Review of Foodborne Viruses in Shellfish and Current Detection Methodologies

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Summary

Foodborne gastroenteritis is a significant cost to society. Norovirus (NoV) is the leading cause of non-bacterial gastroenteritis worldwide and is highly contagious, spread via the faecal to oral route. Immunity to the virus is short lived and new pandemics appear every 2-3 years. Faecal contamination of shellfish production areas, especially near highly urbanised locations, results in increased risk associated with any NoV circulating within the community accumulating in filter feeding shellfish. NoV has been shown to be selectively accumulated and retained within the digestive tissues of oysters, persisting long after bacterial indicators of sewage contamination are no longer detectable. NoV has been implicated as the leading cause of viral gastroenteritis associated with raw or undercooked oyster consumption. Ensuring product safety is crucial in maintaining local consumer confidence, as well as maintaining and growing export markets.

NoVs are highly variable and the genome undergoes frequent changes which are linked to altered viral infectivity. The median infectious dose of NoV is very low (~18 virus particles) which has made detection and estimating risk associated with consumption of contaminated foods difficult. Several diagnostic methods have been developed for NoV detection although serological methods targeting the antigenic viral coat protein have not been sensitive enough for food matrixes. The current gold standard for NoV detection targets the viral genome (real-time RT-PCR), but still lacks the sensitivity to detect very low levels of NoV consistent with an infectious dose. Furthermore, a positive assay is not well related to infectivity as the methodology will also detect degraded and non-infectious viral genomes.

This review will outline the importance of foodborne viruses associated with shellfish, summarise the advantages and limitations of current detection methodologies and outline the requirements for improved new detection technologies.

Oyster Production in Australia

In 2009-2010 oysters ranked in the top five of Australian fisheries production by volume (14,807 tonne), accounting for 6% of the national fisheries production (ABARES, 2011). The three major producers of oysters by state were South Australia (6,123 tonne), New South Wales (4,960 tonne) and Tasmania (3,724 tonne), with production valued at $43 million, $35 million and $21.2 million respectively (ABARES, 2011). The majority of Australian grown oysters are consumed locally. In 2009-2010 only 320 tonne of oysters were exported, predominantly to Australian Pacific Economic Corporation (APEC) countries at a value of $3 million.

Human Pathogenic Viral Diseases Associated with Shellfish Consumption

Foodborne viruses are those which infect via the gastrointestinal tract and are excreted in the faeces and, in some cases, in vomitus. Filter-feeding bivalve molluscan shellfish (oysters, clams, mussels and cockles) have a history of association with viral foodborne disease (FAO/WHO, 2008). The presence of several human enteric viruses (norovirus [NoV], Aichi [AiV], rotavirus [RV], enterovirus [EV], adenovirus [AdV], astrovirus [AV], sapovirus [SaV], Hepatitis A [HAV] and Hepatitis E [HEV]) have been identified in shellfish, although not all have been clearly linked with documented disease outbreaks (Le Guyader *et al.*, 2000; Nakagawa-Okamoto *et al.*, 2009; FAO/WHO, 2008). Based on the symptoms of infection, these viruses can be grouped into those which cause gastroenteritis (NoV, RV, AV, AiV, AdV and SaV), enterically transmitted hepatitis (HAV and HEV) and those which replicate in the human intestine, but only cause illness after they migrate to other organs such as the central nervous system (EV) (FAO/WHO, 2008).

HAV and NoV infection have been shown to be widely associated with the ingestion of contaminated shellfish (Le Guyader *et al.*, 2000), although HEV (Meng, 2011) and AiV infection (Le

Guyader *et al.*, 2008; Ambert-Balay *et al.*, 2008) have also been implicated in the consumption of infected oysters. NoV is the most common cause of foodborne viral gastroenteritis worldwide, while HAV continues to pose an international health threat. In the US the majority of all foodborne illness (58%) are caused by NoV (Scallan *et al.*, 2011). Between 1980 and 2012 there were 368 reported shellfish associated viral outbreaks in the scientific literature (Bellou *et al.*, 2013). The most common viral pathogens involved were NoV (83.7 %) and HAV (12.8 %) with the most frequent shellfish implicated in outbreaks being oysters (58.4 %). In Australia between 2001–2006, there were 13 outbreaks of gastroenteritis associated with oyster consumption reported to OzFoodNet of which half were due to NoV (Huppatz *et al.*, 2008). In some cases, where NoV was confirmed as the cause of illness, frozen imported oysters were implicated.

NoV outbreaks following shellfish consumption are attributed to growing waters contamination by human faeces and consumption of raw or lightly cooked product (Bellou *et al.*, 2013). Recent evidence has shown that NoV bind specifically to shellfish tissue receptor sites and can persist in contaminated shellfish digestive tissue for 8-10 weeks, which would explain why these viruses persist after depuration (Le Guyader *et al.*, 2006; FAO/WHO, 2008).

Economic Cost of Oyster Related Foodborne Viral Disease in Australia

In Australia it has been estimated that 32% of all reported gastroenteritis is foodborne, accounting for 5.4 million cases, 15,000 hospitalisations and 80 deaths annually (Hall *et al.*, 2005). NoV, enteropathogenic *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp. are the cause of most illnesses collectively accounting for 88% of all foodborne gastroenteritis. Of these, NoV accounted for 25% of foodborne gastroenteritis annually (Hall *et al.*, 2005). In the US norovirus is the leading cause of foodborne illness, second leading cause of hospitalisations and fourth leading case of deaths, accounting for 11% of all foodborne pathogen deaths (Batz *et al.*, 2011). In Australia it has been estimated that foodborne gastroenteritis costs $811 million annually largely due to losses in productivity, lifestyle and premature mortality (Abelson *et al.*, 2006). Costs to health care services of foodborne gastroenteritis accounts for an estimated $200 million annually. The cost to governments of public health actions (foodborne illness surveillance and investigation, and maintaining food safety systems) and the non-productivity costs to business are also significant. The estimated cost of public health actions is approximately $10 million annually and the cost of business disruption due to recalls of a food safety nature is approximately $14 million annually. The proportion of cost attributed to NoV is difficult to gauge as it is estimated that only 3% of individuals suffering foodborne gastroenteritis submit a faecal sample for testing to confirm cause (Abelson *et al.*, 2006).

Although the financial cost of foodborne gastroenteritis is difficult to accurately estimate they encompass losses relating to decreased human productivity and burdens on the health sector, as well as impacting directly on primary production, the food industry and trade. One widely reported case of oyster related enteric viral disease in 1997 caused by consumption of oysters from Wallis Lake, NSW, was responsible for an estimated 444 cases of hepatitis A across Australia, including 274 cases in NSW (Conaty *et al.*, 2000). Nearly one in seven cases were hospitalised and one individual died. This outbreak had a multifactorial negative economic impact which included a national health cost of disease estimated at $12.1 million and a 15-20% decrease in the market share for the local oyster industry (net income loss of $500K pa in 1997 and for a few years following). Other broader negative impacts were estimated losses of $0.2 and $1.0 million to the fishing and accommodation sectors respectively in 1997, and reduced public perception of oysters (OzFoodNet, 2006; Handmer and Hillman, 2004).

Noroviral Gastroenteritis and Hepatitis A

Hepatitis A is an acute infectious disease with an average incubation period of 28 days and symptoms lasting for approximately two months. Hepatitis A is a self limiting disease and results in lifelong immunity to re-infection. Since the introduction of an effective hepatitis A vaccine in the 1990s the incidence of disease worldwide has decreased dramatically (Cuthbert, 2001).

