

QUANTIFICATION AND DISTRIBUTION OF HUMAN NOROVIRUSES FROM OYSTER,
WATER, AND PLANKTON SAMPLES OVER A ONE-YEAR PERIOD FROM TWO
GEORGIA ESTUARIES.

by

JENNIFER B. GENTRY

(Under the Direction of Erin K. Lipp)

ABSTRACT

Human norovirus (NoV) has been studied extensively as the cause of gastroenteritis outbreaks worldwide, but only a fraction of NoV studies have examined the virus' distribution in the estuarine environment: including NoV presence in oyster, water, and plankton samples. Here we evaluated two silica-based viral RNA extraction protocols and two norovirus realtime TaqMan RT-PCR assays for the detection of norovirus in shellfish and plankton. The optimized method, a combination of a rapid RNA extraction followed by a TaqMan RT-PCR assay, assisted in detecting 21 NoV-positive samples from the 225 environmental samples analyzed. Twenty-four percent, 28.6%, 31.0%, and 9.5% were from oyster, water, the small plankton fraction, and the large plankton fraction samples and the majority of these samples (90.5%) were from genogroup I. The quantities of NoV found in plankton, water, and oysters provides clues about the presence and distribution of human NoV in the estuarine environment.

INDEX WORDS: norovirus, shellfish, plankton, estuarine, real time RT-PCR

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DEDICATION

To my parents, who always encouraged me to follow my dreams -all the way to the CDC.

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CHAPTER 1

INTRODUCTION

Human norovirus (NoV) causes up to half of all foodborne outbreaks of acute gastroenteritis in the U.S. Bivalve molluscan shellfish have been implicated in outbreaks and human norovirus genogroups I and II have been detected in shellfish harvested from bays and estuaries worldwide. Hence, monitoring of NoVs in shellfish and the surrounding water environment is critical to reducing shellfish-related norovirus illness. In the literature review (Chapter 2), the characteristics, pathogenicity, detection, and prevalence of norovirus are discussed in detail.

The NoV detection methods discussed in Chapter 2 were originally developed for use in stool samples whereas environmental samples, such as those evaluated in this study, tend to have lower viral load as well as higher concentrations of inhibitors; thus, viral detection methods must be adapted accordingly. Chapter 3 presents evaluation of two real-time TaqMan RT-PCR assays and two viral RNA extraction protocols for the detection of NoV in shellfish and plankton. Extraction methods were compared using both NoV RNA run-off transcripts and NoV-contaminated fecal samples. The final detection method includes a rapid viral RNA extraction and quantitative detection of norovirus by real time RT-PCR using RNA run-off transcripts. Efficient detection and enumeration of norovirus in estuarine sample types will allow for a better overall understanding of NoV presence in the water environment and its transmission to shellfish.

As discussed in Chapter 2, no study has yet been able to characterize how NoV may be naturally distributed in an estuarine system, including in water, adhered to particles, and in plankton and shellfish. In Chapter 4, research is presented that explored the distribution of NoV in an estuarine system and begins to explain the transmission of norovirus from human sewage to shellfish. Water, oyster, and plankton samples were collected bimonthly from two Georgia estuaries: Wassaw Sound, near Savannah, GA, and Sapelo Sound, near McIntosh County, GA. Samples were analyzed using the newly optimized detection method outlined in Chapter 3, with a rapid extraction method and real time TaqMan RT-PCR. From the 225 environmental samples analyzed, 21 (9.3%) were positive for NoV with the majority (90.5%) of these samples from genogroup I. Twenty-four percent, 28.6%, 31.0%, and 9.5% were from the oyster, water, small plankton fraction, and large plankton fraction samples, respectively. The quantities of NoV found in plankton, water, and oysters provides information about the presence and distribution of human NoV in the estuarine environment.

In Chapter 5, conclusions are made for advanced shellfish monitoring with the use of an optimized NoV detection protocol. Two key discoveries are discussed that may provide clues as to the distribution of NoV in the environment. Plankton, both small and large size fractions, was found to have high concentrations of NoV and may serve as an environmental reservoir to NoV. Additionally, the surprising finding that NoV GI were found in such high numbers suggests that NoV GI is either more environmentally hardy than GII in the estuarine environment or that this genotype represents strains circulating widely in the population but not associated with the predominantly reported GII outbreaks. Both of these dynamics are significant findings that point to the need for more studies of human norovirus in the water environment in order to prevent NoV-related gastrointestinal disease.

CHAPTER 2

LITERATURE REVIEW

Introduction

Human norovirus (NoV) is the leading cause of viral gastroenteritis worldwide (5). NoV is responsible for 93-96% of outbreaks of nonbacterial gastroenteritis in the United States and 68-80% of all gastroenteritis outbreaks in industrialized nations (31, 39, 50, 52, 67, 84). The Centers for Disease Control and Prevention (CDC) estimate that 23 million cases of acute gastroenteritis due to norovirus occur each year (2). Increased norovirus activity in outbreaks followed by a decrease the next year(s) have been reported in 2002 and 2006-2007 (110, 123).

Shellfish are an important source of NoV infection (13, 76, 78) and are of particular concern because of the ability of shellfish, such as mussels and oysters, to accumulate bacteria and viruses while filter feeding. Between 1994 and 2000, 284 nonbacterial gastroenteritis cases were reported to the CDC and of 233 for which samples were available, 93% tested positive for NoV (31). Of those, 57% were foodborne, 16% were due to person-to-person spread, 3% were waterborne, and 23% were of undetermined transmission. Current data indicate that 33% of total outbreaks are foodborne and 67% are person-to-person transmission (116). Most outbreaks of gastroenteritis go without etiologic diagnosis because detection methods are not widely available in addition to a lack of reporting (31).

Because molecular detection of viruses can be difficult and time-consuming, several epidemiologic criteria have been proposed for use in determining whether an outbreak of gastroenteritis is of viral origin. Kaplan's criteria (2) for this purpose are as follows: 1) a mean

(or median) illness duration of 12 to 60 hours, 2) a mean (or median) incubation period of 24 to 48 hours, 3) more than 50% of people with vomiting, and 4) no bacterial agent previously found.

Norovirus has an incubation time of 24-48 hours after infection and, in most cases, symptoms last for 24-60 hours. Symptoms of a norovirus infection include acute-onset vomiting, watery non-bloody diarrhea, abdominal cramps, nausea, low-grade fever occurring infrequently but more commonly in the elderly, children, and immunocompromised, and rapid dehydration, which is generally the most serious complication (87). No long-term sequelae have been reported.

