

# Norovirus Tracing in Environmental and Outbreak Settings – Experiences of waterborne, foodborne and nosocomial transmission

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To My Family

*Science and everyday life cannot  
and should not be separated.*

– Rosalind Franklin

# Abstract

Noroviruses (NoV), a major cause of acute gastroenteritis in hospital settings, also occur as sporadic infections or periodic non-seasonal community outbreaks. Human NoV replicates to high concentration in the intestinal tract, is readily transmitted by the faecal-oral route, hand-to-hand contact, contaminated food and water, and by aerosols. Large numbers of NoV are discharged into wastewaters and, despite sewage treatment, can cause problems when recycled river waters are used as source of drinking water. Two major groups of NoV are associated with human infections, genogroups (G) GI and GII. Epidemiological studies indicate association of GI with non-seasonal food- and waterborne infections, and GII with person-to-person transmission, particularly nosocomial spread of NoV GII.4.

As NoV detection in filter-feeding bivalves may have a sentinel role in tracing NoV in environmental waters, molecular tools were used to detect and characterize NoV in mussels from Fotö near the plume of sewage effluents from Gothenburg wastewater treatment plant. Sequence analyses of NoV RNA from Fotö mussels revealed GI.1 strains with high similarity (99%, 3.1kb) to strains detected in patients infected in non-seasonal, waterborne outbreaks linked to bathing in Lake Delsjö. Comparative sequence analysis of NoV strains from mussels and patients indicated that human NoV outbreak strains circulate in wastewaters, and can be traced in bivalves.

Molecular methods were used to characterize NoV detected in oysters implicated in a gastroenteric outbreak where only those who ate oysters were affected. Mixed human NoV GI and GII strains were found in the oysters, evidence of faecal contamination of the bivalves, held for several weeks in Strömstad harbour waters. NoV GI.1 strains from the oysters showed high similarity ( $\geq 99\%$ , 285 nt) to the GI.1 detected in faeces obtained from one of the oyster-eating patients. Phylogenetic analyses of GI.1 strains from patient and oysters indicated the contaminated bivalves as point source of infection.

The similarity (99%, 3.1kb) of NoV GI.1 detected in Fotö mussels, patient samples from Delsjö waterborne outbreak, and the Strömstad oyster outbreak, was remarkable. High similarity held also when strains were compared with GenBank references; 96% with L23828, from an oyster outbreak, Japan 1989; 87% with the original Norwalk strain M87661, 1968, point source well water. These findings indicate genomic stability of NoV GI.1 strains over a period greater than 20 years, and dispersal of GI.1 in environmental waters.

Association of NoV GI strains with outbreaks related to sewage-contaminated water was emphasized in the molecular epidemiology of a large, non-seasonal waterborne outbreak affecting Lilla Edet, situated on the River Göta. Molecular studies

revealed marked genomic diversity of NoV GI strains in patient samples. Cloning was used to confirm mixed GI infections including a new genotype, proposed NoV GI.9. Upstream sewage contamination of recycled river water and disinfection problems at the municipal drinking water treatment plant precipitated the outbreak.

In contrast study of NoV infections in hospital settings showed predominance of GII.4 strains in symptomatic patients and their environment. High similarity ( $\geq 99.5\%$ , 1040 nt) was found between GII.4 variant strains from patients, and strains from dust, air, and surfaces in the patient's room. GII.4 strains detected in symptomatic patients in 8 wards during the 5-month study clustered on 11 sub-branches of the phylogenetic tree. One of the wards, a control, was not affected by nosocomial spread of NoV GII.4. High similarity of GII.4 strains from patients and their hospital room environment, in a given ward at a given time, confirmed nosocomial transmission and indicated the need for interventional cleaning studies.

To summarize, NoV tracing provided strong evidence of bioaccumulation of outbreak-related NoV strains in mussels growing near sewage effluents. High similarity of NoV strains from oysters implicated in a NoV outbreak and from an infected patient, indicated transmission of NoV from oysters to humans, confirming high stability of GI.1 strains in oysters, water and mussels. Cloning confirmed mixed NoV GI infections in patients from a waterborne outbreak, strengthening indices of an outbreak caused by sewage-contaminated drinking water. High similarity of NoV GII.4 strains detected in patients and their hospital room environment, confirmed local nosocomial transmission.

### **Keywords**

Norovirus, tracing, environment, outbreak, waterborne, foodborne, nosocomial, mussels, oysters, GI.1, GII.4, dust, air, surfaces, molecular epidemiology

# Sammanfattning

Norovirus (NoV) utgör den vanligaste orsaken till akut gastroenterit som sprids på sjukhus, och påvisas ofta som patogen vid såväl sporadiska maginfektioner som vid kommunala utbrott. NoV förökar sig till stora mängder i magtarm-kanalen och smittar vidare genom fekalt-oralt via förorenad föda och vatten, samt genom aerosol-spridning. NoV utsöndras i stor mängd från infekterade individer till avloppsvatten, vilket ibland kan orsaka omfattande problem när älvvatten återanvänds som dricksvatten. Det föreligger två viktiga genogrupper (G) av NoV som infekterar människa: GI som ofta associerats till icke säsongsbundna vatten- och födorelaterade infektioner, samt GII med person-till-person-smitta där särskilt genotyp GII.4 knutits till nosokomiala utbrott.

Genom att använda molekylära verktyg för att påvisa och karakterisera NoV i musslor vid Fotö i Göteborgs hamninlopp, dit det renade avloppsvattnet från Ryaverken når, skapades möjligheter att spåra utbrott som skett i samhället. Sekvensanalys av RNA från NoV som filtrerats av musslor vid Fotö påvisade NoV GI.1-stammar med hög grad av likhet (99%, 3.1kb) med GI.1-stammar som detekterats hos patienter som infekterats under vattenburna utbrott i samband med badning i Delsjön. Jämförande sekvensanalys av NoV-stammar från musslor med de virus som påvisats hos patienter vid utbrott indikerade att humana NoV cirkulerar i avloppsvatten och kan spåras i skaldjur som musslor.

Liknande molekylär metodik användes även för att karakterisera NoV-stammar som påvisats hos ostron vid ett utbrott av gastroenterit, där endast de restaurang-gäster som ätit ostron blev sjuka. Ostronen, som sumpats i gästhamnen i Strömstad under flera veckor, härbärgerade en blandning av flera NoV, vilket talade för fekal kontamination via havsvatten. Stammar av NoV GI.1 från ostron uppvisade höggradig likhet ( $\geq 99\%$ , 285 nt) med virus av samma genogrupp som påvisats i avföringen hos ett fall som ätit ostron. Jämförande sekvens- och släktskapsanalys av dessa virus indikerade att förorenade ostron utgjorde smittkällan för utbrottet.

NoV-armassa från såväl musslorna från Fotö, patienterna som var involverade i Delsjöutbrottet, samt från det ostron-orsakade restaurangutbrottet i Strömstad, var anmärkningsvärt lika varandra (99% av 3.1kb). Vidare förelåg en stor genetisk likhet även när de lokala stammarna jämfördes med referensstammar i GenBank; 96% identitet med stammen L23828, som associerats med ett ostronutbrott av gastroenterit i Japan 1989, och 87% identitet med originalstammen Norwalk M87661 från 1968 som orsakade ett brunnsvattenrelaterat utbrott. Fynden talar för en höggradig och global genetisk stabilitet hos NoV GI.1-stammar över mer än 20 år, och understryker ett tätt samband mellan dessa virus och vatten i vår livsmiljö.

Den starka associationen av NoV GI-förekomst i avloppsvatten och utbrott beto-

nades ytterligare av ett stort vattenburet utbrott i Lilla Edet vid Göta älv, som undersöktes med molekylär epidemiologi. Sekvenseringsstudier demonstrerade en omfattande genetisk diversitet av NoV GI i avföringsprover från patienterna. Påvisandet av blandinfektioner av GI, inkluderande en nyupptäckt genotyp (föreslagen som NoV GI.9), konfirmerades genom kloning av virussekvenserna. Resultaten talade för att en massiv kontamination med avloppsvatten skett uppströms om intaget av älvvatten, vilken inte kunde hävas genom desinfektion vid det lokala vattenverket.

Vid en studie av nosokomiala utbrott av NoV vid Sahlgrenska universitetssjukhuset återfanns, till skillnad från ovanstående kommunala utbrott orsakade av NoV GI-infektioner, en påtaglig dominans av stammar av genotyp GII.4 både hos inläggande patienter och i miljöprover från deras sjuksalar. Höggradig likhet ( $\geq 99.5\%$ , 1040 nt) demonstrerades mellan GII.4-varianter från patienter och stammar som återfanns i damm, luft, och på olika ytor i patientrummen. GII.4-stammarna som påvisades i patienter med gastroenterit i 8 vårdavdelningar under en 5-månadersperiod placerade sig som 11 distinkta undergrupper på ett fylogenetiskt (släkt-) träd. En avdelningarna som inte drabbades av nosokomial spridning av NoV fungerade som kontroll. Den höggradiga likheten av GII.4-stammar som detekterades hos patienterna och i deras rumsmiljö på en enskild vårdavdelning under en specifik tidpunkt, konfirmerade nosokomial spridning av NoV och väckte frågan om interventionsstudier avseende städning.

Sammanfattningsvis har studien gett starka belägg för att humana utbrottsstammar av NoV ackumuleras hos musslor som lever nära utlopp av renat avloppsvatten. Höggradig sekvenslikhet mellan NoV-stammar hos ostron och det virus som återfanns hos en patient som drabbats vid ett restaurangutbrott talade starkt för en NoV-smitta från ostron till människa, och konfirmerade en genetisk stabilitet av GI.1-stammar i ostron, musslor och i vatten. Påvisandet av blandinfektioner av NoV GI-infektioner vid ett misstänkt vattenburet utbrott, vilket konfirmerades med kloning, stärkte indicierna att utbrottet orsakats av att dricksvattnet kontaminerats av avloppsvatten. Slutligen stärktes hypotesen om en vårdavdelningsbaserad nosokomial smitta av en höggradig sekvenslikhet mellan NoV GII.4-stammar som påvisats hos patienter med de virus som återfanns i miljöprover från patientrummen.

### **Keywords**

Norovirus, smittspårning, miljö, utbrott, vattenburen, födoämnesorsakad, nosokomiell, musslor, ostron, norovirus genogroup I.1, norovirus genogroup II.4, damm, luft, sjukhussalar, molekylär epidemiologi, NoV GI-diversitet, kloning, fylogenetisk analys, nukleotidsekvensering



# List of papers

This thesis is based on the following studies,  
referred to in the text by their Roman numerals:

- I Nenonen NP, Hannoun C, Horal P, Hernroth B, Bergström T.  
Tracing of norovirus outbreak strains in mussels  
collected near sewage effluents.  
*Applied Environmental Microbiology* 2008; 74(8): 2544–2549.
- II Nenonen NP, Hannoun C, Olsson MB, Bergström T.  
Molecular analysis of an oyster-related norovirus outbreak.  
*Journal of Clinical Virology* 2009; 45(2): 105–108.
- III Nenonen NP, Hannoun C, Larsson CU, Bergström T.  
Marked genomic diversity of norovirus genogroup I  
strains in a waterborne outbreak.  
*Applied Environmental Microbiology* 2012; 78(6): 1846–1852.
- IV Nenonen NP, Hannoun C, Svensson L, Torén K,  
Andersson LM, Westin J, Bergström T.  
Norovirus GII.4 detection in environmental samples  
from patient rooms during nosocomial outbreaks.  
*Journal of Clinical Microbiology* 2014; 52(7): 2352–2358.

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# Abbreviations

aa	amino acid
BLAST	basic local alignment search tool
bp	base pair
CD	cluster of differentiation
CDC	United States Centres for Disease Control and Prevention
CMO	county medical officer
Ct value	cycle threshold value
EPS	extracellular polysaccharide substance
FCV	feline calicivirus
gEq	genome equivalent
GI	genogroup I
GII	genogroup II
H	hinge region
HAV	hepatitis A
HAdV	human adenovirus
HAstV	human astrovirus
HBGA	histo-blood group antigen
50% HID	50% human infectious dose
HuNoV	human norovirus
IEM	immune electron microscopy
IFN	interferon
IQR	interquartile range
kb	kilobase
kDa	kilodalton
M cell	microfold cell
MNV	murine norovirus
NA	nucleic acid

NCBI	National centre for biotechnology information
NoV	norovirus
NS	non structural
N/S	N-terminal shell domains
nt	nucleotide
ORF	open reading frame
P	protruding domain
polyA	polyadenylated
PCR	polymerase chain reaction
RIA	radioimmunoassay
RdRp	RNA-dependent RNA polymerase
RHDV	rabbit haemorrhagic disease virus
RT-PCR	reverse transcriptase polymerase chain reaction
rRT-PCR	real-time RT-PCR
RV	rotavirus
S	shell domain
SaV	sapovirus
sgRNA	subgenomic RNA
SMI	Swedish Institute for Infectious Disease Control
TNA	total nucleic acid
UTR	untranslated region
VLP	virus-like particles
VPg	viral protein covalently linked to viral RNA genome
VP1	viral capsid protein 1, major capsid protein
VP2	viral capsid protein 2, minor capsid protein
WTP	drinking water treatment plant
WWTP	waste water treatment plant



# 1. Introduction

## 1.1 Norovirus background

Norovirus (NoV) are a major cause of acute gastroenteritis infections in all age groups in developed and developing countries worldwide [1-5]. With their ubiquitous distribution in environmental settings the NoV present a challenging model of the host-pathogen relationship. Readily transmitted by the faecal-oral route and by direct person-to-person contact, these well-adapted, highly contagious viral pathogens cause dramatic but usually short-term infections in healthy individuals, with rapid spread within the family and the community. In healthy patients NoV infection is usually of short duration (12 to 60 hours) with sudden onset of uncontrollable projectile vomiting, explosive diarrhoea, nausea, stomach cramps, and low-grade fever [6-8]. Ranging from the asymptomatic healthy carrier state in infants, to the extremes of symptomatic chronic excretion in elderly, debilitated, immunosuppressed, or very young patients, NoV infections may cause significant health problems [2, 9-18]. Severe and prolonged excretion is of particular concern in transplant patients and their hospital environment [19-22]. Yet the precise host cells where NoV growth is presumed to occur in humans are not known [23-26].

The cascade vomiting and sudden onset of diarrhoea that typify NoV infections may result in disruptive outbreaks complicated by socio-medical and economic problems. Such outbreaks are particularly difficult to control in semi-closed settings, hospitals and homes for the elderly, preschool nurseries and schools, or cruise ships [27-33]. As the NoV are endemic in the population infections can appear as sporadic cases within the family, or as large non-seasonal community outbreaks [34, 35]. For although nosocomial infections may show seasonal winter distribution, food- and waterborne transmission can occur at any time throughout the year [36]. The sources of contamination are many, including fomites, water, food, surfaces, and hand-to-hand contacts, following exposure to virus contaminated faeces and vomitus [37-39].

The nature of the virus and the infection, with dissemination from cascade vomiting and heavy diarrhoea, favours multiple routes of virus transmission that can complicate epidemiological investigations. Transmission may occur through poor personal hand hygiene on the part of food handlers or restaurant guests recovering from symptomatic or asymptomatic NoV infections, through the contamination of drinking water as occurs when sewage leaks into broken water pipes, when the berry picker confronts poor sanitary conditions, or when virus contaminated water is used to irrigate or freeze vegetables or soft fruits including raspberries and strawberries [34, 40, 41]. Waterborne contamination also affects bivalves such as mussels growing in polluted waters close to estuaries, often long established centres of heavy population and sea-going traffic [42, 43]. As under favourable conditions one mussel

is estimated to filter 2 to 3 litres water in one hour these highly efficient filter feeders readily bio-accumulate enteric viruses such as NoV, adenovirus (HAdV), poliovirus and hepatitis A (HAV) from sewage-contaminated water [44, 45]. These human viruses are concentrated in the digestive glands of the shellfish [46, 47]. As seafood delicacies such as oysters are rarely cooked prior to ingestion, or only lightly cooked or smoked as with the blue mussels or clams, bivalves exposed to sewage-contaminated water can cause severe symptoms and widespread epidemics [48, 49]. Therefore, the routes of NoV transmission are diverse and reflect the high stability of these non-enveloped viruses in the environment, whether in the gastric juices of the digestive tract of infected humans or in contaminated bivalves, in polluted estuarine waters, or in aerosols from vomitus in the patient room (Figure 1).



**Figure 1.** An overview of some of the settings and transmission routes of norovirus infections; food and waterborne dispersal, bivalve contamination, person-to-person contact, and the semi-closed environment of cruise ships and hospitals.

The symptoms of projectile vomiting and sudden onset of diarrhoea were first described as *hyperemesis hiemis* or *winter vomiting disease* by Zahorsky [6]. Reporting from St Louis in 1929 he describes epidemic outbreaks of acute non-bacterial gastroenteric infections that affected families in the community, and young children in particular [6]. Although the children could be quite severely affected they recovered in 48–72 hours without complications. Recurring with biennial periodicity, these outbreaks were most common in late autumn, winter, and early spring, often during extremely cold weather [6]. Hence Zahorsky's choice of name for the condition that he had followed in detailed records from 1904 onwards, *winter vomiting disease*. Light microscopy of faecal samples from affected children showed an absence of pus cells, findings in marked contrast with the pus detected in faeces from children suffering from summer diarrhoeal infections of bacterial origin. However, Zahorsky found several features difficult to explain. These included the concurrence of outbreaks with severely cold weather and an apparent association of the acute gastroenteric symptoms with consumption of milk products, particularly butter, in some of the children. Zahorsky's comments on possible routes of infection indicate the complexity of outbreak investigations where several transmission routes may be involved.

The nature of the infectious agent remained elusive although volunteer challenge studies during the 1940s and 1950s demonstrated that diarrhoeal disease could be transmitted by non-bacterial filtrates from patient faeces and throat washings [50–53]. This suggested a viral aetiology of diarrhoeal infections. The viral aetiology of winter vomiting disease was confirmed in 1972 on immune electron microscopy (IEM) of faecal swab samples derived from study of an acute gastroenteric outbreak that affected young children attending elementary school in Norwalk, Ohio, October 1968 [54, 55]. With an attack rate of 50% in children and teachers, and symptoms of 12–24 hour duration, the outbreak was characterized by sudden onset of vomiting, nausea, low-grade fever, and abdominal cramps, suggesting spread of infection from a common source. However, food did not appear to be implicated. Because the affected school was the only city school with its own well, poorly chlorinated well water was suggested as the point source of infections as bactericidal levels of chlorine were not detected in the water examined on the second day of the outbreak. Despite these indications there was no evidence of bacterial contamination on laboratory examination of the water, nor of sewage leakage on inspection of the well and septic tank. Secondary spread to family members showed an attack rate of 32%, with an incubation period averaging 48 hours, and the typical symptoms described by Zahorsky [6]. No bacteria or parasites were detected in the faecal samples collected from patients. A follow-up survey indicated that the outbreak lasted for about 3 to 4 weeks [54].

Applying the novel approach of direct virology Kapikian et al. (1972) used IEM to examine faecal swabs with paired acute and convalescent serum samples from

patients naturally infected during the Norwalk outbreak, and from volunteers experimentally infected with filtered rectal swab material from an outbreak patient [55]. In outbreak patients, and some of the infected volunteers, IEM of faecal samples treated with pre-challenge, or paired sera, revealed aggregates of antibody-coated viral particles showing cubic symmetry, and indistinct substructure. The virus measured 27 to 32 nm, was non-enveloped, and appeared to be particularly fastidious as it failed to grow in standard cell culture [55]. Although the results of volunteer challenge studies may have been biased because of the high concentrations of virus used to transmit infection, these reports provide valuable information about the role of what were then described as the Norwalk and Norwalk-like viruses in outbreak settings [55, 56]. Besides confirming the viral aetiology of the Norwalk agent, IEM studies indicated the antigenic and genetic diversity of the strains encountered in different outbreaks in the United Kingdom and United States of America. [55-58]. They also revealed the varying susceptibility and apparent resistance of certain individuals in the population to infection with the first recognized Norwalk virus strain. The complexity of clinical immunity to these viruses, now known as the human noroviruses, was also indicated, as most of the adult population was considered to have previous experience of some strain of the noroviruses circulating in the community over time [55, 56, 59].

These and other early volunteer transmission studies demonstrated the non-cultivability of the human noroviruses in standard cell lines, a problem that has proved difficult to resolve despite intensive efforts [60]. To date non-cultivability in standard cell lines has hindered the full characterization of the norovirus strains that infect humans [61-64]. However, recent studies indicate that human norovirus may be cultured in human B cells, that human and murine norovirus may use synergistic cofactors such as extracellular polysaccharide (EPS) from the intestinal microbiota including *Enterobacter cloacae*, or be transported through the microfold (M) cells to pass the intestinal mucosal barrier and to replicate in vivo and in vitro in human or murine B cells [26, 65-67]. The potential of growth in standard cell lines and of a reverse genetic system for human norovirus should advance the characterization of noroviruses and our knowledge of their growth cycle [26, 68].

Immune electron microscopy and radioimmunoassay were for many years the only tools available for laboratory investigation of acute gastroenteric outbreaks, as all attempts at recovering replicative virus in cell culture failed, and there was no small animal model available for study. However, the EM expertise required for interpretation of the demanding, often insensitive, IEM technique was usually based in central laboratories [7, 69]. Outbreak investigations tended to be limited to clinical and epidemiological assessments supplemented by laboratory examination for bacterial and parasitic pathogens.

In these situations acute gastroenteric outbreaks were considered to have the clinical and epidemiological characteristics of a Norwalk-like pattern if they satisfied

the criteria that were adopted by Kaplan in 1982 [70]. Based on review of 642 gastroenteric outbreaks including water- and foodborne infections, outbreaks in nursing homes, cruise ships, and summer camps, Kaplan's criteria for a Norwalk or Norwalk-like outbreak hold to this day [70-72]. When re-evaluated with outbreaks of confirmed NoV or bacterial aetiology, these criteria were found to be 99% specific and moderately sensitive (68%) for the provisional diagnosis of foodborne outbreaks of gastroenteritis due to noroviruses [71].

The criteria are defined as

1. Percentage of cases with vomiting  $\geq 50\%$ ,
2. Mean, or median, duration of illness 12–60 hours,
3. If available mean, or median, incubation period of 24–48 hours,
4. Faeces negative for bacterial and parasitic pathogens.

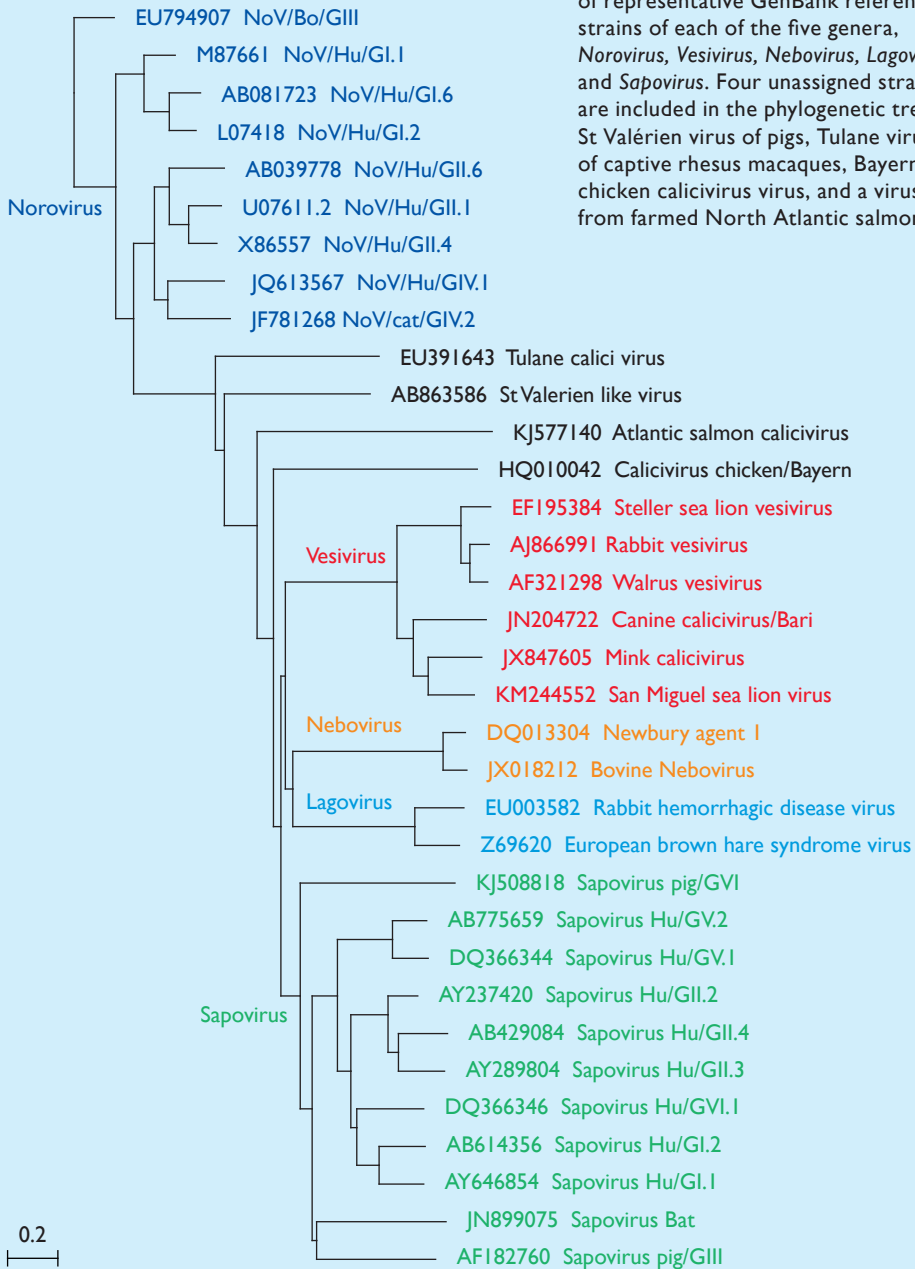
In 1993 Hedberg and Osterholm proposed that having more cases with vomiting than fever in an outbreak could be used as a further epidemiological criterion for NoV outbreaks [73].

The United States Centres for Disease Control and Prevention (CDC), in 2012, recommended that when it is not possible to obtain laboratory confirmation of norovirus in an outbreak of acute gastroenteritis, health departments can use Kaplan criteria to determine if an outbreak was likely to have been caused by norovirus [72]. When all four Kaplan criteria are satisfied, CDC advises that it is very likely that the outbreak was caused by norovirus. However, about 30% of norovirus outbreaks do not meet these criteria. CDC cautions: if the criteria are not met, it does not mean that the outbreak was not caused by norovirus [72]. This is an unsatisfactory conclusion for outbreak investigations, particularly as molecular methods of detection are now readily available for laboratory diagnosis and outbreak investigation. However, successful investigations require adequate and timely sampling of patients and of the possible point source of infections. Hence the need for rapid response from medical, public health, and laboratory authorities in outbreak situations, usually first recognized and reported to authorities by the public and the general practitioner as a sudden onset of acute gastroenteric illness in the community [74].

