at 99°C for 5 min. The first round of nested PCR containing 5 μl cDNA and final concentrations of 10 mM Tris-HCl (pH

8.3), 50 mM KCl, 20 μ M of dNTPs, 2 μ M of each primer, 2.5mM of MgCl₂ and 2.5 U of AmpliTaq® DNA Polymerase (Applied Biosystems, USA). The primers used for the NoV GI and NoV GII reactions were COG1F, G1-SKR and COG2F, G2-SKR, respectively (Kojima *et al.*, 2002) (Kageyama *et al.*, 2003). First round PCR product (1 μ l) was subsequently added to 49 μ l of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 20 μ M of dNTPs, 0.4 μ M of each primer, 2.5 mM of MgCl₂ and 2.5 U of AmpliTaq® DNA Polymerase. In the nested RT-PCR, the pair of primers were used GISKF, GISKR for NoV GI and GIISKF, GIISKR for NoV GII (Kojima *et al.*, 2002). The PCR was performed under the following conditions: an initial denaturation at 95°C for 5 min; 40 amplification cycles with denaturation at 95°C for 1 min, annealing 50°C for 1 min, and extension at 72°C for 2 min; and a final extension of 72°C for 15 min. Amplified DNA fragments were purified using CHROMA SPIN™ columns (Unitech) and products were cloned into pCR®4-TOPO® vector according to the protocol for TOPO TA Cloning® Kit (Invitrogen). Approximately 4 to 6 clones were randomly selected for DNA sequencing. PCR products were sequenced using BigDye Terminator v 3.1 Ready Reaction kit (Applied Biosystems) as per manufacturer's recommendations and analysed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Ten percent faecal suspensions were prepared in Star buffer (Roche) and were extracted (350 μ l) by the MDX automated extractor (Qiagen) using the QIAamp One-For-All protocol as per the manufacturer's instructions. Brome Mosaic Virus (BMV, 0.05pg μ l⁻¹) internal control RNA was added to the lysis buffer prior to extraction. A multiplex real-time RT-PCR was carried out using the Superscript III Platinum one-step Quantitative RT-PCR system Kit (Invitrogen) as per manufacturer's instructions and a 25 μ l reaction volume. The primer and probe sequences for NoV were taken from Rolfe *et al* (Rolfe *et al.*, 2007) and Kageyama *et al* (Kageyama *et al.*, 2003) and were used at concentrations of 400 nM and 80 nM for primers and probes, respectively. BMV was co-amplified by in-house primer and probes at concentrations of 200 nM and 100 nM, respectively. Primer and probes sequences for BMV were as follows: BMV F: 5'-CCT TTT TCA CTG CTT GTT TCG

AGA A-3', BMV R: 5'-TTT CCG ATA GGC ACA ATG AAC CT-3' and BMV Probe: 5' NED – ACT GGC CCT GAA ATC –NFQ (MGB) (Applied Biosystems).

4.2.6 Phylogenetic analysis

The NoV sequences were aligned with reference strains retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/>) by using the ClustalW algorithm of the MegAlign software (DNASTar, Inc., Madison, Wis. USA). The genotype nomenclature of the reference strains was adopted from the online Norovirus Genotyping Tool (<http://www.rivm.nl/mpf/norovirus/typingtool>; National Institute of Public Health and the Environment, Netherlands). The length of the NoV genome region used was 282 bp for NoV GI (5354-5645 of Norwalk virus M87661) and 269 bp for NoV GII (5085-5353 of Lordsdale virus X86557). NoV bovine strain Newbury GIII.2 was used as an outgroup strain in phylogenetic analysis of NoV GI and GII, and the GII.3 Oberhausen AF53944 was used as an outgroup for GII.4 analysis.

A maximum likelihood phylogenetic tree was constructed using PAUP* version 4.0 (Swofford, 2003). Modeltest was used to select the most appropriate model of evolution using the hierarchical likelihood ratio test (Posada and Crandall, 1998). The maximum likelihood phylogenetic tree was based on the TrN+I+G model of substitution for NoV GI, the TrNef+I+G for NoV GII and K80+G for NoV GII.4 (Posada and Crandall, 1998). The reliability of the generated tree was estimated by bootstrap analysis of 1,000 replicates of the sequence alignment, using the neighbour-joining method.

4.2.7 Recombinant analysis

Detection of potential NoV GI and GII recombinant sequences was carried out using the Recombination Detection Program version 4.14 (RDP4) (Martin *et al.*, 2010) comprising the following programmes: RDP, GENECONV, BOOTSCAN, MaxChi, CHIMAERA, SISCAN, and 3SEQ. The default settings for each of the programmes were used in the RDP4, except for the BOOTSCAN and SISCAN, where a window size of 40 and a step size of 20 were used. The potential NoV recombinants were

considered as a putative recombinant sequences if they were detected by at least 3 different programs.

4.2.8 Statistical analysis

The Anderson-Darling test for normal distribution was applied to each data set, and the NoV concentrations in wastewaters and oysters were logarithmically (base 10) transformed to achieve a normal distribution. Minitab statistical software, version 16 (Minitab Inc., State College, PA), was used for the data analysis. The reductions during the wastewater treatment process were calculated on a weekly basis by using the following equation: $\log \text{ reduction} = \log (N_{\text{inf}}/N_{\text{eff}})$, where N_{inf} is the concentration of NoV detected in influent wastewater and N_{eff} is the concentration of NoV detected in effluent wastewater.

4.2.9 Nucleotide sequence accession numbers

The GenBank accession numbers for all sequences analysed during this study are as follows: JQ362499-JQ362594 and JQ280400-JQ280407.

4.3 Results

4.3.1 NoV concentration in the influent, effluent and oysters between January and March 2010

NoV GI and GII were detected in influent, effluent, and oyster samples on all sampling occasions throughout the sampling period. The mean NoV GI and GII concentrations detected in influent wastewaters were 3.93 and 4.58 log genome copies 100 ml⁻¹, respectively (Table 4.1). The mean concentration of NoV GII was 0.66 log genome copies 100 ml⁻¹ higher than the mean concentration of NoV GI in influent wastewater, and this difference was significant (paired t-test; $p < 0.001$). The mean NoV GI and GII concentrations detected in effluent wastewater were 3.01 and 3.44 log genome copies 100 ml⁻¹, respectively, and were not statistically different (paired t-test; $p > 0.05$). The mean concentrations of NoV GI and GII detected in oyster samples were 4.43 and 4.84 log genome copies g⁻¹, respectively, and this difference was statistically significant (paired t-test; $p = 0.005$). The mean NoV GI and GII reductions during the treatment process were 0.92 and 1.14 log genome copies 100 ml⁻¹, respectively.

Table 4.1 Mean concentrations of NoV GI and GII in influent and effluent wastewater and impacted oysters between January and March 2010.

NoV genogroup	Mean concentration \pm SD (range)		
	Influent ^a	Effluent ^a	Oysters ^b
GI	3.93 \pm 0.44 (3.08 – 4.76)	3.01 \pm 0.63 (1.73 – 4.06)	4.43 \pm 0.45 (3.50 – 5.13)
GII	4.58 \pm 0.38 (3.98 – 5.34)	3.44 \pm 0.47 (2.34 – 3.99)	4.84 \pm 0.25 (4.39 – 5.23)

^a Concentration expressed as log genome copies 100 ml⁻¹

^b Concentration expressed as log genome copies g⁻¹

4.3.2 Laboratory-confirmed cases of NoV gastroenteritis

During the study period, NoV GI and GII RNAs were detected in 16 and 931 stool samples, respectively. A peak in laboratory-confirmed cases occurred in week 7 of 2010, with 171 patient samples testing positive for NoV GII (Figure 4.1). The largest number of laboratory-confirmed cases of NoV GI infections (n=4) were detected in week 9 of 2010. Overall NoV GII cases were confirmed approximately 58.2 times more frequently than NoV GI cases ($p < 0.001$). The highest NoV GII concentration was detected in influent wastewater in week 6 of 2010 (2.20×10^5 copies 100 ml^{-1}), which preceded the peak of laboratory-confirmed cases involving NoV GII, in week 7. In the influent, the highest concentration of NoV GI (5.59×10^4 copies 100 ml^{-1}) was detected in week 7.

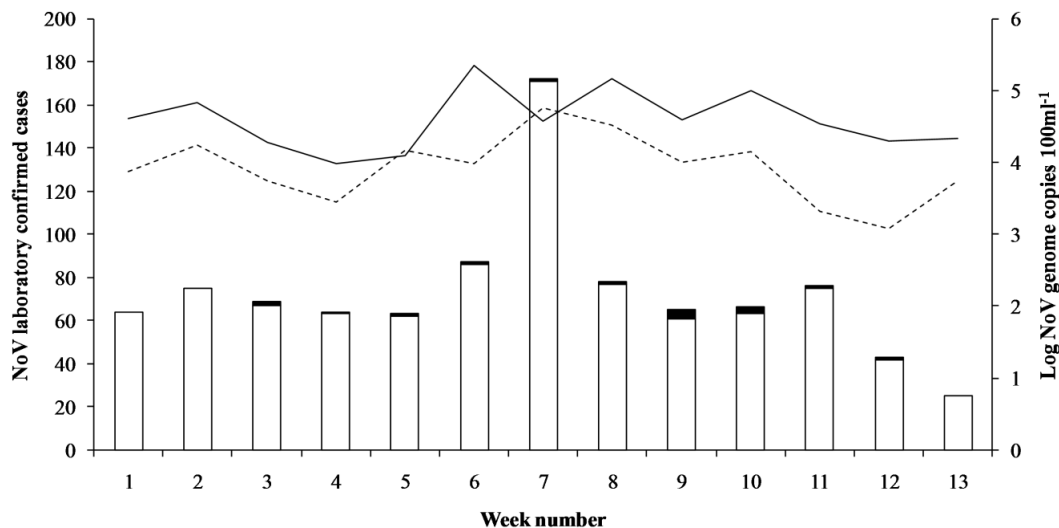


Figure 4.1 Laboratory-confirmed NoV cases and NoV concentrations in influent wastewater.

The numbers of gastroenteritis cases attributed to NoV GI (■) and NoV GII (□) are shown, along with concentrations of NoV GI (---) and NoV GII (—) detected in influent wastewaters between January and March 2010 (week 1 to 13). One NoV-positive stool sample was randomly selected per the NoV outbreak for further sequencing analysis. Eight NoV outbreaks were genetically characterised as follows: in week 5 one outbreak 10IRL03196 identified as GII.4 2010, week 6 two outbreaks 10IRL02653 and 10IRL02654 identified as GII.4 2010, week 7 four outbreaks 10IRL04566 and 10IRL07388 identified as GII.4 2010, 10IRL03903 as GII.13, and 10IRL05499 as GII.4 2008 and in week 9 10IRL06528 identified as GI.6.