Norovirus is highly contagious and an important cause of sporadic gastroenteritis with no treatment for infection (Atmar *et al.*, 2008; Lindesmith *et al.*, 2008). NoV GII.4 strains are the most common cause of global outbreaks (Parra *et al.*, 2012). NoV infection usually results in symptomatic illness (watery diarrhoea and/or vomiting) lasting for one to two days, although asymptomatic infection is also possible. While most people recover within a few days, the very young and old may experience severe disease. Individuals develop short-term immunity following infection but immunity is strain specific. The genetic variability in circulating NoV strains indicates that individuals are likely to be repeatedly infected during their lifetime and like influenza, large outbreaks (epidemics) of NoV infection occur periodically (Lindesmith *et al.*, 2008). The viral genome frequently acquires small changes in its RNA resulting in altered coat protein sequence and hence antigenic drift. Viral antigens (molecules on the surface of infectious virus) stimulate the production of proteins (antibodies) that help the immune system recognise and deal with the foreign invaders. However, if the virus evolves and there are changes in these antigenic sites, new variants may not be recognised by the host. NoV epidemics occur when virus variants emerge to which the human population has no immunity (Lindesmith *et al.*, 2008). The antigenic drift in the virus has also meant that an effective vaccine against NoV has not been able to be developed to date (Atmar *et al.*, 2011). Study of human NoV has also been hampered by the fact that they cannot be cultured in the laboratory (Karst, 2010). Murine NoV is the only group of NoVs that can be artificially cultured in cell lines and has been widely used as a model for human NoV infection. However, these two groups of NoVs have distinctly different genome organisation (Karst, 2010).

The median infectious dose (ID_{50}) of NoV is very low and estimated to be only 18 infectious particles (Stals *et al.*, 2012b; Teunis *et al.*, 2008), whereas the virus is shed at high levels (10^4-10^{11} viral genomic copies/g) in the faeces of infected individuals (Atmar *et al.*, 2008; Chan *et al.*, 2006). In Human NoV challenge experiments susceptible subjects demonstrated a dose-dependent probability of becoming ill, ranging from 0.1 (at a dose of 10^3 genomes) to 0.7 (at 10^8 virus genomes) (Teunis *et al.*, 2008). NoV has been detected in the faeces as early as18 hours post infection and can still be detected 13–56 days following initial infection, long after symptoms have ceased (Atmar *et al.*, 2008). In immune-compromised individuals NoV has been detected in faeces 18 months after infection (R. Ratcliff, personal communication).

Norovirus Genome Organisation

Norovirus is one of four genera within the family *Caliciviridae*. The other three genera are *Lagovirus*, *Vesivirus* and *Sapovirus*. Two of these genera (*Sapovirus* and *Norovirus*) are capable of causing viral gastroenteritis in humans. NoVs are currently classified into five genogroups designated GI to GV. Human strains are classified into genogroups I, II and IV, with porcine and bovine strains classified into genogroups II and III, respectively, and a murine strain classified into genogroup V (Karst, 2010). Genogroups I and II are more commonly known to infect humans than GIV strains.

Noroviruses are single stranded ribonucleic acid (RNA), non-enveloped viruses. Genogroups comprise a range of genetically and antigenically diverse strains which can be further divided into genotypes or genetic clusters (Karst, 2010). Approximately 31 genotypes are currently recognised although this number is steadily increasing. These include eight GI, 19 GII, 2 GIII, 1 GIV and 1 GV genotypes (Karst, 2010). NoVs also display a wide degree of genetic variability, with members within a genogroup differing by 45–61% in amino acid sequence of the capsid protein, members

within a genotype differ by 14–44%, and strains within a genotype differ by 0–14% (Zheng *et al.*, 2006). This high degree of variability is likely one factor that complicates protective NoV immunity.

The human NoV consists of a 7.7 kb RNA genome (excluding the polyadenylated tail) and includes three open reading frames (ORFs) (Figure 1). When visualised by electron microscopy the virus is 26 to 34 nm in diameter, round, with an amorphous surface and ragged outer edge (Donaldson *et al.*, 2010; Le Pendu *et al.*, 2006). ORF1 (ca. 5 kb) is located in the first two-thirds of the genome and encodes a ca. 200 kDa polyprotein that is co-translationally cleaved by the viral protease to yield the non-structural and replicase proteins essential for viral replication (Parra *et al.*, 2012, Donaldson *et al.*, 2010). ORF2 (ca. 1.8kb) encodes the 57 kDa major structural capsid protein (VP1). ORF3 (ca. 0.6 kb) encodes a 22 kDa minor basic structural protein (VP2) present in one to two copies per virion and thought to be associated with stability of the capsid (Le Pendu *et al.*, 2006; Hardy, 2005).

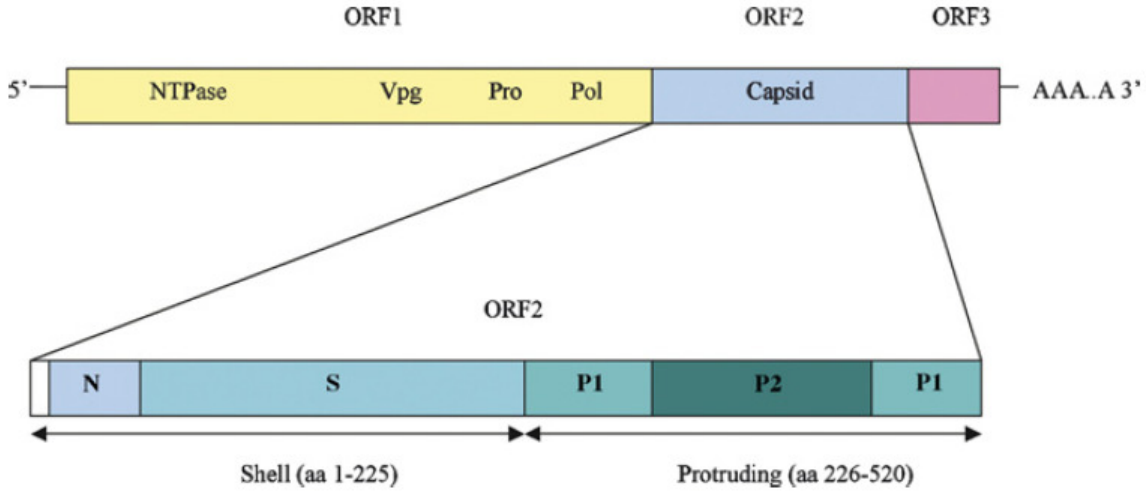

Figure 1: Schematic representation of the NoV genome. ORF1 encodes four nonstructural proteins; nucleoside triphosphate (NTPase), viral protein genome linked (VPg), proteinase (Pro) and polymerase (Pol). ORF2 encodes the virus capsid (VP1) and ORF3 encodes a minor structural protein (VP2). Above is a schematic enlargement of ORF2. N is the NH_2-terminal arm of ORF2 and S refers to the inner shell which is the most conserved part of the capsid. P1 and P2 are the protruding domains, of which P2 is the most variable and exposed. Source Le Pendu *et al.*, 2006.

The NoV capsid is composed of 90 dimers of the VP1 monomer (Figure 2). Each VP1 monomer is composed of two domains, the shell (S) and protruding (P) domains, linked by a flexible hinge. The S domain forms the internal icosahedral scaffold, from which the P domain projects to form arch-like structures. The P domain is further divided into two subdomains known as P1 and P2 (Figure 1 and 2). The P2 domain is the most exposed and variable region of the VP1 and interacts with synthetic carbohydrates corresponding to various ABH histoblood group antigens (HBGA) (Le Pendu *et al.*, 2006; Parra *et al.*, 2012; Donaldson *et al.*, 2010).

The expression of ORF2 (VP1 gene) (Figure 1) in insect cell lines results in the formation of non-infectious (empty) virus-like particles (VLPs) which have been shown to be morphologically and antigenically similar to native NoV virions. Expression of the S domain alone results in smooth particles with a diameter of approximately 30 nm which lack binding function to HBGA (Tan *et al.*, 2011). Expression of the P domain alone is capable of forming 12 dimers which assemble into an octahedral nanoparticle (ca. 20 nm and 840k Da) known as P particles (Tan *et al.*, 2011). These P particles (which can be produced rapidly in bacterial and yeast expression systems) are antigenically similar to the P domain of intact VLPs and have been used in structural and functional studies to map specific amino acid residues in the P2 domain that are involved in carbohydrate interactions (Parra *et al.*, 2012).