The breakthrough came in the early 1990s with the molecular cloning and sequencing of the Norwalk virus agent, and complete genome studies based on nucleotide sequencing of other Norwalk-like strains, including the Southampton and Lordsdale viruses [75-77]. These molecular studies paved the way for improved laboratory diagnosis. Molecular tools revealed a small non-enveloped virion with single stranded RNA viral genome organized into three major open reading frames (ORF), with 5'-leader sequence and 3'-polyadenylated (polyA) tail [75, 76, 78-80]. The ORF1 situated at the 5'-end of the positive strand RNA genome was shown to encode a typical conserved picornavirus-like polyprotein including the putative helicase motif GPPGIGKT (GXXGXGKT), and the RNA-dependent RNA polymerase (RdRp) motifs GLPSG, and YGDD [76]. The second ORF, encoding a single struc-

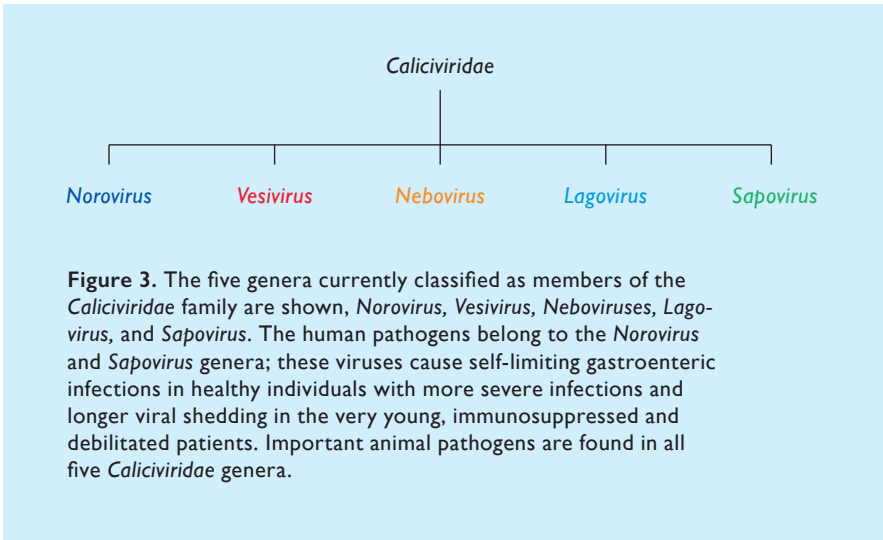
tural protein of molecular weight approximately 59 kDa, was assigned to a major capsid-coding region on the basis of limited similarities (approximately 31% at the amino acid (aa) level to the animal pathogens *Feline calicivirus* (FCV) and *Rabbit haemorrhagic disease virus* (RHDV), both members of the *Caliciviridae* family. As noted previously with the animal caliciviruses, the third ORF encoded a minor capsid protein of unknown function [81]. This organization of the RNA genome with features resembling the animal caliciviruses confirmed the placing of the human Norwalk and Norwalk-like viruses within the *Caliciviridae* family, previously suggested from the *calici- or calyx- cup-like* (from the latin *calix-*) morphology seen in some early EM studies [9, 56, 82, 83].

Sequence analyses of the complete genome of the more than 100 norovirus strains now available for comparative and phylogenetic analyses show that the noroviruses form a distinct clade within the *Caliciviridae*, a family of viruses causing diseases of considerable medical, veterinary, and economic importance in a wide range of animals, birds, marine fish and mammals as well as humans (Figure 2).



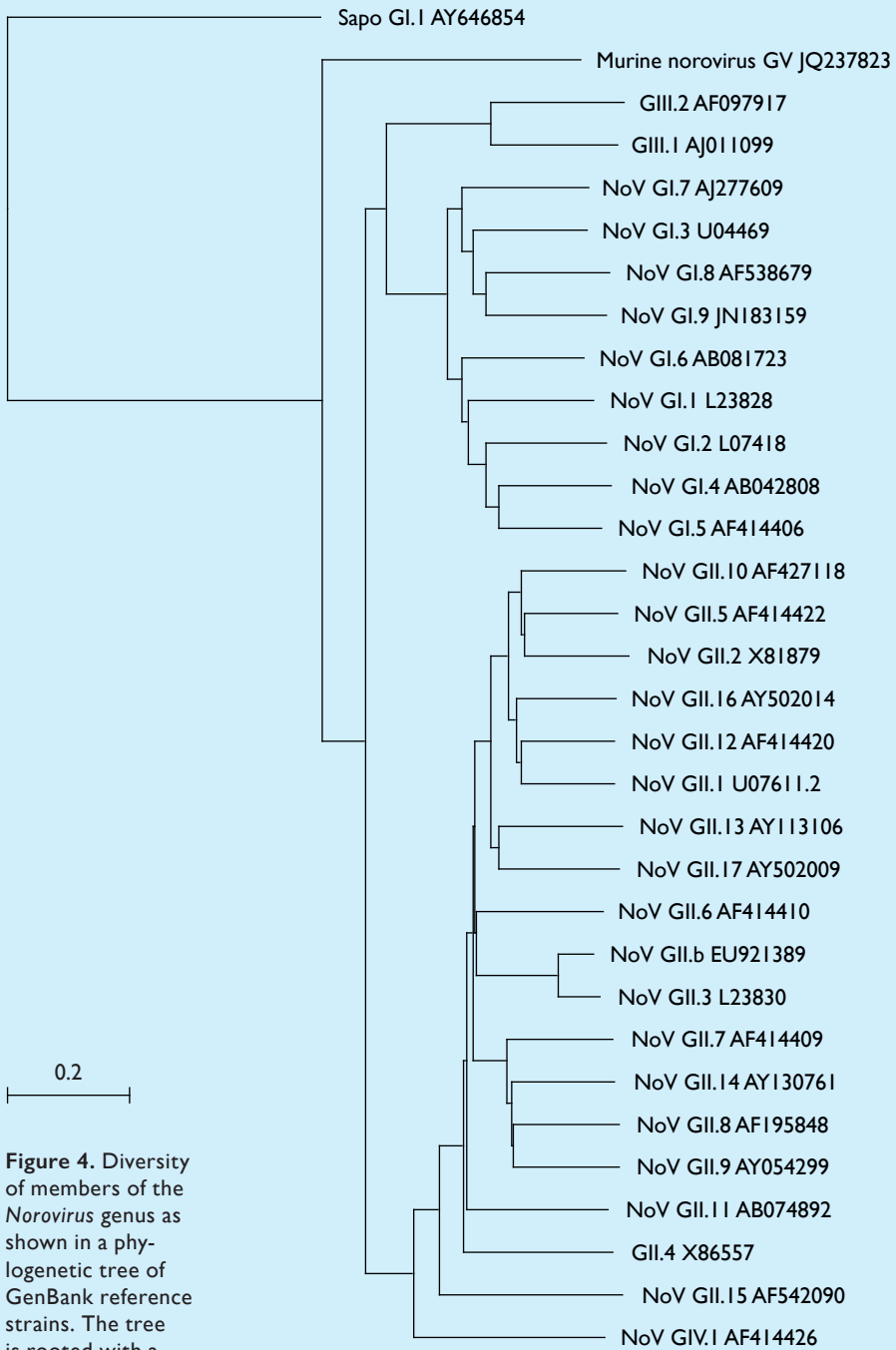
**Figure 2.** Phylogenetic relationships within the *Caliciviridae* family based on neighbour-joining analyses of full-length genome nucleotide sequences of representative GenBank reference strains of each of the five genera, *Norovirus*, *Vesivirus*, *Nebovirus*, *Lagovirus* and *Sapovirus*. Four unassigned strains are included in the phylogenetic tree: St Valérien virus of pigs, Tulane virus of captive rhesus macaques, Bayern chicken calicivirus virus, and a virus from farmed North Atlantic salmon.

Thus phylogenetic analyses confirm the early molecular and EM evidence of close genomic and structural relationship between the noroviruses and other members of the *Caliciviridae* [84]. Now designated as the *Norovirus* genus, this clade is one of five genera currently recognized within the *Caliciviridae* including the *Norovirus*, *Vesivirus*, *Nebovirus*, *Lagovirus*, and *Sapovirus* (Figure 3).



These genera share a common root along with other related viruses that may belong to the *Caliciviridae* family but that have not yet been approved as species; St Valérien virus of pigs, Tulane virus of captive rhesus macaques, Bayern chicken calicivirus virus, and a virus from farmed North Atlantic salmon (Figure 2), [85-88].

Molecular studies show that the strains of caliciviruses detected in human infections cluster on the *Norovirus* and *Sapovirus* branches of the *Caliciviridae* family phylogenetic tree. To date at least five genogroups are recognized within the *Norovirus* genus based on comparative and phylogenetic analysis of the nucleotide sequence of the ORF2 major capsid-coding region of the genome. The strains of norovirus affecting humans cluster in three of these *Norovirus* genogroups, genogroups (G) I, II and IV, denoted as NoV GI, GII and GIV. Although strains of NoV GI and GII affecting humans are frequently detected in sporadic or epidemic infections in waterborne, foodborne, and nosocomial outbreak settings, NoV GIV strains are occasionally reported in routine assays of human faeces. Based on comparative sequence analyses norovirus strains detected in animals may also place in the genogroups: pig strains being found in GII, bovine and ovine forming GIII, canine and lion strains in GIV and at least 19 murine strains in GV (Figure 4), [89, 90].



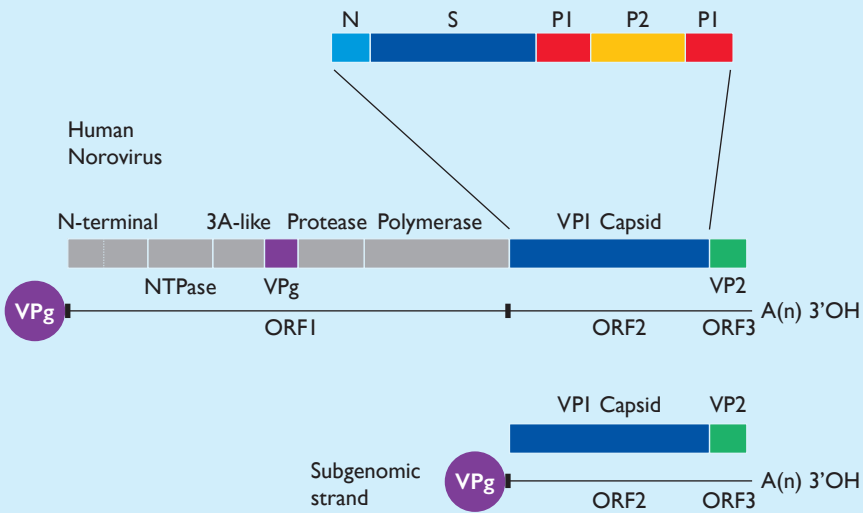
**Figure 4.** Diversity of members of the *Norovirus* genus as shown in a phylogenetic tree of GenBank reference strains. The tree is rooted with a sapovirus strain.

Human sapovirus strains can be genotyped in a similar system based on nucleotide sequencing and phylogenetic analyses of the major capsid-coding region that is encoded in ORF1 of the SaV genome. Human strains are found in SaV genogroups I, II, IV and V [91]. Sapoviruses have been implicated in symptomatic gastroenteric infections in young children under five years, the elderly, and in food and oyster-associated outbreaks [92-97]. Widespread seasonal outbreaks of sapovirus can affect all age groups in the community and may cause nosocomial infections in semi-closed settings as occurred in Gothenburg, winter–spring 2007–08 [98].

## **1.2 Norovirus virion and genomic organization**

Noroviruses are small, non-enveloped viruses with a protein capsid of icosahedral symmetry  $T=3$ , and virion size of 27–45 nm on negative-stain electron microscopy, or 35–40 nm by cryo-electron microscopy [55, 99, 100]. The robust, acid-stable, lipid-free protein capsid encloses and protects the viral RNA genome. As a positive-sense, single stranded RNA virus the NoV genome is a linear molecule of approximately 7.4 to 8.3 kilobases (kb), with a 5'-virus-encoded protein VPg (viral protein genome-linked) attached to the 5'-untranslated (UTR) terminus of the genomic RNA, and a 3'-UTR with polyA tail [85].

The human NoV RNA genome is organized into three open reading frames (ORFs) where ORF1 encodes the non-structural polyprotein, ORF2 encodes the major viral capsid protein known as VP1, and ORF3 encodes the minor capsid protein defined as VP2 (Figure 5), [101].



**Figure 5.** Schematic presentation of the human Norovirus genome showing reading frame usage and gene order. The positive single strand RNA genome of approximately 7.5 kb, shown as a black line, carries a covalently linked 5' virus genome protein VPg (shown as a purple circle). A characteristic short repeated sequence ■ occurs at the beginning of the 5' end of the genome, and again at the start of the VPI gene. The genome is organized into 3 ORFs as indicated. ORF1 encodes a nonstructural polyprotein shown in gray with the VPg coding region shown in purple. The polyprotein is cleaved by the viral protease into at least six proteins which are involved in viral replication. ORF2 encodes the VPI major capsid protein, and ORF3 encodes the minor capsid protein VP2, shown in green. The structural proteins VPI (major capsid protein) and VP2 (minor capsid protein) are produced during replication from the subgenomic RNA transcript that is co-terminal with the 3' end of the genome. The domains of the VPI are denoted by N the N terminal arm, S shell, P protruding domain with subdomains PI, P2 (inserted between), PI. A flexible hinge region occurs between the shell and PI, as shown in Figure 6.

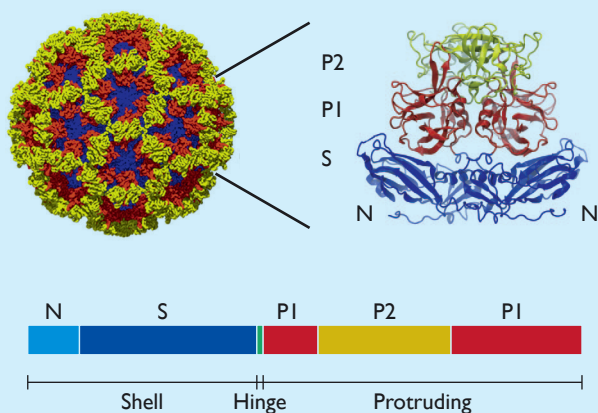
### 1.3 Norovirus translation of gene products and replication

#### *ORF1 and the non-structural proteins*

The first ORF (ORF1) in the NoV genome, as in all genera of the *Caliciviridae*, is located close to the 5'-end of the linear RNA genome (Figure 5). Reading from the 5'-short UTR terminus, ORF1 encodes the non-structural polyprotein which on translation is cleaved by the viral-encoded 3 C-like protease into at least six non-structural proteins: NS1-2 or p48, important in membrane recruitment and suggested scaffolding protein for replication complex; NS3, nucleoside triphosphatase, putative viral helicase involved in membrane-anchorage and replication; p22, a 3A-like protein also implicated in replication; VPg understood to be 5'-covalently linked to the genomic and subgenomic RNAs and involved in translation and replication; 3C-like protease, viral protease, cleaving the ORF1 polyprotein; and the 3D-like RNA-dependent RNA polymerase enzyme (RdRp), NS7, responsible for synthesis of both positive and negative stranded viral RNA in virus replication .

#### *ORF2, the major capsid protein, and virus capsid structure*

In the norovirus, ORF2 encodes a single major viral capsid protein molecule of ~59 kDa known as the NoV virus protein 1 (VP1). This structural protein folds to form the *shell* (S) of the icosahedral capsid, connected through a *flexible hinge* (H) region, to the *protruding* (P) region of the NoV capsid, as shown in X-ray crystallography studies of the three dimensional structure of the Norwalk virus [99]. In these studies Prasad examined the self-assembled, virus-like, but non-infectious, particles (VLP) produced by cloning of the Norwalk virus genome and expression of the virus capsid protein in insect cells transfected with a recombinant baculovirus. These were important advances in the molecular study of the non-cultivable human noroviruses [99, 102, 103], as was expression of the P domain to form P dimers and P particles derived from 12 P dimers [104]. VLP and P particles retain the binding properties of the norovirus in terms of carbohydrate association and have been useful in binding and antigenicity studies in the absence of cell culture [105].



**Figure 6.** X-ray structure of the Norwalk virus capsid. The icosahedral capsid structure shows the typical calicivirus surface depressions ( $n=32$ ) in blue. The capsid is formed by 180 molecules (90 dimers) of the major capsid protein, VP1, colour-coded to show the three domains in the ribbon representation of the dimer, on right. The shell (blue) formed by the N-terminal arm and S domains, the PI (red) and P2 (yellow) subdomains of the protruding P domain (sub domains PI-P2-PI) with the exposed outermost hypervariable P2 (yellow) region. The binding sites for histo-blood group antigens are located in the P2 regions of the dimers. VP1 protein organization is shown in a schematic diagram below the virion. The hinge (H) domain which connects the S and P domains is shown in green. Reproduced with permission from: Venkataram Prasad et al. *Curr Opin Virol* 2014 [106]; Glass et al. *NEJM* 2009 [3], Copyright 2009, Massachusetts Medical Society.

Encoded from the 5'-end of ORF2 the short amino terminal arm (N, aa residues 1–49) and the S region (residues 50–218) of the VP1 protein form the innermost layer and protective shell of the capsid. Known as the amino-shell region (N/S) of the major capsid protein, this is the most conserved region of the VP1. The flexible hinge (H, residues 219–225) connects the S domain with the most externally exposed P domain (residues 226–530, for NoV GI.1). In terms of linear sequence the P domain is composed of two subdomains denoted as P1 and P2, where the P2 region (residues 279–405) is an insertion between the N-terminal and C-terminal regions of the P1 subdomain (residues 226–278, and 406–530) [99, 107]. This P2 region of the VP1 protein is hypervariable. The amino acid sequence shows variation across time, suggested to reflect antigenic changes that may be advantageous to the virus in avoiding herd immunity in the community [108–111]. On folding and capsid assembly (outlined below) the P2 regions form the outermost tips of the virion and therefore are of importance in viral interactions with the host cell, and the environment.

The structure of the norovirus capsid is formed from 180 monomers of the major structural capsid protein molecule, the VP1. These monomers self assemble to form the icosahedral capsid of 90 dimers of the major capsid protein, where each of the dimers forms an arch-like capsomere [99]. The virus capsid, exhibiting T=3 icosahedral symmetry with axes of rotation showing 5:3:2 symmetry, can be modelled from 60 identical equilateral triangles each consisting of three copies of the VP1 protein, identical in amino acid sequence but showing slight differences in conformation, the A, B, and C protomers [99]. The conserved N/S regions at the base of the VP1 dimers form the protective icosahedral shell of the capsid, the icosahedral lattice [103], and subtle conformational changes in the protein dimers, designated as A/B and C/C accommodate the spherical curvature of the icosahedral virus (Figure 6). The P regions of the dimers form the arch-like structures extending outwards from the compact shell. The P2 regions of each dimer forming the outermost tip give a bilobed effect, emphasizing the arch-like structures that are supported by the P1 subdomains extending outwards from the shell. This arrangement of capsomeres around the icosahedral capsid of 20 surfaces and 12 vertices gives the typical appearance of 32 hollows or cuplike (Latin *calix* “cup” or “goblet”) depressions at the icosahedral five and threefold positions of the calicivirus virion. Electron cryo-microscopy and computer imaging reveal the subtle differences in arrangement of the dimers that form the capsomeres in different genera and caliciviral strains [84, 99]. These differences are consistent with the differences in appearance noted in negative stain EM where a wide range of capsid images can be seen, from feathery, seen in many noroviruses including Norwalk virus [55], smooth round structure viruses SRSV, to the sharply defined cups of the classical human caliciviruses, the *Sapporo virus* type species of the *Sapovirus*, and animal vesiviruses such as the *Feline calicivirus* and the *Vesicular exanthema of swine virus* [82, 83, 89].

The 3-dimensional structure, and physical and chemical nature of the virus capsid as a lipid free, non-enveloped virion confer quite remarkable properties on the norovirus. These properties may be important in outbreak settings; viral stability in adverse environments within and outwith the human host, as in resistance to the low pH of gastric juices and bile salts, chlorine, food matrices, recycled river water, wastewaters, on kitchen workbenches, and in the patient’s close environment [112–115]. Similarly the initial contact of virus in host-cell interactions including antigenicity and immune reactions, attachment binding to host cell receptor-like structures such as the human histo-blood group antigens (HBGA), and bioaccumulation in bivalve tissue are all features understood to be initiated at the virion capsid [116–118].

What particular properties of the capsid confer these properties of *resistance* or *robustness* on the norovirus capsid? As the outermost region of the capsid, the protruding P2 subdomain is the region that is most exposed to environmental conditions. Hence this subdomain of the ORF2 major capsid-coding region, and the VP1 protein encoded, is the subject of intensive study. Comparative nucleotide and amino acid

analyses of the P2 subdomain of different NoV strains indicate that this is the most variable region of the capsid protein, and therefore of significant antigenic and molecular epidemiological importance in the typing and tracing of NoV outbreak strains [119–122]. It is also suggested that the P2 region may be involved in initial virus-host cell interactions, as studies of norovirus capsid interactions with the HBGA, putative receptors, locate the virus binding pockets to specific regions in the P2 dimers, in what appears to be a genogroup and strain-dependent manner (Figure 6), [123].

### ***ORF3 and the minor capsid protein***

The third open reading frame of the human NoV genome, ORF3, at the 3' UTR terminus of the RNA genome, encodes a small basic protein known as the minor capsid protein (VP2) of unknown function that is currently undergoing intensive investigation. Predicted to interact with the viral RNA and the major capsid protein, only a few copies of the VP2 protein appear to be incorporated within the virion [101, 124]. The VP2 minor capsid protein, which shows highly basic properties and considerable nucleotide variability between NoV strains, and between the genera of the *Caliciviridae* family may play a role in initiating capsid assembly, in incorporation of the viral RNA genome into the capsid, and in stabilizing the virion during and on completion of virion assembly [125-128]. The VP2 protein of Norwalk GI.1 virus associates with the VP1 at the inner surface of the capsid shell. VP1 and VP2 interactions map to a site within the S domain of the VP1 protein at isoleucine aa 52, in the IDPWI aa motif which is highly conserved across the NoV genogroups [128]. Similar evidence of VP2 association with the IDPWI motif within the shell domain has been shown for NoV GII.4 Houston [128]. As the VP1 capsid protein is quite acidic and negative in charge, encapsidation of the RNA genome may be facilitated by the presence of the highly basic VP2 within the capsid. Although the number of VP2 molecules incorporated into the virion is not known, it is understood to be low, and the authors postulate the possible role of the VP2 in the capsid as *stitching* or locking the VP1 dimers in place during assembly of the curving icosahedral capsid structure [128].

### ***Norovirus replication***

Norovirus molecular biology has proved difficult to study as the human strains of NoV and SaV are non-cultivable and show restricted host range [61-63]. Early studies indicated that the viral RNA was infectious but viral replication was not achieved in cell culture [129]. Recent developments including a plasmid-based human norovirus (HuNoV) reverse genetics system producing reporter-tagged progeny virus containing infectious genomic RNA may permit manipulation of the HuNoV viral genome and production of reporter virions [68]. Advances may follow also on the potential

culture of HuNoV strains in B cells with cofactor HBGA-like EPS from bacterial commensals [26, 65, 67]. Further study of a mouse model for HuNoV infections may also provide more information on the HuNoV life cycle [130].

Despite these methodological limitations, several lines of evidence, such as binding of VLP and co-crystallization studies, suggest that human NoV utilize HBGAs as promoting factors or as initial receptors on target cells [131]. These phenotypically diverse molecules that bind to NoV are complex carbohydrates that are present in body fluids such as saliva and also on the surface of epithelial cells [132]. The findings that another calicivirus, RHDV, utilizes HBGAs as attachment factors in a strain-dependent manner during infection of its natural host can be considered when evaluating the importance of this interaction for human infections of NoV [133]. However, it is still not proven whether HBGAs function as true receptors in humans, or as cofactors as was shown for HBGA-like EPS from bacterial commensals during HuNoV B cell infection [26]. After entry, NoV most likely share many features of other positive-strand RNA viruses such as uncoating in the cytoplasm at an early stage of infection [134].

When calicivirus genomic VPg-5'-RNA is released into the host cell cytoplasm by endocytosis the viral genome, being positive sense single stranded RNA with polyA tail, can directly assume the role of viral messenger RNA. The VPg appears to have a double role, acting as cap-like structure to enable translation of viral genomic RNA, and as protein primer for genomic replication. Host cell ribosomal machinery is used to translate the 5'-VPg-RNA genome, the VPg acting as a cap-like structure [135, 136]. Synthesis of the non-structural ORF1 viral polyprotein is followed by autocatalytic cleavage with release of viral cysteine protease, NS6. This 3C-like viral protease further cleaves the polyprotein (~200 kDa) to its various non-structural components: the p48 protein involved in replication complex formation; nucleoside triphosphatase; p22 (~22 kDa) a 3A-like protein implicated in replication; VPg linked to the 5' end of viral genomes involved in translation and replication; 3C-like cysteine protease (viral protease); and the viral RNA-dependent RNA polymerase (RdRp), NS7 shown in Figure 5, [101, 137].

Replication takes place in the cell cytoplasm [138]. The viral RdRp enzyme, cleaved from the ORF1 polyprotein by viral protease, transcribes the VPg-covalently linked positive sense viral RNA genome through a double stranded RNA hybrid replication complex [138]. This hybrid may dissociate to give one positive strand RNA molecule of viral genome sense, and an intermediate copy of negative sense RNA. The negative sense RNA template can continue to be copied to positive sense full length genomic viral RNA through the activity of the viral RdRp. The full-length covalently linked VPg 5'-genomic viral RNA with polyA tail is encapsidated to give progeny NoV virus.

The intermediate negative-strand RNA can also be transcribed to give a shorter subgenomic polyA RNA (sgRNA) that encodes the major capsid protein, VP1 of

ORF2, and minor capsid protein, VP2 of ORF3. This sgRNA is understood to carry a 5'-covalently linked VPg cap-like structure that acts as protein primer for genome replication and protein cap for translation initiation [139, 140].

The VPg 5' UTR-RNA linked protein is required for initiation of translation and synthesis of the VP1 major capsid protein from the sgRNA in a manner similar to the expression of ORF1 polyprotein from VPg 5'-genomic RNA. The VPg provides preferential translation of viral RNA by a special viral translation initiation mechanism; viral VPg binds to canonical initiating factors including host cell eIF4E cap-binding protein in the cell cytoplasm [136, 141]. This has been shown for FCV, NoV GI.4 Lordsdale, and MNV-1. On translation of the sgRNA encoding the major capsid-coding protein VP1 of ORF2, 180 identical monomers of the VP1 protein dimerize to form 90 dimers that self assemble to create the typical icosahedral capsid (T=3) encapsidating the positive sense viral RNA genome. Or, the 90 dimers can self assemble to form empty, non-infectious, virus-like particles (VLP). Translation of the VP1 capsid protein from the sgRNA is understood to play a role in the efficiency of the viral RdRp reaction and the interplay of non-structural, and structural cognate viral protein production [142, 143]. Apparently the viral structural proteins have regulatory roles in viral RNA synthesis.

The role of the small basic, VP2, protein molecule is less clear [127, 128, 144]. As noted previously only a small number of the highly basic minor capsid VP2 molecules are included in the virion capsid. These VP2 molecules may effect incorporation or packing of the viral RNA genome into, or, within the viral capsid, or stabilize the inner shell region of the capsid protein dimers [126, 128, 144]. Apart from the highly conserved IDPWI aa motif that is suggested to lock the shell domains of the capsid subunits together on the interior surface of the capsid, the VP2 is quite divergent in both size and sequence across the NoV family [128]. There is evidence that the VP2 protein may have a role in increasing the level of VP1 expression in the infected cell, protecting the VP1 protein from disassembly and degradation, and in stabilizing VLPs [145].