4.4 Multiple NoV genotypes detected in wastewater, oyster and stool samples

Following phylogenetic characterisation of samples from week 5 to 10, NoV GI sequences (n=73) were ascribed to five different genotypes, namely, GI.1, GI.4, GI.5, GI.6, and GI.7 (Figure 4.2), and NoV GII sequences (n=74) were ascribed to seven genotypes, namely, GII.3, GII.4, GII.6, GII.7, GII.12, GII.13, and GII.17 (Figure 4.2). Among NoV GII.4 genotypes, three variants were identified: 2006b, 2008, and 2010. NoV sequences with 100% identity detected in the different samples were designated in groups A-E for NoV GI and groups F-G for NoV GII (Figure 4.2).

NoV outbreaks (n=8) that occurred between week 5 and 10 of 2010 (Figure 4.1) were genetically characterised and incorporated in the phylogenetic analysis (Figure 4.2). Outbreaks were associated with NoV GII.4 variant 2010 (n=5), NoV GII.4 variant 2006b (n=1), NoV GII.13 (n=1) and NoV GI.6 (n=1). A NoV GI.6 sequence (10IRL06528)(Figure 4.2) associated with an outbreak that occurred during week 9 had 100% sequence identity with NoV sequences detected in effluent and oyster samples collected during week 8 (group D) (Figure 4.2).



Figure 4.2 Molecular characterisation of NoV GI and GII sequences detected in Ireland in wastewater, oysters and gastroenteritis outbreaks between January and March 2010.

Phylogenetic trees were based on the partial capsid region of the NoV GI and GII genomes (282 and 269 bp, respectively). For NoV GII.4 sequences, a separate phylogenetic tree was built. All phylogenetic trees include bootstrap scores for branches as a percentage of 1000 replicates (bootstrap values of <70% are not shown). NoV sequences were named as INF (influent), EFF (effluent), and oysters (OYS) followed by 00 (sampling day) and 00 (sampling month) followed by consecutive numbers use for laboratory differentiation.

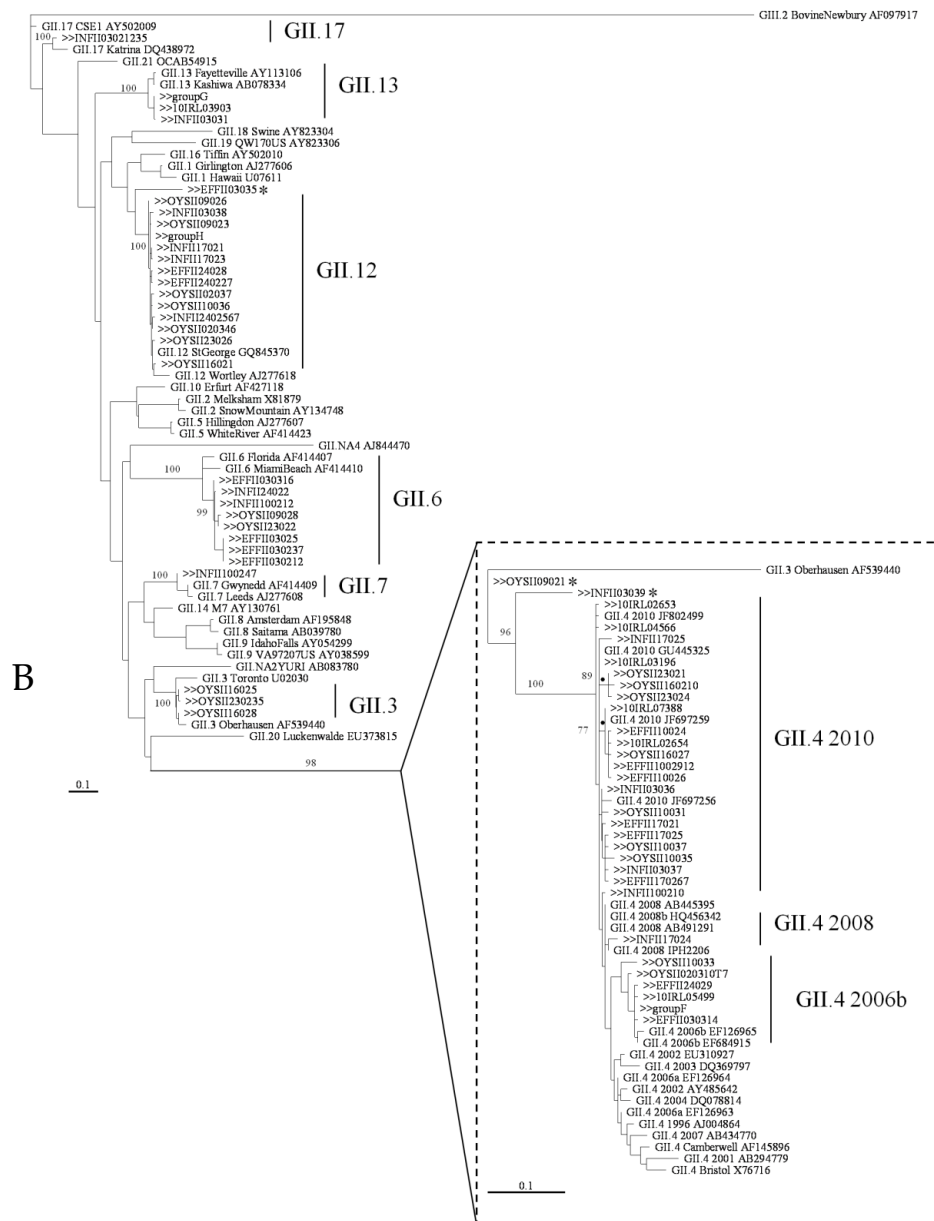


Figure 4.2 continued. Identical sequences of NoV GI detected in different samples were designated in group A (influent sampled on week 5, 6, 7, 8; effluent: week 5, 9 and oysters: week 9), group B (oysters: week 6, 8), group C (influent: week7, 9; oysters week 8), group D (effluent week 8 and oysters week 8), and group E (influent week 5 and 9). NoV GI sequence detected in the NoV outbreak was labelled as 10IRL06528. Identical NoV GII sequences detected in different samples were designated in group F (influent sampled on week 5, 7 and effluent on week 8), group G (effluent week 6 and 7), and group H (oyster week 8 and 9). NoV GII sequence detected in the NoV outbreak was labelled as 10IRL02653, 10IRL02654, 10IRL03196, 10IRL03903, 10IRL04566, 10IRL05499, and 10IRL07388.

(*) represents a putative recombinant.

4.4.1 Norovirus genotype pattern in wastewater and oysters

In five samples of influent wastewater (n=25 sequences), the most frequently detected NoV GI genotype was GI.7 (60.0%; n=15/25) followed by GI.5 (20.0%; n=5/25), GI.4 (16.0%; n=4/25) and GI.6 (4.0%; n=1/25) (Figure 4.3a). In five samples of effluent wastewater (n=25 sequences), NoV GI.4 was the most prevalent NoV GI genotype detected in 44.0% of the sequences (n=11/25) followed by GI.7 (40.0%, n=10/25) and GI.6 (16.0%; n=4/25). In oyster samples, NoV GI.4 was also the predominant NoV GI genotype (56.5%; n=13/23) and other genotypes identified were GI.6 (21.7%; n=5/23), GI.7 (13.0%; n=3/23), GI.5 (4.3%; n=1/23) and GI.1 (4.3%; n=1/23) (Figure 4.3a).

NoV GII.4 was the dominant NoV GII genotype detected (33.3%; n=8/24) in influent wastewater, followed by GII.12 (25.0%; n=6/24), GII.17 (16.7%; n=4/24), GII.6 (12.5%; n=3/24), GII.7 (8.3%; n=2/24) and GII.13 (4.2 %; n=1/24). In the effluent wastewater, the dominant genotype of NoV sequences was GII.4 (54.2%; n=13/24) also, followed by GII.6 (25.0%; n=6/24), GII.12 (12.5%; n=3/24) and GII.13 (8.3%; n=2/24). Genotypes GII.7 and GII.17 were not detected in the effluent despite being detected in the influent wastewater. In oysters, two genotypes; GII.4 (38.5%; n=10) and GII.12 (38.5%; n=10) were detected at the same frequency, followed by GII.3 (15.4%; n=4) and GII.6 (7.7%; n=2). Two genotypes, NoV GI.1 and GII.3 were detected in oysters, but these were not detected in wastewater samples (Figure 4.3b).

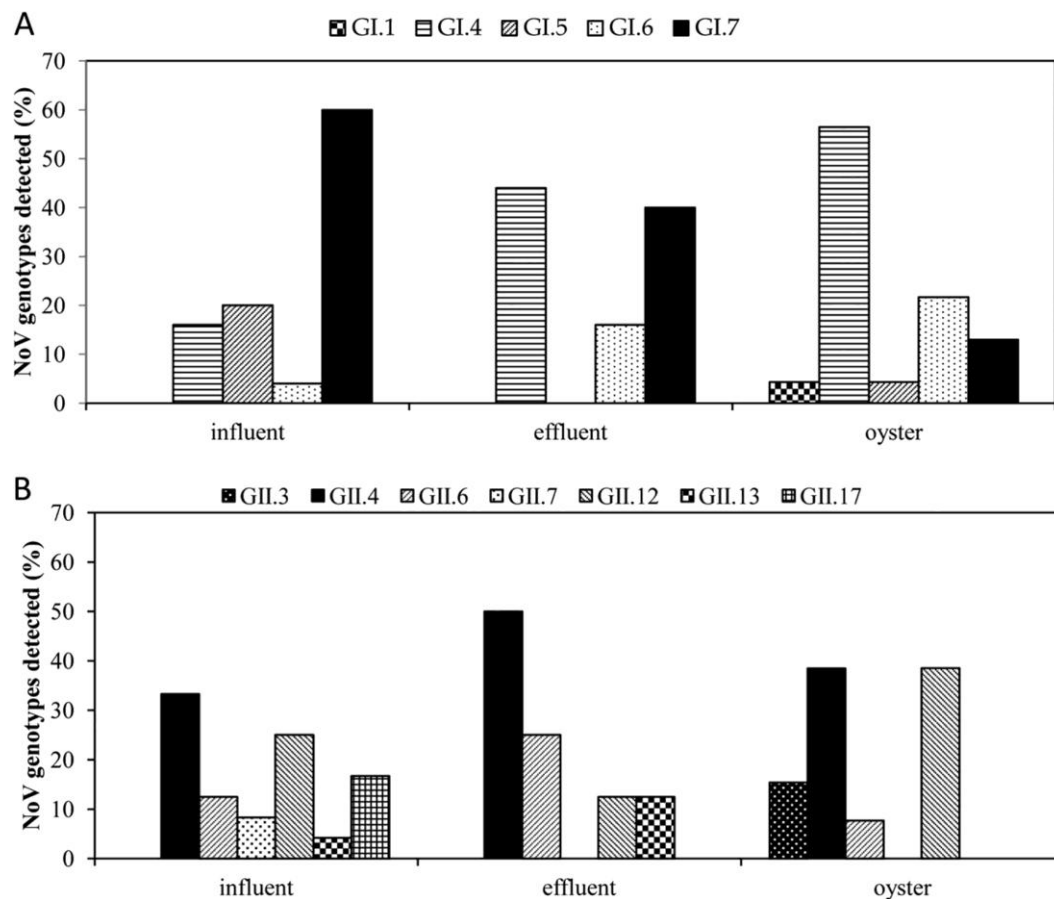


Figure 4.3 NoV genotype profile detected in wastewater and oyster samples.