Outbreaks of norovirus generally occur in institutional settings, including nursing homes, childcare centers, banquet facilities, dormitories, and cruise ships. Norovirus outbreaks are pervasive for many reasons, but particularly because the virus is highly contagious and environmentally hardy. Human NoV is spread primarily through the fecal-oral route when a person consumes contaminated food or water, comes into contact with an infected person, or comes into contact with a contaminated fomite (2). Transmission due to aerosolization of vomitus, resulting from droplets contaminating surfaces or being swallowed, is another possible route of contamination (2). Patients can experience prolonged asymptomatic shedding for up to 2 weeks; allowing for an increased risk of disease spread (36). Also, less than 100 viral particles are required for disease, allowing for person-to-person spread as well as secondary transmission (e.g. by foodhandlers) (2). The virions are stable in the environment, surviving up to 10 ppm of chlorine, freezing, and heating up to 60°C, making decontamination difficult (2). There is also substantial strain diversity; norovirus has multiple genetic and antigenic types, requiring multiple diagnostic tests. In addition, the general population does not have lasting immunity; approximately 50% of persons exposed to norovirus experience illness and acquire short-term

immunity (80).

Norovirus as a Pathogen

Norovirus. Norovirus (NoV), also known as Norwalk-like virus (NLV) and small round structured virus (SRSV), belong to a separate genus of the family *Caliciviridae* (43). Norovirus is divided into five genogroups: GI, GII, GIII, GIV, and GV, which are further divided into 31 genetic clusters (122). Viruses in genogroups I, II, and IV have been detected in humans, but genogroup IV is extremely rare (66). The virus capsid is 30-38 nm in diameter and exhibits a T=3 icosahedral symmetry (2). The genome consists of a single-stranded, positive-sense, 7.7 kb RNA molecule consisting of three open reading frames (ORFs). ORF1 encodes a large polyprotein that forms the helicase, protease, and RNA polymerase; ORF2 encodes the major capsid protein, VP1, and ORF3 encodes the minor capsid protein, VP2 (19, 29, 56, 71, 107).

Norovirus GII predominant genogroup in outbreaks. In reviewing the NoV strains from environmental and clinical samples, La Rosa et al. (69) noticed that despite the broad diffusion of both GI and GII NoV in sewage samples, NoV GII was the predominant cause of outbreaks. Fankhauser et al. (31) reported that of 217 NoV fecal samples received by the CDC between 1997 and 2000, genogroup II was the predominant strain, causing 73% of all NoV outbreaks. Certain GII clusters, such as GII.1 and GII.4 were more commonly associated with outbreaks in nursing homes than other settings (31) and GII/1,4,j were the most common strains in the Netherlands, Japan, and Australia (67, 95, 104, 125).

The majority of norovirus outbreaks since 1995 are caused by one genotype: GII.4. From 1995 to 1996, the NoV strain US95/96 was the predominant strain type identified in the U.S. and The Netherlands, responsible for 55% and 85% of outbreaks, respectively (117). Between 2000

and 2004, this strain was replaced by the Farmington Hills strain in the United States and the GII.4b strain in Europe (31, 83, 88, 99). The Hunter GII.4 strain emerged in Australia, Europe, and Asia in 2004 (16, 68, 99) and in 2006, the Sakai and Minerva strains predominated and co-circulated in the U.S., Europe, and Asia. In late 2007, the GII.4-2006b variant, which is known as the Minerva-like strain in the United States, the Kobe034-like strain in Japan, and V6 in the United Kingdom, became the dominant variant (110). Both GII.4-2006a and GII.4-2006b co-circulate at present, but GII.4-2006b appears to be prevalent in most European countries (110). Of 59 genotyped NoV outbreaks reported in the Netherlands in late 2007, 6 (10%) were GII.4-2006a and 34 (58%) were GII.4-2006b (110).

The prevalence of genogroup II, and specifically GII.4, over genogroup I in outbreaks may be due to several reasons. First, antigenic drift within the human histo-blood group antigen (HBGA) carbohydrate-binding targets may promote GII.4 persistence in human populations (81). HBGAs are a highly heterogeneous group of related carbohydrates on mucosal surfaces. Norovirus is known to infect individuals who express ABH HBGAs; they carry a gene encoding a functional alpha-1,2-fucosyltransferase (FUT2) and are thus designated “secretor positive” (81). A study by Lindesmith et al. (81) argues that GII.4 noroviruses persist by altering their HBGA carbohydrate-binding targets over time. Second, NoV GII has an increased fecal load compared to GI. Chan et al. (20) reported that the median cDNA viral load of norovirus GII is \geq 100 times that of GI in fecal specimens from patients with norovirus associated gastroenteritis. The study suggests that the increased fecal load of GII aids in the transmission of NoV from infected persons to susceptible hosts (20). Third, differences in biological properties such as virulence, routes of transmission, or stability of the two virus genotypes in the environment are

also possible explanations for predominance of GII over GI viruses but have not yet been investigated (15).

Recent studies suggest that the incidence of (or the detection of) NoV GI may be increasing. Fankhauser et al. (31) observed an increase in GI-related outbreaks from 4% between 1996-1997 to 25% between 1997-2000. Additionally, a study by Chapin et al. (21) found that of 54 stool samples collected from 34 traveler's diarrhea cases in Guatemala and Mexico, 65% and 100%, respectively, of those were NoV GI. These results were repeated by a study done on students visiting Mexico (63). NoV GI was present in 22 of 124 samples (15%) as opposed to NoV GII that was present in only 4 stool samples (3%).

An increase in GI detection has also been observed in studies involving sewage samples. In a study by da Silva et al. (24) NoV GI presence was more variable, had higher peaks at all four sites studied, and the average positive influent concentrations were higher than the average positive influent concentrations of NoV GII. This study also found that sewage treatment is less effective for GI than GII, NoV GI and GII were present in 43 and 88% of the influent samples and 24 and 14% of the effluent samples, respectively. This could indicate that NoV GI is more environmentally hardy than NoV GII. However, the authors suggested that NoV GI actually circulates widely in the human population and that physicians see only patients with severe symptoms, which is a small proportion of the actual GI prevalence. The data for wastewater samples may reflect the presence of strains that circulate more broadly in the population and as such are a more powerful tool for public health surveillance (24).

Foodborne Norovirus Infection

Norovirus is estimated to cause two-thirds of the non-bacterial foodborne illness and up to 50% of all foodborne outbreaks of acute gastroenteritis in the U.S. (87). Theoretically, any

food item can potentially be infected with norovirus through fecal contamination, although certain foods are more readily implicated in outbreaks. Shellfish is of particular concern because of the ability of shellfish, such as mussels and oysters, to concentrate bacteria and viruses while filter feeding. Shellfish are able to filter 10-24 liters of water per hour (37).

Gastroenteritis due to contaminated shellfish. The United States attributes approximately 10-20% of its foodborne diseases to consumption of contaminated seafood (18). This percentage increases to 70% in countries such as Japan where seafood is a significant part of the diet (18, 79). For this reason, there are regulations in place to limit the amount of bacterial contamination in shellfish harvesting areas; however, regulations for fecal indicator bacteria often underestimate viral contamination in shellfish and almost half of all seafood-associated gastroenteritis outbreaks are caused by viruses (18, 123).