The mechanism of translation of the VP2 protein has been studied in FCV cultures and is described as a translation termination re-initiation (TTR) mechanism [146]. Post termination ribosomes from the VP1 protein translation events remain associated with the sgRNA through interactions with the termination upstream ribosomal binding sites (TURBS) at the 3'- end of the sgRNA major capsid-coding region of ORF2. This allows recruitment of translation initiating factors to the 5'-end of the minor capsid-coding region of ORF3, with repositioning of ribosomes and reinitiation of translation, to give the minor capsid protein, VP2 [147, 148].

Intense study of the human NoV genome and life cycle continues. New approaches show potential cell growth of HuNoV in B cells of the immune system, studies of these developing culture systems for HuNoV may add to the understanding of NoV infection and viral replication currently based on MNV-1, and FCV [26, 66, 130,

134]. Recruitment of cellular membranes for viral replication and development of virus-induced double membranes in the cytoplasm of infected cells is a common feature of positive sense RNA viruses, also seen in MNV-1 cell cultures [149, 150]. Co-localization of the viral replication complex, the viral non-structural NS7 protein RdRp, and viral double stranded RNA, has been shown in the perinuclear regions of the cell. MNV-1 appears to use the cytoskeletal network to localize the replication complex proximal to the microtubule-organizing centre [150]. Evidence of the importance of long range RNA–RNA interaction between the 5'- and 3'-ends of the positive stranded RNA genome in virus replication is shown for dengue and other positive strand RNA viruses [151, 152]. Reports indicate interactions of the 5'- and 3'- ends of the caliciviral RNA genome through possible complementary sequences that are stabilized by host cell proteins to give genome circularisation and to coordinate essential functions of virus replication [153–155].

#### **1.4 Norovirus detection and genotyping**

Early diagnostic and sequence analyses tended to be based on short lengths of the genome, in particular the ORF1 region encoding the RdRp, NS7, and selected, partial regions of the N/S-major capsid-coding region in ORF2. Different regions of the NoV genome were used in diagnostic testing by different study groups; short amplicons (81–300 bp) detected in classic gel-based reverse transcriptase-polymerase chain reactions (RT-PCR) were confirmed on Southern blotting or sequencing [33, 156–161]. However, the regions chosen for detection and strain typing show different degrees of sequence conservation, and varying sensitivity of detection [159, 162]. Depending on the selection of suitable nucleotide positions for primer design, within conserved regions of the sequence of interest, informative, preliminary analyses of NoV strains implicated in an outbreak could be achieved for some, but not all outbreak strains [163]. Using a battery of primer pairs in RT-PCR provided a better approach to the problem of NoV detection where mixed infections with GI and GII strains are common [164]. These gel-based methods of detection have been replaced by the more sensitive and less time consuming methods of genogroup specific real-time reverse transcriptase (rRT-PCR) detection, where highly conserved regions of the genome are selected for amplification and detection by hydrolysis of fluorochrome-labelled probes. These real-time systems are used in a semi-quantitative approach to detect multiple human enteric viral pathogens including NoV GI, GII, GIV, and SaV GI, II, IV and V. However, gel-based RT-PCR amplification continues to be used for nucleotide sequencing of outbreak strains and in molecular epidemiological studies.

Sequencing of the partial RdRp-complete N/S major capsid-coding region is now the preferred approach for genotyping, phylogenetic analyses, and molecular epidemiology of outbreak strains [90, 165]. This region is particularly informative as it spans the ORF1/ORF2 junction that, along with other regions in ORF2, has been

shown to be a major hot spot for recombination events. Recombinant strains are understood to arise during the replication of a mixture of co-infecting NoV strains in the host cell [166, 167]. Nucleotide sequencing, phylogenetic analysis, molecular cloning, and bootscanning of the potential recombinant against a set of aligned reference strains are necessary steps to confirm and map the sites of recombination [168].

Subtyping or as it is described genotyping of the NoV strains that cluster within the genogroups can be achieved on molecular analysis of the complete major capsid-coding region. However, as yet there is no consensus on the classification of strains at the level of genotype, although Zheng et al. (2006) present an analytical typing system based on a defined pairwise distance cutoff of the complete major capsid-coding region of the genome (VP1, ORF2) with pairwise distance ranges (amino acids) of 0–14.1% for strains within a genotype, 1.3–43.8% between genotypes, and 44.9–61.4% between genogroups [90]. Recently a unified norovirus nomenclature and genotyping based on a phylogenetic approach has been proposed [169]. This system includes a dual nomenclature for the use of both ORF1 and VP1 sequences, as recombination appears to be a common feature of the NoV and identification of recombinant strains may be relevant. Classification of sapovirus strains is currently based on sequencing of the major capsid-coding region of the sapovirus ORF1 [91].

Various tools, known as the NoroNet in Europe, and CaliciNet in USA, were introduced to improve reporting and outbreak strain typing in diagnostic laboratories, to monitor worldwide distribution, and the emergence of new NoV variants [170, 171]. Aiming to improve outbreak control and to limit the impact of future epidemics, these networks encourage reporting of genomic information and try to standardize the nomenclature and information used in defining outbreak strains. Although the tendency is to accumulate quite short sequences of limited information, the overall aim is to monitor the worldwide distribution and emergence of new NoV variants.

### 1.5 Generation of norovirus diversity

NoV are a group of genetically highly diverse single stranded positive sense RNA viruses, and this diversity is reflected at the genogroup and genotype level (Figure 4), [172]. As NoV strains may differ in their epidemiological features, a characterization of their genotypic and phenotypic differences is of interest. Due to lack of reliable serotyping methods, NoV diversity is defined by analysis of gene sequences [90, 169]. The genetic diversity can be generated by point mutations, often as a result of RNA template miscopying by the error prone virus-encoded RdRp enzyme. Thus, genetic variants are constantly circulating in the population and subjected to selection. The rate of this evolutionary process may depend on several factors such as HBGA recognition and herd immunity and may vary between different genogroups and even genotypes [172]. For example, the mutation rate of the rapidly evolving GII.4 genotype was estimated to  $4.3 \times 10^{-3}$  nucleotide substitutions per site per year [110].

Surprisingly, although GII.3 viruses evolve almost as rapidly as GII.4 viruses ( $4.16 \times 10^{-3}$ ) at the nucleotide level, many mutations in the GII.3 genomic RNA revert back to previous amino acid residues, conserving the genetic distance and HBGA binding patterns [173]. This difference in evolution most likely influences the epidemiology of the two genotypes, with GII.3 mainly being linked to paediatric infections while GII.4 cause global pandemics with a 2 to 4 year frequency and selection of new variants in a pattern reminiscent of Influenza A infection.

In addition to mutation, recombination is a powerful tool in creating genetic diversity by concentration of beneficial nucleotide substitutions and dilution of deleterious alterations. A recombinant virus can be described as one virus with genetic information from two or more separate sources [174]. Proposed as an important mechanism for viral survival, recombination enables viruses to replace deleterious mutations that would otherwise result in defective proteins [174, 175]. The ability of NoV to recombine at the ORF1/ORF2 junction enables the virus to maintain its ORF1 genes but to change the viral capsid coat and subsequently adopt a different antigenic profile [174, 175]. This is advantageous for viruses whose host range has been reduced either due to herd immunity, or deleterious mutations at host binding epitopes [174]. Recombination in the NoV was first described by Hardy et al. (1997) in the GII Snow Mountain virus which was shown to have 94% identity in the capsid region with the GII.2 Melksham virus but only 79% in the RdRp region, suggesting recombination with an unknown virus, an orphan RdRp. The strain was denoted at that time as a NoV GII.cGII.2 recombinant [176, 177]. However, much of the early diagnostic genotyping was based on the limited sequence information derived from the ORF1 RdRp. Subsequently, by comparing the phylogeny of the RdRp sequences with those of ORF2 encoding the major capsid protein, VP1, a large number of NoV recombinants were detected and confirmed by other methods, indicating that recombination is an important mechanism in NoV evolution [166]. A new strategy for NoV genotyping based on sequencing across the ORF1 and ORF2 junction has therefore been suggested [169]. Inter- and intra-genotypic recombinants have been described but recombination is rarely identified between the NoV genogroups [178]. Naturally occurring intergenogroup recombinants have only been described for the human NoV and SaV [174]. The rarity of homologous recombination between different viral species may be explained by an incompatibility between heterogeneous viral proteins [174].

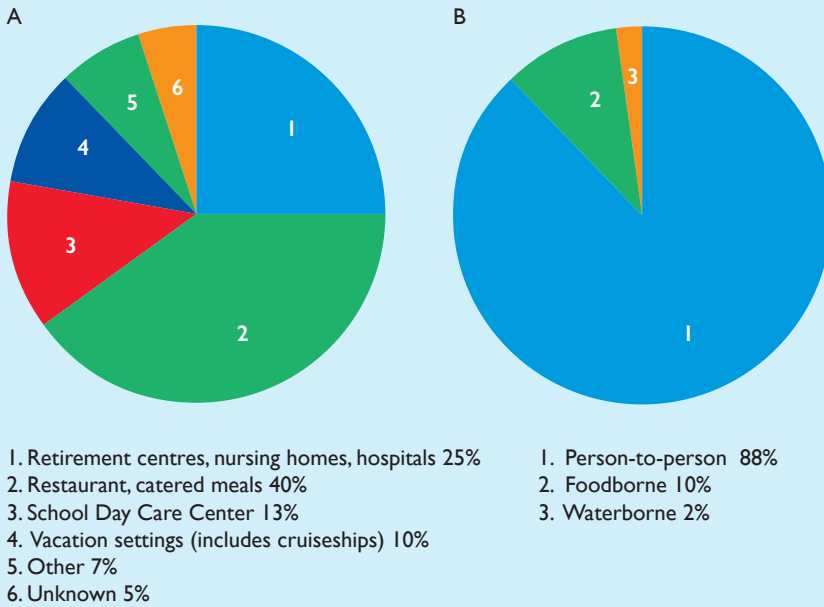
Although most NoV infections are limited in time, prolonged gastroenteric disease and virus excretion is common in immunocompromised hosts. Interestingly, the genetic diversity of NoV GII.4 in such patients was shown to be substantial as revealed by cloning and deep sequencing [172, 179]. Based on these findings, it was suggested that immunocompromised patients may constitute a reservoir and source of new noroviral variants. Transmission, which constitutes a profound genetic bottleneck of NoV, may enhance diversity by promoting transfer of extremely small

minority populations from a donor to a recipient [180]. The real extent of genetic diversity of NoV strains globally is unknown and must involve future extensive sequencing also of strains from asymptomatic infections which may represent the largest genetic pool.

### **1.6 Molecular epidemiology and environmental tracing**

Sequence-based techniques provide the information required for development of molecular diagnostic methods for NoV outbreak investigation. These molecular assays are based on the detection of viral RNA in reverse transcriptase polymerase chain reactions (RT-PCR), by using a selection of primer pairs designed to target conserved regions of the NoV genome [164]. The importance of using a battery of primer pairs, or of degenerate primers, was apparent early in the development of diagnostic methods for noroviral infections, where the genetic diversity of outbreak strains was found to complicate the results of quite demanding detection methods [33, 159, 164, 181, 182]. Moving from gel-based detection systems for amplified products to semi-quantitative analyses where fluorophore labelled sequence-based hydrolysis probes are used in rRT-PCR has enabled the introduction of rapid methods of detection that are now readily accessible to hospital, research, veterinary, and industrial laboratories. Real-time methods of amplification and detection have the advantages of being rapid and sensitive providing laboratory based reports within 3 to 4 hours of receipt of samples, with closed detection systems that reduce the risks of cross contamination between samples and PCR products [183]. Follow-up nucleotide sequencing of outbreak strains has led to the systematic gathering of nucleotide sequence information on human NoV strains implicated in outbreaks, worldwide. This sequence information continues to reveal the great diversity of the norovirus genome, permits detection of emerging variants and recombination events in outbreak strains, and provides a reliable basis for development and refinement of molecular assays for laboratory diagnostics and surveillance. However, there are disadvantages of being entirely dependent on known sequence information, and new approaches are required, such as sequence independent methods of virus detection.

Molecular methods have also improved our understanding of the natural setting of the human noroviruses, their niche within and outwith the human intestinal tract, and revealed their quite ubiquitous dispersal in the environment. Their distribution is widespread, whether in bivalves exposed to sewage water, in ground water, recycled river water, wastewater effluents, the patient's room, or the restaurant kitchen [112, 113, 184-189]. This wider application of molecular methods to environmental monitoring has improved the investigation and understanding of sporadic and epidemic outbreaks of gastro-enteric infections, indicating the settings and routes of transmission of norovirus (Figure 7).



**Figure 7.** Settings and modes of transmission. A: Settings for 233 outbreaks of non-bacterial gastroenteritis, United States, July 1997–June 2000, based on combined detection of noroviruses in RT-PCR and electron microscopy of patient samples from 93% of outbreaks. Data adapted with permission from Fankhauser et al. 2002 [190]. B: Modes of transmission for 5,036 norovirus outbreaks over a five-year period, July 2001–June 2006 in Europe. Data adapted with permission from Kroneman et al. 2008 [191].

Questions of viral agent, point source of outbreak, transmission, and spread of infection require rapid response, and prompt remedial action. The contribution made by the virus laboratory in providing an accurate and rapid diagnosis of patient symptoms, causative organism, route of transmission, and in identifying the point source of contamination whether in human or environmental samples is of immeasurable value in outbreak investigations. As in all laboratory diagnostics the need for rapid response from medical, public health, and environmental officers to ensure adequate patient and environmental sampling is essential. Good liaison with rapid response can restrict an outbreak, and reduce the problems of investigations based only on clinical and epidemiological assessment, as defined by Kaplan's criteria. Moreover, the molecular methods now available for laboratory studies can be used to investigate the point source of viral contamination in environmental samples such as water, bivalves, frozen fruits and vegetables, kitchen and hospital room surfaces, and even food workers' hands [30, 113, 188, 192-194].

The stability of the virion under different environmental conditions such as alcohol or chlorine treatment can also be investigated by the use of molecular methods [114, 195, 196]. Such studies add to our knowledge of environmental contamination and control, and promote the introduction and development of counter measures that can reduce the risk of sporadic or epidemic outbreaks [196]. Yet assessment of NoV stability in different environmental conditions is particularly demanding. We are confronted with the problem of determining the viability of a virus that is detected by the presence of viral RNA, but that cannot be grown readily in cell culture, or replicated in a small animal model. The question remains to what extent demonstration of viral RNA in environmental samples is equivalent to detection of viable, potentially infectious virus. Some workers suggest that an RNA protection assay may help to resolve this question [197]. Others have shown that the persistence of NoV and of NoV RNA in seawater can be measured by rRT-PCR, and that the bioaccumulation of intact NoV greatly exceeds that of free NoV RNA when assessed in oysters during wintertime [198]. Persistence of NoV and the vulnerability of free, naked, NoV RNA have also been shown in experimental assessment of RNA degradation on stainless steel surfaces examined in RT-PCR systems [199].

So, do the NoV RNA signals represent intact, infectious virus? In the absence of standard cell culture, volunteer challenge studies could possibly provide an additional method of detection and analysis of intact, potentially infectious virus [56]. Both RNA protection and human challenge studies require careful controls, and are not necessarily conclusive. However, analysis of NoV outbreaks where contaminated bivalves, drinking or recreational water, or frozen berries, have been implicated, and where NoV RNA with high sequence similarity to that found in affected patients has been detected in the suspect samples of food or water, does help to confirm the significance of NoV RNA detection in environmental samples [30, 41, 187, 200-203]. This molecular approach to laboratory diagnostics and outbreak studies provides the

most reliable tool for investigations when these are based on collaborative efforts to understand the clinical, epidemiological characteristics of an outbreak, and the bioinformatics of the viruses implicated in outbreak settings. To achieve these aims we must collect and analyse the complex biological data of the viral genome, hence the need for adequate and timely sampling.

The comparative sequence-based analysis and tracing of epidemic outbreak strains detected in environmental samples, and in affected patients, is described as molecular epidemiology and forms the basis of virus monitoring in outbreak and environmental settings, where sentinel studies may provide early warning of the onset of seasonal infections [186]. Outbreak investigations rely heavily on good sampling, and the application of nucleotide sequence-based methods to identify the causative agent of patient infections, and to determine the point source of an outbreak. These methods permit an integrated approach to outbreak studies. Based on prompt and informative laboratory results the difficulties and limitations of investigating an outbreak on purely clinical and epidemiological evidence can be reduced or avoided. Derived from the initial molecular studies of the 1990s, and the subsequent systematic gathering and deposition of genomic information in open databank collections such as GenBank, molecular methods provide invaluable information on the huge sequence diversity of the many norovirus strains encountered in outbreaks, worldwide. Sequence-based studies are limited, however, by dependence on or requirement for, sequence information. The approach of sequence independent techniques for gene analysis such as nanosequencing may improve our understanding of these well-adapted pathogens.

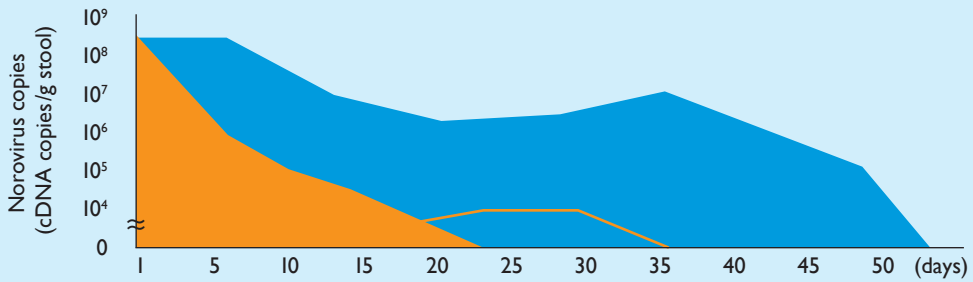
Studies on the physical and chemical nature of the norovirus capsid suggest that the peculiar qualities of noroviral stability in different environmental settings may be conferred by the specialized structure of the capsid protein shell and the P domains forming protruding arches outwards from the shell. Raised proline content in the P domain of the major capsid protein, water channels, and prestress effects may enhance stability on the capsid structure and contribute to the persistence of NoV in the environment [106, 204-206]. The apparent differences in distribution of the GI and GII groups of viruses where GI tends to be associated with waterborne transmission, and GII with the indoor air of hospital wards may reflect inadequate molecular tools. But may also correlate with the marked differences in phylogenetic distance seen between these two major groups of this well adapted human pathogen.

The properties of the noroviruses, their genomic diversity, virion stability, and fastidious nature of replication, continue to challenge the limits of evolving methods for molecular detection of noroviruses in different outbreak and environmental settings.

## 1.7 Shedding of human norovirus

In 1995 Caul stressed the importance of aerosol transmission of NoV during nosocomial outbreaks of acute gastroenteritis in hospital wards [207]. He assessed that the patient who vomits can expel 30,000,000 virus particles to the surrounding area basing these estimates on detection of virus particles in vomitus by EM technique which requires a minimum of  $10^6$  particles per ml [207]. Transmission was related to distance, the closer an individual was to the vomiting event the higher the risk of becoming infected [208]. Recent studies using refined rRT-PCR quantitative methods indicate that Caul's estimates are highly relevant [209, 211]. With the low dose of infection variously calculated for NoV between 10–2800 genome equivalents per ml (gEq/ml) the amount of NoV expelled is huge, and secondary spread is common [207, 210, 212]. In NoV infections the number of secondary cases generated by an infected index case in the absence of control measures is estimated as 14, the basic reproduction number ( $R_0$ ) [213]. For comparison, another highly contagious enteric virus poliovirus has an  $R_0$  estimated to 5–7 while the  $R_0$  for the 1918 pandemic influenza virus was estimated to 2–3 [214, 215]. With effective control measures NoV  $R_0$  can be reduced to 2 [213].

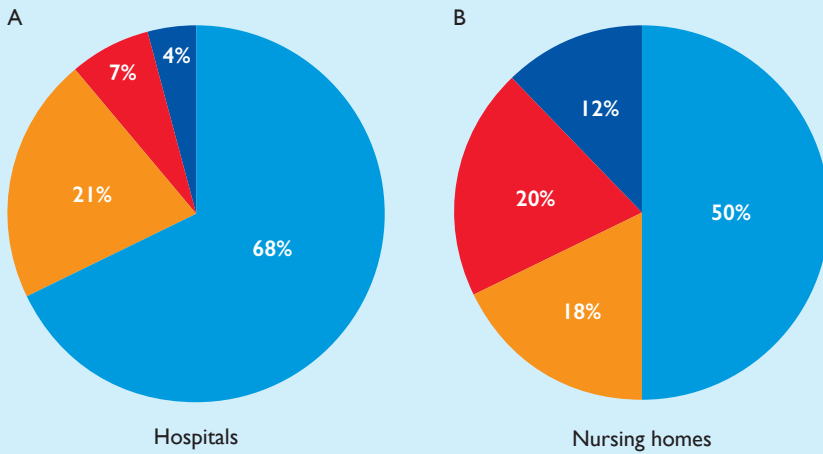
Norovirus is also shed in high quantities in the faeces of the infected patient. Real-time RT-PCR techniques indicate that NoV GII viral loads of  $10^6$ – $10^{11}$  genome equivalents per gram faeces (gEq/g) are common during the symptomatic period [209, 216]. The potential for spread in a closely confined area is high, particularly for the immunocompromised patient, the very young with immature immune system, and the elderly with waning immune defences [217–222]. These patients may show prolonged NoV shedding with severe or intermittent diarrhoeal symptoms and complications as seen in immunocompromised children and adults [223–229]. In individual patients NoV GII.4 shedding has been recorded for 898 days, NoV GII.3 for 670 days, and NoV GII.17 for 433 days [217, 226]. This is exhausting for the patient and a potential source of cross infection and environmental contamination within the medical unit, given the low infectious dose of NoV [228, 230–232]. Strict infection control procedures and rigorous cleaning is required as the impact of long-term shedders is predicted to increase transmission duration by 100% in outbreaks [233].



**Figure 8.** Schematic diagram of norovirus shedding and kinetics of viral clearance. Inpatients and nursing staff were monitored on a weekly basis following detection of symptomatic norovirus infections, during a 2-year surveillance study. The blue area denotes the slow clearance of norovirus infection in a total of 50 debilitated, immunosuppressed, or young hospitalized patients. The orange area shows the rapid clearance of norovirus in 40 healthy nursing staff, infected during hospital outbreaks; one member of staff indicated by extended orange line, showed slow viral clearance. Adapted with permission from Furuya et al. 2011, [218].

NoV viral load decline has been measured with different trajectory in different patient categories and age groups in hospital outbreaks (Figure 8), [218, 219, 234]. In a study of NoV shedding following infections in healthy staff, and different groups of patients, Furuya et al. (2011) show the contrast between the majority of healthy adults who eliminate virus during a median shedding period of 15 days, and inpatients with a median clearance time of 19 days [218]. Patients with high viral load, young individuals, and those on immunosuppressive therapy tended to require a longer time to eliminate virus indicating the need for monitoring and careful management to reduce the risk of nosocomial transmission.

Although NoV GII infections, notably GII.4 and GII.3, appear to dominate in severely ill patients with long-term shedding and reduced immunocompetence, NoV GI infections are reported in 4% of hospital and 12% of nursing home outbreaks recorded in northern England (Figure 9), and in approximately 2% of institutional outbreaks in Norway [36, 235].



**Figure 9.** Diversity and distribution of norovirus strains circulating in outbreaks in A: hospitals, and B: nursing homes in northern England 1998–2001. Diversity of strains cocirculating: NoV GII.4 Grimsby (light blue); GII.1 Girlington (orange); other GII strains (red); GI strains (dark blue). Adapted with permission from Gallimore et al. 2004, [235].

During a prospective study of NoV infection in stem cell transplant patients 6 of 10 patients showed prolonged excretion of NoV GI.3 in faeces for a mean time period of 61 days, with detection of GI.3 RNA in sera for a mean of 33 days [22]. In healthy volunteers, two different studies indicate GI shedding of 33 and 34 days in immunocompetent volunteers who consumed NoV GI.1 spiked oysters, and spiked ground water [236, 237].

The elderly are also particularly vulnerable to NoV and SaV infections, with increased mortality recorded in community-onset NoV infections in elderly individuals compared with hospital-onset NoV in elderly patients, and matched controls [11–14, 95, 238]. NoV has been associated with deaths caused by aspiration pneumonia, necrotizing colitis, gastrointestinal bleeding, septicaemia, cardiac complications and malnutrition [239].

Our understanding of the human NoV and their role in acute gastroenteritis has broadened with the finding that asymptomatic infection can be detected in healthy

individuals, and in patients with underlying disease. The duration of asymptomatic excretion varies remarkably for each individual, and the viability of the virus excreted is difficult to assess. The longer time of shedding noted in studies of young children who continue to excrete moderately high quantities of virus in the weeks or months after symptomatic infection has resolved, suggests an asymptomatic condition with potential to spread from affected, healthy, under 5 year-old children within families, to the day care centre, and the wider community [2, 240–245]. Studies of asymptomatic children indicate the excretion of norovirus in the absence of a recent history of gastroenteric symptoms in approximately 7–29% of children [18, 242, 243]. Asymptomatic excretion also affects the healthy immunocompetent adult recovering from recent NoV infection with important consequences for certain work groups as in the case of the food handler or medical staff [2, 218, 286].

### **1.8 Immune responses and immunity to norovirus**

In essence, immune responses to NoV largely seem to resemble those to other viruses causing acute infections. Since the clinical course of NoV infection has such a short duration, innate immune responses including type I interferons (IFNs) may play an important role during the acute phase as inferred from animal models of MNV infection. In such models, in addition to IFN-producing macrophages and dendritic cells, adaptive immune responses with activation of B cells as well as CD4<sup>+</sup> and CD8<sup>+</sup> T cells all contribute to control of the acute infection [246]. Although detailed studies in humans are lacking, it is noteworthy that immunosuppressed patients often fail to clear NoV infections and therefore develop long-term symptoms and viral shedding [247]. Further studies of this category of patients may help to elucidate which components of the immune response are essential for clearance of NoV infection in humans.

Immunological studies of NoV infection in humans have focused mainly on antibody responses, aiming for a better understanding of NoV epidemiology and for creating a basis for possible vaccine development. In volunteers infected by the GI.1 prototype Norwalk strain, earlier studies indicated a lack of immunity after re-challenge [248]. More recently, after virus-inoculum doses had been standardized, a model of antibody blocking of VLP binding to HGBAs has been used as a surrogate of the commonly used marker of immunity, the neutralization test. Following infection, such blocking antibodies are readily induced, peaking after 14–28 days, and are still present at supposedly protective levels after 6 months [249]. Moreover, high levels of such blocking antibodies were present at infection in most of those individuals who did not develop gastroenteritis. A recent study on volunteers expanded on these findings and showed protective potency of NoV-specific salivary IgA, and reduction of viral load by faecal IgA, suggesting the importance of mucosal immunity at least in NoV GI.1 Norwalk virus infections [250].

Although cross-reactive blocking antibodies are generated during natural infection, levels of genotype-specific blocking antibodies seem to correlate to protection. In a Finnish study of children naturally infected with NoV GII.4 (New Orleans 2010) and using a similar assay, low levels of genotype-specific blocking antibodies were associated with symptomatic infection [251]. This study also demonstrated genotype-specific seroconversion in 6 of 6 children where convalescent sera were analysed. A highly genogroup-specific antibody response was indicated with no concomitant seroconversion to GI VLPs. Interestingly, antibody levels to NoV GI viruses were low in these children, probably reflecting the low grade of circulation of viruses from this genogroup in children in Finland [252, 253].