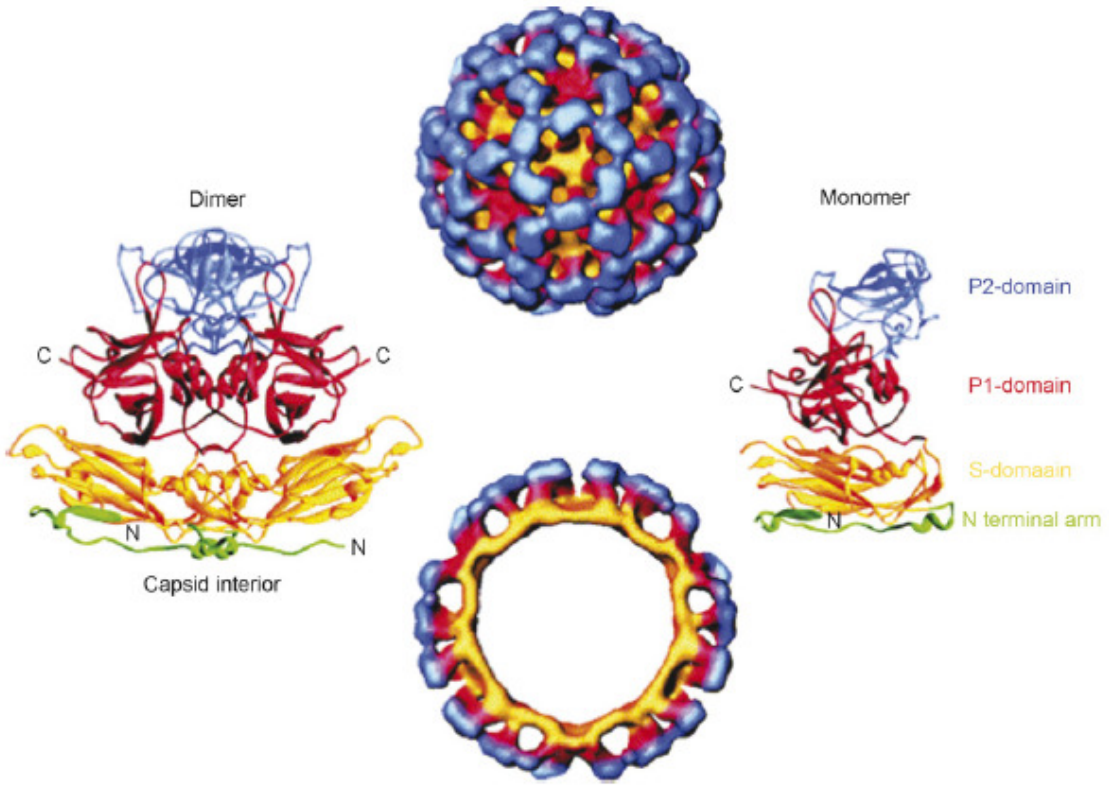

Figure 2: Norovirus-like particles. Surface representation (top) and cross-section (below). The virus-like particles are formed from 90 dimers (left) of the capsid VP1 protein (right). The capsid VP1 protein is divided into an N-terminal region (green) facing the interior of the VLP, a shell domain (S-domain, yellow) that constitutes the surface of the virus, and a protruding domain (P-domain) that emanates from the S-domain surface. The P-domain is divided into P1 and P2 (red and blue, respectively) with the P2-domain at the most distal surface of the particle. Source Le Pendu et al 2006.

Role of Histo Blood Group Antigens (HBGA) in Human NoV Infection and Oyster Bioaccumulation

Binding of NoV capsid to HBGA

Human susceptibility to norovirus infections is determined by the ABH and Lewis HBGAs (Le Pendu *et al.*, 2006). HBGAs are complex glycans present on many cell types including red blood and vascular endothelial cells, as well as on the epithelia of the gastrointestinal, urogenital and respiratory tracts. They are synthesised from a series of precursor structures by stepwise addition of monosaccharide units via a set of glycosyltransferases (Maalouf *et al.*, 2010). Non-secretor (FUT2–/–) individuals, which account for approximately 20% of the population, are highly resistant to symptomatic infections with major strains of NoV although there is now evidence that NoV GII.4 strains have undergone evolutionary changes to recognise Lewis antigens from non-secretors (Nasir *et al.*, 2012; de Rougemont *et al.*, 2011). As different human NoV strains present distinct specificities for HBGAs most strains only ever infect a subset of the population (Maalouf *et al.*, 2010). This suggests a host–pathogen co-evolution driven by carbohydrate–protein interactions (Le Pendu *et al.*, 2006). Binding of NoV to HBGA carbohydrates is through the variable P2 region of the virus VP1 capsid protein and evolutionary pressures on the NoV capsid has affected both antigenic and carbohydrate recognition phenotypes (Parra *et al.*, 2012).

Table 1 shows the binding profiles of different strains of NoV to HBGAs. What is evident is that no single HBGA binds to all NoV and there is also variability in binding profiles among strains of the

same genotype e.g. GII.4 strains. What is not evident from this table is that even when several NoV strains show binding to a common HBGA (e.g. A antigen) the affinity with which they bind can also vary (Lindesmith *et al.*, 2008; Lindesmith *et al.*, 2010; Huang *et al.*, 2005).

Table 1: Synthetic HBGA oligosaccharide and VLP binding assay

Norovirus strain	Genogroup/ genotype	A	B	H1	H2	H3	Le^a	Le^b	Le^x	Le^y	Reference
Norwalk (NV)	I.1	x		x		x		x		x	(Huang *et al.*, 2005)
GI.1 1968	I.1	x		x		x					(Lindesmith *et al.*, 2010)
GI.1 2001	I.1	x		x		x					(Lindesmith *et al.*, 2010)
GI.2 1999	I.2	x				x	x				(Lindesmith *et al.*, 2010)
Desert Shield (DSV)	I.3	No binding									(Shirato, 2011)
GI.3 1999	I.3						x				(Lindesmith *et al.*, 2010)
VA115	I.3	No binding									(Shirato, 2011)
GI.4 2000	I.4	x					x		x		(Lindesmith *et al.*, 2010)
Boxer	I.8							x		x	(Huang *et al.*, 2005)
Hawaii (HV)	II.1	x	x					x			(Huang *et al.*, 2005)
Snow Mountain (SMV)	II.2		x			x			NT	NT	(Tan and Jiang, 2007)
BUDS	II.2	x	x								(Huang *et al.*, 2005)
Mexico (MxV)	II.3	x	x					x			(Huang *et al.*, 2005)
GII.3 TV	II.3	x				x					(Cannon *et al.*, 2009)
VA387	II.4	x	x	x		x		x		x	(Huang *et al.*, 2005)
Grimsby (GrV)	II.4	x	x	x		x		x		x	(Tan and Jiang, 2007)
GII.4 1987	II.4					x				x	(Lindesmith *et al.*, 2008)
GII.4 1997	II.4	x	x			x		x		x	(Lindesmith *et al.*, 2008)
GII.4 2002	II.4					x				x	(Lindesmith *et al.*, 2008)
GII.4 2002a	II.4	x					x		x		(Lindesmith *et al.*, 2008)
GII.4 2004	II.4	No binding									(Lindesmith *et al.*, 2008)
GII.4 2005	II.4	No binding									(Lindesmith *et al.*, 2008)
GII.4 2006	II.4	x	x			x					(Cannon *et al.*, 2009)
MOH	II.5	x	x								(Huang *et al.*, 2005)
VA207	II.9								x	x	(Huang *et al.*, 2005)
Paris island (PiV)	II.13	x	x					x			(Huang *et al.*, 2005)
OIF	II.16						x				(Huang *et al.*, 2005)

NT=not tested

For the past 20 years it has been NoV GII.4 strains which have been predominantly linked with global epidemics (Parra *et al.*, 2012). These GII.4 strains can be divided into variants, and since 2002 they have circulated in the population before being replaced every two or three years by a new variant (de Rougemont *et al.*, 2011). By analysing the VP1 coat protein amino acid sequence of historical NoV GII.4 strains the evolution of various NoV clusters over time are evident (Figure 3) (de Rougemont *et al.*, 2011). Representative strains of these GII.4 clusters have not only demonstrated altered HBGA binding but also altered affinity in their binding to common antigens. Analysis of the binding properties for six NoV GII.4 variants have shown that post-2002 variants (Hunter, Yerseke, Den Haag, and Osaka) presented stronger binding to A and B antigens, suggesting that the GII.4 evolution could be related to an increased affinity for HBGAs for the post-2002 variants (de Rougemont *et al.*, 2011).