Shellfish waters in the United States are classified as approved, conditional, restricted, or prohibited for shellfish harvesting based primarily on the monitoring of fecal coliform levels in shellfish-growing waters. Shellfish harvesting waters in Georgia are required to have levels of fecal coliforms below 14 MPN 100 ml⁻¹ (1). A site will be temporarily closed if water samples reach higher levels for three consecutive sampling periods, but this has not happened since the Georgia Department of Natural Resources began monitoring shellfish harvesting areas (46). Although these standards are thought to be effective in blocking feces-contaminated shellfish from the marketplace, they cannot indicate the level of viral contamination that may persist for a month or longer within shellfish or estuarine sediments after coliform bacterial counts have returned to acceptable levels (28). Furthermore, point source discharge of human waste from commercial and recreational vessels can result in contamination of approved beds without observed increases in fecal coliform counts in marine water samples (6, 55).

Shellfish beds in the vicinity of wastewater treatment plants or septic tanks are likely to be contaminated with viral pathogens as complete inactivation in sewage is rare. Infected individuals can excrete millions of viral particles in feces allowing for large numbers in sewage (32). Without proper removal or inactivation the viruses can be released into recreational and shellfish harvesting water bodies. Contamination of oyster beds has even been reported for geographic locations with high standards of sanitation (10, 32, 33, 74, 100, 109). Moreover, the contamination of one shellfish bed can be responsible for multiple NoV outbreaks as the disease risk for consumption of raw oysters is high (75). A study by Le Guyader et al. (73) used both epidemiologic and molecular analysis to track multiple NoV strains involved in foodborne outbreaks in both France and Italy to one shellfish bed in southern France.

Norovirus in Oysters. The persistence of NoV in oyster tissue is well documented (6, 7, 40, 74, 75, 77). Bivalve molluscan shellfish in waters contaminated with human sewage accumulate bacteria and viruses while filter-feeding large amounts of water, but while oysters are able to depurate some bacteria, they have demonstrated only 7% virus removal (106). A study on the persistence of NoV in oysters was performed by Ueki et al. (115). The study monitored the persistence of norovirus GII in artificially contaminated oysters (*Crassostrea gigas*) during a 10-day depuration. NoV GII was detected in shellfish tissues for the entire 10-day period (115). While GI was not tested in this study, a previous study determined that GI is poorly depurated compared to *E. coli* (106). NoV persistence in shellfish tissues is most likely due to the virus' ability to attach to oysters' carbohydrate structures that have a terminal N-acetylgalactosamine residue in an alpha linkage (75). NoV attaches to the digestive tract of oysters using the P2 subdomain of its capsid, which is the same binding site used for recognition of human HBGAs (75). Norovirus accumulation in oysters may depend on factors such as water temperature,

mucus production, glycogen content of connective tissue, or gonadal development, though temperature is thought to be the most significant factor (17). In a study by Le Guyader et al. (74), NoV was detected mainly during cold months, from November to March. Prevalence was low in spring, increasingly slightly in the summer, and highest in November (74).

Norovirus Detection

Until recently, norovirus could not be cultured *in-vitro* (112); detection currently focuses on either the viruses themselves or their nucleic acids (112), both of which have limitations. Detection of viral particles by electron microscopy requires at least 1×10^6 virions ml^{-1} for detection (44, 58). While detection limits are lower for conventional RT-PCR to detect viral RNA, this method requires examination by gel electrophoresis or a lengthy hybridization step (2). Recently, rapid and sensitive real-time RT-PCR assays have been developed (58). However, many laboratories continue to use laborious RNA extraction methods of varying efficiencies from environmental samples (44), which counteracts the goal of a rapid detection system.

Norovirus RNA extraction. The common methods of viral RNA extraction and purification from environmental samples involve acid adsorption-elution (53, 111), direct glycine buffer elution (79), silica bead extraction (12, 79), virus precipitation using Cat-Floc (102) or polyethylene glycol (47, 53), and solvent extraction using chloroform (7, 91) or chloroform/butanol (6). The drawback to most of these methods is the time required for extraction and the loss of viral genomes during successive steps (44). To date, the most reliable and straightforward method for purifying viral nucleic acids from shellfish and water samples is the silica bead extraction method on which most commercial kits are now based (44). Arnal et

al. (4) found the silica bead extraction method to have the lowest limit of detection compared to more laborious extraction methods.

Recently, a virus extraction method from shellfish was developed by de Roda Husman et al. (26). This protocol includes concentration of viruses using Zirconia beads and extraction of viral RNA using the Qiagen RNeasy Mini kit following the Plant and Fungi Method (26). The authors found this extraction method to be the most time- and recovery-efficient virus extraction protocol when comparing five different extraction methods, including in-house silica-bead extraction, qiashredder silica bead extraction, RNeasy Mini Kit (Qiagen), ultracentrifugation followed by qiadshredder-RNeasy Mini Kit, and chloroform-PEG precipitation (26). These results were confirmed in the study by Schultz et al. (2007) who compared several methods of NoV detection in oysters (105). They compared PEG precipitation followed by phenol/chloroform extraction, the RNeasy Mini Kit (Qiagen), and the QIAamp Viral RNA Mini Kit (Qiagen) to find that the RNeasy Mini Kit was the most rapid and recovery-efficient extraction method.

Molecular detection. Viral RNA extraction methods can be followed by variants of the RT-PCR assay such as nested RT-PCR, semi nested RT-PCR, RT-PCR followed by hybridization, and real-time RT-PCR, which, when paired with RNA run-off transcripts, can be used to quantify the amount of viral RNA. However, detection of NoV RNA by RT-PCR has been extremely difficult to due the strain heterogeneity (61). Katayama et al. (61) analyzed the full-length norovirus genome of genogroups I and II using four genogroup I strains and ten genogroup II strains and found a low level of sequence similarity across the entire genome. The region with the highest nucleotide similarity (most conserved) is found in the ORF1-ORF2 junction region. Most current real-time RT-PCR primers and probes target this region (57, 58).

The first RT-PCR assays for norovirus were developed after the cloning and sequencing of Norwalk and Southampton virus (54, 71); however, these first assays showed poor performance because the genetic diversity of NoV strains was much greater than initially anticipated (27, 55, 90). A study by Griffin et al. (45) examined the presence of microbial indicators and viral pathogens in the canals of the Florida Keys using two early prototype primer and probe sets to detect Norwalk virus and SRSVs. NoV was found in 10% of the samples, but they detected only a small fraction of the NoV strains and were inefficient, requiring $>10^5$ virions per reaction for detection (45). The authors speculated that assays with increased sensitivity and applicability would have identified a higher percentage of samples positive for NoV.