NoV GI genotypes (a) and NoV GII genotypes (b) are shown as a percentage of all genotypes detected in either the influent or effluent wastewater or oyster samples.

4.4.2 Recombinant analysis

Four potential NoV recombinant strains were identified following analysis by the RDP4. A sequence was considered to be a putative recombinant if a recombination event was detected by at least 3 recombinant detection methods. The parent genotype and putative recombinants detected were as follows: GII.12/GII.4 2006b (EFFII03035), GII.4 2010/GII.13 (INFII03039), and GII.4 2010/GII.3 (OYSII09021) for NoV GII and GI.4/GI.6 for NoV GI (Figure 4.4). Potential parental sequences and possible recombination break points are listed in Table 4.2.

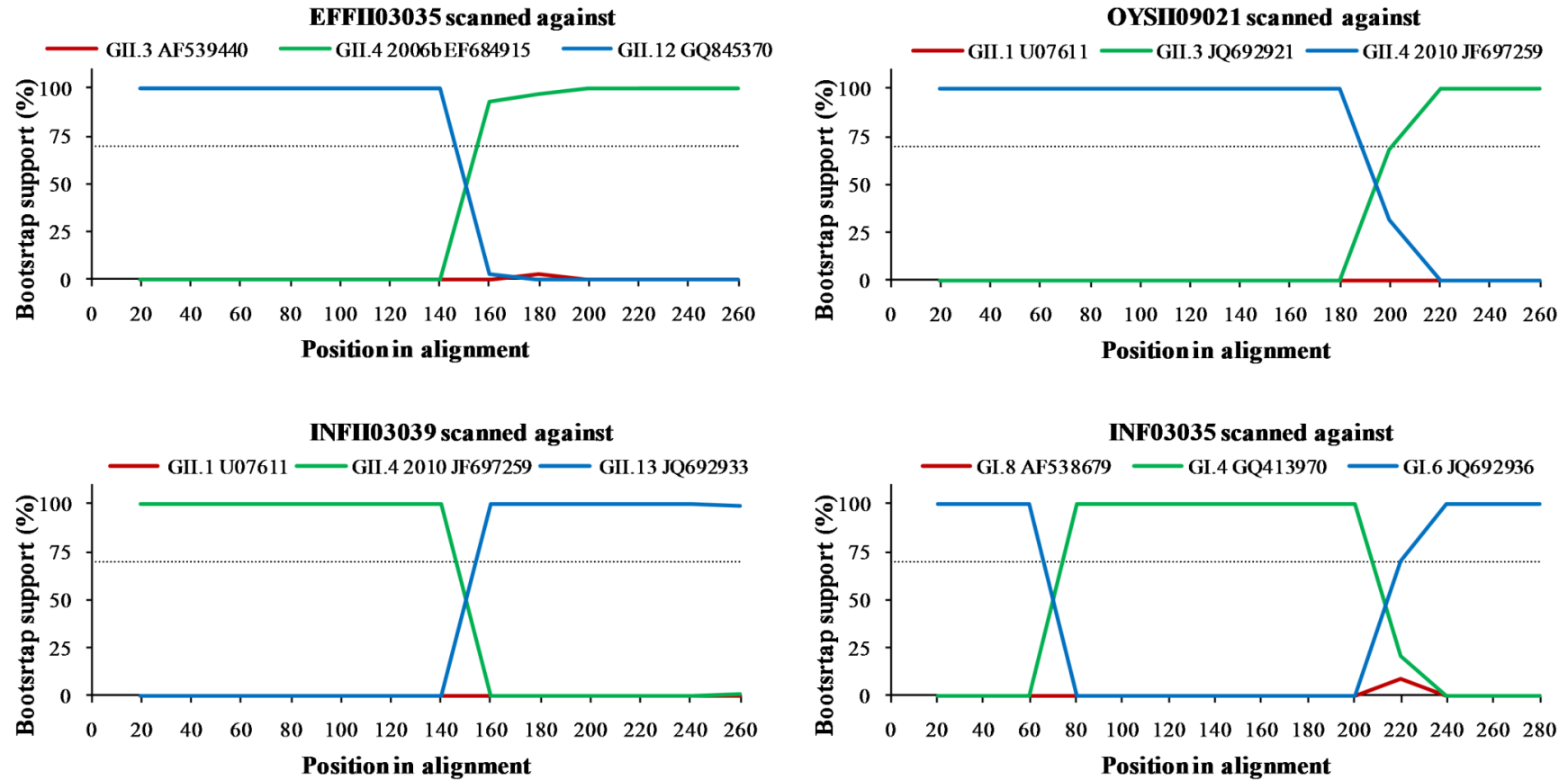


Figure 4.4 BOOTSCAN evidence for the recombination events.

BOOTSCAN analysis was performed based on pairwise distance with a window size of 40, step size of 20, and 100 bootstrap replicates.

Table 4.2 Recombination events with possible recombination breakpoints and P values for the different recombination detection methods.

NoV	Recombinant Sequence	Breakpoint ^a		<i>P</i> value for detection method ^b					
		Begin	End	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
GII	EFFII03035	Unidentified	152 (5236)	1.10×10^{-4}	4.19×10^{-7}	7.15×10^{-7}	7.15×10^{-7}	NS	6.55×10^{-17}
GII	INFII03039	Unidentified	147 (5231)	4.29×10^{-3}	9.29×10^{-6}	2.72×10^{-6}	2.68×10^{-6}	NS	2.19×10^{-13}
GII	OYSII09021	Unidentified	201 (5285)	NS	1.01×10^{-4}	5.02×10^{-6}	4.95×10^{-6}	NS	1.05×10^{-13}
GI	INF03035	75 (5428*)	219 (5582*)	NS	1.17×10^{-3}	4.87×10^{-6}	1.01×10^{-5}	2.11×10^{-12}	2.60×10^{-12}

^aBreakpoint position in alignment (breakpoint position relative to strain X86557 or M87661 [strain M87661 where marked with an asterisk]).

^bNS, not significant.

4.5 Discussion

In 2010, 1789 NoV illness notifications were reported in Ireland. This was the highest number of NoV notifications compared to previous years. A particularly high frequency NoV notifications (1309) was recorded between January and March (Cloak *et al.*, 2010). In the first three months of 2010 an increased number of NoV infections were also reported in other parts of Europe including Scotland (McAllister *et al.*, 2012), England and Wales (HPA, 2012), and Belgium (Mathijs, 2011). Higher-than-average NoV activity in epidemic years has been associated with cold, dry weather, lower population immunity and the emergence of new NoV GII.4 variants (Lopman *et al.*, 2009). The winter of the 2009–2010 (December to February) was the coldest winter since the winter of 1962–63 in Ireland, but it was drier and sunnier than usual, as reported by Met Éireann (<http://www.met.ie/>).

The national peak of NoV GII infections as judged by laboratory-confirmed cases occurred approximately 1 week after the highest concentration of NoV GII was detected in influent wastewater. However, given that NoV shedding can commence before the onset of clinical symptoms (Atmar *et al.*, 2008) and that there is a slight time lag between infection and laboratory confirmation, it is likely that the peak concentration of NoV GII in wastewater occurred concurrently with the peak number of infections in community. Detection of elevated NoV GII concentrations in wastewater prior to the higher-than-normal reports of NoV gastroenteritis has been described previously (da Silva *et al.*, 2007). Few NoV GI-associated laboratory-confirmed cases were reported compared to those involving NoV GII, and 7 of a total 16 cases occurred between week 9 and 10. However, given the small number of overall NoV GI-associated cases, it is not possible to determine whether this represented a peak of infections.

Concentrations of NoV GII were significantly greater than NoV GI concentrations detected in influent wastewater, indicating either a greater frequency of NoV GII-associated infections in the community or greater virus shedding by NoV GII-infected patients than by NoV GI-infected patients. During the wastewater treatment process, both NoV GI and GII underwent comparable reduction over the

study period, and the achieved reductions (approximately 1 log) are consistent with reductions in previously reported studies (Nordgren *et al.*, 2009 and Ottoson *et al.*, 2006). Larger numbers of laboratory-confirmed cases of NoV GII were detected in the community. It appears that the greater concentrations of NoV GII in the influent wastewater were a result of NoV GII cases in the community. Nevertheless, despite the small number of laboratory-confirmed cases of NoV GI infection during the study period, relatively high concentrations of NoV GI were detected in influent wastewater, and concentrations peaked in week 7. This suggests that during the study period, an increased number of community infections associated with NoV GI occurred. Unless a significant number of NoV GI-associated infections are asymptomatic, it appears that NoV GI-associated infections were underreported in Ireland during this study period. The majority of stool samples analyzed in this study were obtained from outbreaks that occurred in healthcare settings. Given the previously described prevalence of NoV GII infections in such settings (Kelly *et al.*, 2008 and Matthews *et al.*, 2012), the large number of laboratory-confirmed cases involving NoV GII was expected. It is possible that NoV GI infections are underreported to public health authorities more often than NoV GII infections due to sampling bias in healthcare settings, their more-varied transmission mode (Matthews *et al.*, 2012 and Verhoef *et al.*, 2010), or the severity of illness (Desai *et al.*, 2012).

NoV outbreaks were genetically characterized, and three different genotypes were detected: GII.4 (variants 2010 and 2006b), GII.13, and GI.6. All of these NoV sequences were highly similar to NoV sequences found in the influent wastewater, effluent wastewater and oysters. The newly emerged GII.4 2010 variant (Belliot *et al.*, 2009 and Mathijs, 2011) was the most frequently detected variant of GII.4 in the outbreaks as well as in the environmental samples. The GII.4 2010 variant was first identified in France in February 2009 (Belliot *et al.*, 2009), and it became the dominant GII.4 variant in Europe during the winter of 2009–2010 (Mathijs, *et al.*, 2011, McAllister *et al.*, 2012, and van Beek *et al.*, 2012). In the United States, GII.4 2010 replaced GII.4 2006b as the predominant variant in the winter of 2009–2010 (Vega *et al.*, 2011 and Yen *et al.*, 2011). GII.4 2010 gained a global distribution in 2009

and 2010, being detected in Taiwan, China, Australia, Japan, India, Cameroon, and the United States (BLAST search). It is clear that a similar GII.4 variant replacement took place in Ireland, as supported by its high rate of detection in wastewater and of laboratory-confirmed cases of NoV infections reported in this study.

Interestingly, all of the NoV GII.12 sequences detected in the environmental samples were highly similar to the recombinant strain GII.g (polymerase)/GII.12 (capsid) StGeorge (GenBank accession no. GQ845370) (Eden *et al.*, 2010), which was reported in a large number of non-GII.4 NoV outbreaks in the United States (Takanashi *et al.*, 2011 and Vega and Vinje, 2011) and Europe (van Beek *et al.*, 2012) in the winter of 2009–2010. However, this strain was not identified in any of the outbreaks samples analyzed in this study. It is possible that some of the NoV genotypes frequently identified in the influent, the effluent, and impacted oysters, such as NoV GII.12, were responsible for infections in the community at the beginning of 2010, but were detected due to limited sequencing carried out on the outbreak samples.