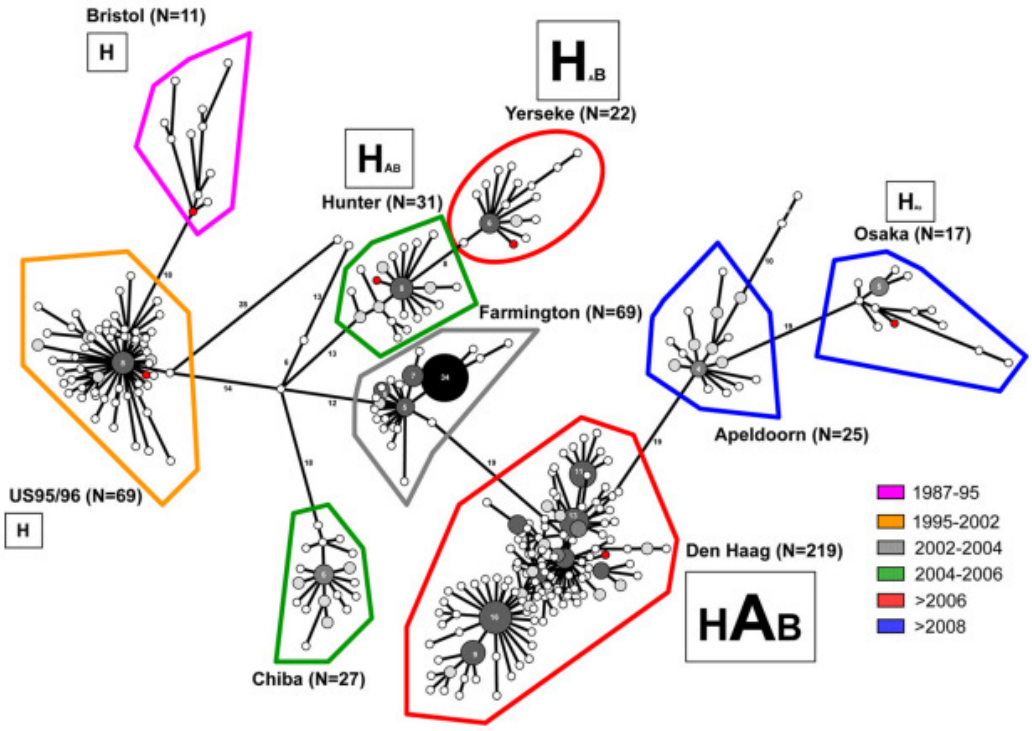

Figure 3: Minimum spanning tree of 496 ORF2 amino acid sequences of NoV GII.4 strains from GenBank corresponding to Bristol, US95/96, Farmington, Hunter, Chiba, Yerseke, Den Haag, Apeldoorn and Osaka variants. The number of sequences for each group of variants is indicated in parentheses. Identical sequences are represented by gray-shaded circles which are scaled according to member count. The white and light gray circles represent one and two sequences, respectively. For dark gray and black circles, the number of identical sequences is indicated inside each circle. The six GII.4 isolates analysed for HBGA binding are indicated by red circles. Each group of variants is colour coded according to the year of circulation. For the six variants that were analysed, the strength of binding profile to A, B and H antigens are indicated by scale of lettering. Source de Rougemont et al 2011.

Role of HBGA and other ligands in NoV bioaccumulation by oysters

Analysis of global shellfish-related NoV outbreak data has indicated a high proportion of NoV GI strains (Le Guyader *et al.*, 2012). Studies have indicated that oysters are not just passive filter feeders but can selectively accumulate NoV strains based on viral carbohydrate ligands (HBGA) shared with humans. These observations contribute to a possible explanation for the GI bias observed in shellfish-related outbreaks compared to non-shellfish related outbreaks where GII.4 strains dominate (Le Guyader *et al.*, 2012).

Le Guyader et al (Le Guyader *et al.*, 2006) demonstrated specific binding of the Norwalk virus strain (GI.1) to oyster digestive tissue through A-like carbohydrate structures which were indistinguishable from the human blood group A antigen. Subsequently, this observation was confirmed in different oyster species and for other NoV strains (Maalouf *et al.*, 2010). However, as NoV strains show differing specificities for HBGAs in humans, not all strains may be captured equally well in oysters. For example, NoV GI.1 and GII.4 have been shown to bind to different types of ligands within the oyster resulting in spatial variation in the localisation of virus within the host tissues (Maalouf *et al.*, 2010). Both GI.1 and GII.4 bind to A-like antigen ligands expressed within the oyster's digestive tissues, but GII.4 also bind to sialic acid containing ligands expressed in the gills and mantle. NoV strains which bind sialic acid containing ligand are thought to be less well retained due to unknown mechanisms (Le Guyader *et al.*, 2012).

Specific selection and persistence of GI NoVs in oysters have been demonstrated both in bioaccumulation and environmental studies (Le Guyader *et al.*, 2012). NoV GI have been shown to be concentrated to a greater degree than GII strains in oysters. It has been shown that NoV GI requires 30 viral RNA copies/L water to bioaccumulate 1 viral RNA copy/g oyster tissue as compared to ~1200 viral copies/L of water to bioaccumulate 1 viral copy/g oyster tissue for NoV GII. Furthermore, studies investigating a NoV contaminated oyster production area showed that NoV GI persisted at a higher level for longer that GII in the oysters. The initial percentage of oyster samples positive for NoV GI and GII were 59% (10/17) and 70% (12/17), respectively. Yet, after four weeks the prevalence decreased to 41% (7/17) and 17% (3/17) of GI and GII respectively, suggesting a greater persistence in oyster tissues of GI NoVs compared to GII strains (Le Guyader *et al.*, 2008).

The theoretical selective transmission of NoV strains to the human population through oysters accumulating and retaining NoV from the environment is shown in Figure 4. Ligands that facilitate bioaccumulation (the A-like antigen) or that contribute to the elimination of the virus (the sialic acid-containing ligand) may both influence NoV accumulation and survival in oysters (Figure 4). Other NoV genogroups present within the environment, such as bovine GIII, will not bioaccumulate efficiently in oysters due to lack of the specific glycan ligand required for attachment.

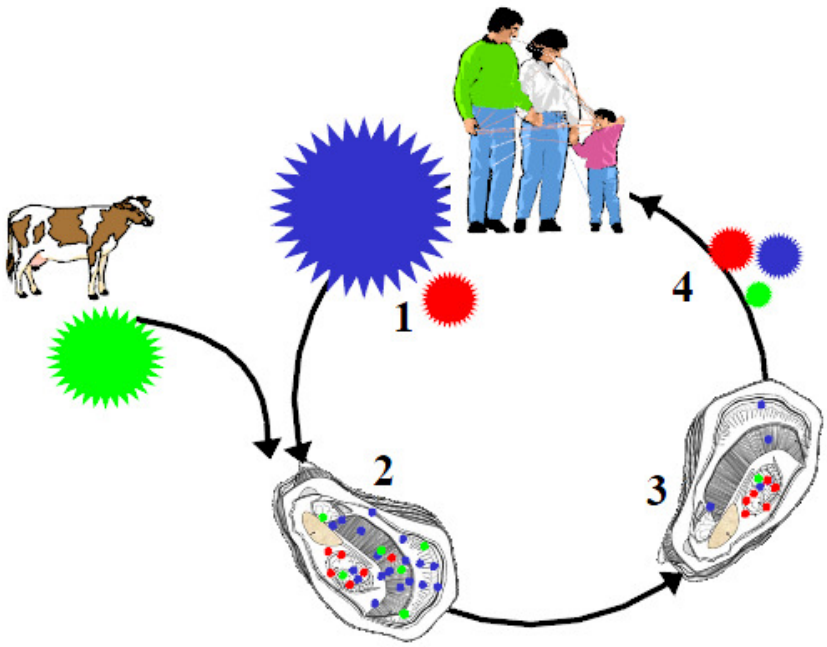

Figure 4: Influence of oysters in the selection of NoV transmission. 1: Shedding in the environment of large amounts of human NoV GII (blue), lower amounts of human NoV GI strains (red) and bovine NoV GIII (green). 2: Viruses present in seawater are ingested by oysters. GI NoVs particles are mainly directed to the gut, whereas GII particles are retained in mantle or gills. GIII NoVs are probably randomly distributed. 3: NoV

GI and GII are accumulated in the gut via an HBGA A-like ligand, most GII and GIII particles outside the gut are presumably destroyed. 4: Upon consumption of a NoV-contaminated oyster, infection caused by GI and GII strains occur with similar frequency because of the selective accumulation and retention of GI viral particles. GIII NoV transmission is unlikely to happen as few particles persist in oysters and humans do not express the glycan ligand. Source Le Guyader et al 2102.