With the cloning and sequencing of additional strains, RT-PCR assays were developed targeting more specific regions of the norovirus genome. Four main genomic regions have been targeted: regions A and B in ORF1 and regions C and D in ORF2. To date, only one study has been reported that utilized primers adhered to region D (118). The majority of the reported studies target region A (3, 41, 77, 119) or region C (41, 65, 94, 120). First generation conventional RT-PCR assays were designed targeting the RdRp region in ORF1 because this area was thought to contain the most conserved nucleotide sequence in the genome, the 'YGDD' motif, which is widespread among viral RdRp (121). The 5'-end of ORF2 is also commonly used because this region is relatively conserved within either GI or GII (70). The most widely used primer pairs for NoV GI and GII were designed by Kojima et al. (65) and target the 5'-end of ORF2. One study, by Yan et al. (126), used the primers designed by Kojima et al. (65) in a multiplex format to simultaneously detect norovirus GI and GII, sapovirus, and astrovirus in fecal samples (126).

While in recent years NoV detection in fecal samples has improved, detection in environmental samples still poses a number of problems. Environmental samples, such as estuarine water and oysters, generally have a low concentration of viruses, which requires the use of nested or semi nested RT-PCR assays (48). Most of the nested and semi nested assays produce short fragments of no more than 158 bp (42, 96, 97, 121). While short PCR products allow for a rapid identification assay in addition to enhancing detection, they are less useful for back tracing and genotyping of strains by phylogenetic analysis. Additionally, studies of gastroenteritis outbreaks associated with NoV-contaminated oysters reveals that mixed infections often occur (34, 113). In one study of a 2001 oyster-associated gastroenteritis outbreak, three of eight cases were positive for multiple norovirus genotypes; one case was co-infected with three genotypes and two cases were co-infected with two genotypes (14). Gallimore et al. (35) found a mixed GI/GII infection from fecal samples and discovered a recombinant genotype, rGII.3a, in shellfish-related gastroenteritis fecal samples. Accordingly, sewage samples are often co-contaminated with multiple genotypes (69). This presents a problem for NoV detection in environmental samples: a co-infected sample may appear as a “single band” in an electrophoresis gel. Cloning before sequencing samples is a necessity, despite the extra time and cost required.

Another challenge with NoV detection in environmental samples is the amplification of nonspecific products during RT-PCR of shellfish samples (108). The use of a probe in TaqMan real-time RT-PCR can alleviate this problem. The assay described in Kageyama et al. (58) includes two sets of degenerate primers and probes: one set for the detection of genogroup I and one set for the detection of genogroup II. These new sets were able to detect NoV in previously negative stool specimens as determined by electron microscopy (58) and also in shellfish samples (93). Non-degenerate primers targeting the same region of the genome were designed

by Jothikumar et al. (57). The study tested the new primers and probes on shellfish samples to determine that the assays were sensitive enough to detect the low concentration of NoV present in environmental samples. Additionally, multiplexing is an emerging area in NoV detection in environmental samples. A triplex real-time RT-PCR assay was designed by Wolf et al. (124) to detect human NoV genogroups I, II, and III in a variety of matrices, including stool, treated and raw sewage, source water, and treated drinking water (124).

An important factor to consider in the use of RT-PCR for detection of norovirus is that PCR detects both infectious viruses and viral genomes derived from non-infectious viruses, such as defective virions or naked viral genomes. Taking viral replication into consideration, there is the possibility that naked viral genomes represent a large percentage of all viral genomes in aquatic environments (49). Haramoto et al. (49) modeled the recovery of naked viral genomes in water samples by various virus concentration methods. The study found that the concentration method for water samples had an effect on the ratio of complete virions to naked viral genomes detected by PCR. The Mg-method (developed by the group) was the most efficient concentration method for detection of virions, rather than naked viral genomes, as compared to two conventional concentrations methods (1MDS-method and HA-method) and the Al-method (also developed in-house) (49). Future studies could use this comparison in their analyses to better determine the risk of virus infection from environmental samples.

Quantitation. Several methods of quantitation have been described for norovirus, including endpoint dilution (38, 55, 62, 106), most-probable number RT-PCR (76), and TaqMan real-time RT-PCR assays. The use of real-time RT-PCR is more advantageous over conventional RT-PCR because it provides rapid results, avoids post-PCR hybridization analyses, and cross-contamination due to handling of amplified samples (44). TaqMan real-time PCR also

allows for the use of standards for within and between-test reproducibility, in addition to quantitation. However, quantitation of NoV in environmental samples, such as shellfish, can be difficult if not impossible due to the high level of inhibitory substances as discussed earlier.

There are relatively few studies quantifying NoV from shellfish and water using real-time PCR. Most rely upon detection of fluorescence from SYBR Green, which binds to double-stranded DNA, so that as DNA is amplified, fluorescence increases (9, 72, 89, 92). TaqMan technology has also been used, which employs fluorescent probes to bind specific sites on amplified DNA (51, 58, 59, 93). Kageyama et al. (58) used serially diluted standard DNA plasmids (10^7 copies to 10^1 copies) containing the ORF1-ORF2 junction sequence in detecting and quantifying NoV cDNA. Similar DNA plasmids were used by Jothikumar et al. (57) to quantitate the limit of detection of their newly developed primers and probes. The use of RNA run-off transcripts has not yet been reported but would take into account the reverse transcription step in addition to the amplification stage and allow for single-step RT-PCR.

Norovirus Detection in Estuarine Environments

Human NoV infection due to contaminated oysters has been investigated extensively, and shellfish have been implicated in food-borne outbreaks of enteric viruses (25, 30, 64). However, transmission from contaminated sewage to shellfish has not yet been reported. Urban estuaries can, and often do, have elevated contaminant levels in their sediments, water, and associated biota. It is assumed that norovirus contamination results from human carriers via domestic sewage, treated wastewater, and illegal dumping of human waste. However, transmission has not been elucidated because it is difficult to collect NoV from feces, environmental water samples, and oysters in geographically close areas during the same time period (114). Many pathogens, such as norovirus, are not routinely monitored because of the lack of widely available

detection and quantitation methods (44).

Detection in water environment. Before 1997, when norovirus was first detected in a water source, NoV outbreaks were confirmed using epidemiology studies (8, 11, 60). While there are now methods to detect NoV in water, there are still limited reports on the fate of noroviruses in estuarine water (69). In 2001, when detection of norovirus in water samples was still relatively novel, NoV was identified in contaminated groundwater that was supplying a tourist saloon in Wyoming at the center of a NoV outbreak. Investigators found both GI.3 and GII.6 present in the well water but only GII.6 in one stool sample (98). Groundwater was the cause of many NoV outbreaks in Finland in a study by Maunula et al. (85). Of 18 NoV-positive fecal samples from which waterborne contamination was implicated from 1998-2003, NoV was found in 10. Most norovirus contaminations occurred in groundwater systems and only 3 epidemics were due to contaminated surface, lake, and river water with equal numbers of NoV GI and GII detected. A more recent study by La Rosa et al. (69) examined NoV presence in seawater, estuarine water, and sewage effluent in Rome, Italy to evaluate the role of environmental surface contamination as a vector for NoV transmission. The authors found that river, estuarine, and seawater samples were scarcely contaminated while, to no surprise, all sewage samples were contaminated (69).