Seroprevalence of IgG antibodies against NoV GI.4, GII.3 and GII.4 was assessed in healthy persons of different age groups from Korea, reaching seropositivity rates of 80-90% for all three and suggesting frequent exposure to these viruses [254]. However, a seroepidemiological study of American tourists travelling to Mexico who developed tourist diarrhoea (9 patients with NoV GI, 3 with GII) revealed that pre-existing IgA and IgG antibodies, as well as blocking antibodies, were significantly more often directed to GII than to GI, which might explain the susceptibility to GI in these subjects [374]. Another argument for the existence of antibody-dependent protective immunity is provided by studies of genetic drift and IgG response to GII.4 over time in humans. Concurrent with the pandemic emergence of the NoV GII.4 strain Sydney in 2012, antigenic differences occurred and at least two key epitopes of blocking antibodies were altered. It was suggested that these changes allowed an escape from herd immunity, which promoted global spread of the virus [255]. Taken together, these studies suggest that B cell immunity is prevalent to diverse NoV, targets HBGA-binding epitopes on the virus and may stand for evolutionary selection pressure on rapidly (as regards amino acid alteration) evolving genotypes such as GII.4. Moreover, memory B-cells are readily induced and correlate to protection [250], and vaccine candidates have recently been shown to activate such clones in anamnestic response [256]; there is an immunological basis for development of vaccines against norovirus.



## 2. Aims

### **General aim:**

To investigate the role of environmental contamination of noroviruses in spread of infection and disease.

### **Specific aims:**

- To detect NoV RNA in bivalves, and to compare sequences obtained to those found in patients.
- To investigate the molecular epidemiology of noroviruses in outbreaks of food- and waterborne infections.
- To assess norovirus contamination in the patient's room during nosocomial outbreaks.

*The most beautiful adventures  
are not those we go to seek.*  
– Robert Louis Stevenson

# 3. Materials and Methods

## 3.1 Environmental and outbreak settings

The transmission of NoV infections in different outbreak and environmental settings described in Papers I, II, III, and IV of this thesis is outlined in Table 1. Additional sequence-based molecular studies of a confectionary foodborne outbreak from February 2004, a small-scale investigation of norovirus in wild mussels exposed to sewage contamination from March 2004, and an oyster outbreak that affected students at Tjärnö marine biology station in February 2008, are shown in Table 1. These investigations are included as complementary studies of NoV distribution in the marine eco-system that underpin the concepts of this thesis.

### *Foodborne transmission of NoV infection: the infected food handler*

In February 2004, a widespread foodborne outbreak of acute gastroenteritis associated with contaminated confectionary from a local bakery was reported in Gothenburg (population ~490,000). Many telephoned complaints were received at the Infectious Disease Control Unit from adults describing severe gastroenteric symptoms related to consumption of complimentary cakes eaten at workplaces across the city. Stool samples were obtained from 9 patients including the baker, an office worker and a shop assistant at the bakery, 5 adults who had consumed the confectionary, and a 6-months old infant. Faecal samples were examined for enteric viruses and bacterial pathogens. No food samples were available for examination.

**Table I.** Overview of sampling from environmental and outbreak settings: details of number, type, and timing of samples examined as described in Papers I, II, III, and IV, including additional outbreak and bivalve monitoring over the study period 2004–2012

	Outbreak/Environmental investigation	Sampling month	Environmental samples	Patient/Staff samples	Background
Outbreak	FBO Feb 2004 Frölunda confectionary	Feb 2004		P (9)	Extensive FBO across city. All age groups Marzipan cream cakes. Point source baker
Pilot study	Bivalve monitoring	March 2004	Mussels (10)		Monitoring mussels near WWTP plume
Paper I	RWBO Aug 2004 Lakes Delsjön + Aspen	Aug 2004	Water samples examined at SMI	P (19+18)	Non-seasonal recreational WBO After rainy summer
	Bivalve monitoring	Nov 2004	Mussels (40)		Monitoring mussels near WWTP plume
Paper II	Bivalve monitoring Jan 2007 Restaurant opening Strömstad	Jan 2007	Oysters (5)	P (1)	Oysters stored in corves sunk in harbour DV in 30 oyster eaters of 100 guests, ICN telephone interviews No oyster eating, no DV. Oysters from restaurant
Complex outbreak	Complex OB Feb 2008 WBO Pump-house repairs Japanese oysters PTP Tjärnö	Feb 2008	Oysters (20) Water samples (6)	P (11)	Pump-house problems at marine institute station One student opened all oysters served DV onset 24–48 hr after eating oysters Students who did not eat oysters not affected
Paper III	WBO Sept 2008 Re-cycled river water heavily polluted Contaminated MuDW Lilla Edet	Sept 2008	Water samples examined at SMI	P (50)	MuDW contaminated. Upstream WWTPs breakdowns, emergency releases, heavy rainfall Sudden onset DV in down stream community Public concern, primary care response, BWO Media appeals for patient samples
Paper IV	NOSOs Jan–May 2012 7 hospital wards PTP 1 control ward	Jan – May 2012	Patient room environment (135)	P (125)	In-patient sampling based on DV symptoms Environmental swabs from patient rooms Patient tables, wash basins, air vents, dust Air samples, virus air trap device
Staff survey	Personnel Jan–May 2012 7 wards with NOSO	Jan – May 2012	NRS (133)	NRS (133)	Rectal swabs from staff attending patients in 7 wards with NOSO

FBO foodborne outbreak; (n) number of samples; P samples of faeces from patients; WWTP wastewater treatment plant; RWBO recreational waterborne outbreak; COYo contaminated oyster outbreak; ICN infection control nurse; WBO waterborne outbreak; SMI Swedish Institute for Infectious Disease Control; PTP person-to-person spread; MuDW municipal drinking water; DV diarrhoea and vomiting; BWO boil water orders issued; NOSO nosocomial outbreak; NRS nursing staff rectal swab samples.

### **Pilot bivalve studies**

A small-scale pilot study was carried out to investigate the prevalence of human NoV in blue mussels (*Mytilus edulis*) growing close to sewage effluents, Table 1. The aim was to determine to what extent the NoV strains detected in these mussels reflect the strains of human NoV that circulate in the population during community and hospital outbreaks. Wild mussels (n=10) were collected from the coastal waters around Fotö, a small island (population 617) in the northern archipelago of Gothenburg (Figure 10). Fotö lies close to the plume of wastewater from Rya wastewater treatment plant (WWTP), processing sewage from the city of Gothenburg and surrounding regions (population ~650,000 in 2004). All mussel samples were transported on ice to the bivalve laboratory, to be processed and examined for NoV RNA.



**Figure 10.** Map of the greater Gothenburg area showing Fotö island; Rya wastewater treatment plant; the arrow shows the plume of effluents from the wastewater treatment plant at Rya; city of Gothenburg; River Göta; Lake Delsjö; lake Aspen in the Lerum area.

### ***Bivalve studies and recreational waterborne outbreaks (Paper I)***

In an environmental study carried out in November 2004, wild mussels (n=40) were collected from Fotö. Mussels sampled in November were collected 9 months after the confectionary foodborne outbreak of February 2004, and 4 months after widespread outbreaks of waterborne infections in August 2004. These August outbreaks of acute gastroenteritis were associated with recreational bathing in the inland lakes of Delsjö and Aspen. Large numbers of bathers (>400) complained of symptoms of diarrhoea and vomiting. Faecal samples were obtained from patients who bathed in Delsjö (n=19), and Aspen (n=18) in Lerum region (Figure 10). Water samples from Delsjö were examined at the Swedish Institute for Infectious Disease Control (SMI) [257].

### ***Contaminated oysters implicated in the transmission of norovirus infections (Paper II)***

An outbreak of norovirus infections associated with oyster eating occurred in January 2007, following the opening of a seafood restaurant in Strömstad, a small town (population 11,572) on the west coast of Sweden. Thirty of 100 invited guests complained of severe symptoms of acute gastroenteritis including vomiting, diarrhoea, headache, and muscle pains, within 12 to 24 hours of consuming raw oysters served at the evening shellfish buffet. Guests, invited from across Sweden, dispersed rapidly after the event; only one person who had severe symptoms provided a faecal sample. Other guests were interviewed by telephone, but few secondary cases were noted. No illness was reported in the 70 guests who had not eaten oysters. Five unopened Swedish oysters (*Ostrea edulis*) were retrieved from the restaurant and transported on ice for examination in the virus bivalve laboratory.

### ***Outbreak linked to consumption of Japanese oysters***

Complex routes of virus transmission involving sewage contaminated bivalves, waterborne infections, and person-to-person spread were indicated in a severe outbreak of gastroenteritis that affected students at Tjärnö marine station in February 2008 (Table 1). Reassured that the surrounding waters and Japanese oysters (*Crassostrea gigas*) growing around the station were free of toxin-producing algae, the students arranged an oyster eating evening. Of the 24 students, 16 ate raw oysters. Approximately 36 hours later students (n=16) who had eaten oysters complained of severe symptoms of acute gastroenteritis. Those (n=8) who had not eaten oysters were not affected.

Faecal samples were obtained from 11 symptomatic students. Freshly collected oysters (n=10) and depurated oysters (n=10) from the collection area were examined

for enteric viruses, but no oysters were left over from the batch served at the party. Samples of water (n = 3) were obtained from oyster banks close to the pump station, and from other oyster collection areas (n = 3). Total nucleic acid (TNA) extracts of samples from patients, oysters, and water were examined for enteric viral pathogens by using real-time RT-PCR (rRT-PCR) designed for detection of enteric viruses in faecal samples.

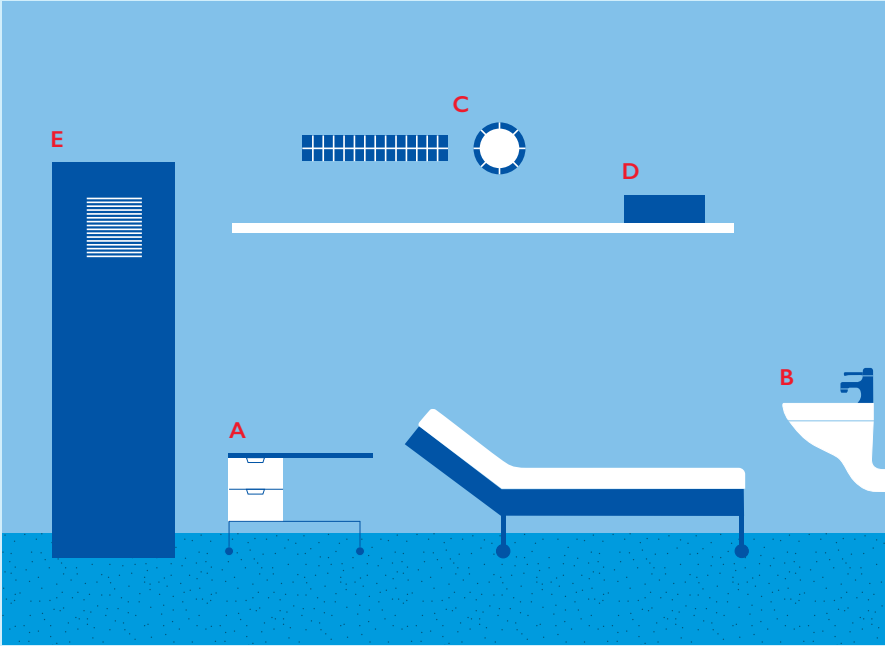
### ***Waterborne transmission (Paper III)***

A large outbreak of acute gastroenteritis affected the municipal of Lilla Edet in September 2008 (Paper III). Situated on the River Göta, Lilla Edet has a population of 13,000 inhabitants. Telephone reports from the public describing sudden onset of acute gastroenteritis, widespread absenteeism at workplaces, schools and day care nurseries, alerted local primary health care staff and the county medical officer (CMO) of a large community outbreak of enteric infections. Abrupt onset of the outbreak suggested a common point source of infection. Waterborne transmission was strongly suspected as river conditions had deteriorated causing heavy turbidity during the first week of September [258, 259]. Boil water orders were issued by the CMO as cases were not restricted to a single region of the town, or to intake of a specific food such as bivalves, but were associated with consumption of municipal tap water, although not with private well water [32]. Following appeals made in local media, faecal samples were obtained from 50 inhabitants who had experienced symptoms of vomiting and diarrhoea in the first three weeks of September. Patient samples (n=50) were examined for presence of viral, bacterial and parasitic pathogens by using rRT-PCR technique, microbial culture, and wet film microscopy.

### ***Nosocomial transmission and environmental investigations (Paper IV)***

Environmental and patient sampling was carried out in 8 hospital wards located in 4 different hospital buildings during the period January to May 2012, Table 1. Seven of the wards were affected by nosocomial outbreaks of NoV infection over the period. An outbreak ward was defined as a ward in which two or more cases of suspect or verified NoV gastroenteritis occurred in patients, and the infection spread to other patients within the ward. Samples of faeces and, or, vomitus were obtained from in-patients who developed symptoms of acute gastroenteritis during their hospital stay.

Environmental samples were collected from surfaces by swabbing with sterile cotton swabs pre-wetted with PBS. A standardized procedure was used to swab over-bed tables, wash hand basins, air vents, and dust from locker tops in patient rooms (Figure 11).



**Figure 11.** Patient room and sampling sites: A, patient's over-bed table; B, wash hand basin; C, in and out air vents; D, shelf with virus air trap device; and E, locker top dust sampled.

A simple virus air trap device, designed to capture virus particles from the air, was placed on a shelf approximately 2–3 metres above the patient's bed, and exposed for 3 h (Figure 12). Control virus air trap sampling was carried out in an administrator's office, in a separate hospital building, with no patient wards (Figure 12). Environmental samples (n=135) were extracted and examined in a separate laboratory from patient samples (n=125).



**Figure 12.** Virus air trap sampler designed to capture virus particles from the air.

When a nosocomial outbreak of NoV was reported, the ward was visited and all members of staff were given the opportunity to participate in a questionnaire-based study on risk factors for NoV transmission in hospital setting. Self-sampling of faeces using a rectal swab was carried out by the individual nurse according to instructions included with the swab pack [260]. The sample was returned by post to the virus laboratory, along with the questionnaire reply giving information on current state of health/symptoms of infection.

### **3.2 Sample preparation and nucleic extraction**

To avoid cross contamination sample preparation, extraction, and virus detection were carried out in different rooms, within different laboratories, on different floors in the virology department. Patient samples were examined in the routine diagnostic department, oysters and mussels were dissected and examined in the bivalve laboratory, and environmental swabs were processed in a separate laboratory independent of the routine and bivalve facilities. All procedures were carried out in dedicated rooms with separate UV light cabinets. Water controls, barrier filter tips, and dedi-

cated pipettes were used according to the principles of good PCR laboratory practice recommended by Kwok and Higuchi [183]. These precautions are particularly important when *nested* PCR systems are used to detect viral RNA.

### ***Patient samples***

Samples of faeces or vomitus obtained from patients with symptoms of acute gastroenteritis were emulsified as 10% suspensions in 4 ml of Hank's medium, or PBS and prepared for extraction on a MagNA Pure LC instrument as described in Papers I, II, and III. In the studies described in Paper IV, TNA extracts (110  $\mu$ l) were prepared from 250  $\mu$ l of supernatant fluid lysed in 2 ml NucliSENS lysis buffer before isolation of TNA on the EasyMAG (BioMerieux) instrument. TNA extracts were stored at  $-80^{\circ}\text{C}$  before assay.

### ***Bivalve samples***

Bivalve samples were collected from the mussel banks off Fotö island in March (n=10), and November (n=40), 2004. In the Strömstad investigation, oysters (n=5) were retrieved from the restaurant within two days of outbreak reporting to the health authorities, January 2007. Bivalves were transported to the laboratory in freezer-cooled bags, then brushed and washed in sterile distilled water before being shucked. Digestive glands were dissected out of individual bivalves, emulsified and homogenized individually in the MagNA lyser (Roche) instrument as described in Papers I and II. RNA was extracted from tissue lysates by using a MagNA Pure LC (Roche) instrument according to the RNA III protocol, Papers I and II.

### ***Water samples***

Water samples were centrifuged 2 times at  $4000 \times g$  for 20 minutes at  $4^{\circ}\text{C}$  to remove sediment. Pooled supernatant fluids and sediments were stored separately at  $-20^{\circ}\text{C}$  prior to ultracentrifugation of 300 ml supernatant at 50,000 rpm overnight (16–18 hours) in the Beckman Ultracentrifuge. Pellets were solubilized overnight at  $4^{\circ}\text{C}$  in 200  $\mu$ l sterile PBS, and pooled to give 1.5 ml, prior to isolation of TNA in the EasyMAG extractor (BioMerieux). NA extracts were stored at  $-80^{\circ}\text{C}$  before analysis.

### ***Environmental swab samples***

The pre-wetted environmental swabs collected from patient rooms during nosocomial outbreaks were transported to the laboratory in 2ml NucliSENS lysis buffer, and vortexed vigorously prior to TNA preparation on the EasyMAG extractor (BioMerieux), Paper IV.

### ***Rectal swabs from nursing staff***

Rectal swabs were vortexed in 1.5 ml of PBS and 250  $\mu$ l aliquots were lysed in 2 ml BioMerieux lysis buffer prior to TNA preparation (110  $\mu$ l) on the EasyMAG extractor (BioMerieux). TNA extracts were stored at  $-80^{\circ}\text{C}$  until assay in the rRT-PCR for enteric viral pathogens.

### **3.3 Combined nested RT-PCR for detection of NoV RNA**

#### ***Amplification and detection of short products from environmental and patient samples***

Combined nested single-tube RT-PCR was carried out with master mixes using a battery of primer pairs for detection of short NoV products as described in Paper I [45, 261]. Three individual master mixes were prepared using primer pairs NI/NVp110, NVp36/NVp110, NVp69/NVp110 for amplification of RNA extracts [107, 157, 159]. To reduce the risk of RT or PCR enzyme inhibition by inhibitors co-extracted from the environmental samples (bivalve tissue or water) serial dilutions ( $10^0$ ,  $10^{-1}$ ,  $10^{-2}$ ) of NA extracts were prepared in RNase-free water immediately before testing. Inoculated first round RT-PCR master mixes were amplified as described for short products, Paper I and II. Master mixes for nested PCR included the inner primers JV12Y/JV13I, [45, 262], with amplification conditions for short products as described in Papers I and II. Nesting procedures were carried out by transfer of 5 or 8  $\mu$ l of the amplified 1st round RT-PCR reaction to the nesting master mixes. Amplicons were separated by gel-electrophoresis using 1.8% agarose depending on amplicon size, with 0.25  $\mu$ g ethidium bromide per ml for staining and UV-detection.

#### ***Long-fragment detection in environmental and patient samples***

Combined RT-PCR was used to detect products (1–3.1 kb) from amplification of the RdRp-N/S major capsid-coding region of NoV strains detected in bivalves, patients, dust, and other environmental samples (Papers I, II, III, and IV). RT-PCR master mixes were prepared as outlined previously, but with increased AmpliTaq polymerase concentration (1 unit per reaction tube) and selected outer primer pairs NVp36/TVN<sub>25</sub>, NI/TVN<sub>25</sub>, RJ12Y/TVN<sub>25</sub> and NVp69/TVN<sub>25</sub>. Long-fragment products were amplified with the RT-PCR programmes described in Papers I, II, III and IV. Nested PCR was carried out with a battery of primers, amplified with the nested PCR programmes as detailed (Paper I, II, III, and IV). Products detected by gel-electrophoresis on 1% agarose were cut from the UV-illuminated ethidium bromide stained gel, and purified on Qiagen columns (Qiagen, GmbH).

### 3.4 Real-time RT-PCR detection assays

Validated semi-quantitative taqMan rRT-PCR or rPCR assays were used for detection of NoV GI and NoV GII, SaV GI+II+IV+V, human adenovirus (HAdV), rotavirus (RV), and astrovirus (HAstV) in environmental swabs, and in samples from patients and nursing staff (Paper II, III, and IV).

#### *Principles of real-time RT-PCR*

The rRT-PCR is a refined tool that has greatly improved the approach to quantitative and diagnostic detection of viral agents over a wide dynamic range of template concentrations, permitting template amplification from a broad variety of sample types with a large, yet unpredictable, range of genome copy number. This allows rapid processing of clinical samples, and is well suited to the quick decision-making required for microbiological investigations and outbreak studies [263].

In the rRT-PCR system each master mix included RT-enzyme, taq DNA polymerase, primers designed to amplify a short, highly conserved region (60–150 nt) of the RNA virus genome, and a fluorophore-labelled hydrolysis probe detection system based on the 5'-exonuclease activity of taq DNA polymerase enzyme [264]. Short-product amplification and inclusion of specific dual-labelled hydrolysis probes ensure rapid, sensitive, sequence-based detection of a specific PCR product as it accumulates during the reaction in a sealed system, designed to reduce the risk of cross contamination. With duplex or multiplex detection, where a series of optimized specific primers and probes are included in one master mix to detect more than one viral agent in the sample, the rRT-PCR assay based on fluorogenic 5'-exonuclease chemistry has revolutionized molecular diagnostics.

Short, synthetic, fluorescent-labelled oligonucleotide probes complementary to specific sequences downstream from a primer-binding site are included in the PCR or rRT-PCR master mixes. Dual-labelled with 5'-reporter dye (R) and 3'-quencher dye (Q), the probe is non-extendable at the 3'-end of the molecule. The 5'-reporter signal from the short intact probe is effectively silenced by the 3'-quencher [264, 265]. This quenching effect holds as long as the R and Q fluorophore dye molecules are in close spatial proximity within the intact oligonucleotide probe.

Labelled probe/s are designed with higher melting temperature (approximately +10°C higher) than the primer melting temperatures (56° to 60°C), an essential requirement as the probe must anneal to the complementary, denatured strand of target DNA, or amplicon, before primer annealing during each PCR cycle. On primer annealing to the complementary strand, the DNA polymerase elongates the primer *en route* to synthesising a new double stranded amplicon. But the presence of hybridized labelled probe on one of the denatured target strands elicits the 5'-exonuclease activity of the DNA polymerase, cleaving the hybridized dual-labelled probe from the strand by hydrolysis. The enzyme *noses off* the probe releasing R fluorophore

and Q dye as free molecules; the 5'-reporter fluorophore is liberated from the silencing effect of the 3'-quencher dye.

If target viral NA is present in the sample, exponential increase of specific amplicon with each PCR cycle is paralleled by hydrolysis of labelled probe through the polymerase 5'-exonuclease activity. Accumulating release of R dye is recorded by the sequence detection system as an increasing fluorescence signal at the optimal excitation wavelength for the R dye. This permits *real-time* detection of specific amplified products at the end of the extension phase of each PCR cycle. As amplification and detection are carried out in a closed system, with non-template and water controls, the risk of cross contamination is reduced. Moreover, in rPCR viral DNA detection master mixes that include a heat-labile uracil-DNA glycosylase can be used to prevent re-amplification of dUTP labelled carry-over PCR products, reducing the risk of cross contamination.

In a well-designed optimized rRT-PCR assay amplicon size is short (60–120 bp); sensitivity, specificity, efficiency and speed of amplification reaction is greatly improved compared with nested gel-based nested RT-PCR end-point systems, where great care is required to reduce the risk of cross contamination. However, as NoV rRT-PCR is based on the choice of short, highly conserved regions, gel-based RT-PCR amplification of longer, more variable regions of the NoV genome is relevant for sequencing and comparative analysis of strains.

### ***Cycle threshold value***

The rRT-PCR is characterized by the cycle threshold value (Ct), the point in time during cycling when amplification of a target is first detected, rather than by the amount of target accumulated at the end of the PCR, as in end-point gel-based PCR. Ct value is defined as the number of cycles required for the fluorescent signal from probe cleavage to cross the threshold, to exceed the background level noise of non-specific fluorescence effects in the inoculated reaction mix. In the presence of target NA the positive reaction is detected by accumulation of R dye fluorescent signal, and is expressed as the Ct value. The Ct value varies inversely with the amount of virus in the sample, the higher the viral copy number concentration in the input sample of NA, the lower the Ct value for the given sample. Ct value can be used as a proxy value for viral load particularly in non-uniform samples such as faeces [266], although target containing plasmid preparations or armoured RNA of known concentration may be used to prepare standard curves for direct quantification of target sequences, and quantification of viral load. For RNA viruses, and all RNA and DNA detection, the Ct value is dependent on the efficiency of a well optimized PCR or RT-PCR system [267].

### **PCR Inhibition**

Successful rRT-PCR amplification of specific products is dependent on quality of NA extraction, including removal of PCR inhibitors from the preparations. Inhibition can be a particular problem in assay of food or environmental samples where multiple humus products, and metal ions may be co-purified with NA, and interfere with enzyme activity. Inhibition may also affect amplification of patient samples particularly faeces, although in a hospital outbreak study <1% of patient faeces samples were shown to be affected by inhibitors when internal controls were included in the rRT-PCR [219, 268]. Inhibitory effects can be overcome by improved extraction methods, by inclusion of internal amplification controls, and by dilution of extracts prior to amplification [269]. Inhibition is a particular problem in detection of NoV in bivalves, frozen fruit and foods such as raspberries or salad [41, 43, 201].

### **3.5 Nucleotide sequence analyses**

Purified RT-PCR products were sequenced in unilateral cycle sequencing reactions by using the Big Dye terminator kits v 1.1 or 3.1 (Applied Biosystems), with appropriate primer, and cycle sequencing programme as outlined in Papers I, II, III, and IV. Cycle sequencing products were precipitated and washed prior to analysis on the ABI Prism 3100-Avant Genetic Analyzer, or the 3130xl Genetic Analyzer (Applied Biosystems).

### **3.6 Comparative nucleotide sequence analysis**

Sequence analysis was carried out first by inspection of individual uni-directional sequences, then by sequence alignment of sample sequences with inclusion of appropriate and adequate reference sequences from GenBank, by using the Sequencher version 4.9, software (Gene Codes Corp. Ann Arbor, MI) for Papers III and IV, and Sequence Navigator (Applied Biosystems) in Papers I and II. Individual sample sequences were examined in BLAST (NCBI), the Basic Local Alignment Search Tool (National Centre for Biotechnology Information). The BLAST programme finds regions of local similarity between sequences, compares nucleotide or protein sequences to sequence databases, and calculates the statistical significance of matches [270].

### **3.7 Phylogenetic analysis**

Phylogenetic analysis provides a means of simultaneously describing the relationship between all sequences in a given sample of sequences, and may reveal the ancestral roots, evolutionary development, and diversification of the group of viral sequences being studied.

Phylogenetic relatedness among the sequences presented in Papers I, II, III, and IV was estimated by using MacVector 7.2 software (Accelrys Inc., San Diego, CA). Distance matrix methods were used to calculate the similarities by Neighbour-joining method. The Kimura K2P or Tamura-Nei nucleotide substitution models were used to estimate the nucleotide distances. These models allow for different scores for the probability of transition and transversion events [271, 272]. The similarity between each pair of sequences is used to quantify the evolutionary changes and find the common ancestor, to provide the tree that best reflects the relatedness among sequence data in a matrix of pairwise sequences values.

Bootstrap analysis is used to evaluate the reliability of the inferred tree, or more precisely to evaluate the reliability of specific clades in the tree [273]. Bootstrapping is a form of significance testing based on resampling from the original data set. A new alignment is obtained by randomly choosing columns from the original, to make a new alignment of the same length as the original. Usually resampling is repeated 100 to 1000 times; sub-trees and an optimal tree are reconstructed each time. After bootstrapping a consensus tree is constructed from all optimal trees, and a bootstrap value is presented at each internal node. This value indicates how many times the data was branched into the two clusters that lie on either side of the node. So bootstrap values shown in the constructed phylogenetic trees are a measure of the robustness of the branches of the tree, a confidence value, where values are calculated for the 100 or 1000 re-samplings of the data set. Only values  $\geq 70$  are shown at the nodes of branches in the phylogenetic trees presented in Papers I, II, and III.