NoV Immunogenicity and Specificity of Antibodies

It is known that the NoV genome undergoes frequent change and there is evidence that some of these changes are linked to altered viral infectivity (Marshall and Bruggink, 2006).The NoV capsid protein has evolved over time by antigenic drift. It has been shown that GII.4 viruses evolve through a series of changes in the protruding and immunogenic P2 domain of the capsid protein sequence which occurs sporadically after periods of stability. These changes in the capsid protein result in new clusters of the virus based on immunogenic evolution (Figure 5) (Lindesmith *et al.*, 2011). Some cross reactivity of antibodies generated from one NoV strain against another is apparent, however, specificity within NoV clusters are generally greater than among cluster (Table 2).

No universal serological test exists for the detection of all known NoV due to the large variability within the amino acid sequences of the immunogenic viral capsid. Commercially available serological tests are available for clinical norovirus testing which do discriminate between GI and GII genogroups. These assays have been shown to be less sensitive for NoV detection than nucleic acid based detection (78% vs 91%) but have the similar specificity for the two genogroups (Kele *et al.*, 2011).Various polyclonal and monoclonal antibodies have been shown to detect a broad range of NoV genotypes with varying specificities (Okame *et al.*, 2007). Polyclonal antibodies have the advantage that they are relatively quick and inexpensive to produce compared to monoclonal antibodies and are generally less specific in that they are capable of recognising multiple epitopes on any one antigen. This capability can provide advantages such as increased signal produced by the target antigen as the antibody will bind to more than one epitope. Furthermore, polyclonal antibodies are less sensitive to antigen changes than monoclonal antibodies.

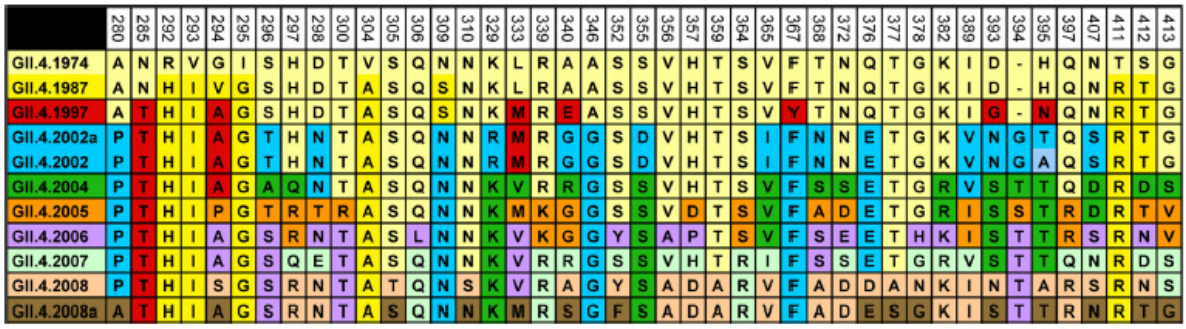

Figure 5: Norovirus GII.4 P2 sub-domain antigenic variation. Eleven representative amino acid sequences were selected from each major phylogenetic cluster, beginning with strains originating in 1974 through to 2008. Each colour represents amino acid changes that occurred within a sub-cluster. Ancestral 1974 GII.4 (light yellow); GII.4-1987-Camberwell (yellow); GII.4-1997-Grimsby (red); GII-2002 & GII-2002a-Farmington Hills (blue); GII-2004-Hunter (green); GII-2005-Sakai (orange); GII-2006-Minerva (purple); GII.5-2007 (light green); GII.4-2008-Stockholm (tan); and GII.4-2008a-Appledorn (brown). Source Lindesmith et al 2011.

Polyclonal antibody raised against the VLP of a NoV GII.3 genotype showed broad-range cross reactivity, reacting both with intra-genogroup strains and inter-genogroup strains (five NoV GI and 11 NoV GII genotypes investigated (Okame *et al.*, 2007). Several laboratories have also reported the production of broadly reactive monoclonal antibodies, which recognise the linear GI and GII

cross-reactive epitopes or the conformational GI specific epitope. Shiota *et al.* (2007) demonstrated that a single monoclonal antibody (MAb14-1) could recognise 15 different VLPs representing GI.1, 4, 8, and 11 and GII.1-7 and 12-15, while also showing weak affinity to GI.3 making it the broadest existing NoV monoclonal antibodies generated to date. Despite this broad recognition range MAb14-1 generally showed lower affinity to the GI genotypes tested. The MAb12-1 recognition epitope has been identified as the terminal antigenic regions (amino acid positions 418 to 426 and 526 to 534) on the C-terminal P1 domain (Shiota *et al.*, 2007).

Table 2: Percent cross-reactivity between representative GII.4 strain VLPs and the mouse antisera generated against each strain. Camberwell and Grimsby share similar antigenicity as indicated by the yellow squares. All others are distinct. Farmington Hills, blue; Hunter, green; Sakai, orange. Two different shades of blue indicate two different antigenic types in the Farmington Hills cluster. Source (Donaldson *et al.*, 2008).

	GII.4 antisera					
VLP	GII.4.1987	GII.4.1997	GII.4.2002a	GII.4.2002	GII.4.2004	GII.4.2005
GII.4.1987	100	60	10	16	12	22
GII.4.1997	100	100	17	46	25	19
GII.4.2002a	2	1	100	4	3	4
GII.4.2002	21	7	19	100	21	28
GII.4.2004	17	8	10	18	100	27
GII.4.2005	24	9	6	17	32	100

Currently Available Detection Methodologies for NoV

As human NoV have been unable to be cultured (Straub *et al.*, 2011), early laboratory detection methodologies have depended on visual detection using electron microscopy (EM) and enzyme immunoassays (EIA) such as the enzyme-linked immunosorbent assay (ELISA). Although both these methods are appropriate for detection of NoV in clinical samples (e.g. faeces) their level of sensitivity is not appropriate for detecting low levels of NoV contamination in food samples (EFSA, 2012; Stals *et al.*, 2012b). Commercial ELISA (RIDASCREEN and IDEIA) assays have been successfully used to rapidly detect NoV GI and GII strains in clinical faecal samples but these assay do not detect all genogroups nor all strains within a genotype (Kirby *et al.*, 2010). Since the 1990s more sensitive methodologies have been developed for NoV detection based on the polymerase chain reaction (PCR) and isothermal amplification methodologies targeting detection of the genomic viral RNA (Stals *et al.*, 2012b; Marshall and Bruggink, 2006).

Several conserved genomic regions have been identified for targeting nucleic acid based detection of NoV, including the RNA-dependent RNA polymerase (RdRp) gene, the ORF1-ORF2 junction, or regions within the capsid encoding gene (ORF2) (Mattison *et al.*, 2009; Stals *et al.*, 2012b). Unfortunately, no single sensitive and specific method for detecting all human and animal NoVs has yet been developed despite targeting these highly conserved regions of the genome (Stals *et al.*, 2012b; Mattison *et al.*, 2009). The most conserved region in the genome of human NoVs is the ORF1/ORF2 junction and this region has been used as the preferred target region for molecular detection using techniques such as reverse transcription (RT) PCR, nucleic acid sequence-based amplification (NASBA), and transcription loop-mediated isothermal amplification (RT-LAMP) (Stals *et al.*, 2012b). The most widely used techniques for the detection of NoV RNA are based on RT-PCR methods, and at present quantitative or real-time RT-PCR is considered to be the gold standard for molecular detection of NoVs in clinical, food, and environmental samples. Real-time RT-PCR has several advantages in that it is the most sensitive method currently available, eliminates the need of post-PCR processing, is able to be multiplexed and can quantify genome copies present within the sample (Stals *et al.*, 2012b). However, the technique has two major drawbacks in that it does not discriminate between the presence of infectious versus non-infection

virus particles and its level of sensitivity (10^2-10^4 genome copies/gram) is not low enough to detect low doses (e.g. ID_{50} = 18 infectious particles) (Stals *et al.*, 2012b; Liu *et al.*, 2011).