Detection in shellfish. Despite the known importance of norovirus as the major etiologic agent of shellfish-borne gastroenteritis, direct detection and identification of viruses in shellfish tissue has been challenging. Shellfish samples generally have low levels of viral contamination and high levels of PCR inhibitors while the extraction methods for shellfish tend to have low virus recovery. The development of concentration, extraction, and detection of NoV from shellfish in a rapid, sensitive, and accurate molecular method has been difficult but is critical to

NoV disease reduction worldwide. Every continent (except Antarctica) has reported NoV gastroenteritis outbreaks due to contaminated oysters. Cheng et al. (22) investigated raw oysters from 11 countries in Europe, Asia, Australia, and America. NoV was found in oysters from every continent and 53 out of 507 (10%) oyster samples were positive for NoV (22).

Similar NoV positive rates in oysters have been reported in other studies (10, 74, 92, 93, 109). One of these studies, Nishida et al. (93), found NoV in 17 of 191 (9%) Japanese oysters collected between December 2001 and February 2002. Genogroup I was present in 3 samples and genogroup II was present in the other 14 with both genogroups displaying a wide genetic diversity (93). The researchers were also able to quantify the NoV present in the oysters and observed levels higher than 10^2 copies of the NoV genome in 11 of 17 oysters. Conversely, a study by Costantini et al. (23) showed much higher NoV persistence in raw market oysters. The study scanned for both human and non-human norovirus strains in oysters from 45 bays in the Gulf, West, and East coasts of the United States during the summer and winter of 2002 and 2003. Human norovirus was detected in 20 of 45 samples (44%) with all of these representing genogroup II (23).

The majority of shellfish-related norovirus studies find a higher proportion of NoV GII than GI. However, Boxman et al. (14) is one of the few studies where genogroup I predominated in either shellfish or human fecal samples. The study focused on detection of norovirus in local and imported shellfish to the Netherlands and human fecal samples from a 2001 oyster-associated outbreak of gastroenteritis (14). Of ten positive sequences from oyster samples, seven (70%) of these were GGI.4 (genogroup I, genotype 4), two (20%) were GIIB and one (10%) was GII.4. The genotypes present in the positive fecal samples were GI.1 (2 cases), GI.4 (3), GIIB (1), GII.7 (1), GIV (14).

Currently, real-time RT-PCR is being used more frequently to detect NoV GI and GII in shellfish samples (57, 59). Loisy et al. (82) evaluated 150 naturally contaminated shellfish extracts and found 27% of the samples displaying inhibition and all samples displaying high Ct values (30-41). While the GII primers and probes outperformed the conventional RT-PCR method, the GI primers and probes performed poorly comparatively. The authors suggested that there was poor homology of these oligonucleotides, which were designed for the strains circulating in Japan, to the strains circulating in France, leading to a higher limit of detection (82).

Detection in multiple estuarine samples. There is a marked association of shellfish-transmitted diseases to sewage pollution and illegal overboard sewage discharge into shellfish-harvesting areas (101). However, very few studies have conducted a comprehensive analysis of norovirus transmission in the water environment. The study by Ueki et al. (114) detected NoV presence in patients with gastroenteritis, domestic sewage, treated wastewater, river water, and cultivated oysters (from geographically close areas in Japan). In samples from the Takagi River and Matsushima Bay, 18 of 30 (60%) oysters, 6 of 8 (75%) river water samples, 8 of 9 (89%) treated wastewater samples, and 9 of 9 (100%) wastewater samples were positive for norovirus, indicating that treated wastewater could be one of the main sources for NoV pollution in the area.

Saito et al. (103) performed a similar study, evaluating oyster and water samples once or twice a month for 30 months from October 2001 to March 2004. The researchers evaluated changes in viral occurrence among the 208 isolates collected to find that NoV was present year-round in untreated sewage but found a seasonal occurrence in processed sewage, river water, seawater, oysters, and children's feces. Additionally, there was a clear genetic relationship

between NoVs detected in children's feces and environmental oysters and water, suggesting transmission from the feces of infected children to oysters by the flow of water from farm sewage to rivers, the sea, and finally accumulating in the mid-gut of oysters (103).

Prevention of Outbreaks

Human waste is believed to be the predominant cause of NoV contamination of shellfish-harvesting growing areas and the source of contamination in many studies has been approved shellfish harvesting areas (25, 108). Loisy et al. (75) suggests three possibilities for illnesses resulting from the consumption of shellfish harvested in sanitary growing areas. First, the source of pollution in harvesting areas may be a sporadic or non-point source, which would be difficult to track with the current indicator organism, fecal coliforms. Second, the source of pollution may be a non-contained point source, such as a malfunctioning sewage disposal system. Lastly, fecal coliform monitoring systems may not accurately reflect the presence of viruses in shellfish in the estuarine environment (75).

Current detection methods for viral pathogens, including norovirus, are not adequate for routine monitoring by local regulatory laboratories (108). Anthropogenic sources of contamination will continue to invade shellfish growing waters, and shellfish will continue to bioaccumulate pathogens, including enteric viruses. While research continues into acceptable (viral) pathogen indicators and adequate detection methods for the viruses themselves (26, 69, 105, 112, 124), a more practical approach to reduce NoV gastroenteritis includes enhanced vigilance, monitoring, and education.

Caution on behalf of the industry, regulatory agencies, and the consumer could substantially reduce the incidence of illness. First, updating sewage treatment facilities and

continuous inspection, especially during periods of increased rainfall, would be a major step to reducing viral pathogens in sewage effluent. Second, improved monitoring along the chain of shellfish production, from harvesting to distribution, would help to reduce viral illnesses. This would include shoreline surveillance, mandatory restrictions on waste dumping, improved indicators, and public education (108). Educating local citizens about waste dumping around growing sites and enforcing those regulations is critical. Lastly, many current post harvest controls, such as mild cooking, do not eliminate viruses such as NoV and HAV from oysters (86). New processing and analytical technologies, such as high hydrostatic pressure processing and molecular biological assays may be able to enhance shellfish safety.

With the inception of shellfish monitoring using fecal coliforms, the level of bacterial gastroenteritis (from shellfish) dropped dramatically (108). The prevailing pathogens related to shellfish-borne gastroenteritis are now viruses, such as hepatitis A and norovirus (44). The actions required to reduce the incidence from these diseases are the development of improved detection methods, the development of improved indicators, the rigorous monitoring of shellfish production, from harvest to distribution, and, most importantly, the education of citizens, both growers and consumers.