### **3.8 Molecular cloning**

Molecular cloning was carried out to confirm the sequences of individual members of the mixtures of NoV strains detected in patient samples from the Lilla Edet outbreak, and to determine the nucleotide sequence of the genotype I strains identified in the outbreak (Paper IV). The Topo TA kit (Invitrogen, Carlsbad, CA) was used in the cloning procedures according to the instructions.

*Ne dites pas: J'ai trouvé la vérité,  
mais plutôt: J'ai trouvé une vérité.*

– Gibran Khalil Gibran

# 4. Results and Discussion

The concept of this thesis is grounded on studies of the widespread distribution of human NoV in the environment, and the different routes of transmission of NoV infections. These epidemiological features are exemplified below in a diverse set of NoV outbreaks that occurred in the Gothenburg area during the years 2004 to 2012. Some of the outbreak settings are published, and some are added here for completeness. Together, these outbreaks and the tracing of NoV in the environment, illustrate the distinct and divergent contagiousness of this virus family. The NoV transmission studies presented in this thesis are based on the molecular epidemiology of the NoV strains detected in patients and in the different environmental settings encountered in outbreaks referred to in Table 1, section 3.1.

## 4.1 Experiences from a foodborne outbreak

This study describes an early experience of NoV outbreak tracing where molecular epidemiology confirmed the point source of a widespread foodborne outbreak. The confectionary outbreak that affected more than 300 people in Gothenburg in February 2004 was linked to consumption of marzipan cream cakes, ordered by one firm for their business clients (Table 1). The source of the large order was readily traced to one bakery in the suburb of Frölunda. The baker succumbed to an influenza-like illness with symptoms of fever, diarrhoea and vomiting during the second week of February, but returned to work within a few days of recovery. Inspection of the bakery on two occasions indicated poor standards of hygiene; the premises were closed for cleaning following the second visit. Cases occurred from late February through to the first week of March. People who ate complimentary cakes at their work fell ill within 12 to 24 hours with feverish diarrhoea and vomiting. All age groups were affected, including a 6 month-old infant, a secondary case within a family where the father developed acute gastroenteritis after consuming confectionary at work. No food samples were obtained for analyses. However, 9 patient samples were examined including a faecal sample from the baker. Returning to work too early proved to be an unfortunate example of misplaced loyalty, one of many factors that can erode good hygiene practices [192, 275, 276].

NoV GII strains were detected in 7 of 9 faecal samples examined, the baker, one member of the baker shop staff, 4 adults who consumed the cakes, and one infant from an affected family. Initial testing in diagnostic gel-based RT-PCR amplifying short regions of the RdRp in three parallel master mixes with primers NI/NVp110, N36/NVp110, and N69/NVp110, revealed NoV in one sample. This was from the 6-month old infant secondary case whose sample amplified a product in the NI/

NVp110 master mix. However, NoV was not detectable in 8 of the 9 samples examined in this initial routine diagnostic test. No other enteric viruses or bacterial pathogens were detected in the samples.

To check the validity of the *Norovirus not detectable* result in what appeared to be a large non-bacterial foodborne outbreak, nested PCR was carried out using primer pair JV12Y/13I amplifying the RdRp region [262]. Although described as a *nesting* technique (Paper I and II) the term *carry-over* PCR might be more appropriate as 2nd round products (328 bp) amplified by primers JV12Y/JV13I were longer than those amplified by the 1st round RT-PCR primers NI/NVp110, and N69/NVp110 (product size ~120 bp, 150 bp respectively). The third master mix used in diagnostic routine testing contained primers NVp36/NVp110 amplifying an outer product of 379 bp in the RdRp, which can be nested into the JV12Y/JV13I master mix giving a 328 bp product. In the nesting detection system a small volume (5  $\mu$ l) was transferred in parallel from each of the amplified 1st round RT-PCR master mixes to three individual JV12Y/JV13I PCR master mixes for 2nd round PCR. This method proved to be more sensitive. A 328 bp product was amplified and subsequently sequenced from 7 samples, including those from baker and infant. A longer time-period had passed between illness and patient sampling in the adults, perhaps explaining the insensitivity of the 1st round RT-PCR, and the need for a nesting technique.

In explanation, degenerate reverse primer NVp110 gave a good yield of cDNA but forward primers NI and NVp69 had sequence mismatches. However, primers JV12Y/JV13I amplified a 320 bp product from the cDNA produced by the NVp110 primer. The reverse nesting primer JV13I lies at nt 4878–4858 (GenBank M87661) just within the outer reverse primer NVp110 (nt 4884–4865, M87661). The forward primer JV12Y is positioned at nt 4552–4572 (M87661), (Paper I).

Subsequently, two of the patient samples were examined in the group specific NoV GI and GII rRT-PCR detection systems developed for laboratory diagnostics in 2005. These samples showed NoV GII Ct values of 19 in the infant, and 25 in the adult, confirming the positive NoV GII findings, and indicating a high viral load in the patient faeces.

The molecular epidemiology linking the baker to the outbreak and the NoV infections detected in outbreak patients was good. NoV strains detected in samples from baker and 6 patients showed 100% nucleotide sequence similarity (RdRp region, 285 nt). Comparative sequence analysis with GenBank reference strains indicated that the confectionary NoV outbreak strains belonged to the GII.b cluster of recombinant GII strains with no known *own* capsid genes, as designated in 2004 [277]. Confectionary strains showed equal similarity (84%) to GII.1 and GII.4, the closest GenBank reference strains. Similar NoV GII.b strains ( $\geq 97.5\%$ , 285 nt, GenBank accession no. AF365989) were detected in outbreaks in Gothenburg in 2001, in a waterborne outbreak in Stockholm county 2003 (AY240939), and closely related strains are still circulating in the local community and worldwide [40, 278–285]. The NoV GII.b.II3

recombinant was confirmed in two of the confectionary strains following sequence and molecular analyses of longer products of 1- and 2-kb spanning the RdRp-N/S capsid-coding major region (GenBank accession nos. EU085479.2, EU085491.2). A proposed renaming of recombinant NoV strains suggests that these GII.b recombinants now be defined as NoV GII.P21\_GII3 [169].

The confectionary outbreak where NoV strains with high similarity were detected in the food handler and affected consumers is typical of the transmission of NoV infections in food that has been prepared and contaminated by one individual food handler [276, 286, 287]. The Frölunda baker returned to work too early before full recovery because of the heavy workload of oncoming Easter celebrations requiring detailed marzipan decoration. This is another example of the food handler who should have known better [276]. The consequences were widespread, aggravated by poor standards of hygiene on the premises. Conditions of work where basic hygiene regulations are not strictly adhered to are common in foodborne outbreaks; careless use of disposable gloves, poor planning, and inadequate cleaning of work areas may add to the problem [42, 288, 289]. These are recurring situations described in early studies of NoV foodborne infections in the confectionary trade, and yet still actual [289, 290]. Good standards of health education for food handlers and staff dealing with the public, and with food processing, are essential. But to be effective, understanding and regular reinforcement of instructions on basic hand and personal hygiene is required, to reduce the risk of foodborne outbreaks. Where wedding cakes with hand-spread strawberry filling appeared to be the source of a NoV outbreak, Friedman suggested that food handlers should wait at least 72 hours after symptoms have ceased before return to work [289]. Sound advice, but not yet regulatory in most countries, where 48 hours are recommended [288]. Self-quarantine may be advantageous for all.

One immediate problem is the unsuspecting food handler who is actively and uncontrollably sick on the premises, and does not understand the need to report directly to management, leave the site of food preparation, and go home [276]. Good communications are important. But identifying the *asymptomatic* carrier and his role in foodborne outbreaks also poses problems. The importance of the asymptomatic worker who prepares food during the pre- or post-symptomatic period of infection is clear from many outbreak reports, including the Frölunda baker with recent history of symptomatic infection [287, 291]. However, asymptomatic food workers with no pre- or post-preparation symptoms may also transmit foodborne infections [292–295]. Danish studies estimate that 64% of outbreaks where food has been contaminated during preparation or serving are caused by an asymptomatic food handler [291]. Careful interview may reveal gastric illness in the worker's home; the food handler may remain asymptomatic, be post symptomatic, or develop symptoms after food preparations [286, 291]. Even the sick, recovering, or asymptomatic child in nappies, may add to the risk of infection in parenting food workers [2, 240,

296–298]. However, recommendations on exclusion of workers when household members have gastric illness are difficult to implement because of socio-economic repercussions [291]. Detailed molecular studies of NoV in serial mouthwash samples during and post infection may also be relevant for advice and regulation of *return to work policies* following acute gastroenteric infections, particularly for food handlers and medical personnel [35, 299].

Experience from the Frölunda confectionary outbreak indicates that such an exclusion policy, and a clearance of 72 hours following the end of symptoms in the food handler, may reduce the risk of NoV foodborne outbreaks [287, 289, 291]. Sporadic cases of NoV infection may occur at any time in the population, the low dose of infection of the NoV, direct hand contact with food, surfaces, and fomites, fellow workers, and clients makes the food worker, his work environment, his products, and customers, particularly vulnerable. Risks can be reduced by better education and adherence to good standards of personal hygiene and environmental cleaning, as the robust non-enveloped NoV has a record of persistence on kitchen and toilet surfaces [188, 199, 300–306].

Although the Frölunda confectionary outbreak occurred at the height of the seasonal NoV infections, foodborne outbreaks can occur at any time of the year, and are not restricted to infections with a given NoV or SaV genogroup, or genotype [36, 41, 93, 97, 307, 308]. Laboratory investigations should include examination of samples from *all* food handlers involved, whether symptomatic or asymptomatic, to determine virus shedding. The rRT-PCR methods that have replaced nested RT-PCR systems are highly sensitive, more specific, and target conserved regions of the viral genome. This is illustrated by the detection of high viral loads in 2 confectionary outbreak samples when examined in follow up NoV GII rRT-PCR analyses. Prompt sampling of all food workers, affected patients, and suspect foods may ensure relevance of viral findings. Rapid processing and nucleotide sequencing is essential in outbreak investigations, to identify and characterize the infectious agent, to determine point source of transmission, and to trace molecular epidemiology. Early retrieval of suspect food and food dishes, such as salads or berries, should be the aim as examination at the molecular level is important in tracing routes of transmission [194, 309, 310]. Rapid communication of informative and timely laboratory analyses may support medical and public health officers in the choice of countermeasures.

In contrast to the foodborne infections caused by the individual food handler, a spectrum of mixed NoV strains may be encountered where food products have been contaminated by wastewater, or by the picker working in poor sanitary conditions. Imported contaminated fresh or frozen soft fruit such as raspberries, salad greens, vegetables, or shellfish, have been traced across continents following detection of multiple strains of NoV, HAV, or HAdV in food or patients [194, 201, 311–313]. Molecular epidemiology of food borne outbreaks caused by products contaminated by pickers or sewage contaminated waters resembles outbreaks with point source contaminated water or bivalve molluscs.

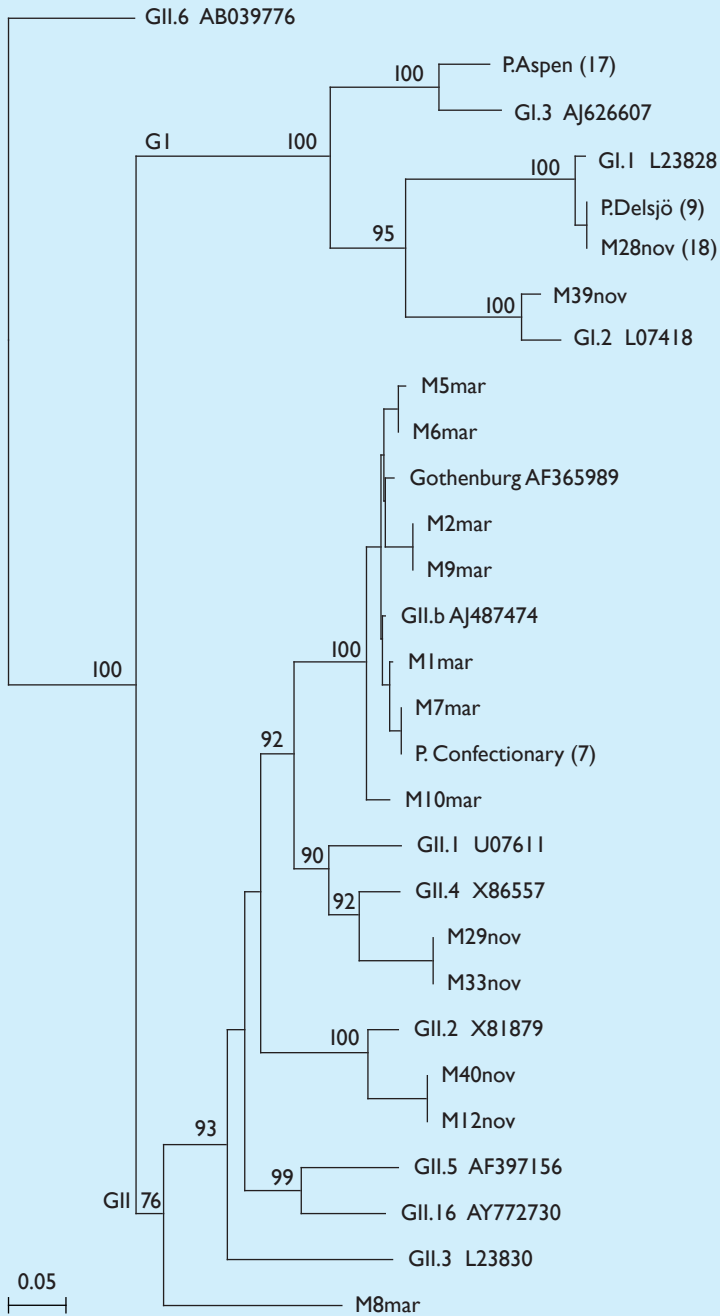
## 4.2 Norovirus detection in bivalves: a pilot study

Bivalves are now known to contribute to the reduction of water eutrophication, and their filter-feeding activities make them good bio-indicators or *sentinels* of aquatic environmental contamination [314–317]. However, bivalve shellfish have long been recognized as a common but specialized source of foodborne outbreaks of gastroenteric infections [43]. Correlation of ingestion of shellfish with hepatitis A infections is well established, as is the marked stability of hepatitis A virus in aquatic environments [45, 46, 301, 318]. Furthermore, huge outbreaks of gastroenteritis across USA, Europe, and Australia have focused attention on the special role of bivalves in transmission of NoV [187, 319–322].

This small-scale study was designed to determine if human NoV could be detected in wild mussels growing near sewage effluents, and to assess the potential use of mussels in environmental tracing of human NoV strains (Table 1). Tracing would require detection, characterization, and correlation of the NoV strains found in contaminated mussels with NoV strains detected in patient outbreaks occurring in west Sweden. Wild mussels were collected from Fotö island close to the plume of wastewaters from Rya WWTP, in March 2004 (Figure 10). In 2004, Rya WWTP served the greater Gothenburg region and the municipalities of Ale, Härryda, Kungälv, Mölndal and Partille, a population of approximately 650,000.

Molecular methods were used to examine mussels (n=10) in the combined RT-PCR nested system for detection of the RdRp (ORF1) region of the NoV genome [45]. This method was selected following the experience gained of improved sensitivity in the detection of NoV infections linked to the confectionary outbreak. Also, the nesting method could be expected to reduce the risk of RT-PCR inhibition from co-extracted inhibitors, such as humus, salts and heavy metals [323, 324]. In addition, serial dilutions of bivalve NA extracts, prepared in RNase-free sterile distilled water, were used in the 1st round combined RT-PCR NoV test system to reduce inhibition problems. NoV specific RNA was detected in 8 of 10 mussels and nucleotide sequencing (285 nt) confirmed the presence of NoV GII strains.

Comparative sequence and phylogenetic analyses showed that 7 of the March mussel strains belonged to the NoV GII.b, and grouped with the confectionary outbreak strains of February 2004 (94–100%, 285 nt), and the Gothenburg 2001 strain (AF365989), (Figure 13).



**Figure 13.** Neighbour-joining tree based on analysis of partial NoV RNA-dependent RNA polymerase coding region (285 nt). Mxmar and Mxnov identify individual mussel strains from the March and the November 2004 studies. P represents individual patient strains linked to outbreaks. (n) indicates the number of strains analysed and shown to be identical. Genotype sub-groups are defined on branches beside GenBank accession numbers of reference strains. Bootstrap values are shown at the nodes.

One mussel strain *M8* differed from all other reference strains, showing equidistance (73%) from the closest reference strains GII.3, GII.5 and GII.16. This suggested recombination between an unknown NoV strain, now with proposed name GII.P22 over the RdRp and NoV GII.5 [169]. In 2014 a complete genome with 99% similarity to strain *M8* (285 nt) was reported to GenBank (accession no. KJ196277) NoV GII/Hu/JP/2001/GII.P22\_GII.5/Saitama/T49 denoting a recombinant strain.

The finding in mussels of NoV GII.b strains with high similarity to the confectionary outbreak strains suggested that NoV GII.b strains had been circulating in the population for some time. Strains similar to the recombinant confectionary NoV GIIb.GII.3 (GII2.P21\_GII.3) have caused widespread outbreaks, being found in raw and treated sewage across Europe, and globally [169, 252, 278, 284, 285, 325–328].

This small-scale study of NoV prevalence in bivalves confirmed that mussels growing near sewage effluents bioaccumulate human NoV strains from environmental waters contaminated by sewage effluents. Some of the NoV strains detected in mussels showed high similarity to clinical strains from outbreak settings in the region, indicating that mussels may be used to trace patient outbreak strains of NoV circulating in polluted environmental waters. This study also showed that the RNA extraction procedures and nested NoV detection systems could be used to trace human NoV strains in filter feeding bivalves, preparing the way for subsequent environmental and outbreak studies of NoV in mussels and oysters.

#### **4.3 Detection of human NoV strains in mussels growing near sewage effluents (Paper I)**

The source of the NoV strains detected in environmental studies of river water and of bivalves is understood to be human, from wastewater effluents, land runoff of excrement during heavy rainfall, illegal wastewater discharge such as sewage dumping overboard, or sewage flooding [329–332]. The bivalve studies were designed to extend information on prevalence of NoV in environmental waters, to correlate NoV strains detected in the aqueous environment with outbreak strains that circulate in the population, and indirectly in the wastewaters. This was highly relevant in a year

disrupted by widespread community outbreaks of NoV infections linked to the February confectionary outbreak, and the recreational waterborne outbreaks of August 2004, Table 1, (Paper I).

#### **Recreational waterborne outbreaks:**

The summer of 2004 was notable for heavy rainfall at the height of the tourist season. A sudden change in conditions to extremely hot weather precipitated mass bathing expeditions to inland lakes close to the city of Gothenburg. During August complaints were received from the public about bathers vomiting on the shores and unsanitary conditions around the public toilets [257]. Symptoms of diarrhoea and vomiting were reported from bathers, approximately 400, who had been swimming and playing in the shallow waters of lakes Delsjö and Aspen in the Lerum area (Figure 10). As Lake Delsjö provides the reserve city water supply, further bathing was banned, and chlorination procedures were immediately increased at the city WTP.

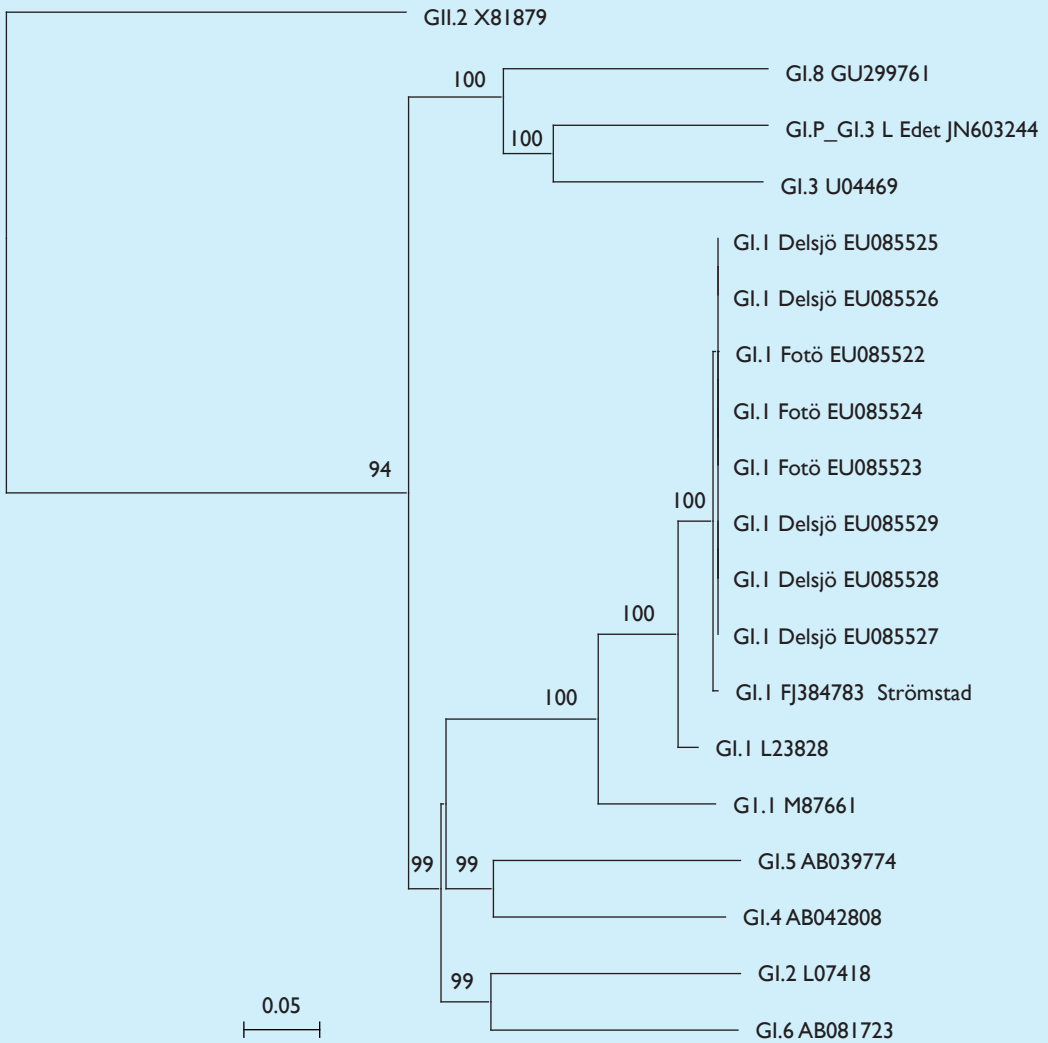
Patient faeces samples (n = 37) were examined in gel-based nested RT-PCR for detection of the NoV RdRp region of the genome. A battery of primer pairs, NI/NVp110, NVp36/NVp110, N69/NVp110 in individual master mixes, was used in 1st round RT-PCR, followed by nesting with primers JV12Y/JV13I. Nucleotide sequencing of the 320 bp products amplified confirmed NoV GI infections in the bathers. Comparative nucleotide sequencing studies characterized the NoV strains detected in 9 of 19 Delsjö bathers as NoV GI.1. In contrast, strains from 17 of 18 Aspen bathers sequenced as NoV GI.3. One child showed a weak positive reaction for NoV GII, and *Campylobacter spp* was isolated from his faeces. Microbiological examination of Delsjö and Aspen waters showed *E. coli* levels of 100 most probable number (MPN)/ml and 120 MPN/ml on different days, indicating fresh faecal contamination of the waters; an unrelated NoV GII strain was detected in Delsjö water examined at the SMI laboratory [257].

#### **November mussel studies:**

The November 2004 bivalve study was carried out to monitor and characterize NoV in bivalves growing close to sewage effluents following foodborne NoV GII.b infections in February, and waterborne outbreaks of GI in August 2004. Mussels (n=40) were collected from Fotö, close to the plume from the Rya WWTP (Table 1). Mussel glands were examined for presence of NoV by using the gel-based nested RT-PCR detection system for the NoV RdRp region (Paper I). Sequencing of the amplified product (328 bp) confirmed the presence of NoV in 23 of 40 mussels (57%). NoV GI.1 strains were detected in 18 mussels, with one strain of GI.2 detected in another. Four other mussels showed the presence of NoV GII strains, confirmed on sequencing as GII.2 (n=2), and GII.4 (n=2). NoV GI.1 strains from November mussels showed

100% sequence similarity (285 nt) to the Delsjö patient strains of NoV GI.1, good correlation across the short RdRp region (Figure 13). The GI.3 Aspen strain, however, was not detected in the mussels. The absence of NoV GI.3 Aspen strains from November mussels was somewhat surprising, but may be explained by the greater speed of river flow in the River Göta than in its tributary, Sävån from Aspen, by distance and dilution factors, and the alternative wastewater disposal in the Lerum area in 2004 (Figure 10).

NoV GI.1 strains from mussels and from Delsjö patients were further characterized by sequence analysis of the long product amplified from the RdRp-N/S major-capsid coding region of the genome (3.1 kb). The sequence similarity (99%, 3082 nt) of NoV GI strains from mussels and the Delsjö patients was high. The NoV sequences were most similar to GI.1 reference sequences, and showed unique GI.1 deletions in capsid amino acids compared with sequences from other GI genotype strains (Paper I). Delsjö patient strains and the mussel strains showed 96% similarity (2516 nt) to GenBank reference strain L23828, SRSV-KY-89/89/J, a patient strain from an oyster outbreak in Japan, 1989, and 86% similarity (3082 nt) to the original Norwalk strain M87661, associated with a school outbreak and contaminated well water (Figure 14).



**Figure 14.** Neighbour-joining tree based on analyses of sequences from the RNA-dependent RNA polymerase-N/S capsid-coding polyA-3' region (3.1 kb) of NoV strains from five patients infected in Delsjö waterborne outbreak, Aug 2004; three mussels from Fotö, Nov 2004; and one Strömstad patient who developed gastroenteritis after eating oysters, Jan 2007. Relevant GenBank reference strains are included, where GI.3 reference (U04469, 3kb) and GI.1 (L23828, 2.5 kb) are of shorter length. The bar shows genetic distance per nucleotide per site per year.

The high sequence similarity (99%, 3082 nt) of NoV GI.1 strains from mussels and patients provides strong molecular evidence of the tracing of clinical NoV strains in environmental waters (Paper I). The molecular and phylogenetic analyses indicate bioaccumulation of NoV strains of human origin in mussels growing near the plume from communal wastewater effluents, and mirror the circulation of GI.1 outbreak strains of high sequence similarity to those detected in the Delsjö waterborne outbreak. Although GI infections and mixtures of NoV GI and GII strains have been implicated previously in outbreaks associated with shellfish consumption and waterborne infections [333–335], few studies have succeeded in demonstrating the long stretches of NoV GI.1 genome (3.1 kb) that were found in Fotö mussels in November following the Delsjö waterborne outbreak [257, 284]. Ueki, however, in a Japanese study based on phylogenetic analysis of 241 nt of the NoV capsid gene, showed that NoV strains from patients at the Matsushima city hospital were traceable in river water, wastewaters, and oysters growing close to the estuary flowing into the Matsushima bay [185]. This Japanese study supports the underlying concept of this thesis that bivalve and wastewater studies can be used to trace the dispersal of human NoV outbreak strains in communal wastewaters.