Evaluation of different RT-PCR NoV detection assays targeting the ORF1/ORF2 junction have confirmed that this region allows amplification of a broad range of human NoV genotypes (Mattison *et al.*, 2011). Different primers and probes targeting this region allow for the detection of NoV GI or GII strains (Figure 6). It is also this region that has been targeted for the development of an international standard for quantitative RT-PCR detection of NoV GI and GII in food and feed (ISO/TS15216, 2012; Lees, 2010).

In addition to detection of NoV, five genomic regions have frequently been used for genoyping or classification of NoV strains by conventional RT-PCR amplification and sequencing. These are situated within the polymerase gene (regions A and B) and major the capsid protein VP1 gene (regions C, D, and E) (Figure 7) (Mattison *et al.*, 2009).

The choice of detection technique is dependent on the sample type and they all have their own advantages and disadvantages (Table 3). The level of sensitivity reported for different methodologies varies from 10^6-10^7 viruses/gm faeces for EM to 10^2-10^4 virus copies per sample or gram of sample for nucleic acid based methodologies (Fukuda *et al.*, 2008; EFSA, 2012; Stals *et al.*, 2012b; Glass *et al.*, 2000).

Numerous RT-PCR assays have been developed and evaluated for detection of NoV, however, these are largely variants on the same technique (Boxman, 2010; EFSA, 2012). These have included either one or two step RT in combinations with nested, semi-nested and real time PCR, done as either a singleplex (detecting one virus) or multiplex (detecting multiple enteric viruses in one assay) reaction (Stals *et al.*, 2012b). Real-time RT-PCR assays based on sequence-independent chemistries (intercalating dyes) and sequence-dependent chemistries (hydrolysis probes, hybridisation probes and molecular beacons) have been described for detection of human and animal NoV (Stals *et al.*, 2012b). Although sensitivity of these assays may vary slightly, reliable NoV detection has been reported in the range of 10^2-10^4 viral copies per gram of sample for real time RT-PCR assays (Liu *et al.*, 2011; Stals *et al.*, 2012b).

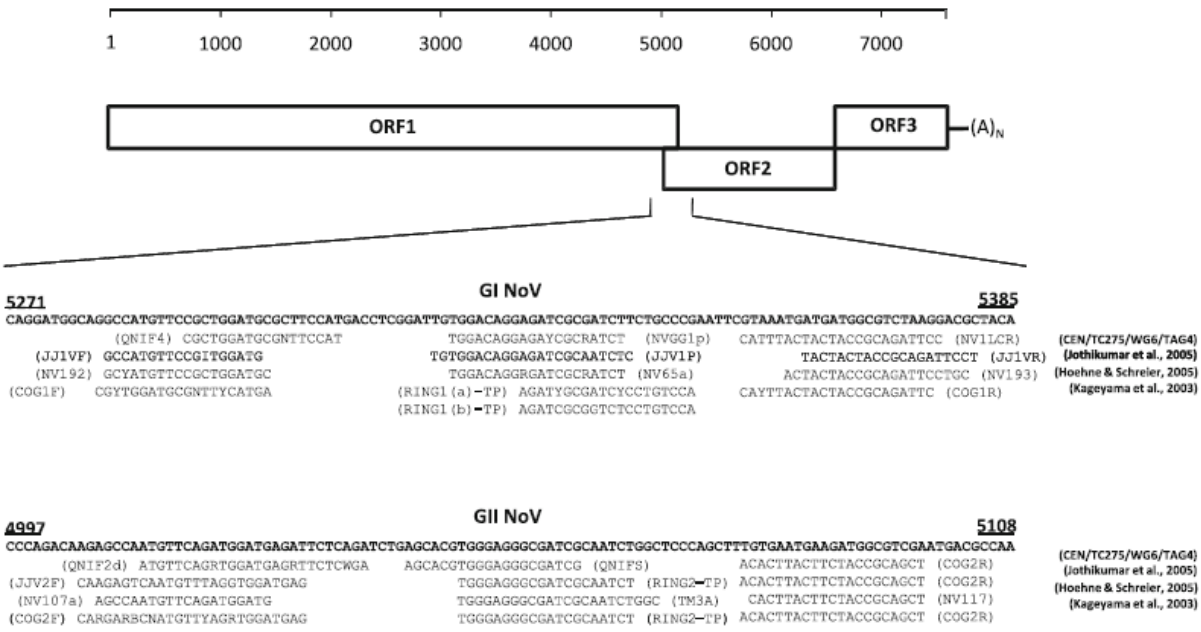

Figure 6: Overview of various real-time RT-PCR primers and hydrolysis probes targeting the ORF1–ORF2 junction of the NoV genome. NoV GI and GII sequences shown are deduced from EMBL/Genebank accession numbers M87661 and X86557, respectively. Names of primers and hydrolysis probes are given

between brackets in front or behind the primer or probe sequence. Reverse primers are shown in reverse complement sequence. Source Stals et al 2012b.

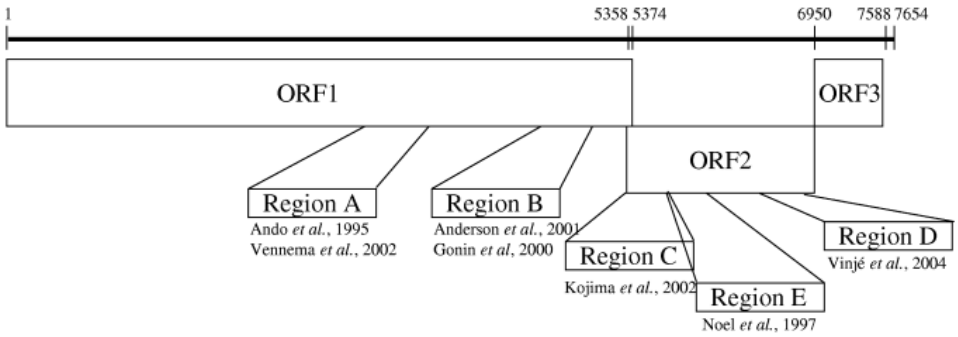

Figure 7: Schematic representation of the NoV genome and the positions of several representative regions (regions A–E) that have been used for genotyping. Numbers refer to positions in the Norwalk GI.1 virus genome (GenBank accession number M87661). Source Mattison et al.2009

Table 3: Currently available viral detection methodologies for NoV. Summarised (Stals *et al.*, 2012b; EFSA, 2012; Liu *et al.*, 2011; Fukuda *et al.*, 2008; Glass *et al.*, 2000).