The NoV genotype profiles detected in the influent, effluent and oyster samples varied. NoV GI.7 and NoV GI.4 were the most frequently detected NoV GI genotypes from environmental samples during this study. This is consistent with the findings of the Foodborne Viruses in Europe (FBVE) network, which reported these genotypes as the most frequent causes of NoV GI outbreaks throughout 2010 (van Beek *et al.*, 2012). In our study, NoV GI.7 was detected predominantly in the influent, less frequently in effluent wastewater, and only sporadically in oyster samples. In contrast, NoV GI.4 was the dominant genotype detected in effluent wastewater and oysters. This is consistent with a previous study by this laboratory in which NoV GI.4 was the predominant genotype detected in oysters originating from a commercial harvest area (Rajko-Nenow *et al.*, 2012). In this study, the NoV GI.1 and GII.3 genotypes were absent in wastewater but were detected in oyster samples, and this could possibly be linked with their preferential accumulation in oyster tissues, which has been reported previously (Maalouf *et al.*, 2011).

Recent studies on virus-like particles (VLP) have shown that the overall virion structures of GI.1, GII.4, GII.10, GII.12, and GV.1 VLPs are similar; however, the

differences in the flexibility between their P and S capsid domains can influence binding of VLP to monoclonal antibodies (Chen *et al.*, 2004 and Hansman *et al.*, 2012). It has also been shown that NoV GI.1 particles can bind specifically to a histo-blood group antigen (HBGA) A-like ligand present in the oyster digestive tract, and this binding facilitates accumulation within oysters (Maalouf *et al.*, 2011). Therefore, it is reasonable to suggest that some NoV genotypes may accumulate in oysters more efficiently than others because of their binding properties, which explains why some NoVs have been detected more commonly in shellfish-related outbreaks (Le Guyader *et al.*, 2012).

We identified four putative NoV recombinants: GI.4/GI.6 and GII.4 2010/GII.13 in the influent, GII.12/GII.4 2006b in the effluent, and GII.4 2010/GII.3 in the oysters. Although a short fragment (291 bp for GI and 302 bp for GII) of the NoV genome was used to identify recombinants, the detection of each recombinant was supported by at least three detection methods available in the RDP4. The GII.4/GII.3 recombinant has been described previously by Vidal *et al.* (Vidal *et al.*, 2006); however, to our knowledge, the three other recombinants detected here have not been identified before. Most of the parental sequences identified, such as GI.6, GII.4 2010, GII.4 2006b, and GII.13, were detected in the clinical samples during this study. As the putative recombinants were detected in the environmental samples only, it is not possible to comment on their virulence. It has been shown experimentally (Mathijs *et al.*, 2010) that a novel murine NoV recombinant can exhibit different biological properties from those of its parental viruses. As oysters can accumulate different NoV genotypes (including recombinants), the potential exists for the reintroduction of these new or their subsequent consumption.

In summary, we suggest that during this study, allowing for the delay in laboratory confirmation, there were concurrent peaks in NoV GII concentrations in wastewater and NoV infections in the community in Ireland in February 2010. We demonstrated that a diverse range of NoV GI genotypes were present in environmental samples (wastewater and oysters), despite the fact that the overwhelming majority of laboratory-confirmed cases were attributed to NoV GII. Therefore, we suggest that the number of NoV GI-associated infections may be

underreported. Finally, different genotype profiles were observed in influent wastewater and oysters, possibly indicating differing survival characteristics through the treatment process for different genotypes or preferential accumulation of some genotypes in oysters.

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4.7 Supplemental material

Table 4.3 NoV concentrations detected in weekly wastewater samples.

Date sampled	Influent wastewater		Effluent wastewater	
	NoV concentration in genome copies / 100 ml			
	GI	GII	GI	GII
10-Jun-09	6830	10900	173	432
17-Jun-09	3060	10400	1340	2130
23-Jun-09	909	982	72	129
1-Jul-09	2000	5200	294	117
8-Jul-09	4330	7920	96	165
15-Jul-09	832	2340	889	403
22-Jul-09	197	107	334	259
30-Jul-09	349	828	203	372
5-Aug-09	916	7210	184	54
12-Aug-09	133	737	215	207
19-Aug-09	1180	891	265	545
26-Aug-09	814	284	92	122
2-Sep-09	581	926	186	43
9-Sep-09	407	158	99	1
16-Sep-09	773	154	112	96
23-Sep-09	468	913	18	48
30-Sep-09	9060	3450	475	95
7-Oct-09	2260	2900	397	584
14-Oct-09	663	297	404	32
22-Oct-09	113	347	45	340
28-Oct-09	1470	3340	247	147
4-Nov-09	1130	3840	148	192
11-Nov-09	130	123	60	269
18-Nov-09	1250	585	104	140
25-Nov-09	1360	1550	236	320
2-Dec-09	1400	65	696	33
9-Dec-09	1430	1080	279	322
16-Dec-09	16200	13300	808	527
4-Jan-10	7320	40400	1970	5680
13-Jan-10	17700	67000	2560	3790
20-Jan-10	5620	21800	1920	7290
27-Jan-10	2810	9450	1090	1900
3-Feb-10	14900	12500	11300	534
10-Feb-10	9730	220000	374	5780
17-Feb-10	57900	36600	11400	3250
24-Feb-10	33500	146000	2020	9760
3-Mar-10	10100	39000	1180	5210
10-Mar-10	13800	87700	557	3720
18-Mar-10	2100	34700	154	5410
24-Mar-10	1200	19300	54	675
1-Apr-10	5550	21800	215	219
7-Apr-10	4050	26900	1500	11900
14-Apr-10	7810	9180	446	954
21-Apr-10	1390	1630	163	213
29-Apr-10	814	284	265	545
5-May-10	757	5440	170	101
19-May-10	1160	3680	1	354
26-May-10	19100	8290	218	35

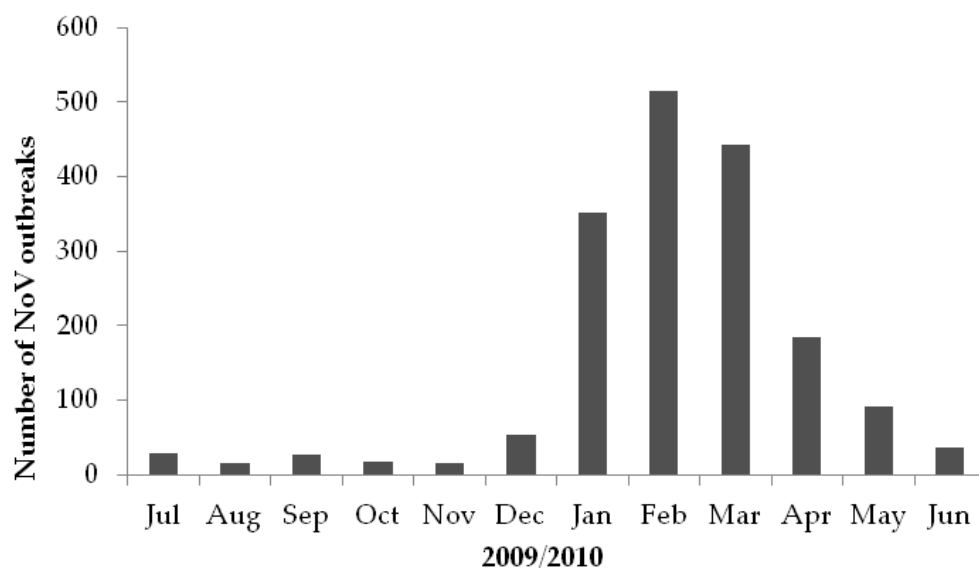


Figure 4.5 Number of NoV outbreaks notified in Ireland from June 2009 to June 2010.

**Chapter 5 Norovirus genotypes
implicated in two oyster-related
illness outbreaks in Ireland.**

Abstract

We investigated norovirus (NoV) concentrations and genotypes in oyster and faecal samples associated with two separate oyster-related outbreaks of gastroenteritis in Ireland. Quantitative analysis was carried out using RT-qPCR and phylogenetic analysis was conducted to establish the NoV genotypes present. For both outbreaks, the NoV concentration in oysters was >1000 genome copies g^{-1} digestive tissue (DT) and multiple genotypes were identified. In faecal samples, GII.13 was the only genotype detected for outbreak 1, whereas multiple genotypes were detected in outbreak 2 following the application of cloning procedures. While various genotypes were identified in oyster samples, not all were successful in causing infection in consumers. In outbreak 2 NoV GII.1 was identified in all four faecal samples analysed and NoV GII concentrations in the faeces were $> 10^8$ copies g^{-1} . This study demonstrates that a range of NoV genotypes can be present in highly contaminated oysters responsible for gastroenteritis outbreaks.

5.1 Introduction

Norovirus (NoV) is the most commonly reported viral cause of foodborne outbreaks in the European Union, affecting 3784 people in 111 outbreaks in 2007 (EFSA, 2009). Food can be contaminated during its production by washing or growing in faecally contaminated water, or through preparation by an infected food handler. Oysters can accumulate NoV in their tissues (Tian *et al.*, 2007) and are often implicated in foodborne NoV gastroenteritis outbreaks as they are traditionally consumed raw or lightly cooked (Ang, 1998).

Worldwide, the majority of NoV outbreaks have been associated with NoV genogroup II (GII) genotypes, in particular genotype 4 (GII.4) (Siebenga *et al.*, 2009). Most food handler-related outbreaks or person-to-person outbreaks are associated with a single NoV genotype (Noda *et al.*, 2008 and Matthews *et al.*, 2012). In contrast, multiple NoV GI and GII genotypes are frequently identified in shellfish-related outbreaks, being detected in the faeces of infected patients and implicated shellfish (Noda *et al.*, 2008 and Le Guyader *et al.*, 2012). Furthermore, a significantly higher attack rate has been reported in oyster-related outbreaks than in food handler-associated outbreaks (Noda *et al.*, 2008).

In Europe the sanitary quality of shellfish harvest areas is assessed by *E. coli* monitoring under regulation (EC) No. 854/2004. Harvest areas are classified into categories on the basis of *E. coli* concentrations and the level of treatment required prior to consumption determined. Despite these controls, oysters compliant with EU regulations have been implicated in outbreaks of NoV illness (Dore *et al.*, 2010 and Le Guyader *et al.*, 2010). Recently, a standardised real-time quantitative reverse transcription PCR (RT-qPCR) for the detection of NoV and hepatitis A in shellfish has been developed by the European Committee for Standardization (CEN) working group (TC275/WG6/TAG4) (Lees, 2010). Although the standardised RT-qPCR method has been considered suitable for the detection and quantification of NoV in oysters by the European Food Safety Authority, guidance or regulatory limits for NoV concentrations in oysters have yet to be established (EFSA, 2012).