Evidence of the detection of NoV strains in environmental settings such as water or bivalve samples tends to be limited to short sequence information ( $\leq 300$  nt) and the question remains are the NoV RNA signals found in bivalves, or other environmental samples, representative of potentially viable, infectious NoV [185, 200, 325, 336–338]. This recurring question cannot be readily answered apart from volunteer studies. However, detection of long or full-length NoV genomes is understood to indicate the presence of intact infectious NoV virion in environmental samples, as free viral RNA is particularly susceptible to RNase hydrolysis activity [198, 305, 306]. Long template PCR assays have been used to estimate the inactivation of NoV and of poliovirus in the chlorine disinfection of water [114]. Detection of long stretches of NoV GI.1 RNA (3.1 kb) in Fotö mussels collected in November 2004, provides molecular evidence of the stability of human NoV GI strains in marine and estuarine waters, the bioaccumulation of human NoV GI.1 in bivalve digestive glands, and indirectly reflects the circulation of outbreak strains in the communal waters (Paper I). In agreement with this hypothesis the NoV GI.1 strains from wild mussels growing close to the plume from Rya WWTP showed high nucleotide similarity ( $> 99\%$ , 3085 nt) to the NoV GI.1 strains detected in bathers infected during the recreational waterborne outbreaks of August 2004, and 96% similarity (2516 nt) to the nearest GenBank reference strain L23828, SRSV-KY-89/89/J from Japan. These findings indicate the environmental and geographical spread of human NoV strains in the plume from wastewaters, and the stability of human NoV in environmental waters.

The sentinel studies of wild mussels growing near sewage effluents suggest different temporal patterns of bioaccumulation in bivalves. Mussels can grow under a broad range of environmental conditions tolerating salinity of 0 ppt to 31 ppt, and

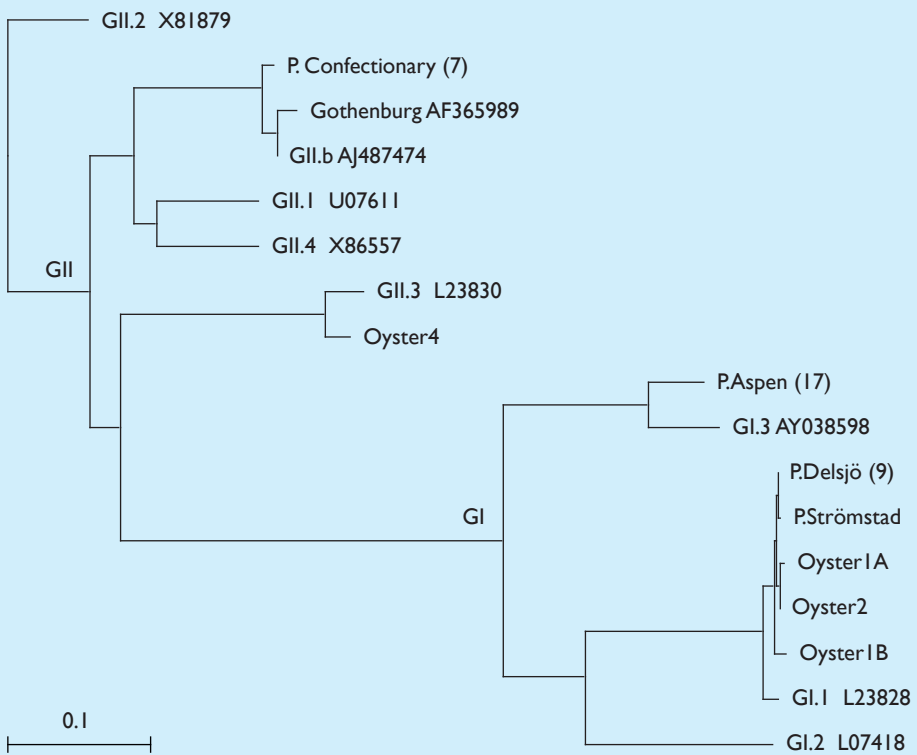
temperatures up to 25°C [339]. Although growth rate is reduced at low temperatures, mussels survive temperatures below zero and significant growth may still occur in cold waters between November and April where food availability is high [339]. In a 2-year study of bivalve mollusc growing areas Lowther et al. (2012) showed that the levels of NoV concentration in oyster tissue increased markedly in the cold months with lower air temperature [193]. The human NoV GIIB strains predominating in March mussels, 2004, appear to reflect the seasonal NoV outbreaks of 2003–2004 and earlier, as the 2001 Gothenburg strain (GenBank accession no. AF365989) places on the same cluster of the tree with the foodborne confectionary outbreak strain (Figure 13). In contrast, the strains detected in mussels collected in November 2004, at the beginning of the 2004–2005 season, reflect the late summer outbreaks of NoV GI.1 recreational waterborne infections (Paper I). Similar findings have been noted in studies of NoV, SaV, and HAV strains from wastewaters and clinical settings [186, 340]. The Fotö mussel studies indicate that NoV strains found in mussels growing near sewage effluents mirror the shifts in NoV strains circulating in communal wastewaters, and can be traced back in time to patient outbreak strains (Paper I).

#### 4.4 Molecular analysis of an oyster outbreak (Paper II)

The NoV contamination detected in oysters that were held sunk for three weeks in Strömstad harbour prior to the Strömstad restaurant outbreak provides further evidence of the stability of the potentially infectious NoV GI strains in marine waters (Paper II). This oyster-related outbreak confirms the bioaccumulation of human NoV GI strains in bivalves growing in contaminated waters, and the transmission of NoV GI from contaminated bivalves to healthy individuals. The NoV outbreak that affected Strömstad (population 11,572) in 2007 echoes Roos' early classic epidemiological study of hepatitis A infections caused by oysters sunk in corves in the contaminated waters of Havstensund harbour, 1955 [318]. *Sumping* of oysters in harbour waters was a long-standing tradition with local oyster fishermen and the NoV oyster outbreak in Strömstad, January 2007, appears to have been the result of the same malpractice.

The Strömstad outbreak was a typical shellfish outbreak. Of 100 guests, only those who ate the raw oysters were affected. Of 30 guests in the party who enjoyed the oysters but subsequently suffered from acute gastroenteritis, only one individual provided a faecal sample for laboratory examination. Indeed, and perhaps naturally, healthy individuals tend to be reluctant to offer samples during the acute or convalescent period, particularly when they have been invited guests. In contrast to many other shellfish outbreaks, however, 5 unopened oysters from the batch consumed, were retrieved from the restaurant kitchen. NoV GI.1 was detected in the one patient faeces sample available for laboratory examination. This NoV GI.1 patient strain was characterized by nucleotide sequencing of a 3.1-kb product spanning the RdRp-N/S

major capsid-coding region of the genome (Paper II). NoV GI.1 (285 nt) was detected in 2 oysters, and NoV GII.3 (285 nt) was identified in a third, reliable molecular evidence that the oysters were contaminated with human faeces (Figure 15). In the case of the Strömstad outbreak the NoV GI.1 strain from the single patient sample showed  $\geq 99\%$  similarity (285 nt) to the GI.1 strains detected in the two oysters, evidence linking the oysters to the acute gastro-enteric infections experienced by the oyster-eating guests.



**Figure 15.** Neighbour-joining tree based on analyses of the partial NoV RNA-dependent RNA polymerase-coding region (285 nt). Four NoV sequences obtained from three of the Strömstad oysters are defined as Oyster IA and Oyster IB from one oyster, Oyster 2, and Oyster 4. P represents sequences from patient strains linked to outbreaks Strömstad, Delsjö, Aspen, and Frölunda Confectionary. (n) indicates number of sequences analysed and shown to be identical. Genotype subgroups are denoted on branches besides GenBank accession numbers of reference strains. Genetic distance per nucleotide per site is shown by the bar.

Moreover, comparative sequence analysis of the NoV GI.1 strains from the Strömstad patient, from patients affected in the Delsjö recreational waterborne outbreaks, and from November mussels 2004, indicated high similarity (> 99%, 3085 nt) across the RdRp-N/S major capsid-coding region (Figure 14).

The genomic similarity of NoV GI.1 strains from the Strömstad outbreak patient, the Delsjö bathers, the Fotö November mussels, and the Strömstad oysters is striking. As noted the NoV GI.1 strains detected in patients affected in the Delsjö waterborne outbreak of 2004, and in the patient from the oyster outbreak Strömstad, 2007, revealed > 99.0% similarity across 3085 nucleotides of the complete major capsid-coding region. This shows high similarity between outbreak strains of NoV GI.1 over a 2 to 3 years time difference. Further, the NoV GI.1 strain from the Strömstad oyster-eating patient showed 96% nucleotide similarity to the nearest GenBank reference, NoV GI L23828 (SRSV-KY-89/89/J) originally detected in an adult male affected during an oyster outbreak in Japan, 1989. The 96% nucleotide similarity between the Strömstad and Japanese strains indicates high similarity between NoV GI.1 strains world wide, and remarkable nucleotide stability of NoV GI.1 over time, 18 years. Moreover, comparative sequence analysis of the Japanese strain L23828 from 1989, and the original Norwalk Ohio 1968 strain, M87661, shows 87.2% nucleotide similarity, amounting to 96–98.9% amino acid similarity [107]. This genomic stability of NoV GI.1 strains over a time interval of more than 20 years is clearly shown in the molecular, comparative sequence and phylogenetic analyses of the GI.1 strains from these different patient outbreaks, and from the bivalve studies (Papers I and II). This characteristic of high similarity between NoV GI.1 strains contrasts most markedly with the highly variable nucleotide sequences detected in GII viruses, particularly the seasonal GII.4 outbreak strains described in the nosocomial report (Paper IV).

The Fotö and Strömstad findings are consistent with the huge capacity of bivalves to filter water, whether polluted waters from the plume of effluents close to Fotö, or the suspect contaminated waters of Strömstad harbour. As filter feeders, mussels trap natural phytoplankton and organic particles including viruses from the water that passes over the fine intricate net of their gills in the processes of respiration and feeding. The filtration rate depends on a range of factors including bivalve size, water temperature, and concentration of suspended particles but in favourable conditions one mussel can filter 2–3 litres of water per hour, or 1 kg of mussels can filter 100 litres of water [44]. Therefore, the filtration and concentration capacity of mussels may give them a sentinel role in the monitoring of viruses in wastewater effluents and estuarine waters. This may allow detection of previous virus dispersal in *compact* time; our finding of outbreak-related strains suggests that NoV detection in mussels reflect recent events. This concept is well illustrated in the bivalve studies described in Papers I and II.

The environmental and outbreak settings encountered in the epidemiology of NoV

infections are numerous, and often unexpected. Occasionally the settings seem familiar, recurring situations where close parallels can be drawn with past outbreaks. This was the case in the Strömstad outbreak (Paper II) where the practice of using corves sunk in harbour waters to maintain oysters over the festive season had re-emerged, despite the problems recognized many years before by Roos [318]. Yet the Strömstad oysters were collected in a clean area as defined by European regulations. But holding them in the guest harbour over the festive season was bad practice, and is forbidden according to the European regulation 54/2004/EC. Similar incidents occurred in Bari, Italy, where shellfish held in sweat tanks for long periods of time were implicated in large outbreaks of HAV infections in 1996 [341]. Lack of understanding of the reasoning behind the rules and regulations indicates an information gap that requires better education and regulation of the fisherman and his produce.

The recurring problems of oyster-associated outbreaks of NoV, HAstV, and HAV infections are difficult to accept in a region where shellfish are considered a delicacy and an enrichment of the culinary culture [342]. Challenging problems have to be met in the regulatory control of growing sites, control of discharge of sewage at sea, and education on the handling of bivalve molluscs at each point along the route from fishing to eating. Regulatory controls are needed for the control of sewage discharges from sea traffic of all size scales, but may be difficult to apply, and compliance may be poor.

Yet no two outbreaks are exactly alike; every outbreak situation is unique. Unrecognized or unexpected outbreak settings may arise which require intensive epidemiological and laboratory investigations before the point source and routes of transmission can be traced or understood, requiring an open-minded approach.

#### **4.5 Outbreak linked to Japanese oysters – mixed Sapovirus and Norovirus infections**

Outbreaks of viral gastroenteritis may be multifactorial events where identification and significance of viral transmission routes is difficult to assess when sewage contaminated water, bivalves and person-to-person spread may be implicated. The outbreak of acute gastroenteritis that affected students at Tjärnö marine station, University of Gothenburg, in February 2008, was complex. The local pump house serving the marine station had broken down, the Japanese oysters (*Crassostrea gigas*) consumed were collected from surrounding waters, the dining room kitchen and restaurant were under renovation, one student opened the majority of the oysters, and several students had been affected in the previous weeks by the seasonal *winter-vomiting* infections (Table 1).

Mixed SaV, NoV GI, and NoV GII infections were detected in students who developed symptoms of severe gastroenteritis following an oyster-eating evening where raw Japanese oysters collected from waters around the marine station were con-

sumed. Of 24 students, 16 ate raw oysters. One male student opened the oysters for the oyster eaters. Approximately 36 hours after consuming the oysters the students complained of acute symptoms of stomach pains, vomiting, nausea, headache, muscular pains, light fever and diarrhoea, and tiredness. Two female students described milder symptoms of exhaustion and poor appetite, but no vomiting and diarrhoea. The eight students who did not eat oysters were not affected.

Eleven acutely ill students provided faecal samples. Eight samples tested positive for *caliciviruses* when examined in rRT-PCR detection systems for NoV GI, GII and SaV I, II, and IV. Three samples were negative. The sample from the student who acted as chief oyster shucker and who ate the greatest number of oysters ( $n = 20$ ), showed a mixture of SaV and NoV GII. Single SaV infections were found in samples from 4 students. These SaV strains sequenced as SaV GI. 2 ( $n=2$ ) and SaV GII.1 ( $n=2$ ). NoV GI infection was detected in 2 students, and NoV GII in one other. The two female students with milder symptoms, but no vomiting or diarrhoea had recovered from an episode of seasonal *calicivirus* infection approximately 3 and 9 weeks prior to the Tjärnö outbreak. The faecal sample from the first of these two students showed infection with SaV GII.1, the second student tested negative for NoV GI, GII, and SaV. The median Ct value for SaV in the faecal samples was 24.3, interquartile range 2-25.

The outbreak occurred at the height of the *winter vomiting* season in February 2008 when GII and SaV infections were implicated in nosocomial and seasonal widespread community outbreaks (Virus Laboratory reports 2007–08), [343, 344]. No viruses were detected in oyster or water samples collected from waters around the marine station, near the pump station, where the suspect oysters were collected. Samples of depurated oysters from the collection area were also negative for NoV. Negative bivalve findings are common in shellfish outbreaks and detection of viruses in shellfish outbreaks is often limited to detection in the patient samples. The oysters examined are rarely representative of those consumed, and virus detection may be complicated by co-extraction of enzyme inhibitors in the NA preparation of oyster tissue. Mixed SaV, NoV and enteric virus infections have been reported, however, in patients affected by gastroenteritis following consumption of oysters in Japan [345]. SaV has been detected also in commercially packed oysters, and in environmental studies of oysters, wastewaters and estuarine waters in Japan [346, 347]. Wastewater studies indicate a high all year round concentration of SaV in incoming effluents as compared to the more seasonal fluctuations associated with NoV GI and GII [185, 348]. Infections with mixtures of NoV strains tend to be a common finding in outbreaks where sewage contaminated bivalves are implicated as point source of infection, and are also detected in waterborne outbreaks caused by sewage polluted drinking water (Paper III), [38, 333–335, 349]. Possibly, the dominance of SaV findings in the student samples reflected the seasonal prevalence of SaV gastroenteritis detected in 2007–2008.

For the students in the Tjärnö incident symptoms were quite marked, but resolved

within a few days. The variety of strains found in their samples, however, resembles the remarkable mixtures that affected diners and staff at a renowned restaurant in the United Kingdom, 2009, one of the most infamous NoV outbreaks recorded [42]. Although SaV was not implicated, the long-drawn-out wave of mixed viral infections is typical of the complexity of shellfish-derived outbreaks, and of the diversity of NoV strains encountered when contaminated bivalves appear to be the point source of an outbreak. Multiple NoV GI and GII strains including GII.3, GII.4 and GII.6, were detected in 10 diners, and in subsequent batches of oysters obtained from the growing area. This is the typical pattern for a bivalve-associated outbreak [49]. Moreover, food handlers involved in preparation of elaborate and exotic razor clam *soud of the sea* shellfish dishes, and waiters serving the public, were also infected, predominantly with NoV GII strains of II.2, II.4 and II.6 [42]. The mixed infections and variety of NoV strains detected in restaurant diners, and in staff, resemble the bivalve outbreak at Tjärnö and the Lilla Edet waterborne infections described in Paper III.




The Tjärnö and U.K. oyster restaurant outbreaks contrast markedly with the Frölunda food borne infections transmitted by the confectioner, where a single genotype of NoV GII.b was traced back to the food handler. But the poor standards of hygiene, and lack of adherence to food regulations showed similar negligence or disregard for standards required in the food industry [288]. The need for education of the public and restaurant managers on the importance of rapid notification of a food associated outbreak to public health authorities was underlined in the U.K. report [42].

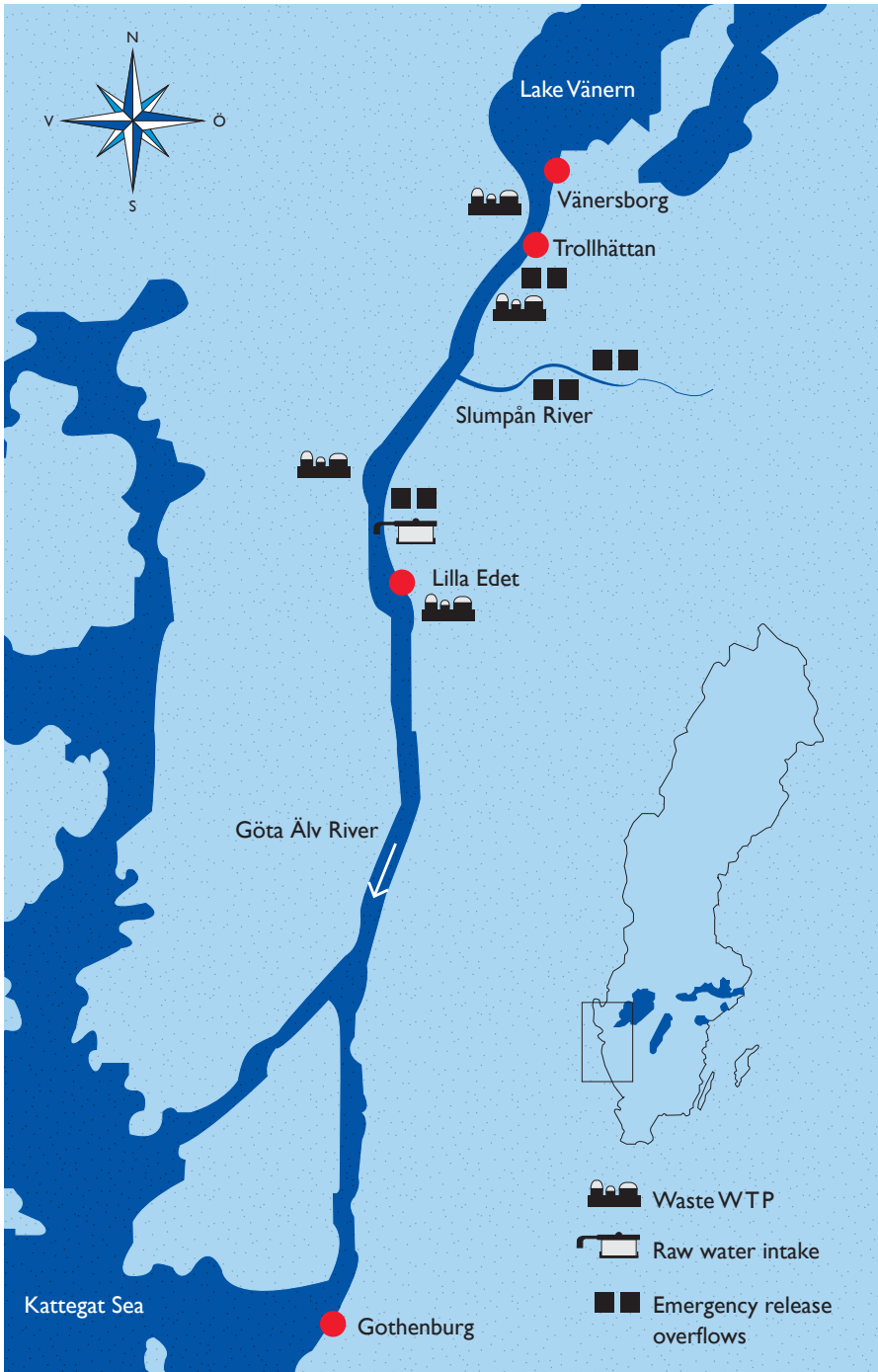
#### **4.6 Waterborne infections and norovirus diversity (Paper III)**

Waterborne outbreaks are often multifactorial events associated with a combination of heavy rainfall, early release of untreated wastewaters, and disinfection failure. Sudden change in conditions can overwhelm the drinking water treatment system where altered treatment processing, technical breakdowns, and severe weather conditions are common risk factors [350, 351]. Flash flooding, heavy rainfall, overflows, runoff from melting snow, high temperatures, contamination from upstream incidents, and human errors can precipitate an outbreak [74, 352–355]. High temperatures and heavy rainfall in the 30 days preceding waterborne outbreaks caused by surface water contamination are commonly reported [355, 356], just such conditions held for the Lilla Edet outbreak (Table 1), (Paper III).

### *The outbreak*

The remarkable diversity of NoV GI strains that may circulate in the general population during late summer is demonstrated by the waterborne outbreak that affected Lilla Edet (population 13,000), September 2008, when extreme weather conditions precipitated contamination at the municipal water treatment plant (WTP), Figure 16 map, (Paper III).

**Figure 16.** Map of River Göta, showing location of Lilla Edet, Trollhättan, Vänersborg, and Gothenburg, the upstream wastewater treatment plants at Lilla Edet, Trollhättan, Vänersborg are denoted as Waste WTPs . Raw water intake at drinking water treatment plants are indicated . Emergency releases are shown as .



The rapid medical and public health response in issuing boil water orders and ensuring increased chlorination procedures at the drinking WTP helped to bring the outbreak under control as indicated in the epicurve, but 17 days elapsed before boil water orders could be lifted [32]. The epicurve showed sudden onset of infections that could not be correlated with consumption of bivalves, or a common food (Figure 17A). The spread of infections in areas served by the municipal water supply, but not affecting private well owners, or private drinking water plant users, indicated problems at the municipal WTP. This evidence was strengthened by reports that members of a visiting football team drank water, but ate no food during their short visit to Lilla Edet. Only those who drank tap water fell sick [32]. Further, the questionnaire-follow up indicated that symptoms of gastroenteritis correlated with the number of cups of water consumed [32].

The Lilla Edet epicurve showed a protracted course (Figure 17A). This could be due to secondary spread from primary cases, in parallel to what was described for the original Norwalk outbreak where primary and secondary cases were registered (Figure 17B), [54]. During Lilla Edet outbreak the CMO, in daily media contact with the public, re-emphasized the need to boil all drinking water. However, later cases of infection could also be explained by direct transmission from the environment, the drinking water, if the public did not comply carefully and promptly with initial boil water orders. In a study of 74 outbreaks with waterborne, foodborne or person-to-person transmission, outbreaks generally ended in about one week although secondary person-to-person transmission was common [7]. As Kaplan explained longer outbreaks occur only when new groups of susceptible persons are introduced, usually in the setting of a persistent common source of infection [7].

**Figure 17A.** Lilla Edet community outbreak, 2008. Epicurve shows the distribution of 379 cases with acute gastroenteritis reported by date of onset of symptoms through a questionnaire survey. Municipal and private water household users are indicated. Adapted with permission from [32, 122].

**Figure 17B.** Norwalk, Ohio, 1968. Outbreak in an elementary school associated with well water. Epicurve shows the distribution of primary and secondary cases over time. Adapted with permission from [54].

Figure 17A.

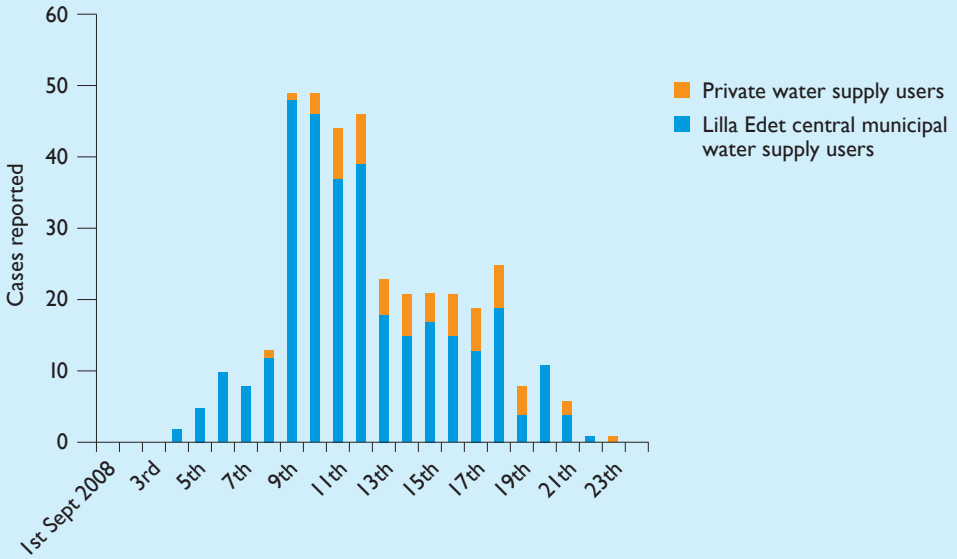
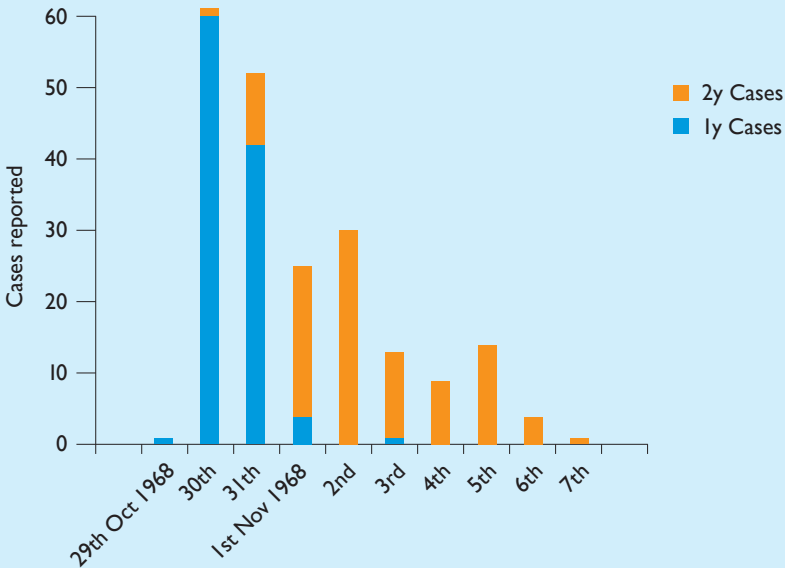
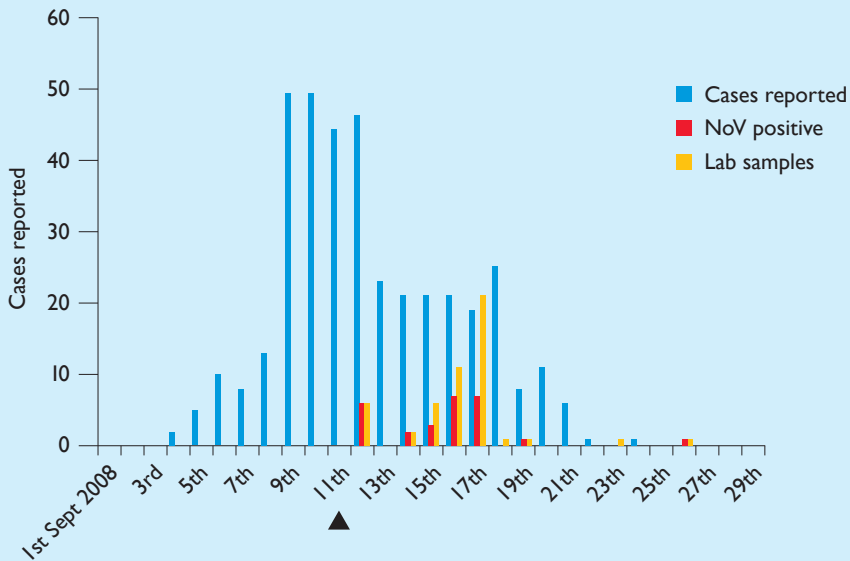


Figure 17B.