REFERENCES

1. **Georgia Department of Natural Resources, Coastal Resources Division.** Georgia Shellfish Product Safety Guidelines for Shellfish Harvesters.
www.dnr.state.ga.us/dnr/coastal
2. **Centers for Disease Control and Prevention** 2008. Norovirus: Technical Fact Sheet.
<http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus-factsheet.htm>
3. **Ando, T., S. S. Monroe, J. R. Gentsch, Q. Jin, D. C. Lewis, and R. I. Glass.** 1995. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and Southern hybridization. *J. Clin. Microbiol.* **35**:64-71.
4. **Arnal, C., V. Ferre-Aubineau, B. Besse, B. Mignotte, L. Schwartzbrod, and S. Billaudel.** 1999. Comparison of seven RNA extraction methods on stool and shellfish samples prior to hepatitis A virus amplification. *J. Virol. Methods* **77**:17-26.
5. **Atmar, R. L., and M.K. Estes.** 2006. The epidemiologic and clinical importance of norovirus infection. *Gastroenterol. Clin. N. Am.* **35**:275-290.
6. **Atmar, R. L., F.H. Neill, J.L. Romalde, F. LeGuyader, C.M. Woodley, T.G. Metcalf, and M.K. Estes.** 1995. Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. *Appl. Environ. Microbiol.* **61**:3014-3018.
7. **Atmar, R. L., T. G. Metcalf, F. H. Neill, and M. K. Estes.** 1993. Detection of enteric viruses in oysters by using the polymerase chain reaction. *Appl. Environ. Microbiol.* **59**:631-635.
8. **Beller, M., A. Ellis, S. H. Lee, M. A. Drebot, S. Jenkerson, E. Funk, M. D. Sobsey, O. D. S. III, S. S. Monroe, T. Ando, J. Noel, M. Petric, J. P. Middaugh, and J. S. Spika.**

1997. Outbreak of viral gastroenteritis due to a contaminated well. International consequences. *JAMA* **278**:563-568.
9. **Beuret, C.** 2004. Simultaneous detection of enteric viruses by multiplex real-time RT-PCR. *J. Virol. Methods* **115**.
 10. **Beuret, C., A. Baumgartner, and J. Schluep.** 2003. Virus-contaminated oysters: a three-month monitoring of oysters imported to Switzerland. *Appl. Environ. Microbiol.* **69**:2292-2297.
 11. **Blackburn, B. G., G. F. Craun, J. S. Yoder, V. R. Hill, R. L. Calderon, N. Chen, S. H. Lee, D. A. Levy, and M. J. Beach.** 2004. Surveillance for waterborne-disease outbreaks associated with drinking water- United States, 2001-2002. *MMWR Surveill. Summ.* **53**:23-45.
 12. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. W.-v. Dillen, and J. v. d. Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**:495-503.
 13. **Bosch, A., G. Sanchez, F. Le Guyader, H. Vanaclocha, L. Haugarreau, and R. M. Pinto.** 2001. Human enteric viruses in coquina clams associated with a large Hepatitis A outbreak. *Water Sci. Technol.* **43**:61-65.
 14. **Boxman, I. L. A., J. J. H. C. Tilburg, N. A. J. M. t. Loeke, H. Vennema, L. Jonker, E. d. Boer, and M. Koopmans.** 2006. Detection of noroviruses in shellfish in the Netherlands. *Intl J Food Microbiol* **108**:391-396.
 15. **Buesa, J., B. Collado, P. Lopez-Andujar, R. Abu-Mallouh, D. J. Rodriguez, D. A. Garcia, J. Prat, S. Guix, T. Llovet, G. Prats, and A. Bosch.** 2002. Molecular

- epidemiology of caliciviruses causing outbreaks and sporadic cases of acute gastroenteritis in Spain. *J. Clin Microbiol.* **40**:2854-2859.
16. **Bull, R. A., E. T. Tu, C. J. McIver, W. D. Rawlinson, and P. A. White.** 2006. Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis. *J. Clin Microbiol.* **44**:327-333.
 17. **Burkhardt, W., and K. R. Calci.** 2000. Selective accumulation may account for shellfish-associated viral illness. *Appl. Environ. Microbiol.* **66**:1375-1378.
 18. **Butt, A. A., K. E. Aldridge, and C. V. Sanders.** 2004. Infections related to the ingestion of seafood. *Lancet Infect. Dis.* **4**:201-212.
 19. **Cauchi, M. R., J. C. Doultree, J. A. Marshall, and P. J. Wright.** 1996. Molecular characterization of Camberwell virus and sequence variation in ORF3 of small round-structured (Norwalk-like) viruses. *J Med Virol* **49**:70-76.
 20. **Chan, M. C. W., J. J. Y. Sung, R. K. Y. Lam, P. K. S. Chan, N. L. S. Lee, R. W. M. Lai, and W. K. Leung.** 2006. Fecal viral load and norovirus-associated gastroenteritis. *Emerg. Infec. Dis.* **12**:1278-1280.
 21. **Chapin, A. R., C. M. Carpenter, W. C. Dudley, L. C. Gibson, R. Pratdesaba, O. Torres, D. Sanchez, J. Belkind-Gerson, I. Nyquist, A. Karnell, B. Gustafsson, J. L. Halpern, A. L. Bourgeois, and K. J. Schwab.** 2005. Prevalance of norovirus among visitors from the United States to Mexico and Guatemala who experience traveler's diarrhea.
 22. **Cheng, P. K. C., D. K. K. Wong, T. W. H. Chung, and W. W. L. Lim.** 2005. Norovirus contamination found in oysters worldwide. *J Med Virol* **76**:593-597.

23. **Constanini, V., F. Loisy, L. Joens, F. S. Le Guyader, and L. J. Saif.** 2006. Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. *Appl. Environ. Microbiol.* **72**:1800-1809.
24. **da Silva, A. K., J.-C. L. Saux, S. Parnaudeau, M. Pommepuy, M. Elimelech, and F. S. L. Guyader.** 2007. Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: Different behaviors of genogroups. *Appl. Environ. Microbiol.* **73**:7891-7897.
25. **David, S. T., L. McIntyre, L. MacDougall, D. Kelly, S. Liem, K. Schallie, A. McNabb, A. Houde, P. Mueller, P. Ward, Y. L. Trottier, and J. Brassard.** 2007. An outbreak of norovirus caused by consumption of oysters from geographically dispersed harvest sites, British Columbia, Canada, 2004. *Foodborne Pathog. Dis.* **4**:349-358.
26. **de Roda Husman, A. M., F. Lodder-Verschoor, H. H. J. L. V. d. Berg, F. S. L. Guyader, H. V. Pelt, W. H. M. V. d. Poel, and S. A. Rutjes.** 2007. Rapid virus detection procedure for molecular tracing of shellfish associated with disease outbreaks. *J. Food Prot.* **70**:967-974.
27. **DeLeon, R., S. M. Matsui, R. S. Baric, J. E. Hermann, N. R. Blacklow, H. B. Greenberg, and M. D. Sobsey.** 1992. Detection of Norwalk virus in stool specimens by reverse transcriptase-polymerase chain reaction and nonradioactive oligoprobes. *J. Clin. Microbiol.* **30**:3151-3157.
28. **Deng, M. Y., S. P. Day, and D. O. Cliver.** 1994. Detection of hepatitis A virus in environmental samples by antigen-capture PCR. *Appl. Environ. Microbiol.* **60**:1927-1933.