In this study, we investigated two NoV oyster-related outbreaks that occurred during the winter of 2010 and 2012 in Ireland. We used the newly standardised RT-qPCR method to determine the concentrations of NoV in the implicated oysters. In addition, sequencing procedures were used on oyster and faecal samples to identify the causative NoV GI and GII genotypes.

5.2 Methods

5.2.1 Outbreak descriptions and sampling

Outbreak 1, 2010: From January to March 2010, 334 cases of NoV infection were linked to the consumption of raw oysters originating from several harvest areas in Europe (Westrell *et al.*, 2010). Approximately 70 of these cases originating at restaurants throughout Ireland and the UK were linked to a single commercial harvest area (harvest area 1) in Ireland. The implicated oysters were harvested before 5th February from commercial harvest area 1 in Ireland. The area was classified as a category A production area, which means that the oysters could be sold directly for consumption. However as an additional precaution, oysters were also depurated prior to sale (Dore *et al.*, 2010). Following the reports of NoV gastroenteritis, an oyster sample was collected and analysed from two sites (site 1 and 2) within harvest area 1 on 14th of February. The remains of oysters directly linked to illness were received and analysed from two Irish restaurants (A and B) on 18th of February. A stool sample from an infected consumer that had an oyster meal in restaurant B on the 5th of February was analysed by the National Virus Reference Laboratory (NVRL) on the 17th of February and sequence data was available for further phylogenetic analysis.

Outbreak 2, 2012: On 11th of January 2012, the competent authorities in Ireland were informed that 18 people had reported gastroenteritis symptoms following consumption of oysters served as a portion of up to six oysters each per person at restaurant C. These oysters originated from a harvest area in the west of Ireland (harvest area 2). An oyster sample obtained directly from the batch of oysters causing illness and served at restaurant C was collected and analysed on 11th of January for NoV. In addition to the restaurant-oysters, an oyster sample was collected from the implicated harvest area (harvest area 2) on 12th of January. Harvest area 2 was classified as a category B harvest area and depuration was routinely undertaken by the producer prior to dispatch. *E. coli* concentrations detected in oysters from restaurant C and harvest area 2 were 20 and <20 MPN 100

g⁻¹, respectively. Four stool samples were collected from infected consumers and were analysed for NoV.

5.2.2 Sample preparation and RNA extraction

Upon arrival into the laboratory, ten oysters (*Crassostrea gigas*) from each sample were opened and the digestive tissues (DT) dissected out. To prepare the shellfish extract, 2 g of oyster DT was weighed to which 2 ml of 100 µg ml⁻¹ proteinase K solution (30 Umg⁻¹; Sigma-Aldrich) was added (Flannery *et al.*, 2012). Mengo virus strain MC₀ was used as an internal positive control (IPC) virus. Viral RNA was extracted using NucliSENS® miniMAG® platform and NucliSENS® magnetic extraction reagents (bioMérieux, Marcy l’Etoile, France) according to the manufacturer’s protocol.

For each stool sample, 1 ml of PBS (Oxoid, Basingstoke, England) was added to a 2-ml tube containing between 73 to 218 mg of faecal material (neat) and vortexed vigorously. Then, 100 µl of the re-suspended faecal material (neat) was transferred into a fresh tube containing 900 µl of PBS (10⁻¹) and serial dilutions were prepared up to 10⁻⁵. Virus RNA was extracted from 500 µl of each dilution using NucliSENS® magnetic extraction reagents (bioMérieux) and eluted into 100 µl of elution buffer.

5.2.3 Quantification of NoV using one-step real time RT-qPCR

A previously described RT-qPCR was performed using RNA Ultrasense™ one-step quantitative RT-PCR system (Invitrogen, Carlsbad, CA) on an AB7500 real-time PCR instrument (Applied Biosystems, Foster City, CA) (Rajko-Nenow *et al.*, 2012). For NoV GI analysis, forward primer QNIF4 (da Silva *et al.*, 2007), reverse primer NV1LCR, and probe NVGG1p (Svraka *et al.*, 2007) and for NoV GII forward primer QNIF2 (Loisy *et al.*, 2005), reverse primer COG2R (Kageyama *et al.*, 2003), and probe QNIFS (Loisy *et al.*, 2005) were used. Primers Mengo209, Mengo110 and probe Mengo147 used were the same as those described by Pintò *et al.* (Pintó *et al.*, 2009) to analyse the internal positive control (IPC) virus. A log dilution series (range 1 × 10¹ to 1 × 10⁵ copies per µl) of plasmids carrying the GI and GII target sequences were

included in duplicate on each RT-qPCR run and the number of NoV RNA genome copies per μl was determined. The limit of quantification (LOQ) and the limit of detection (LOD) for NoV GI and GII was calculated as 100 and 20 genome copies g^{-1} of DT, respectively. All oyster samples were assessed for both RT-PCR inhibition using external control RNA and extraction efficiency using IPC.

For quantification of stool samples, five dilutions of each (from 10^{-1} to 10^{-5}) were analysed on a separate RT-qPCR runs to avoid cross contamination and the geometric mean of dilutions ($>\text{LOD}$) were calculated and expressed as genome copies g^{-1} faeces. The LOD for NoV GI and GII was calculated as 20 genome copies ml^{-1} .

5.2.4 NoV molecular characterisation and genotyping

Reverse transcription (RT) was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The nested PCR was carried out as described previously (Rajko-Nenow *et al.*, 2013). Briefly, sample cDNA (5 μl) was added to a first round of PCR mixture, containing primers COG1F, G1-SKR for NoV GI and COG2F, G2-SKR for NoV GII (Kojima *et al.*, 2002 and Kageyama *et al.*, 2003). The primers used in a second round of PCR were GISKF, GISKR for NoV GI and GIISKF, GIISKR for NoV GII (Kojima *et al.*, 2002). The nested RT-PCR products were cloned into pCR[®]4-TOPO[®] vector according to the protocol for TOPO TA Cloning[®] Kit (Invitrogen). Approximately 5 clones were randomly selected for DNA sequencing with BigDye Terminator v 3.1 Ready Reaction kit (Applied Biosystems) and analysed on ABI Prism 310 genetic analyzer (Applied Biosystems).

5.2.5 Sequence alignment and phylogenetic analysis

Nucleotide sequences were aligned using the ClustalW algorithm of the MegAlign software (DNASTar, Inc., Madison, Wis. USA) and compared with reference strains retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Percent identity was calculated for each pair of sequences in the nucleotide alignment. The length of

NoV alignment was 285 bp for NoV GI (5354-5645 of Norwalk virus M87661) and 294 bp for NoV GII (5085-5353 of Lordsdale virus X86557). Phylogenetic trees were computed using the maximum likelihood method with PAUP* software version 4.0 (Swofford, 2003). Using the hierarchical likelihood ratio test in Modeltest (Posada and Crandall, 1998), the F81+G and GTR+I+G model of evolution were selected for NoV GI and NoV GII phylogenetic analysis, respectively. The reliability of the generated tree was estimated by bootstrap analysis of 1000 replicates of the dataset using PAUP*. NoV genotypes were assigned based on the clustering in the phylogenetic tree (> 70% bootstrap support).

Accession numbers. The Genbank nucleotide sequence accession numbers for all the sequence analysed during this study are as follows: KC954402-KC954472.

5.3 Results

5.3.1 Outbreak 1, 2010

Concentrations of NoV GI and GII in the oyster sample (REST79) collected from restaurant A were <LOQ and 2350 genome copies g^{-1} DT, respectively (Table 5.1). Similar concentrations of NoV GI and GII were detected in oysters (REST80) from restaurant B; <LOD and 2040 genome copies g^{-1} DT, respectively. NoV GI concentrations in the two oyster samples collected from site 1 and 2 following notification of the outbreak, were 195 and <LOD genome copies g^{-1} DT, respectively. NoV GII concentrations detected in site 1 and 2 were 2890 and 1920 genome copies g^{-1} DT, respectively (Table 5.1). For all oyster samples, NoV GII concentrations were at least 10 fold greater than NoV GI concentrations.

Although NoV GI was below the LOD of the RT-qPCR assay, in two oyster samples (REST80 and HAR70, Table 6.1) NoV GI was amplified for genotyping by the nested RT-PCR assay and NoV GI.4 genotype was detected. A single NoV GII.3 genotype was detected in the oyster sample (REST79) collected from restaurant A, whereas four NoV genotypes (GI.4, GII.3, GII.4 New Orleans 2009 and GII.13) were detected in the oysters (REST80) from restaurant B (Figure 5.1) (Figure 5.2). Multiple NoV genotypes were also detected in oysters from site 1 (GI.4, GII.3, and GII.13) and site 2 (GI.4, GII.12, and GII.13) in harvest area 1.

A single NoV GII.13 genotype was detected by direct sequencing in the stool sample by the NVRL (Table 5.1). This NoV GII.13 sequence (STOOL) had 100% identity to a GII.13 sequence detected in harvest area 1 site 2 (HAR70.2) and 99.7% identity to sequences REST80.5 (restaurant B) and HAR69.13 (harvest area 1 site 1).

5.3.2 Outbreak 2, 2012

The NoV GII concentration in the oyster sample from restaurant C (REST10) was 2380 genome copies g^{-1} DT, whereas the NoV GI concentration was <LOD of the RT-qPCR assay (Table 5.1). NoV GI and GII concentrations of 1790 and 4000 genome copies g^{-1} DT respectively, were detected in the oyster sample from harvest area 2 (HAR13).

In the oysters that were consumed at restaurant C (REST10) and directly linked to illness, six NoV genotypes were detected; GI.1, GI.4, GII.1, GII.3, GII.4 Den Haag 2006b, and GII.6 (Figure 5.1) (Figure 5.2). NoV GI.4 was detected in 74% (n=14/19) and NoV GI.1 in 26% (n=5/19) of the NoV GI clones in this sample. For NoV GII, the most frequently detected genotype was GII.4 Den Haag 2006b (41%, n=9/22) followed by GII.3 (27%, n=6/22), GII.1 (23%, n=5/22), and GII.6 (9%, n=2/22). Multiple NoV genotypes (GI.2, GI.11, GII.1, GII.6, and GII.7) were also detected in oyster samples (HAR13) from harvest area 2, including an unidentified NoV strain that was designated GI.NA (Table 5.1).

Four stool samples were cloned prior to sequence analysis and multiple genotypes were detected in samples; 2937, 2915, 2906 and 2761 (Table 5.1). All stool samples contained NoV GII and concentrations ranged from 2.2×10^8 to 5.66×10^9 genome copies g^{-1} faeces, while only one stool sample (2906) had similarly high NoV GI concentrations (1.32×10^8 genome copies g^{-1} faeces). NoV GII.1 was the only genotype detected in all stool samples. NoV GII.1 sequences 2761.1, 2906.3, 2915.1, 2915.3 and 2937.1 detected in the stool samples shared 99.7% identity to a NoV GII.1 sequence found in the oyster sample from restaurant C (REST10.22) and harvest area 2 (HAR13.7). In addition to NoV GII.1, GII.7 genotype was also identified in the faeces of one patient (2906).