Stormy weather, upstream overflows caused by technical breakdowns, combined sanitary overflows, and disinfection problems relating to chlorine contact times in the presence of heavy turbidity in recycled river waters, added to the difficulties at the municipal WTP (Figure 16), [258, 259]. NoV was not detected in water samples examined at the Virus Laboratories, SMI, Sweden, although coliphages were demonstrated in raw and drinking water from the distribution network on one sampling day [32]. No bacterial indicators of faecal contamination were detected in the drinking water sampled. However, the difficulties of catching a water sample at the right time-point in the course of a short contamination event following a sudden burst of heavy faecal contamination are recognized [49, 203, 352, 357].



**Figure 18.** Lilla Edet epicurve indicates the distribution of 379 cases with acute gastroenteritis by date of onset, issue of boil water recommendations (black arrow), and positive Norovirus GI findings (red) in incoming patient samples (orange). Adapted with permission from [32, 122].

### *Virological findings*

Initial laboratory examination of Lilla Edet patient samples included rRT-PCR assays for the major human enteric viruses: NoV GI, NoV GII, SaV GI+II+IV+V, RVA+B+C, HAstV, and HAdV (Paper II), [260]. NoV GI was detected in the first five samples received as shown in Figure 18 and Table 2.

**Table 2.** Norovirus detection in first five patient samples received for virus analyses in real-time RT-PCR.

Patient	Age	NoV rRT-PCR <sup>a</sup>	Ct value <sup>b</sup>	NoV genotype <sup>c</sup>
P1	27	NoV GI	31	NoV GI.4
P2	57	NoV GI	27	NoV GI.9 <sup>d</sup>
P3	61	NoV GI	27	NoV GI.3, GI.4 <sup>e</sup>
P4	52	NoV GI	30	NoV GI.7
P5	40	NoV GI NoV GII	25 33	NoV GI.4, GI.7 NoV GII.6 <sup>e</sup>

<sup>a</sup> NoV genogroup specific real-time reverse transcriptase PCR

<sup>b</sup> Ct cycle threshold value, cycle number at which fluorescence signal crosses threshold, correlates inversely with initial template concentrations

<sup>c</sup> Genotype confirmed on results of sequencing and, or, cloning studies

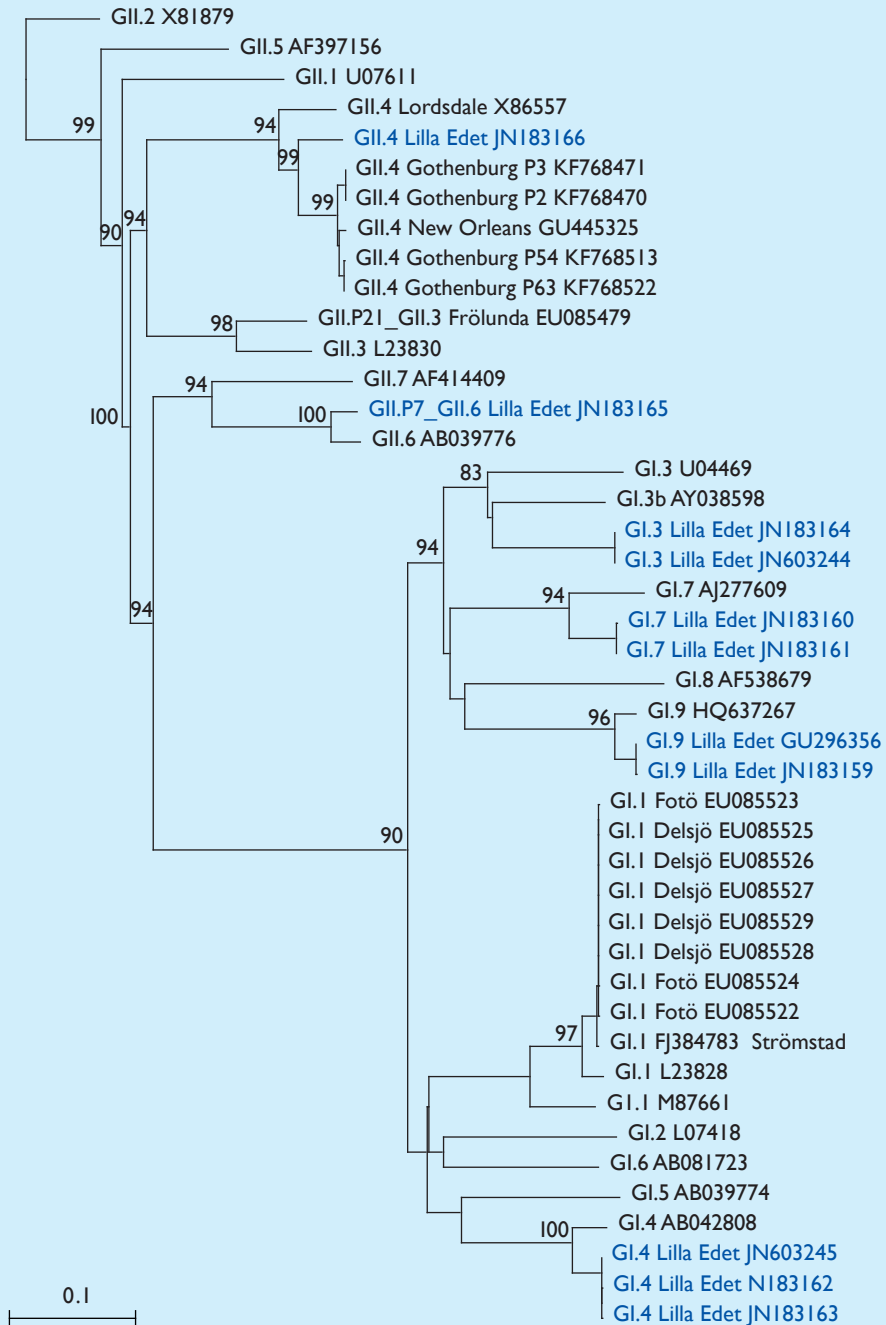
<sup>d</sup> GI.9 new genotype

<sup>e</sup> Genotype mixes

These positive NoV GI results were reported to the medical authorities within 5 hours of receipt of samples, a rapid response made possible by the use of rRT-PCR detection methods. A total of 50 patient samples were examined for enteric pathogens following local media appeals for faecal samples from affected individuals. NoV GI strains were detected in samples from 31 patients, including mixed GI strains in 5 patients. Stray cases of NoV GII (n=3), SaV (n=1), RV (n=3), HAdv (n=1), *Campylobacter spp.* (n=2), but no parasites, were detected (Paper III). Nucleotide sequencing confirmed a predominance of diverse NoV GI strains; GI.3, GI.4, GI.7 and a new strain, proposed GI.9. The diversity of NoV strains detected in the Lilla Edet outbreak is shown in the phylogenetic analysis of representative sequences obtained from patient samples (Figure 19).

**Figure 19. Diversity of norovirus strains detected in Lilla Edet outbreak**

Neighbour-joining tree of norovirus strains from Lilla Edet (L Edet) outbreak showing the diversity of strains detected in patient samples. Sequences are 2.1 kb long starting at nt 4800 (GenBank reference strain M87661). Norovirus strains from Frölunda, Gothenburg, Fotö, Delsjö and Strömstad, from the various outbreak and environmental studies are shown. Adequate GenBank reference strains are included. The tree is rooted with X81879, a NoV GII.2 reference strain. Distance bar indicating the number of nucleotide changes per site is shown.



Molecular cloning of the major capsid-coding gene (ORF2) was used to confirm the nucleotide sequence of the new NoV GI.9 strains detected, and to identify individual strains in mixtures of NoV GI found in 5 patients. Cloning is an essential step in identification and sequencing of individual NoV strains in mixed viral infections. Cloning is also required to confirm the sequence of new genotypes, although classification of noroviruses below the group level is unresolved as yet [85, 90]. To date, molecular information on NoV GI genotypes is limited to nine genotypes based on the classification system proposed by Zheng *et al.* (2006), [90, 358]. In this classification genogroups and genotypes are defined on the basis of percentage pair-wise distance ranges across the amino acids of the major capsid-coding region (ORF2). NoV genogroups show percentage distance ranges over the capsid amino acids of between 44.9–61.4% (inter-genogroup range), while genotypes show a range of 14.3–43.8% (inter-genotype range), and strains within a genotype may show 0–14.1% (intra-genotype) [90].

The Zheng classification requires comparative sequence analysis of the complete nucleotide sequence of the major capsid-coding region (ORF2) of the NoV strain being examined with NoV GenBank reference strains. One of the NoV GI genotypes detected in samples from three Lilla Edet patients was a new genotype proposed as NoV GI.9 on the basis of 26% amino acid dissimilarity from all other NoV GI genotype reference strains (NoV GI.1–GI.8), and on cloning of the major capsid-coding region (Figure 19), (Paper III). Lilla Edet strains (accession numbers GU296356, JN183159) showed high similarity (96–98%) to two strains in GenBank; one of these strains, accession number HQ637267, is now designated as GI.9, [84].

Cloning studies also confirmed detection of NoV GI.3 strains that placed on a new sub-branch of the NoV GI.3 cluster, showing 13 to 15% dissimilarity to GenBank reference strains. These GI.3 strains were found in three of the Lilla Edet patients.

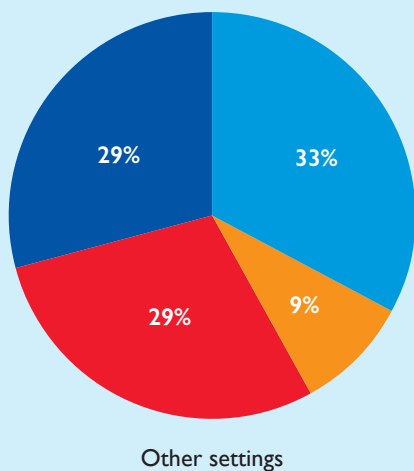
The molecular epidemiology of NoV strains detected in patients during the non-seasonal Lilla Edet waterborne outbreak provides detailed information on the variety of GI strains circulating in the community and in wastewaters in late summer, 2008 (Figure 19). The diversity of GI strains detected in all age groups was remarkable: NoV GI.3 (n=3), GI.4 (n=21), GI.7 (n=9), and GI.9 (n=3), with a scattering of NoV GII.6 (n=2), GII.4 (n=1), and SaV II.2 (n=1), where (n) indicates the number of patients infected with the given strain (Paper III). Yet the prevalence of NoV GI strains detected in community and in hospital outbreaks where person-to-person transmission may be anticipated, tends to be low, except in the context of waterborne, bivalve or foodborne transmission of infections where the significance of GI, and mixed GI and GII infections is recognized [203, 308, 349, 359–361], (Paper II and III). This adds to the paradox as the source of most waterborne or shellfish contamination is assumed to be from sewage wastewaters of human origin [74, 362].

### *Prevalence of NoV in the community*

Lilla Edet waterborne outbreak raises questions on prevalence and significance of NoV GI and GII infections in the community. The source of NoV GI contamination in the Lilla Edet outbreak remains an enigma. Although sewage contamination of the municipal drinking supply was the apparent explanation of the non-seasonal outbreak, there was no laboratory or epidemiological evidence of ongoing NoV infections in the region over the summer months of 2008. Nor was there an obvious prevalence of GI infections in the spring of that year, when SaV and NoV GII infections dominated in seasonal outbreaks, 2007–2008. The combined role of asymptomatic and symptomatic NoV excretion from long-term virus shedders in the healthy community, and from severely affected hospital patients or outpatients, may contribute to the levels of NoV circulating in communal wastewaters. Although speculative, these sources may be implicated indirectly in waterborne infections caused by sewage-contaminated drinking water.

But in the case of the Lilla Edet outbreak these were widespread NoV GI waterborne infections. Few, if any, studies have explored the contribution or significance of GI infections in asymptomatic NoV, although the link between NoV GII and asymptomatic infections is recognized [2, 240]. Underreporting may affect the level of sporadic and endemic NoV GI and GII infections recorded in the healthy community, where recovery is rapid and uncomplicated [363, 364]. In the absence of a community outbreak with specific cause such as food, bivalves, or drinking water, affected families may not contact primary care or public health authorities unless a young child or elderly member develops severe illness [363]. Sensitivity of laboratory tests may also affect reporting, some studies indicate that NoV GI infections multiply to lower viral load compared to GII, although caution is advised in interpretation as the GI and GII rRT-PCR systems may not be of equivalent efficiency [266, 365]. However, examination of the Lilla Edet outbreak epicurve suggests limited spread of GI infections within the family and the community once boil water orders were introduced and the contamination event at the drinking water treatment plant was brought under control (Figures 17A, 18).

The marked diversity of NoV GI strains detected in Lilla Edet outbreak suggests a background endemic circulation of NoV GI strains in the community. A study of NoV diversity in strains co-circulating in the north of England, 1998 to 2001, showed 29% prevalence of NoV GI in sporadic cases of gastroenteritis in healthy adults and children in community outbreaks (Figure 20) as compared with 4% in hospital settings, and 12% in nursing homes (Figure 9), [235]. Moreover, mixtures of three NoV GI strains, GI.2, GI.3 and GI.4 were detected in September 1999.



**Figure 20.** Prevalence of NoV strains detected in community outbreaks of gastroenteritis during a study of NoV strains cocirculating in northern England, 1998–2001. NoV GI infections are indicated in dark blue; GII.4 (Grimsby) in light blue; GII.1 (Girlington) orange; and other GII strains (red) in outbreaks 1998–2001. Adapted with permission from Gallimore et al. 2004 [235].

Gallimore et al. (2007) noted that inter-seasonal genotyping of NoV outbreak strains showed greater diversity of NoV genotypes in September, at the beginning of seasonal outbreaks when compared to strains circulating towards the end of the outbreak season in the following spring, when NoV GII.4 strains predominated (> 90%) [366]. The recreational waterborne outbreaks in August 2004, and the Lilla Edet waterborne outbreak in September 2008, suggest a prevalence and spread of GI strains in the healthy susceptible community during late summer. These non-seasonal waterborne outbreaks strengthen indices of a background endemic circulation of NoV GI strains in the population that may be masked by GII.4 or GII.3 nosocomial infection reporting during winter seasonal outbreaks. Apparently, NoV GI infections are more common in the community than is indicated by hospital orientated surveillance statistics [235], and, or, current sequence-based systems limit the range of norovirus strains being detected (Figure 20). Sequence-based detection systems are only as good as the information available in public gene banks. New techniques may reveal an even greater variety of viral genomes. The genomic diversity and preponderance of NoV GI strains detected in the Lilla Edet outbreak adds to the information on circulation of NoV GI strains in the community and communal wastewaters, and challenges our understanding of outbreak settings.

### ***Contamination from wastewaters and land run off***

Communal wastewaters and land run-off from human or animal waste are major pathogen sources of sewage contamination in drinking water supplies [40, 367]. Human or animal faeces are a microbiota of many different viruses, bacteria and parasites that are discharged into communal wastewaters. Therefore, recycled river waters are susceptible to viral contamination dependent on the epidemiology, incidence, distribution, pattern of infections in the population, and the stability of the virus in environmental waters. Moreover, human enteric viruses NoV, SaV, HAV, HAdV, HAstV, polio, and enteroviruses may persist in effluents from WWTP unless specific disinfection steps such as chlorination, UV, ozone treatment, and membrane bioreactor filtration are included in WWTP processing to reduce viral content prior to release of effluents to recipient waters [348]. Apart from UV treatment, these are costly but necessary improvements in infrastructure with public health concerns for safety of drinking water supplies, and of shellfish growing areas. Investment is slow in the face of environmental changes.

NoV levels in the order of  $10^2$  to  $10^4$  genome equivalents/ml of wastewater are common in incoming wastewaters, even in the absence of clinical symptoms in the general population [340, 368, 369]. NoV GI and GII strains can be detected with a lower but steady prevalence of GI during summer months and throughout the year [340, 370]. Irregular spiking of GI levels has also been described as compared to maintained GII levels throughout the year [369]. NoV GI wastewater tracing contrasts with the higher concentrations of GII seen in samples during winter months when GII levels maximize following the trend of GII dominance in seasonal infections [370, 371]. SaV has been detected in influent wastewaters throughout the year but NoV GI and GII concentrations varied temporally being highest between February and May [348]. Combined wastewater and outbreak studies from Japan indicate that NoV and SaV strains detected in raw sewage reflect the viruses circulating in the community, where subclinical NoV infections are common [340]. Further, the risk of sewage being contaminated with NoV may be linked to the size of population contributing to incoming wastewaters [372]. This is relevant in the Lilla Edet outbreak where extensive overflows were recorded at the upstream WWTP serving Trollhättan (population 46,457) in the days prior to the outbreak (Table 3), [258, 259].

**Table 3.** Overflows from Trollhättan WWTP. Wastewater releases from Trollhättan WWTP 6th Sept ~ 30,000 cubic metres over an 18 h period [259].

Date	Overflow releases (m <sup>3</sup> )
2008-09-04	114
2008-09-05	10 890
2008-09-06	29 819
2008-09-07	12 277
2008-09-08	12 043
2008-09-09	6 028
2008-09-10	7 996

### **Conditions precipitating the Lilla Edet waterborne outbreak**

A quantitative microbial risk model of the Lilla Edet outbreak showed build up of conditions contributing to drinking water contamination at the Lilla Edet WTP [258, 259]. Hot dry summer weather resulted in sedimentation and blockage of sewage in wastewater tunnels during the last week of August 2008 (personal communication, Olof Bergstedt). Sudden change in weather with heavy rainfall caused emergency overflows of untreated wastewaters from upstream WWTPs at Trollhättan and Lilla Edet. Conditions at the Trollhättan plant serving 43000 inhabitants were particularly difficult with combined sanitary overflows and early release of untreated wastewater over a period of 18 h, and technical problems requiring emergency diversion of wastewaters to Slumpån a tributary entering the River Göta, 8 kilometers upstream of Lilla Edet WTP (Figure 16), [373]. In the week before the outbreak heavy turbidity was recorded on the River Göta that supplies six drinking WTPs (approximately 700,000 people) along the riverside. Gothenburg city closed the water intake drawing on the city reserve water supply, but this option was not available for Lilla Edet. Gross turbidity was detected in river water on 6th September with high *E. coli* counts (1200 MPN/100 ml) recorded on 8th September at monitoring points 5 and 40 km downstream of Lilla Edet [375]. These river conditions would have challenged the efficiency of any modern WTP, including the Lilla Edet plant system of aggregation, sedimentation, and filtration. Westrell *et al.* (2003) pinpoint *sub-optimal particle removal or ineffective disinfection as the main risk incidents in water treatment processing* [376]. Failures in the distribution system may also feature in outbreak investigations when accidental cross connections cause microbial contamination of

networks or storage reservoirs [34, 377, 378]. So vigilance, immediate reporting of incidents at treatment plants, and early recognition of warning signals are essential in WTP management.

Land run-off associated with heavy rainfall may have aggravated the situation at Lilla Edet WTP. However, the effects of land run-off on WTP systems are not readily assessed by direct examination of water for viral pathogens such as NoV. Although somatic coliphages were demonstrated in water samples on one day, NoV was not detected in the raw or drinking water samples collected at the Lilla Edet WTP, perhaps because of the difficulties of *flash* contamination. The contaminated water has passed by before the event is recognized [329]. To overcome these difficulties and improve raw and treated water monitoring in areas where WTP are prone to contamination events, a daily or weekly *rolling* sample system is recommended [203]. Detection of NoV in raw and treated drinking water is a challenge not easily accomplished even in specialized laboratories with access to the best molecular tools [379].

The link between land run-off and contamination of the aqueous environment was clearly shown however in the huge outbreaks of bivalve-related NoV gastroenteritis caused by contamination of shellfish-growing areas in Sydney, Australia 1978 and 1990, and New York State, 1982 [48, 362, 380]. Molecular studies of NoV from oysters implicated in international outbreaks also indicate the importance of land run-off following heavy rainfall in contamination of oyster-growing regions in France [187]. Mixed strains of NoV GI.4, NoV GII.4 and GII.8 were detected in oysters, and patients were infected with NoV GI.4, GI.6, GII.4, GII.8, and GII.b strains of high similarity > 98.5% to those found in the shellfish [187]. Several years later outbreaks of oyster-related gastroenteritis were traced to the same growing areas again affected by heavy flooding; mixtures of NoV GI, NoV GII, Aichi virus, HAAdV, and RV were detected in oysters and patients [49]. These oyster studies indicate the dangers associated with human faecal contamination of water following heavy rainfall and land run-off, and resemble the findings of mixed NoV GI strains in Lilla Edet patients where sewage contaminated drinking water, not bivalves, was understood to be the point source of infections (Paper IV).

### **Countermeasures**

The food and waterborne outbreaks described in this thesis indicate the importance of prompt response to incoming reports of gastroenteric infections from the public and the medical health community, and the need for molecular epidemiological investigation for pathogen tracing to reduce widespread infections [381]. Good communications between experts in public health medicine, water and environmental engineering, and microbial diagnostics is essential. Similarly, communication of relevant findings to health authorities and the public is important. Investigation and follow-up studies of outbreak settings should lead to improvements in maintenance

and management of treatment plants, and introduction of countermeasures that may reduce the risk of future incidents.

After the Lilla Edet waterborne outbreak a UV water-treatment system was introduced to improve water quality at the municipal WTP (Paper IV). The completion in 2012 of a tunnel system, operational in 2014, that carries wastewater from Lerum area to Rya WWTP is part of a large-scale plan to improve the quality of the River Göta water, and to reduce the risks when recycled river water is the source of drinking water supplies for large cities and smaller communities. Control of wastewater effluents, introduction of ultrafiltration or microfiltration membranes at raw water intakes where recycled river waters are the source of drinking water should reduce the risks of contamination of drinking water. This was a major concern during the NoV GI outbreaks associated with recreational bathing in Lakes Delsjö and Aspen, and Lilla Edet waterborne outbreak. These are problems of increasing public health and economical concern following local experience and the waterborne cryptosporidial infections in Östersund, 2010 [32, 382]. The effects of climate changes require investment in infrastructure and more research into safe water management.

The waterborne outbreak that affected Lilla Edet was one of the largest NoV outbreaks in Sweden, affecting at least 2400 (18.5%) of 13000 inhabitants, with total costs estimated at SEK 8700000 (~ €0.8) [32]. Rapid detection of multiple and diverse NoV GI strains in the first samples analysed, strengthened indices of a waterborne outbreak caused by NoV, and raised pertinent questions about dispersal of NoV in wastewaters, and recycled river waters.

#### **4.7 Nosocomial infections, NoV GII.4, dust and patient room environment (Paper IV)**

Early epidemiological studies of nosocomial outbreaks emphasize the importance of dispersal of NoV in air and fomites around the infected patient, as well as direct person-to-person or surface-to-hand contact in the rapid transmission of NoV within the hospital community [383]. The explosive onset of vomiting and diarrhoea affected patients and nursing staff, non-medical hospital workers and visitors alike. Similar situations continue to disrupt medical care in hospital settings during nosocomial outbreaks, when aerosolized or airborne virus may be transmitted by vomiting, coughing, flushing of a toilet, or disturbing of dried contaminated faeces or vomitus. Recurring nosocomial outbreaks, where NoV GII.4 or GII.3 strains appear to dominate, have costly social, economic, and medical effects on hospital and health care services [31, 384]. This was the background for molecular investigation of the environmental contamination and airborne dispersal of NoV in hospital wards during seasonal outbreaks, (Paper IV).

The nosocomial study was designed to evaluate possible routes of viral transmission during outbreaks and to improve understanding of NoV dispersal in the patient

room environment (Figure 11), (Paper IV). Investigations included patient and environmental sampling from eight hospital wards in four buildings during a five-month period of nosocomial outbreaks. One of the eight wards where NoV infection in the index case did *not* spread to other patients was monitored as the control, outbreak-free ward. A sample survey of staff was included in an attempt to trace the direction of transmission of NoV infections in patients and staff during nosocomial outbreaks (Table 1).

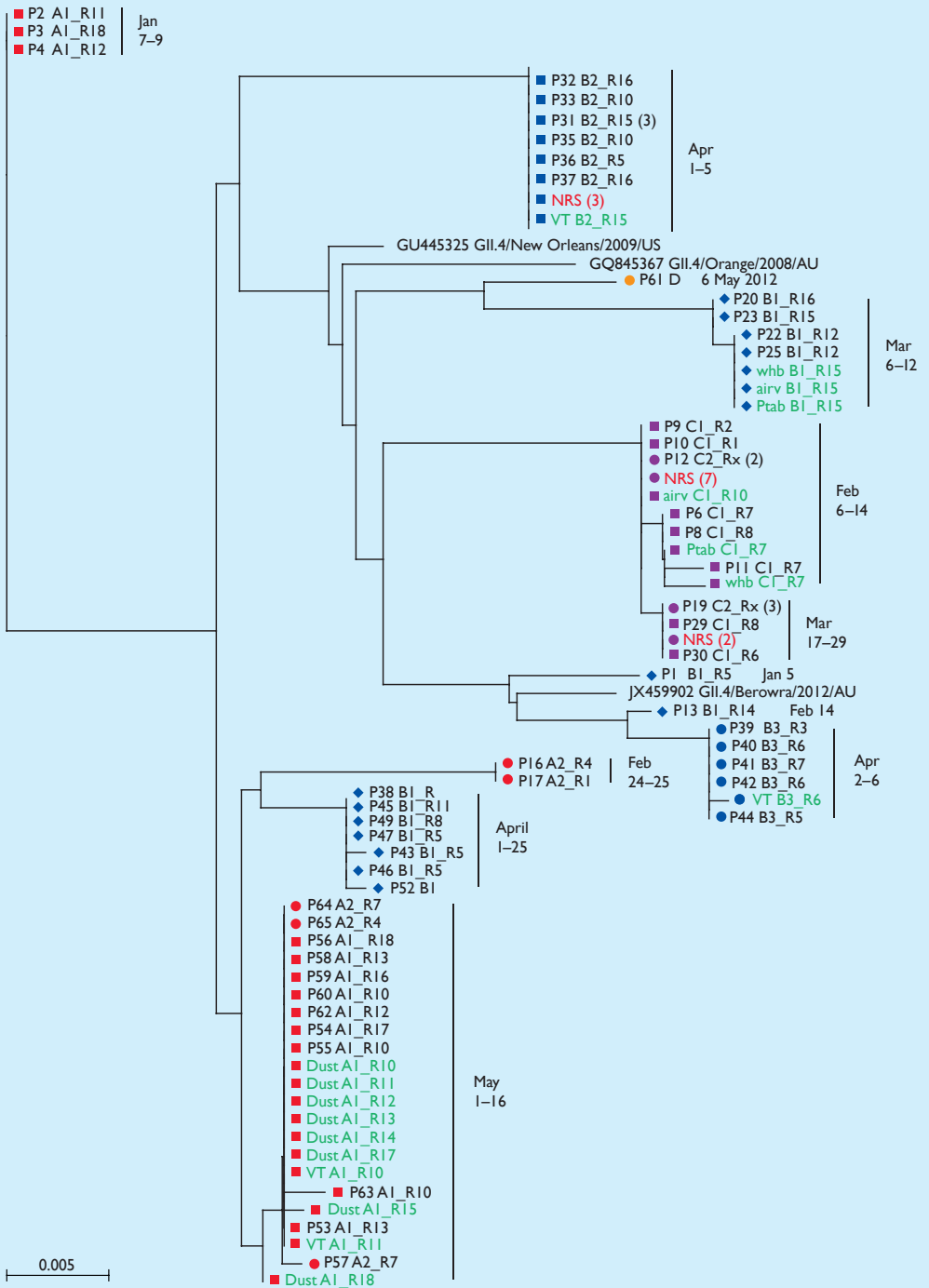
Molecular epidemiology based on nucleotide sequencing of NoV, phylogenetic analysis and ward outbreak reports, was used to trace NoV strains detected in patients, and in their hospital room environment. A total of 65 patients from the 8 wards showed symptomatic infection with NoV GII. NoV GII infections were detected by rRT-PCR in 63 of 108 (58%) symptomatic patients from the 7 outbreak wards caring for geriatric, elderly medical, or substance abuse patients. In these outbreak wards, rRT-PCR revealed NoV GII contamination in 48 of 101 (47%) environmental samples, including dust (10/10), surfaces (29/79), and virus air trap samples (9/12). These NoV GII results from environmental samples suggested significant NoV GII contamination in patient rooms in wards during nosocomial infections. In contrast, in the outbreak-free ward caring for cardiac and lung transplant patients, 2 of 17 (12%) patients were infected with NoV GII, and NoV GII signals were detected in 2 of 28 (7%) environmental samples. The environmental and medical care situations in the outbreak and control wards contrasted sharply, raising questions on training and cleaning strategies in different medical situations.