29. **Dingle, K. E., P. R. Lambden, E. O. Caul, and I. N. Clarke.** 1995. Human enteric *Caliciviridae* - the complete genome sequence and expression of virus-like particles from a genetic group II small round structured virus. *J. Gen. Virol.* **76**:2349-2355.
30. **Dowell, S. F., C. Groves, K. B. Kirkland, H. G. Cicirello, T. Ando, Q. Jin, J. R. Gentsch, S. S. Monroe, C. D. Humphrey, C. Slemp, D. M. Dwyer, R. A. Meriwether, and R. I. Glass.** 1995. A multistate outbreak of oyster-associated gastroenteritis: implications for interstate tracing of contaminated shellfish. *J. Infec. Dis.* **171**:1497-1503.
31. **Fankhauser, R. L., S. S. Monroe, J. S. Noel, C. D. Humphrey, J. S. Bresee, U. D. Parashar, T. Ando, and R. I. Glass.** 2002. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. *J. Infec. Dis.* **186**:1-7.
32. **Formiga-Cruz, M., A.K. Allard, A.C. Conden-Hansson, K. Henshilwood, B.E. Hernroth, J. Jofre, D.N. Lees, F. Lucena, M. Papapetropoulou, R.E. Rangdale, A. Tsibouxi, A. Vantarakis, and R. Girones.** 2003. Evaluation of potential indicators of viral contamination in shellfish and their applicability to diverse geographical areas. *Appl. Environ. Microbiol.* **69**:1556-1563.
33. **Formiga-Cruz, M., G. Tofino-Quesada, S. Bofill-Mas, D. N. Lees, K. Henshilwood, A. L. Allard, A. C. Conden-Hansson, B. E. Hernroth, A. Vantarakis, A. Tsibouxi, M. Papapetropoulou, M. D. Furones, and R. Girones.** 2002. Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden and the United Kingdom. *Appl. Environ. Microbiol.* **68**:5990-5998.
34. **Gallimore, C.** 2002. Norwalk-like viruses and an oyster associated outbreak in Sheffield. *Communicable Disease Report Weekly* **12**.

35. **Gallimore, C. I., J. S. Cheesbrough, K. Lamden, C. Bingham, and J. J. Gray.** 2005. Multiple norovirus genotypes characterised from an oyster-associated outbreak of gastroenteritis. *Intl J Food Microbiol* **103**:323-330.
36. **Garcia, C., H. L. DuPont, K. Z. Long, J. I. Santos, and G. Ko.** 2006. Asymptomatic norovirus infection in Mexican Children. *J. Clin Microbiol.* **44**:2997-3000.
37. **Gastoff, P. S.** 1964. The American oyster, *Crassostrea virginica*, Gmelin, vol. 64. The U.S. Government Printing Office, Washington, D.C.
38. **Gilpatrick, S. G., K. J. Schwab, M. K. Estes, and R. L. Atmar.** 2000. Development of an immunomagnetic capture reverse transcription-PCR assay for the detection of Norwalk virus. *J Virol. Methods* **90**:69-78.
39. **Gonin, P., M. Couillard, and M. A. d'Halewyn.** 2000. Genetic diversity and molecular epidemiology of Norwalk-like viruses. *J. Infec. Dis.* **182**:691-697.
40. **Gouvea, V., N. Santos, M. d. C. Timenetsky, and M. K. Estes.** 1994. Identification of Norwalk virus in artificially seeded shellfish and selected foods. *J. Virol. Methods* **48**:177-187.
41. **Green, J., C.I. Gallimore, J.P. Norcott, D. Lewis, and D.W.G. Brown.** 1995. Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis. *J. Med. Virol.* **47**:392-398.
42. **Green, J., K. Henshilwood, C.I. Gallimore, D.W. Brown, and D.N. Lees.** 1998. A nested reverse transcriptase PCR assay for detection of small round-structured viruses in environmentally contaminated mollescan shellfish. *Appl. Environ. Microbiol.* **64**:858-863.

43. **Green, J., J. P. Norcott, D. Lewis, C. Arnold, and D. W. Brown.** 1993. Norwalk-like viruses: demonstration of genomic diversity by polymerase chain reaction. *J. Clin. Microbiol.* **31**:3007-3012.
44. **Griffin, D. W., K. A. Donaldson, J. H. Paul, and J. B. Rose.** 2003. Pathogenic Human Viruses in Coastal Waters. *Clin. Microbiol. Rev.* **16**:129-143.
45. **Griffin, D. W., C. J. G. III, E. K. Lipp, K. Riley, J. H. Paul, and J. B. Rose.** 1999. Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the canals of the Florida Keys. *Appl. Environ. Microbiol.* **65**:4118-4125.
46. **Guadagnoli, D.** 2007. Georgia DNR Coastal Resources Division (personal communication).
47. **Guillard, R. R., and J. H. Ryther.** 1962. Studies of marine planktonic diatoms. *Can. J. Microbiol.* **8**:229-239.
48. **Hafliger, D., M. Gilgen, J. Luthy, and P. Hubner.** 1997. Semi-nested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood. *Intl. J. Food Microbiol.* **37**:27-36.
49. **Haramoto, E., H. Katayama, K. Oguma, and S. Ohgaki.** 2007. Recovery of naked viral genomes in water by virus concentration methods. *J. Virol. Methods* **142**:169-173.
50. **Hedlund, K. O., Rubilar-Abreu, and L. Svennson.** 2000. Epidemiology of calicivirus infections in Sweden, 1994-1998. *J. Infec. Dis.* **181**.
51. **Hohne, M., and E. Schreier.** 2004. Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. *J. Med. Virol.* **72**:312-319.