NoV GI concentrations of 1.32×10^8 and 3.46×10^5 genome copies g^{-1} faeces were detected in stool samples 2906 and 2937, respectively. In these stool samples, NoV GI.4 was the only GI genotype identified and sequences 2906.1 and 2937.2 shared 100% identity with the GI.4 sequence detected in the oyster sample from restaurant C (REST10.21). In contrast, the concentration of NoV GI detected in stool sample 2761 that contained three GI genotypes (GI.4, GI.2, and GI.6) was 2.45×10^3 genome copies g^{-1} faeces. In stool sample 2915, concentrations of NoV GI were not detected using the RT-qPCR assay yet were positive using the nested RT-PCR assay and NoV GI.1 and GI.6 were identified. NoV GI.6 was identified in two of the stool samples despite not being detected in the oyster samples collected from the restaurant or harvest area.

Table 5.1 Norovirus genogroup I (GI) and genogroup II (GII) concentrations and genotypes detected in outbreak samples.

Outbreak	Type	Sampling point	Lab ID	Real-time RT-qPCR		Genotypes (number of clones)		Comment
				GI†	GII †	GI	GII	
February 2010	Oyster	Restaurant A	REST79	<LOQ	2.35×10^3	n.t.	II.3 (5)	Oyster consumed at restaurant associated with gastroenteritis illness
	Oyster	Restaurant B	REST80	<LOD	2.04×10^3	I.4 (5)	II.3 (2), II.4 (1), II.13 (2)	Oyster consumed at restaurant associated with gastroenteritis illness
	Oyster	Harvest area 1 site 1	HAR69	1.95×10^2	2.89×10^3	I.4 (4)	II.3 (5), II.13 (2)	Oysters harvested after outbreak occurred
	Oyster	Harvest area 1 site 2	HAR70	<LOD	1.92×10^3	I.4 (4)	II.12 (3), II.13(2)	Oysters harvested after outbreak occurred
	Stool*	Patient	STOOL	*	*	*	II.13	Sequence data obtained by direct sequencing
January 2012	Oyster	Restaurant C	REST10	<LOD	2.38×10^3	I.1 (5), I.4 (14)	II.1 (5), II.3 (6), II.6 (2), II.4 (9)	Oyster consumed at restaurant associated with gastroenteritis illness
	Oyster	Harvest area 2	HAR13	1.79×10^3	4.00×10^3	I.NA (2), I.2 (3), I.11 (2)	II.1 (1), II.6 (2), II.7 (2)	Oysters harvested after outbreak occurred
	Stool	Patient	2937	3.46×10^5	4.85×10^8	I.4 (5)	II.1 (5)	Cloning of the nested RT-PCR products was performed prior to sequencing
	Stool	Patient	2915	<LOD	9.81×10^8	I.1 (4), I.6 (1)	II.1 (4)	
	Stool	Patient	2906	1.32×10^8	2.21×10^8	I.4 (5)	II.1 (4), II.7 (1)	
	Stool	Patient	2761	2.54×10^3	5.66×10^9	I.2 (1), I.4 (2), I.6 (2)	II.1 (5)	

n.t., Not typed as nested RT-PCR amplification was unsuccessful.

*Sample tested by National Virus Reference Laboratory (NVRL) in Dublin.

†Real-time RT-qPCR results are expressed in genome copies g^{-1} faeces for stool samples and in genome copies g^{-1} digestive tissues (DT) for oyster samples.

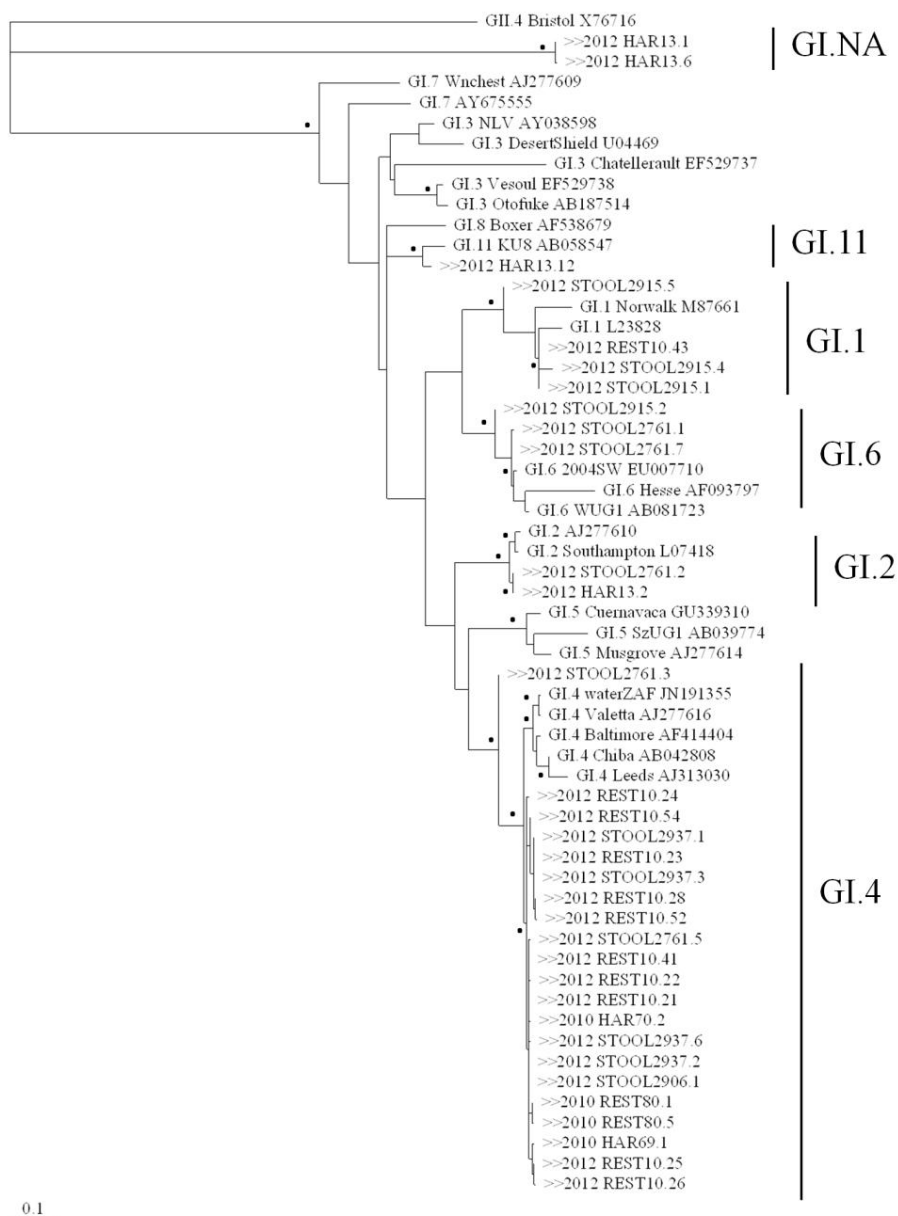


Figure 5.1 Maximum-likelihood tree based on capsid N/S domain (285 bp) of the NoV GI sequence alignment.

Bootstrap analysis was carried out for 1000 replicates of the data set and values of >70% are indicated by the black dots beside the appropriate branch. NoV GI sequences detected during outbreak 1 and 2 are preceded by '>>2010' and '>>2012', respectively. The lower scale represents genetic distances in nucleotide substitutions per site.



Figure 5.2 Maximum-likelihood tree based on capsid N/S domain (294 bp) of the NoV GII sequence alignment.

Bootstrap analysis was carried out for 1000 replicates of the data set and values of >70% are indicated by the black dots beside the appropriate branch. NoV GII sequences detected during outbreak 1 and 2 are preceded by '>>2010' and '>>2012', respectively. The lower scale represents genetic distances in nucleotide substitutions per site.

5.4 Discussion

We analysed samples from two oyster-related outbreaks of NoV gastroenteritis that occurred in Ireland in 2010 and 2012. The implicated oysters were harvested from production areas designated as category A (outbreak 1) and B (outbreak 2) under EU regulation 854/2004 (Anonymous, 2004). According to EU regulations, category A oysters are suitable for direct human consumption, whereas category B oysters require post-harvest treatment prior to consumption. Oysters harvested from both areas were routinely depurated prior to sale. Despite this, depurated oysters from both harvest areas contained total NoV (GI+GII) concentrations in excess of 1000 genome copies g^{-1} DT. This is consistent with a previous study that demonstrated that oysters containing a total NoV concentration >500 genome copies g^{-1} DT had an significantly increased risk of causing illness outbreaks when consumed than oysters containing concentrations <500 genome copies g^{-1} (Lowther *et al.*, 2012). Concentrations of NoV GI were close to the LOQ of the RT-qPCR in almost all oyster samples during the two outbreaks. One exception was the oyster sample collected on January 12th from harvest area 2 following notification of the outbreak. This sample contained 1790 genome copies g^{-1} DT and showed a different NoV genotype profile compared with the oysters that were previously harvested and were implicated in illness amongst consumers. It is likely that harvest area 2 underwent further contamination with NoV during the period 2nd to 12th January 2012. In particular, on 4th January 2012 a significant rainfall (16.4 mm reported by Met Éireann, <http://www.met.ie>) event occurred, which may have caused the additional NoV contamination from the nearby wastewater treatment plant approximately 1 km from the oysters growing area.

Oysters causing illness in the two outbreaks investigated here contained multiple strains of NoV GI and GII. In outbreak 1 a single NoV GII.13 strain was detected in the stool sample. However, as only direct sequencing was carried out, it is possible that other NoV strains were present in this faecal sample. Baker *et al.* attempted direct sequencing of oysters associated with the wider outbreak and also originating from harvest area 1, but phylogenetic analysis was incomplete due to

the presence of several NoV strains (Baker *et al.*, 2010). During outbreak 2, nested PCR products were cloned prior to sequencing and showed that all patients were infected with a mix of NoV GI and GII genotypes. Oysters served at restaurant C were contaminated with multiple NoV genotypes (GI.1, GI.4, GII.1, GII.3, GII.6 and GII.4 Den Haag 2006b). However, not all of these genotypes were present in each of the individual stool samples. NoV GII.1 was present in all four, GI.4 in three and GI.1 in only one stool sample. Some NoV genotypes (GI.2, GI.6, and GII.7) were shed by patients, but were not detected in the restaurant-oysters (REST10). As asymptomatic NoV infections have been reported in 12% of the healthy population (Phillips *et al.*, 2010), it is possible that the detection of these genotypes in faeces could not be attributed to the consumption of oysters. It appears that NoV GII.1 genotype was the most virulent genotype as it was detected in all stool samples along with high concentrations of NoV GII in faeces ($> 10^8$ copies g^{-1}) determined by RT-qPCR. It has been demonstrated previously that different genotypes of NoV seem to cause differences in severity of disease (Friesema *et al.*, 2009). NoV GII.4 strains in particular were found to be associated with more frequent vomiting and a higher attack rates among individuals.