Assay	Detection	Sample type	Detection limit	Advantages	Disadvantages
EM	Visual	Clinical	10^6- 10^7 virus/gm faeces	Independent of NoV specific reagent	Low sensitivity Non-specific (identification based on morphological characteristics)
EIA/ELISA	Serological	Clinical	10^4-10^6 virus/gm faeces	Rapid and simple	Less sensitive and specific than methods based on nucleic acid detection
Hybridisation probes	Nucleic acid	Clinical	10^4-10^6 virus/gm faeces	Can distinguish among strains	No universal assay available
(RT-LAMP)	Nucleic acid	Clinical, food and environmental	$>10^3$ genome copies/oyster		Use of different primer sets may hinder development of long-term use due to antigenic drift NoV genome Not quantitative
(NASBA)	Nucleic acid	Clinical, food and environmental	$>10^3$ genome copies/oyster	RNA amplification method (direct amplification of viral genome. No RT step required).	Low incubation temperature may increase non-specific amplification As RNA is the final amplification product, the possibilities for post-amplification analyses may be limited Not quantitative
NASBA-RT-LAMP	Nucleic acid	Clinical, food and environmental	10^2 -10^3 genome copies/oyster	As above	As above Detection sensitivity and specificity determined by primers and sample preparation/nucleic acid extraction
RT-PCR	Nucleic acid	Clinical, food and environmental	10^2-10^4 genome copies/oyster	Low detection limit	Not quantitative Detection sensitivity and specificity determined by primers and sample preparation/nucleic acid extraction
Real time RT-PCR	Nucleic acid	Clinical, food and environmental	10^2 -10^4 genome copies virus/oyster	Absence of post-PCR processing, ability for multiplexing, ability to quantify NoV level	Detection sensitivity and specificity determined by primers and sample preparation/nucleic acid extraction Not reflective of number of infectious particles present in sample as detects non-encapsidated RNA and degraded viral genomic RNA Not sensitive enough to detect an infectious dose

Progress Towards a Standardised Method for NoV Detection in Food

The numerous RT-PCR based assays for detecting NoV in shellfish reported in the literature have been difficult to compare due to lack of standardisation and availability of a reference method (Boxman, 2010). Furthermore, many assays reported in the literature for detection of NoV in shellfish only reported semi quantitative data which has made estimating the level of absolute detection of assays difficult. To compare NoV real time RT-PCR methodologies a ring trial coordinated by the European Community Reference Laboratory, Cefas UK was run in 2008. The ring trial consisted of laboratory constructed samples of NoV GI, GII and HAV in lenticule format which were distributed to 28 participating international laboratories (Boxman, 2010). Results of the trial indicated that 67% or participating laboratories obtained intended qualitative (positive or negative) results from lenticlules loaded with 10^3 NoV copies (GI or GII) and 10^4-10^5 HAV copies. It was found that 41%, 48% and 72% of participating laboratories returned a correct result for NoV GI, GII and HAV respectively. False positive and negative results ranged from 4-6% and 6-11% respectively.

In 2004 the European Committee of Standardisation (CEN) initiated the development of a standard method for detection of NoV in foodstuffs, including bivalve molluscs, based on quantitative RT-PCR targeting the ORF1/ORF2 junction of the NoV genome (CEN/TC275/WG6/TAG4 working group) (Lees, 2010). This standard (ISO/TS 15216: *Microbiology of food and animal feed - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR*) was published in late in 2012 and is outlined in Figure 8.

NoV Extraction and Concentration Methodologies Suitable for shellfish

A particular difficulty in working with molluscan shellfish is the sample matrix. Viruses become concentrated and trapped in the digestive tissue creating additional difficulties for recovery of the virus for subsequent detection. By seeding oyster samples with a heterologous nonenveloped positive-sense ssRNA virus (Mengovirus) it has been estimated that virus extraction efficiency is <20% from oyster tissue (EFSA, 2012). Extraction of enteric viruses from shellfish is based on several steps: virus elution from shellfish tissues, recovery of viral particles and then virus concentration (Table 4) (Stals *et al.*, 2012a). In RT-PCR based applications the weight analysed generally ranges from 1.5 to 2 g of digestive tissues. Some methods propose larger weights for the first step but thereafter analyse only a fraction of the extracts (Table 4). Viruses are eluted from shellfish digestive tissues using various buffers (chloroform/butanol or glycine) before being concentrated either by polyethylene glycol precipitation (PEG) or ultracentrifugation. Direct lysis of virus particles has also been used, including methods utilising proteinase K or Trizol to destroy shellfish tissues, or mechanical disruption (Zirconia beads) and a denaturing buffer for virus and/or nucleic acid elution (Table 4). These virus elution, concentration and RNA extraction methodology can all influence the recovery of virus and the sensitivity of downstream RT-PCR assays. For example, the proteinase K methods has been shown to be better for NoV virus recovery than PEG precipitation or ultracentrifugation in shellfish (Comelli *et al.*, 2008) and it is this method that has been selected for use in the ISO/TS15216 standard. However, proteinase K treatment, or other direct lysis methods are not appropriate if intact virus particles are required for serological capture as the viral capsid is damaged (Stals *et al.*, 2012a; Mormann *et al.*, 2010). Table 5 summarises the function of various virus elution methodologies which may be appropriate for recovery of intact NoV from shellfish, while Table 6 outlines the advantages and disadvantages of methods which may be used to concentrate NoV following elution.

Table 4: Overview of methods used for virus elution, concentration and nucleic acid (NA) extraction based on RT-PCR detection in shellfish. (EFSA, 2012)

Shellfish mass	Virus elution	Virus conc.	Mass extracted	NA extraction	% analysed	Reference
Oysters						
25g	Glycine	PEG	0.5g	QIAamp (Qiagen)	3	(Shieh *et al*., 1999)
50g	Water	PEG, precipitate	0.5g	Boiling	nc	(Chung *et al*., 1996)
18g	Glycine	Ultracentrifugation	1g	GuSCN	10	(Muniain-Mujika *et al*., 2003)
50g	Sonication	PEG	nc	GuSCN	nc	(Green *et al*., 1998)
25g	Glycine	PEG	0.4g	Tri-reagent (Sigma)	10	(Kingsley and Richards, 2001)
1.5g DT	CHC13-but, Catfloc	PEG	1.5g	Prot. K, CTAB	20	(Atmar *et al*., 1995)
1.5g DT	Gycine-threonine	PEG	0.12g	GuSCN+ QIAamp	16	(Beuret *et al*., 2003)
1.5g DT	Chloroform-but, Catfloc	PEG	1.5g	Prot. K, CTAB	20	(Schwab *et al*., 2001)
1.5g DT	PBS pH7.4, CHC13-but, Catfloc	Ultracentrifugation	1g	QIAamp	25	(Nishida *et al*., 2003)
1.5g DT	Zirconia beads	nc	0.09g	RNEasy (Qiagen)	6	(Lodder-Verschoor *et al*., 2005)
DT of 1 oyster	Stainless-steels beads	nc	nc	Silica & guanidium	17	(Ueki *et al*., 2005)
2g DT	Proteinase K	nc	0.01g	GuSCN	4	(Jothikumar *et al*., 2005)
10g DT	TRIzol (Gibco)	nc	0.08g	GuSCN	8	(Boxman *et al*., 2006)
1.5g DT	Zirconia beads	nc	nc	RNeasy	5	(Schultz *et al*., 2007)
nc	Glycine, pH 10	Ultracentrifugation	nc	Silica-method	10	(Hernroth and Allard, 2007)
DT of 3 oysters	Phosphate saline buffer	PEG	1g	QIAamp	5	(Fukuda *et al*., 2008)
5g DT	Buffer pH 8 + Prot. K	nc	0.05g	Silica & guanidium	10	(Greening and Hewitt, 2008)
25g flesh	Adsorption-alkaline elution	PEG (twice)	5g flesh	RNeasy	3	(Kittigul *et al*., 2008)
DT of 6 oysters	Buffer pH 8 + Prot. K	-	nc	Silica & guanidium	11	(Lowther *et al*., 2008)
1.5g DT	Glycine pH 9	PEG	1.5g	Nuclisens (BioMerieux)	20	(Le Guyader *et al*., 2009)
5g DT	Finely chopped + Prot K	-	0.15g	RNeasy	4	(Gentry *et al*., 2009)
25g flesh	Adsorption/elution	PEG (twice)	25g flesh	RNeasy	6	(DePaola *et al*., 2010)
2g DT	TPB and CHC13-but	PEG	nc	Silica & guanidium	nc	(Rigotto *et al*., 2010.)
Mussels						
20g	Glycine, Catfloc	Antigen capture	0.04g	QIAamp	21	(Lee *et al*., 1999)
50g	Glycine	PEG	nc	Guanidium, CsCl	100	(Croci *et al*., 2000)