Nucleotide sequencing and comparative analyses of the RdRp-N/S major capsid-coding region (1040 nt) of NoV GII.4 strains detected in symptomatic inpatients and their hospital room environment supported the finding of patient-related NoV GII.4 strains in environmental samples. Nucleotide sequencing of patient strains from the 8 wards confirmed NoV GII.4 infections in 57 of the 58 patients whose strains could be sequenced (Supplemental material, Table S1). In the 7 outbreak wards NoV GII.4 was detected in 56 patients, and in 18 of 48 NoV GII positive environmental samples from five of the outbreak wards where NoV GII positive samples were sequenced across the 1040 nt region. In contrast, the control outbreak-free ward D showed minimal environmental contamination; short fragments (285 nt) of NoV GII.4 being detected in 2 (dust) of 28 environmental samples. Of the 2 patients detected in the control ward the first had symptomatic infection with a NoV II.6 recombinant (GII.P7\_GII.6), the second developed GII.4 infection [169]. The results of these investigations raise relevant questions on the role of persistence of NoV in the patient's close environment, and transmission of NoV infections during nosocomial outbreaks.

Eleven NoV GII.4 genomic variants (differing by 0.7–3.7%, 1040 nt) were detected in symptomatic patients from the eight wards monitored during the five-month study (Figure 21).

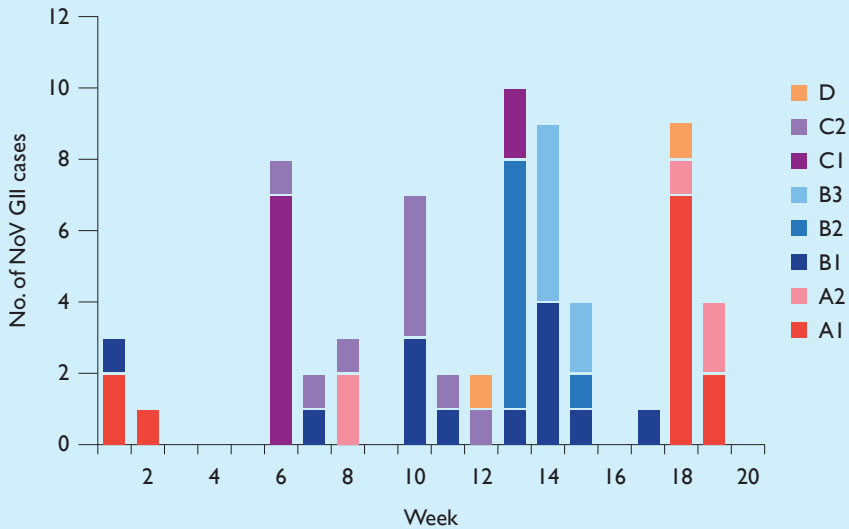
**Figure 21.** Neighbour-joining tree based on nt analysis of the NoV RNA-dependent RNA-polymerase N/S capsid-coding region (1040 nt). A1, A2, B1, B2, B3, C1, C2, D = wards; R = patient room; Rx = patient room not defined. Sequences from environment in green type: VT = virus trap; ptab = patient over-bed table; whb = washbasin; airv = air vent. P = patient strain sequence. NRS = nursing staff sequence shown in red type. (n) indicates number of sequences analysed. Hospital buildings are represented by the four colours red, blue, purple, and orange; wards are indicated by symbols ●, ◆, ■. Relevant Genbank reference strains are included. The bar indicates genetic distance per nucleotide per site per year.

Figure 21.



These NoV GII.4 variants showed high similarity ( $\geq 97.9\%$ , 1040 nt) to the recombinant GII.4 New Orleans 2009 strain (GenBank reference GU445325). Five of the GII.4 variants detected in patients from different wards at different times were also found in dust, air, or room surfaces from the corresponding patient's room. The NoV GII.4 strain detected in an individual patient and in his/her hospital-room environment in a given ward, at a given time, showed high nucleotide similarity ( $> 99\%$ , 1040 nt). Moreover, the GII.4 variant detected grouped with strong correlations to time and place of outbreak, as shown in the phylogenetic tree particularly for the January variant in ward A1, February variant in A2, March variant in B1, April variant in B2 (Figure 21). This sequence-based evidence strengthens and complements the descriptive epidemiology of clustering in place, time, and person, as noted in the daily infection control reports. Careful analysis of the molecular epidemiology may provide information for improved understanding and control of transmission routes during nosocomial outbreaks [230, 385, 386]. These molecular studies indicate extensive, patient-related NoV GII.4 contamination in patient rooms during nosocomial outbreaks, and raise issues of hygiene awareness, hygiene education, and improved cleaning strategies in hospital settings.

The epicurve confirms that several wards experienced recurring episodes of NoV GII.4 infections, findings which justify interventional cleaning studies (Figure 22). Wards A1, A2, and B1 were particularly affected, with different NoV GII.4 variants occurring in different outbreaks, in different wards, over time. In ward A1, with patients requiring haematology and immunosuppression monitoring, outbreaks occurred in January and May. The adjacent medical ward A2, where patients shared dining and shower areas with A1, reported outbreaks in February and May. NoV GII.4 strains detected in wards A1 and A2 in January and February clustered on different sub-branches of the phylogenetic tree with 98.8% similarity (1040 nt), but the strains detected in wards A1 and A2 in May showed 100% similarity (Figure 21).



**Figure 22.** Epicurve showing distribution of NoV GII cases (n=65) in eight wards A1, A2, B1, B2, B3, C1, C2, and D, during seasonal nosocomial outbreaks, weeks 1 to 20, 2012. NoV GII.4 was detected in 57 of 58 NoV GII patient strains that were sequenced. The one exception was a case of GII.6 infection detected in outbreak-free ward D, week 12.

Patients in wards C1 and C2 situated on floors 4 and 6 of the same hospital building showed NoV GII.4 variants of high similarity (100%, 1040 nt). Exchange of personnel or relocation of patients may be the most likely reason for the high similarity of NoV GII.4 strains involved in the C1 and C2 ward outbreaks. After the outbreaks in wards C1 and C2, thorough environmental sampling of the ventilation system from air intake to outlet failed to detect NoV with the exception of one sample that was positive by rRT-PCR at Ct 39 for NoV GII.

Situated in a different hospital building, the geriatric general medical ward B1 experienced outbreaks in January, February, March and April (Figure 22). Possibly ward B1, with debilitated incapacitated geriatric patients, and wards C1 and C2, caring for patients with dementia and substance abuse, presented the greatest difficulties in infection control. Medical units caring for the mentally infirm and incapacitated patients are recognized as being particularly vulnerable to increased NoV outbreak rates [299, 387]. Nursing staff, endeavoring to maintain good standards of patient care and hygiene in these wards may have been confronted by challenging conditions requiring an improved approach to environmental and patient management.

The environmental studies emphasize the need for improved standards of education for all members of nursing and medical staff, calling for increased awareness of personal hygiene practices for staff and patients, particularly soap and warm water hand-washing, and targeted daily cleaning of the patient's close environment [196, 388–390].

Evidence of the importance of surface contamination in transmission of NoV in nosocomial outbreaks was provided by early environmental studies where molecular methods were used to identify contaminated areas and possible reservoirs of NoV infection in an outbreak ward [299]. Those studies in a ward for the long-term mentally infirm supported *cohorting* of sick patients, and indicated that environmental sampling can be used to evaluate methods for removal of virus, to assess infection control measures, and the significance of airborne and other routes of virus transmission. Yet reports on detection and significance of environmental contamination in transmission of NoV infections during nosocomial outbreaks are limited [231, 299]. However, environmental swabs have been used to monitor surface contamination in general and specialized paediatric units during outbreak-free periods [20, 113, 120, 391], and in tracing NoV contamination during prolonged outbreaks in nursing homes, houseboats, cruise ships, hotels and restaurants [28, 37, 231, 299, 300, 302, 392].

Few reports detail methods for molecular detection of NoV as airborne or aerosolized virus, or dispersed in dust, as outlined in Paper IV. A literature search failed to recover references describing detection of airborne NoV using an ionizing device similar to the virus air trap (Figure 12) designed to capture NoV from air in the hospital ward. However, use of an impactor air sampler is described in attempts to detect NoV GI and GII, and other viruses including Torque teno virus (TTV), in air samples from hospital settings [393]. TTV was found in air samples from paediatric and haematology units, but NoV was not detected in air samples (n=62) from sites examined during a 2-year period. However, surface contamination with NoV was limited to one of a total of 114 samples, indicating low levels of NoV environmental contamination in areas examined. On the question of dust and air sampling, the use of heating, ventilation, and air conditioning (HVAC) filters for indoor environment investigations of airborne microbes, suggested that these filters can serve as passive long-term samplers in indoor airborne studies of bacterial and fungal concentrations, but their usefulness in virus monitoring is not known [394].

Real-time RT-PCR and nucleotide sequencing of NoV strains detected in environmental samples from nosocomial settings confirmed NoV GII.4 RNA contamination of patient rooms suggesting direct surface contact spread and airborne dispersal of NoV during outbreaks (Paper IV). Environmental swabs showed NoV GII median Ct values of 34 on room surfaces including over-bed tables, wash hand basins, and air vents; Ct 32 median in virus air trap samples; and Ct 31 median in dust. Those NoV GII median Ct values from environmental samples can be compared with NoV GII

Ct values from symptomatic patients in the 7 outbreak wards. Faeces samples from outbreak patients showed a Ct range of 13–38, median Ct 20, with GII Ct values of 20 estimated to approximate  $10^8$  gE/ml, Paper III, [209].

As up to 16% of healthy individuals with no recent infectious intestinal disease may be NoV GII rRT-PCR positive, a diagnostic cut-off value of Ct 31 (approximately  $10^5$  viral genomes per gram faeces) in patient samples was proposed as indicative of active NoV GII infection in the diagnostic rRT-PCR [266]. These workers also suggested that Ct values could be used as a proxy measure of viral load, a reasonable approach to the problems of assessing viral load in non-uniform samples such as patient faeces or vomitus. At the Virology Department, Sahlgrenska University Hospital, patient samples with Ct values  $\leq 39$  in validated in-house rRT-PCR NoV GI and GII detection systems are reported as positive. Ct values are not issued with the official laboratory report. The test is run for 45 cycles, with Ct values  $\leq 39$  registered in the laboratory protocol (Papers II, III and IV).

With no consensus strict guidelines on *predictive* Ct values for patient diagnostics, or for environmental samples, each in-house diagnostic test system must be validated with control panels and plasmids, and patient results have to be considered on an individual basis. Although no Ct values are presented, Morter *et al.* (2011) comment briefly on rRT-PCR findings from environmental samples examined in hospital settings: *Sensitivity of the RT-PCR assay used suggests that when NoV RNA was detected, about  $10^3$  to  $10^5$  virus particles were present, as extrapolated from the PCR cycle when product was first detected. This is well in excess of the infectious dose of  $\sim 10$  virus particles* [231]. However, no nucleotide sequencing results are provided for these environmental samples, possibly indicating that virus concentrations were below the limit for successful sequencing [231]. Neither do other studies of environmental contamination in paediatric wards, semi-closed institutions, house-boat, restaurant and hotel outbreaks, provide quantitative or semi-quantitative results for comparison with Ct data obtained from the environmental studies reported in the Supplemental material Table S1, Paper IV, [20, 28, 37, 113, 299, 300, 302]. The NoV Ct values detected in 18 environmental samples from different wards during nosocomial outbreaks described in Paper IV were confirmed as NoV GII.4 specific reactions on nucleotide sequencing. Comparative sequence analysis with sequences from patient strains indicated patient-related NoV GII.4 contamination of surfaces, dust and air in the patient rooms (Paper IV).

However, the question of detection of potentially *infectious* NoV in environmental samples cannot be resolved until a robust standard cell culture technique is developed, or volunteer studies are undertaken. Yet the molecular evidence in dust and air of long RNA genomic fragments (1040 nt) of NoV GII.4 variants with high sequence similarity to those detected in the infected patient occupying the room, indicates dispersal of patient-related NoV strains in hospital rooms (Figure 21). These long-fragment studies also suggest that potentially infectious, intact virions with encapsidated

genomic RNA are being detected [114]. When considered against the background of the low viral dose of NoV infection, variously estimated as from 10 to 2800 virus particles or gEq in different NoV reports, the exploratory studies of environmental contamination described in nosocomial settings require further investigation, Paper IV, [207, 211, 212, 395]. Dust and virus air trap sampling may offer a novel approach for investigating the airborne dispersal of NoV in the patient's environment, and for monitoring the course of nosocomial outbreaks in semi-closed settings where interventional cleaning studies are motivated and performed.

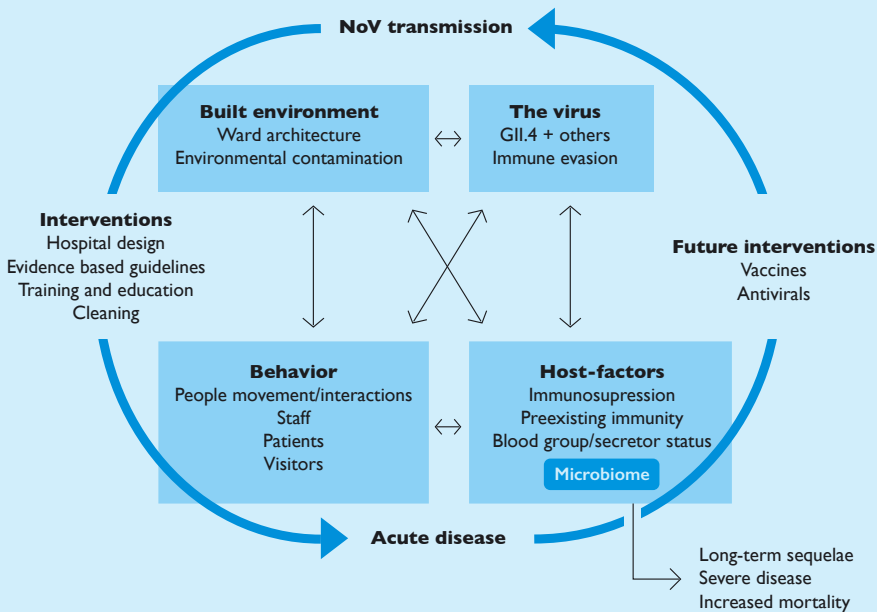
In the staff survey 133 nurses completed the questionnaire and contributed rectal swab samples for NoV assay during the nosocomial outbreaks [260]. NoV GII was detected in 26 (19.5%), and 2 were positive for SaV; almost all the nurses that were NoV positive reported gastroenteric symptoms. Strains from nurses in 3 of the wards genotyped as NoV GII.4 variants with high genetic similarity (100%, 1040 nt) to those from patients and environment in the respective ward, (Figure 21). In no case did NoV GII positive findings in staff precede infection in the index patients for each ward, indicating that transmission occurred in the direction from patients to staff, or alternatively that the staff were part of the nosocomial outbreaks, although not initiating them. Harris et al. (2010) in a review of infection control noted that some investigations show evidence of infections beginning in the staff then being transmitted to patients, and vice versa; others show clear associations between the amount of staff contact with patients and risk of illness [396, 397].

Although NoV is commonly described as being transmitted by the *faecal-oral* route, Greenberg et al. (1979) extended the description to include the *vomit-oral* route following RIA and IEM detection of NoV in vomitus from volunteers experimentally infected with NoV GI.1 [398]. As the virus was found in material collected more than 24 h after ingestion, it was unlikely that the NoV represented original inoculum. Microscopy of small-bowel and gastric biopsy samples from volunteers infected with NoV GI.1 shows alterations in upper bowel, villi blunting and disturbance of enzyme production, but not in stomach [23, 25, 399]. Whether virus in vomitus represents refluxed material from small bowel, or whether replication occurs in the stomach, cannot be determined until virus infected cells have been localized [398]. In recent volunteer studies, however, NoV GI.1 was detected in 15 (56%) of 27 vomitus samples at a median concentration of 41,000 gEq/ml [209, 211]. When considered in terms of HID50 for GI.1 infections, estimated as 2800 gEq these volunteer studies suggest that vomiting is a significant route of viral transmission [211]. These levels of virus reaffirm Caul's original assessment of the importance of vomitus in transmission and emphasize the need for prompt cleaning and disinfection of sites contaminated by vomitus [400].

Significant levels of NoV are detected in vomitus from naturally infected, symptomatic hospital patients as shown in the nosocomial study, where NoV GII.4 RNA was detected at a median Ct value of 26 (IQR=7.45) in vomitus from 28 infect-

ed patients (Paper IV, Supplemental material, Table S1). Confirmed by nucleotide sequencing of vomitus from 26 patients, the NoV GII rRT-PCR findings re-assert the importance of vomiting in the dispersal of GII.4 in the patient's room (Figure 21), Paper IV, Table S1. The forcibly expelled NoV-containing particles may settle to contaminate surfaces and fomites, be picked up on hands, or be dispersed as airborne virus. If protocols for effective cleaning and disinfection are not followed meticulously these levels of virus may be relevant in virus transmission, confirming the importance of vomiting and airborne dispersal of NoV as suggested in outbreaks from aeroplane, hospital, hotel, restaurant and school settings [208, 300, 383, 401–403]. Those studies on symptomatic patients and volunteer experimental infections underline the importance of vomiting and environmental contamination in transmission of NoV within semi-closed settings where the very young, elderly, and immunosuppressed are at greatest risk [11, 19, 22, 28, 219, 404]. The extent of environmental contamination detected by molecular methods provides new insight on the costly problems of recurring nosocomial outbreaks, and indicates that interventional cleaning studies and reinforced hand washing regimes are justified (Paper IV) [299, 405, 406].

Functional studies of projectile vomiting with a simulated vomiting system known as the *Vomiting Larry* model, show that splashes and droplets produced during an episode of projectile vomiting may travel with forward spread of more than 3 m, and lateral spread of 2.6 m [407]. Droplet spread is not readily seen with the naked eye and UV examination of experimental emesis with fluorescent tracers shows that cleaning of contaminated surfaces is difficult. Cleaning and decontamination of surrounding areas of at least 7.8 m is recommended after projectile vomiting [407]. Disposable aprons, gloves and cleaning cloths should be used as fingers contaminated with faeces or vomitus, and soiled cleaning cloths may transfer viruses to contact surfaces such as taps, door handles, and telephones, and fingers can transfer NoV sequentially to seven clean surfaces [20, 28, 196, 300, 388]. To achieve good hygiene surfaces should be cleaned using a cloth soaked in detergent before applying combined hypochlorite/detergent (5000 ppm of available chlorine), otherwise NoV is transferred to new surfaces and to the cleaner's hands [196]. This is important information that has to be explained to cleaning and nursing staff before being implemented at all levels of infection control praxis. The effect of intensive cleaning policies and environmental disinfection was demonstrated in the counter measures that reduced recurring NoV outbreaks onboard cruise ships from 195 cases to less than 10 on subsequent cruises [405].



**Figure 23.** Overview of norovirus infections in healthcare settings. Many aspects of the semi-closed medical care setting, building and ventilation, quality of cleaning, medical care, the patient’s susceptibility may influence the persistence of the highly variable norovirus GI.4 strains in the hospital environment. Adapted with permission from Iturriza-Gomara and Lopman 2014 [408].

The infectious dose for NoV GI.1 infection has been estimated as from 10–2800 infectious virion or gEq [207, 211, 212, 395]. This low dose raises questions of NoV transmission, virus stability, aerosol formation in toilet areas, and dispersal in air including cross-contamination from soiled fomites such as carpeting, dust rising from floor coverings, architectural features of ward units, and cleaning procedures (Figure 23). First suggested as important in the spread of infections in closed and semi-closed settings in early studies of school, hospital, and cruise ship outbreaks these areas of virus study are being reinvestigated in cooperation with engineers engaged in aerobiology [27, 208, 383, 401, 405, 409–412]. Understanding of airborne transmission of viruses, generation of droplets of different size, atomization of virus containing samples such as faeces or vomitus, and the role of aerosols in infections has grown, stimulated by outbreaks of severe acute respiratory syndrome (SARS).

Tracing transmission of airborne SARS virus has reopened areas of common interest with studies of *re-entrainment* of virus from contaminated carpeting following SARS spread through a vomiting incident, multi-zone modelling of probable SARS virus transmission by airflow between flats, and investigation of possible atomization of SARS from sewage aerosols transported in building downpipe systems during the largest nosocomial outbreak in Hong Kong [410, 412–414].

Explosive, uncontrollable, projectile vomiting is a prominent symptom in NoV gastroenteritis seen in up to 90% of cases, and exposure to a vomiting event is a significant risk factor for infection in crowded, poorly ventilated, indoor environments [39, 208, 402, 415]. High energy vomiting increases the likelihood of dispersal of aerosol droplets containing NoV with potential for widespread environmental contamination [411, 416]. This is why scientists working in aerobiology and virology are interested in studying ventilation systems and NoV transmission in indoor environments. Droplet size is understood to be important for droplet dispersal, deposition on surfaces, and survival of pathogens within the droplets. The infectivity of viruses found in droplets, and the chemical and physical properties of the droplets, are influenced by temperature, absolute and relative humidity, exposure to chemical effects such as UV light and sunshine, and to physical conditions such as air flow [410, 417, 418].

Several transmission routes have been suggested depending on droplet size. Although speculative, these routes may apply in acute NoV infections where larger droplet aerosols ( $\geq 5 \mu\text{m}$ ) may be inhaled by individuals in the vicinity of the vomiting event, deposited in the respiratory tract, and swallowed along with respiratory mucous [411]. Or, these larger aerosols containing virus may settle quickly onto toilet or kitchen surfaces, be transferred to hands and ingested by exposed individuals, particularly if surfaces and hands are not washed regularly. Also, patient mouthwash studies indicate that oral-to-oral transmission of NoV may occur beyond the immediate period of symptomatic infection in GII.3 and GII.4 outbreaks, findings relevant in food handling and medical care [35, 299]. After vomiting events fomites such as soiled bedding or carpeting may become secondary sources of infectious particles, released into the indoor air mechanically through re-suspension or *re-entrainment* in heavily trafficked areas, to be inhaled directly, dispersed by air currents, or to re-settle on other surfaces for subsequent hand-to-mouth transmission [411]. Questions of aerosol and droplet transmission are also being investigated in the spread of Ebola virus where standard, contact, and droplet precautions are recommended [418].

Smaller droplet nuclei ( $\leq 5 \mu\text{m}$ ) rapidly affected by evaporation give rise to dried-out residues that may contain virus [419–420]. Dried-out droplet nuclei may remain in indoor air for hours, a source of airborne virus dispersed in clouds around the infected patient, to be breathed in, and swallowed by nearby individuals [419, 421]. Much depends on the chemical and physical nature of the virus, and host immune response, as described recently in murine-based studies of the rhinovirus in the common cold infections [422]. Lipid containing enveloped influenza and SARS respi-

ratory viruses in these sub-micron droplet nuclei may penetrate deeper into the respiratory tract causing infection of the lower respiratory tract with systemic spread. Less is known about the airborne dispersal of the non-enveloped lipid-free NoV. Early interest in airborne dispersal was intense following reports that vomiting can generate aerosols of NoV that may pose a significant risk of virus transmission [208, 383]. However, expelled particles carrying viral pathogens such as NoV do not exclusively disperse from infected patients by airborne or droplet transmission but avail of both methods simultaneously [423]. Collaboration between indoor engineers and virologists may improve understanding of the multiple effects of indoor environment on virus transmission at the molecular level.

Therefore, aerosol transmission of a virus may be considered in terms of aerosol generation, virus stability in the environment, and access to target tissue [418]. These properties are highly relevant in the spread of noroviruses. NoV GI and GII strains share common features being robust, environmental stable viruses, inducing similar symptoms in their host though differing greatly in genomic sequence and environmental distribution. Our studies of GI viruses reassert the remarkable genomic stability of these strains, their particular stability in water, and ready dissemination in the aqueous environment. In contrast, the GII noroviruses in particular the GII.4 types exhibit greater genomic diversity, temporal variation and are more closely associated with the indoor air, and semi-closed settings, where aerosol transmission plays an important role in nosocomial outbreaks. These are interesting and contrasting features of a family of well-adapted virus pathogens. The noroviruses are candidates worthy of intensive study.

## 5. Conclusions

- Molecular tracing of human NoV outbreak strains in bivalves was possible.
- Such tracing in mussels could be used as sentinel of human NoV strains circulating in contaminated environmental waters.
- Sequencing of human NoV strains from oysters, the food handler, or patients with NoV infections contracted from contaminated drinking water was helpful in defining outbreak point source.
- Molecular epidemiology of multiple NoV GI strains was a useful indicator of massive faecal contamination of drinking water in a large community outbreak of gastroenteritis.
- Multiple GI strains detected in the waterborne outbreak indicated endemic circulation in the healthy population.
- Nosocomial outbreaks of NoV GII.4 were local to a given ward, and initiated by symptomatic patients; the molecular-based evidence indicated NoV GII.4 contamination of the patient's close environment.
- These findings demonstrate the ubiquity of the norovirus, and may provide a platform for new strategies for prevention and intervention in outbreak settings.

## 6. Future perspectives

- If the human norovirus can be grown in cell culture a challenging new era of *entero-virology* may be anticipated, precipitating novel changes in the direction of norovirus investigations. Until a robust cell culture is established, renewed interest in human volunteer challenge studies, cell imaging, electron microscopy, whole genome sequencing, sequence independent detection such as nanopore-based sequencing revealing new genomic variants, may be the most direct way of assessing virus viability, and the significance of virus findings in dust and air and water.
- Such new methodology may provide powerful tools to evaluate strategies for prevention and intervention of NoV outbreaks related to environmental contamination.



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