The NoV GII.1 genotype detected in oysters and faecal samples during outbreak 2 shared high identity to the NoV GII.g/GII.1 recombinant (JF697289) (Mathijs, 2011) that caused a number of NoV outbreaks across Europe during 2010 and 2011 (Van Beek *et al.*, 2012). It is possible that NoV GII.1 was a newly circulating strain in Ireland at the time of outbreak 2. If so it is likely that the population was highly susceptible to this particular strain due to lack of existing immunity. In addition, high concentration of NoV GII in the oysters, presumably including a correspondingly high GII.1 concentration, could contribute to the effective shellfish-borne spread of the GII.1. During both investigated outbreaks, oysters directly linked to illness were contaminated with multiple NoV genotypes, including GII.3. However, this genotype was not detected in any of the stool samples which is in contrast with a previously study that demonstrated that NoV GII.3 had a greater attack rate in oyster-related outbreaks than any other genotype (Noda *et al.*, 2008).

This may be indicative of pre-existing immunity within the oyster consumers for NoV GI.3 during these outbreaks.

During outbreak 2, NoV GI was not detected in the restaurant-oysters by the RT-qPCR assay, but was detected using the nested PCR and indicates that NoV GI was present at very low concentrations. Despite this, three out of the four consumers contained NoV GI.4 in their faeces. In two of these three stool samples NoV GI.4 was the only NoV GI strain present and these contained NoV GI concentrations at 10^5 and 10^8 genome copies g^{-1} which could be indicative of an active infection. Comparable NoV GI.4 concentrations in faeces have been previously reported during a waterborne outbreak affecting more than 2400 people in Sweden (Nenonen *et al.*, 2012). These findings concur with a recent study that estimated a median infectious dose for NoV GI as low as 7.5 genome copies per oyster (Thebault *et al.*, 2013). It is possible that there may be considerable differences between the infectious doses of NoV genotypes and suggests caution is required when ascribing the risk associated with oyster consumption based on total NoV concentrations. However, given the relatively low concentration and sporadic occurrence of NoV GI detected in stool samples it remains unclear from our investigations what role NoV GI.4 detected during this outbreak played in causing illness. In addition, a part of the NoV genome (~300 bp) encoding for the N/S domain was sequenced; therefore, it cannot be excluded that the 100% identity shared between NoV GI.4 sequence detected in the stool and the oyster samples could significantly differ in other parts of NoV genome, including the P2 domain.

Despite the continued occurrence of oyster associated NoV outbreaks, viral standards for shellfish do not exist in EU food legislation and it is clear that *E.coli* monitoring of shellfish is unsuitable to indicate the risk posed by NoV contaminated shellfish. In this study, two NoV related outbreaks were caused by the consumption of highly contaminated oysters, containing multiple NoV strains and total NoV concentrations >1000 genome copies g^{-1} DT. It has been demonstrated previously that such highly contaminated oysters can pose a significant risk to human health (Lowther *et al.*, 2012). While multiple NoV genotypes were present in oysters, not all appeared to be successful in causing NoV infection in consumers as judged in this

study by the phylogenetic analysis. This suggests that factors such as pre-existing population immunity and differences in the infectious dose may impact on the ability NoV strains present in oysters to cause illness. This may have implications when considering setting acceptable concentrations for NoV standards in oysters.

5.5 Reference

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5.6 Supplemental material

During outbreak 2 discussed in Chapter 4 of this thesis, an oyster sample collected from harvest area 2 on 12 January 2012 contained high concentrations of NoV GI and GII, 1790 and 4000 genome copies g^{-1} , respectively. For NoV GI, the nested RT-PCR products were cloned, and one of the sequenced clones was identified as bovine NoV GIII (HAR13.27) using BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To confirm the detection of NoV GIII, the nested RT-PCR targeting the N/S domain of NoV GI genome was repeated prior to cloning. Five clones were selected at random for sequencing, two of which were confirmed as NoV GIII. These clones had high sequence identity to the previously identified NoV GIII sequence (HAR13.27).

The NoV GIII HAR13.27 sequence had 75.1% identity to NoV GIII.2 AF097917, and length of 278 bp (5110 – 5359 nt, AF097917), and 77.3% identity to NoV GIII Bo/CV521-OH/2002/US (AY549161.1). Amino acid similarity between NoV GIII HAR13.27 and NoV GIII Bo/CV521-OH/2002/US AY549161.1 strain was 84% (Figure 5.3).

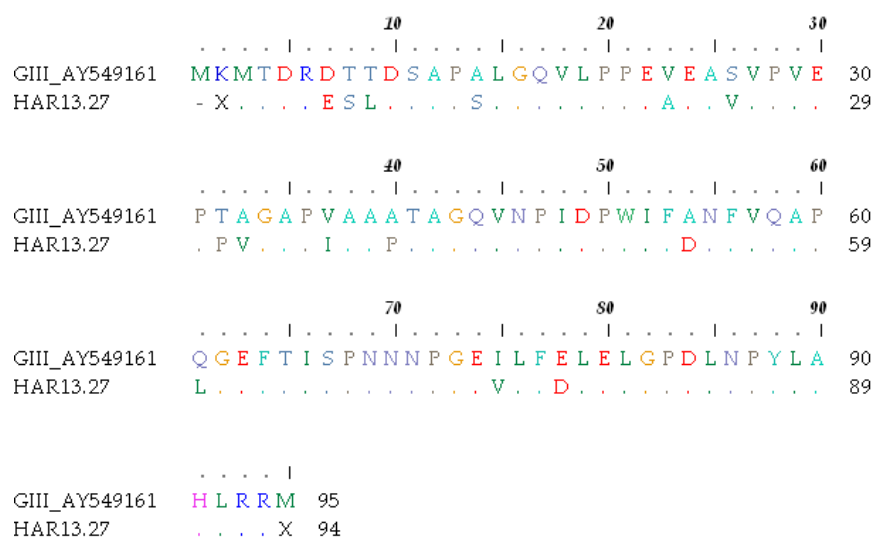


Figure 5.3 Pairwise sequence alignment of GIII HAR13.27 and GIII AY549161.1.

The detection of NoV GIII can be associated with a period of intense rainfall leading to agricultural runoff that occurred at this time. The simultaneous detection of bovine NoV GIII and human NoV raises concerns about human infection or co-infection with human and animal strains that could lead to genomic recombination, and the emergence of new strains. This finding highlights a possible route for zoonotic transmission of NoVs through the food chain.

Chapter 6 Conclusions and future recommendations

6.1 Conclusion remarks

Despite current EU regulations, NoV-contaminated oysters continue to cause local and international outbreaks, affecting many consumers and bringing financial losses for the shellfish industry. During this research project, the RT-qPCR assay and the nested RT-PCR assay were applied to quantify and molecularly characterise the NoV contamination in oysters and wastewater. The research outputs from this research project will provide information on the diversity of NoV genotypes that are circulating in the community, and those that may be preferentially accumulated in oysters.

A wide diversity of NoV strains was detected in wastewater and oyster samples throughout the course of this study. All NoV genotypes detected in all types of samples are listed below:

- ❖ GI.2, GI.3, GI.4, GII.4, GII.b, GII.12, and GII.e/2007 (Chapter 3).
- ❖ GI.1, GI.4, GI.5, GI.6, GI.7, GII.3, GII.4 (variants 2010 and 2006b), GII.6, GII.7, GII.12, GII.13, GII.17, and four putative recombinants GI.4/GI.6, GII.12/GII.4 2006b, GII.4 2010/GII.13, and GII.4 2010/GII.3 (Chapter 4).
- ❖ GI.1, GI.2, GI.4, GI.6, GI.11, GII.1, GII.3, GII.4 (variants 2010 and 2006b), GII.6, GII.7, GII.12, GII.13, GIII, and GI.NA (Chapter 5).

The standardised RT-qPCR assay used to quantify NoV was reliable and robust; however, the RT-qPCR product could not be used for genotyping due to its specificity towards the most conserved region of the NoV genome. In chapter 3, the nested RT-PCR assay targeting the part of RdRp gene of NoV (region A) was used for sequencing and genotyping. To enable the detection of multiple NoV strains in a single oyster sample, a cloning procedure was introduced, and a number of clones were randomly selected prior to sequencing. The phylogenetic analysis of NoV sequences (region A) allows the NoV genotypes to be categorised; however, its usefulness to distinguish between different NoV GII.4 variants is limited. For that reason, the nested RT-PCR assay targeting the N/S domain of NoV capsid gene (region C) was employed to generate a longer fragment of NoV genome that allows better differentiation between NoV strains, and genotyping based on this assay was

carried out in Chapter 4 and 5. In addition, the implementation of this nested RT-PCR assay (region C) enabled interlaboratory comparisons of the NoV sequences detected in wastewater and oyster samples with those detected in NoV gastroenteritis outbreaks reported Ireland.

Initially during this study NoV contamination was characterised in oysters from a single area over a two year period (Chapter 3). The harvest area was closed because of previous incidents of NoV illness associated with the site. A standardised RT-qPCR assay was used to determine concentrations of NoV in oysters over a two year period, and NoV genotypes were determined based on phylogenetic classification of the NoV genome region A. Mean concentrations of NoV GI and GII detected in oysters from the closed harvest area were 1569 and 1837 genome copies g^{-1} DT, respectively. This was despite compliance with a category B classification determined by *E. coli* monitoring. Bacteriological monitoring alone therefore indicated a moderate risk to consumers and that oysters required minimal treatment (depuration) before consumption.

In Chapter 5, the consumption of NoV-contaminated oysters (>1000 genome copies g^{-1} DT) originating from the harvest areas resulted in a number of NoV gastroenteritis cases that were reported in Ireland and throughout Europe. Therefore, it can be concluded that the sale of oysters investigated in Chapter 3 would probably have led to cases of NoV gastroenteritis among consumers. This, plus the fact that there had previously been outbreaks of illness following consumption of oysters from this area demonstrates that NoV contamination in shellfish harvest areas can be effectively monitored using the standardised RT-qPCR assay. RT-qPCR monitoring provides a more accurate assessment of the risk to consumers than current bacterial controls. In this case it would have been possible to inform the shellfish farmer and advice against harvesting shellfish at this time based on the RT-qPCR results. Such intervention may provide a suitable mitigation strategy for reducing NoV illness in shellfish consumers.

In Chapter 4 it was concluded that the peak of laboratory-confirmed cases of NoV GII infections coincided with the peak of NoV GII concentrations detected in influent wastewater. During a 13-week period, concentrations of NoV GI and GII

were detected in the influent and effluent wastewater at comparable levels, despite the few laboratory-confirmed cases of NoV GI infections reported to the public health authorities at that time. The reasons for this discrepancy may be that NoV GI infections are underreported in Ireland as a consequence of sampling bias in healthcare setting, their more-varied transmission mode, the (possibly less) severe illness produced leading to asymptomatic shedding.