Shellfish mass	Virus elution	Virus conc.	Mass extracted	NA extraction	% analysed	Reference
100g	Glycine	Ultracentrifugation	1g	GuSCN	5	(Pina *et al*., 1998)
10g	Glycine	PEG	0.8g	RNeasy	50	(Chironna *et al*., 2002)
25g	Threonine	PEG	nc	GuSCN	2.5	(Mullendore *et al*., 2001)
25g DT	Glycine	Ultracentrifugation	1.5g	TRIzol (Gibco) + Boom	6	(Myrmel *et al*., 2004)
2g DT	Glycine	Ultracentrifugation	0.1g	GuSCN	10	(Hernroth *et al*., 2002)
75xg total	Glycine buffer pH 9.2	PEG	8.3g	Guanidium	100	(Croci *et al*., 2007)
1.5g DT	Glycine buffer pH 9.5	PEG	1.5	Nuclisens	5	(Vilarino *et al*., 2009)
25g DT	Glycine buffer pH 9.2	PEG (twice)	0.75g	Nucleospin RNA (M-N)	4	(Serracca *et al*., 2010)
Clams						
25g	Glycine, CHC13	Ultracentrifugation	1.25g	Nucleospin RNA	10	(Sunen *et al*., 2004)
1.5g DT	CHC13-but, Catfloc	PEG	0.07g	RNeasy	6	(Costafreda *et al*., 2006)
1g DT	PBS	Ultracentrifugation	nc	QIAamp	1.5	(Hansman *et al*., 2007)

Table 5: Elution buffers and additional components used for virus extraction from food matrices. (Stal et al 2012a)

Component/condition	Detail	Function
Alkaline–neutral pH	pH 9.5 to 10.5 (alkaline)or pH 7 (neutral)	Detaches viral particles from food surface
pH buffer system	Tris(–HCl) buffer Phosphate buffer	Prevents pH reduction caused by acidic fruit/ vegetable juices
Beef extract	1% to 3% (w/v)	Facilitates flocculation of NoV on PEG
Glycine	0.05 M to 0.5 M	Reduces non-specific adsorption of protein or virus
Cat-floc® TL		Improves flocculation of food solids
Chloroform and n-butanol		separate organic (lipid) and aqueous (virus) phases

Table 6: Advantages and disadvantages of diverse methods used to concentrate eluted virus particles. Stal et al 2012a

Concentration method	Advantage	Disadvantage
Polyethylene glycol precipitation	Cheap and simple	pH neutralization of virus eluate necessary. Absolute quantitation is likely to be questionable.
Ultracentrifugation	Consistent results	Requires virus eluates free of vegetable matter. Need for specialized equipment. Absolute quantitation is likely to be questionable
Immune-capture	Simple	Capture is dependent on specificity of antibody. Absolute quantitation is likely to be questionable

Need for Improved Methods to Detect Infectious NoV in Shellfish

Despite the increased sensitivity of quantitative RT-PCR methods for detecting NoV, the risk associated with illness from consuming contaminated food is not clear for two reasons: (1) the limit of detection is not sensitive enough to detect a theoretical infectious dose; and (2) viral copies detected by PCR are not directly related to infective viral particles as non-encapsidated RNA and degraded viral RNA are also detected (EFSA, 2011). Furthermore, the relationship between the number of infectious virus particles and the number of virus genome copies detected by real time RT-PCR is not a constant and may vary depending on environmental conditions including time from the initial release from the host (EFSA, 2012). As the number of genome copies detected by real time RT-PCR are not directly related to infectious NoV particles these methods can only be used to provide an indirect measure of risk. When considering what is an acceptable level of NoV in oysters it is also important to realise that the infection risk associated with low level contaminated oysters as determined by real time RT-PCR may be overestimated (EFSA, 2011). Detection of high levels of norovirus RNA in oysters is indicative of a significantly elevated health risk. However, illness may not necessarily be reported after consumption when detection of norovirus RNA indicates low levels in the consumed food (Lowther *et al*., 2010). This may be due to the fact that the low levels of NoV RNA detected may not have been obtained from infectious virus particles or that the epidemiological surveillance systems used were not sensitive enough to capture associated illness. Despite these short fallings real time RT-PCR is the most advanced and sensitive methodology currently available.

Key Criteria Successful New Methodology Should Meet

A key criteria that any new methodology will need to meet to be truly successful in detecting NoV in oysters (and other food matrices) is that it will need to have a level of sensitivity which is greater than, or at least equal to, the best methodology currently available (quantitative RT-PCR). The detection limit should to be in line with what constitutes a median infective dose and ideally <10 viral particles. Any new method should also give a clearer association between detection of NoV and the presence of infectious particles. Other criteria desirable would be that the methodology would be quicker to perform compared to quantitative RT-PCR. Currently the best case scenario for NoV testing is three to four working days due to the multistep process required (dissection of the digestive tissues, purification of the virus from tissue matrix, extraction of nucleic acid and RT-PCR setup). New methodologies should also be less costly than current detection techniques to encourage uptake by industry and pro-active monitoring by industry. Achieving all these criteria would be desired, however, achieving any one or a combination of these criteria will be a significant advance on existing methodology. It is anticipated that alongside any new detection methodologies based on antigen capture that confirmation would still be required using nucleic acid based approaches. If a biosensor approach using antibodies was developed than it should be linked to a surveillance system which monitors how the virus is changing so that effective detection against new norovirus strains is being achieved.

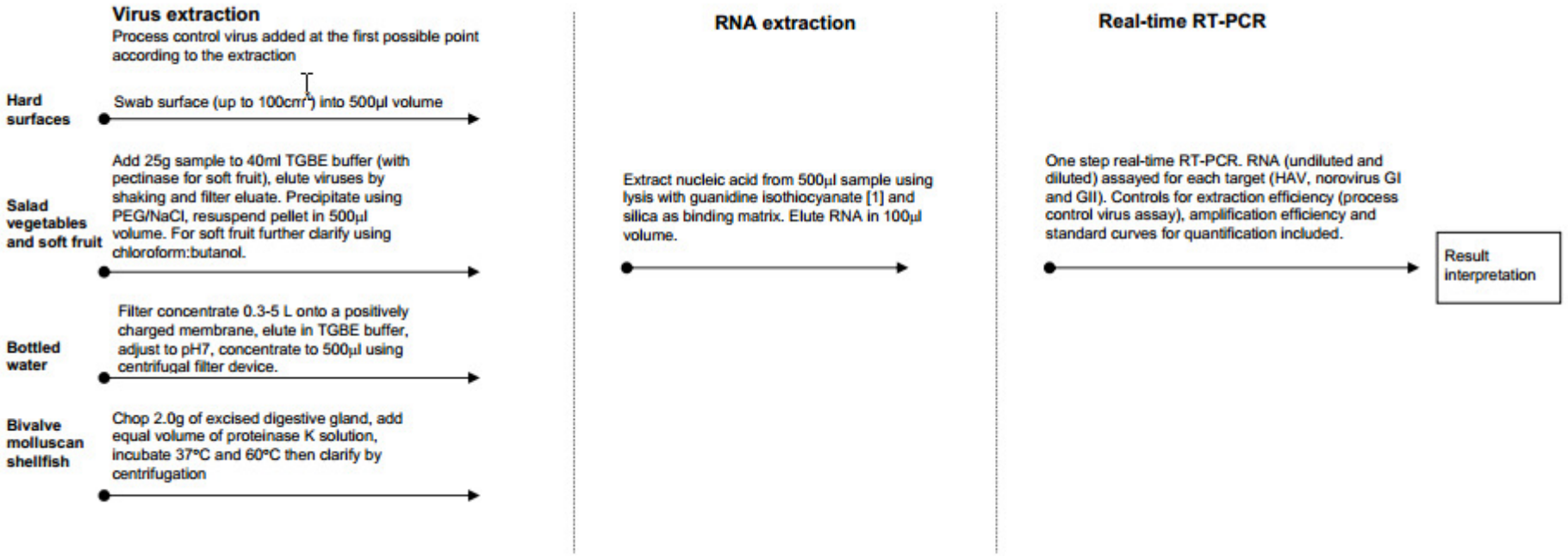

Figure 8: Horizontal method for detecting NoV and HAV in foodstuff by quantitative RT-PCR. Taken from ISO/TS 15216 “Microbiology of food and animal feed - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR

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