During this thesis, a direct link was established between NoV GI and GII genotypes present in the wastewater and oysters placed near the WWT outfall studied in Chapter 4. A variety of NoV GI and GII strains were detected in these oysters that were either highly similar or identical to NoV sequences found in both the influent and effluent wastewater. In addition, the NoV genotypes detected in the oyster were similar to those implicated in NoV outbreaks reported at the time of the study. For instance, the newly emerged NoV GII.4 2010 variant (recently renamed GII.4 2009 New Orleans) was the most frequently detected variant of NoV GII.4 in the outbreaks as well as in the wastewater and oyster samples. However, in Chapter 3, NoV GII.b was frequently detected in oysters during the first year (2007–2008), which was then replaced by NoV GII.4 2006b in the second year (2008–2009). The NoV variant replacement observed during this work in oysters has also been reported in a number of other studies tracking NoV genotype variants responsible for illness in the general population. In addition, the recombinant NoV GII.e/GII.4 2007 strain was detected in oysters in November 2008, and was detected in epidemiological surveillance/studies in other countries at exactly the same time. On this basis, it can be inferred that oysters are capable of bioaccumulating NoV strains responsible for current infections in the population. Therefore, it is demonstrated here that oysters, because of their ability to concentrate and retain NoV, represent a useful composite of the NoV genotypes circulating in the population. Ongoing temporal monitoring of NoV contaminated oysters may be a useful tool to study the evolutionary dynamics of NoV. In particular, the epidemiology of NoV GII.4 variants currently circulating the population can be reconstructed using oyster samples exposed to wastewater effluents.

The NoV genotype profiles detected in the influent, effluent, and in oyster samples varied, indicating that some strains can persist better throughout WWT process or possibly be preferentially accumulated in oysters. In Chapter 4, NoV GI.4 was the most prevalent genotype detected in oysters (52.5%), but constituted only 16% of genotypes detected in influent and 44% detected in effluent wastewaters. Between October 2007 and September 2009, NoV GI.4 was the most frequently detected genotype in oysters sampled from the closed shellfish harvest area, and was present in 88.9% of the samples. Despite this, no gastroenteritis outbreaks of NoV GI.4 were notified in Ireland at that time. On the other hand, NoV GI.4 is generally more frequently detected in foodborne outbreaks than those transmitted by person-to-person. In Chapter 5, NoV GI.4 was detected in faeces of individuals that consumed NoV-contaminated oysters, and in the implicated oysters. This may either be explained by the greater survival of this strain during wastewater treatment process or its preferential bioaccumulation by oysters.

In Chapter 4, four putative NoV recombinants were detected in the influent (GII.4 2010/GII.13 and GII.4 2010/GII.3), in the effluent (GII.12/GII.4 2006b), and in the oysters (GI.4/GI.6) suggesting that the NoV recombination commonly occurs in human population. In Chapter 5, bovine NoV GIII and an unidentified NoV GI.NA strain in addition to a wide range of human NoV genotypes (GI.2, GI.11, GII.1, GII.6, and GII.7) were detected in the oyster samples, which contained high total NoV concentrations (5790 genome copies g^{-1} DT). The simultaneous detection of bovine NoV GIII and human NoV in highly contaminated oysters raises concerns about co-infection with human and animal strains that could lead to genomic recombination, and the emergence of new or possibly more virulent strains. This finding highlights a possible route for zoonotic transmission of NoVs through the oyster consumption.

In an attempt to establish a link with illness and the presence of particular NoV genotypes two oyster-related outbreaks of gastroenteritis were studied during this thesis (Chapter 5). In the first outbreak (occurring in 2010), NoV GII concentrations detected in oysters served at restaurant A and B were 2350 and 2040 genome copies g^{-1} DT, respectively, and were high compared with the NoV

concentrations detected in Chapter 3. In the second outbreak (occurring in 2012), NoV GII concentrations detected in oysters were just as high (2380 genome copies g^{-1} DT). The oysters responsible for both outbreaks were contaminated with multiple NoV GI and GII genotypes. In outbreak 2010, a single NoV GII.13 strain was detected in the stool samples. However, as only direct sequencing was performed, it is possible that other NoV strains were present in this stool sample. During outbreak 2012, the nested RT-PCR products were cloned prior sequencing, and showed that all patients were infected with multiple NoV GI and GII genotypes. It was concluded that NoV GII.1 was the most virulent genotype as it was identified in all four faecal samples analysed along with high NoV GII concentrations in faeces ($>10^8$ copies g^{-1}). NoV GI.4 was detected in three out of four individuals, despite the fact that NoV GI concentrations detected in oysters were at LOD of the RT-qPCR assay. The detection of multiple genotypes was possible due to the cloning procedure undertaken during this study and highlights the importance of this step when genotyping during outbreak investigations. Only one faecal sample was analysed by direct sequencing during the outbreak 2010; therefore, the connection between the consumption of NoV contaminated-oysters and gastroenteritis illness was more difficult to establish than in outbreak 2012.

In outbreak 2012 (Chapter 5), the NoV genotypes detected in the oyster consumed at the restaurant included the following genotypes: GI.1, GI.4, GII.1, GII.3, GII.6, and GII.4 Den Haag 2006b. Highly similar or identical NoV sequences of NoV GI.1, GI.4 and GII.1 were detected in the faeces of symptomatic individual during the outbreak investigation. However, an oyster sample collected from the same harvest area, but not involved in the outbreak, contained different NoV GI genotypes such as GI.NA, GI.2 and GI.11, and increased NoV GI concentrations indicating an additional NoV GI contamination. In view of this, it can be concluded that the phylogenetic analysis of NoV sequences can be used a supportive tool in tracking the origin of NoV oyster-related outbreaks.

In conclusion, sequencing of the N/S capsid domain of NoV genome followed by cloning is an appropriate approach to identify multiple NoV strains in the influent and effluent wastewater, in oysters and in faecal samples. A wide diversity

of NoV GI and GII genotypes and high concentrations of both genogroups were detected throughout the course of this study; however, it remains unclear why only a small number of laboratory-confirmed NoV GI infections was notified in Ireland. Highly NoV-contaminated oysters frequently contain multiple NoV strains (including recombinants, animal strains, and new strains) highlighting the possibility for the reintroduction of these new or possibly more virulent NoV strains into the human population through the food chain. Better control measures are required to improve the quality of shellfish placed on the market in Ireland and ensure a viable industry into the future; RT-qPCR monitoring of shellfisheries could be a valuable tool to achieve that.

6.2 Future recommendations

The results presented in this thesis will feed into the wider understanding of NoV contamination in shellfish harvest areas and the epidemiological profile of NoV circulating in the population as measured by the analysis of NoV in wastewater. The following recommendations are proposed based on the findings and conclusions presented in this thesis.

- During NoV shellfish-related outbreaks, cloning of nested PCR products is recommended prior to sequencing of NoV-positive faecal samples since multiple NoV GI and GII genotypes can be identified in the faeces of individuals.
- Further studies should be undertaken to investigate the effectiveness of sequencing in tracing the origin of NoV oyster-related outbreaks.
- Further studies to determine why NoV GI is so common in wastewater.
- Further studies on how significant shellfish are as a vector for NoV recombination.
- Further studies on specific NoV genotype differential survival and binding.
- Investigate the potential of using wastewater or oyster samples for assessing NoV evolution in the general population.
- Determine whether prohibiting harvesting oysters with NoV contamination exceeding 1000 genome copies g⁻¹ reduces incidence of illness in consumers.

**Appendix A. Author publications,
presentations and posters**

List of author publications

- Rajko-Nenow, P.**, Keaveney, S., Flannery, J., O'Flaherty, V., Doré, W., 2012. Characterisation of norovirus contamination in an Irish shellfishery using real-time RT-qPCR and sequencing analysis. *International Journal of Food Microbiology* 160, 105–112.
- Rajko-Nenow, P.**, Waters, A., Keaveney, S., Flannery, J., Tuite, G., Coughlan, S., O'Flaherty, V., Doré, W., 2013. Norovirus genotypes present in oysters and in effluent from a wastewater treatment plant during the seasonal peak of infections in Ireland in 2010. *Applied and Environmental Microbiology* 79, 2578-2587
- Rajko-Nenow, P.**, Keaveney, S., Flannery, J., McIntyre, A., Doré, W., 2013. Norovirus genotypes implicated in two oyster-related illness outbreaks in Ireland. *Epidemiology & Infection*, FirstView Article, pages 1-9
- Flannery, J., **Rajko-Nenow, P.**, Keaveney, S., O'Flaherty, V., Doré, W., 2012. Simulated sunlight inactivation of norovirus and FRNA bacteriophage in seawater. *Journal of Applied Microbiology* 115, 922–6.
- Flannery, J., Keaveney, S., **Rajko-Nenow, P.**, O'Flaherty, V., Doré, W., 2012. Concentration of Norovirus during Wastewater Treatment and Its Impact on Oyster Contamination. *Applied and Environmental Microbiology* 78, 3400–6.
- Flannery, J., Keaveney, S., **Rajko-Nenow, P.**, O'Flaherty, V., Doré, W., 2012. Norovirus and FRNA bacteriophage determined by RT-qPCR and infectious FRNA bacteriophage in wastewater and oysters. *Water Research* 47, 5222–5231.
- Dore, B., Keaveney, S., Flannery, J., **Rajko-Nenow, P.**, 2010. Management of health risks associated with oysters harvested from a norovirus contaminated area, Ireland, February–March 2010. *Eurosurveillance* 15, 1–4.
- Dore, B., Flannery, J., Keaveney, S., **Rajko-Nenow, P.**, 2013. STRIVE 109 Noroviurs in Wastewater and Shellfish. *Environmental Protection Agency Programme 2007-2013*

Oral presentations

Rajko-Nenow, P., Keaveney, S., Flannery, J., Doré, W., (2013) Characterisation of norovirus genotypes in wastewater and shellfish. *10th Irish Shellfish Safety Scientific Workshop, Marine Institute, Galway, Ireland*

Rajko-Nenow, P., Keaveney, S., Flannery, J., Doré, W., (2010) Characterisation of norovirus contamination in a shellfishery using real-time RT-qPCR and sequencing analysis. *SEGH 2010 International Conference and Workshop, Galway, Ireland*

Rajko-Nenow, P., Keaveney, S., Flannery, J., Doré, W., (2011) Diversity of Norovirus strains present in wastewater and contaminated shellfish in Ireland. *40th annual UCC Food Research Conference, University Collage Cork, Cork, Ireland*

Poster presentations

Rajko-Nenow, P., Waters, A., Keaveney, S., Flannery, J., O'Flaherty, V., Doré, W., (2012) Characterisation on norovirus genotypes present in a wastewater treatment plant and in impacted oysters. *3rd Food and Environmental Virology Conference, Lisbon, Portugal*

Rajko-Nenow, P., Keaveney, S., Flannery, J., Doré, W., (2012) Characterization of norovirus in oysters associated with gastroenteritis. *SGM Spring Conference, Dublin, Ireland*

Rajko-Nenow, P., Keaveney, S., Flannery, J., Doré, W., (2011) Diversity of Norovirus strains present in wastewater and contaminated shellfish in Ireland. *EPA post graduate seminar day, Dublin, Ireland*

Rajko-Nenow, P., Keaveney, S., Flannery, J., Doré, W., (2010) Characterisation of norovirus contamination in a shellfishery using real-time qRT-PCR and sequencing analysis. *EPA post graduate seminar day, Dublin, Ireland*