52. **Inouye, S., K. Yamashita, S. Yamadera, M. Yoshikawa, N. Kato, and N. Okabe.** 2000. Surveillance of viral gastroenteritis in Japan: pediatric cases and outbreak incidents. *J. Infec. Dis.* **181**:Suppl 2:S270-S274.
53. **Jaykus, L. A., R. DeLeon, and M. D. Sobsey.** 1996. A virion concentration method for detection of human enteric viruses in oysters by PCR and oligoprobe hybridiation. *Appl. Environ. Microbiol.* **62**:2074-2080.
54. **Jiang, X., D. Y. Graham, K. Wang, and M. K. Estes.** 1990. Norwalk virus genome cloning and characterization. *Science* **250**:1580-1583.
55. **Jiang, X., J. Wang, D.Y. Graham, and M.K. Estes.** 1992. Detection of Norwalk virus in stool by polymerase chain reaction. *J. Clin Microbiol.* **30**:2529-2534.
56. **Jiang, X., M. Wang, K.N. Wang, and M.K. Estes.** 1993. Sequence and genomic organization of Norwalk virus. *Virology* **195**:51-61.
57. **Jothikumar, N., J. A. Lowther, K. Henshilwood, D. N. Lees, V. R. Hill, and J. Vinje.** 2005. Rapid and sensitive detection of norovirus by using TaqMan-based one-step reverse-transcription-PCR assays and application to naturally contaminated shellfish samples. *Appl. Environ. Microbiol.* **71**:1870-1875.
58. **Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama.** 2003. Broadly reactive and highly sensitive assay for norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J. Clin. Microbiol.* **41**:1548-1557.
59. **Kageyama, T., M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, S. Kojima, R. Takai, T. Oka, N. Takeda, and K. Katayama.** 2004. Coexistence of multiple

- genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to *Norovirus* in Japan. *J. Clin Microbiol.* **42**:2988-2995.
60. **Kaplan, J. E., R. A. Goodman, L. B. Schonberger, E. C. Lippy, and G. W. Gary.** 1982. Gastroenteritis due to Norwalk virus: an outbreak associated with a municipal water system. *J Infect Dis* **146**:190-197.
61. **Katayama, K., A. Shimasaki, and S. Ohgaki.** 2002. Development of a virus concentration method and its application to detection of enterovirus and Norwalk virus from coastal seawater. *Appl. Environ. Microbiol.* **68**:1033-1039.
62. **Kingsley, D. H., G.K. Meade, and G.P. Richards.** 2002. Detection of both Hepatitis A virus and Norwalk-like virus in imported clams associated with food-borne illness. *Appl. Environ. Microbiol.* **68**:3914-3918.
63. **Ko, G., C. Garcia, Z.-D. Jiang, P. C. Okhuysen, J. Belkind-Gerson, R. I. Glass, and H. L. DuPont.** 2005. Noroviruses as a cause of traveler's diarrhea among students from the United States visiting Mexico. *J. Clin Microbiol.* **43**:6126-6129.
64. **Kohn, M. A., T. A. Furley, T. Ando, M. Curtis, S. A. Wilson, Q. Jin, S. S. Monroe, R. C. Baron, L. M. MacFarland, and R. I. Glass.** 1995. An outbreak of Norwalk virus gastroenteritis associated with eating raw oysters. Implications for maintaining safe oyster beds. *J. Am. Med. Assoc.* **273**:466-471.
65. **Kojima, S., T. Kaheyama, S. Fukushi, F. B. Hoshino, M. Shinohara, K. Uchida, K. Natori, N. Takeda, and K. Katayama.** 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J. Virol. Methods* **100**:107-114.
66. **Koopmans, M., C. von Bonsdorff, J. Vinje, D. de Medici, S. Monroe.** 2002. Foodborne viruses. *FEMS Microbiol. Rev.* **26**:187-205.

67. **Koopmans, M., J. Vinje, M. de Wit, I. Leenen, and W. v. d. Poel.** 2000. Molecular epidemiology of human enteric caliciviruses in the Netherlands. *J. Infect. Dis.* **181**:S262-S269.
68. **Kroneman, A., H. Vennema, J. Harris, G. Reuter, C. H. von Bonsdorff, K. O. Hedlund, K. Vainio, V. Jackson, P. Pothier, J. Koch, E. Schreier, B. E. Bottiger, M. Koopmans, and F.-b. v. i. E. network.** 2006. Increase in norovirus activity reported in Europe. *Euro. Surveill.* **11**:E061214.1 061211.
69. **La Rosa, G., S. Fontana, A. Di Grazia, M. Iaconelli, M. Pourshaban, and M. Muscillo.** 2007. Molecular identification and genetic analysis of norovirus genogroups I and II in water environments: comparative analysis of different reverse transcription-PCR assays. *Appl. Environ. Microbiol.* **73**:4152-4161.
70. **Lambden, P. R., B. Liu, and I.N. Clarke.** 1995. A conserved sequence motif at the 5' terminus of the Southampton virus genome is characteristic of the Caliciviridae. *Virus Genes* **10**:149-152.
71. **Lambden, P. R., E. O. Caul, C. R. Ashley, and I. N. Clarke.** 1993. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. *Science* **259**:516-519.
72. **Laverick, M. A., A. P. Wyn-Jones, and M. J. Carter.** 2004. Quantitative RT-PCR for the enumeration of noroviruses (Norwalk-like viruses) in water and sewage. *Lett. Appl. Microbiol.* **39**:127-136.
73. **Le Guyader, F., F. Bon, D. DeMedici, S. Parnaudeau, A. Bertone, S. Crudeli, A. Doyle, M. Zidane, E. Suffredini, E. Kohli, F. Maddalo, M. Monini, A. Gallay, M. Pommeppy, P. Pothier, and F. M. Ruggeri.** 2006. Detection of multiple noroviruses

- associated with an international gastroenteritis outbreak linked to oyster consumption. *J. Clin Microbiol.* **44**:3878-3882.
74. **Le Guyader, F., L. Haugarreau, L. Miossec, E. Dubois, and M. Pommepuy.** 2000. Three-year study to assess human enteric viruses in shellfish. *Appl. Environ. Microbiol.* **66**:3241-3248.
75. **Le Guyader, F., F. Loisy, R. L. Atmar, A. M. Hutson, M. K. Estes, N. Ruvoen-Clouet, M. Pommepuy, and J. Le Pendu.** 2006. Norwalk virus-specific binding to oyster digestive tissues. *Emerg. Infect. Dis.* **12**:931-936.
76. **Le Guyader, F., F. H. Neill, E. Dubois, F. Bon, F. Loisy, E. Kohli, and M. Pommepuy.** 2003. A semiquantitative approach to estimate Norwalk-like virus contamination of oysters implicated in an outbreak. *J. Food Microbiol.* **87**.
77. **Le Guyader, F., F. H. Neill, M. K. Estes, S. S. Monroe, T. Ando, and R. L. Atmar.** 1996. Detection and analysis of a small round-structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. *Appl. Environ. Microbiol.* **62**:4268-4272.
78. **Lees, D. N.** 2000. Viruses and bivalve shellfish. *Int. J. Food Microbiol.* **59**:81-116.
79. **Lees, D. N., K. Henshilwood, and W. J. Dore.** 1994. Development of a method for detection of enteroviruses in shellfish by PCR with poliovirus as a model. *Appl. Environ. Microbiol.* **61**:2999-3005.
80. **Lindesmith, L., C. Moe, J. LePendou, J.A. Frelinger, J. Treanor, and R.S. Baric.** 2005. Cellular and humoral immunity following Snow Mountain Virus challenge. *J. Virol.* **79**:2900-2909.

81. **Lindesmith, L., E. F. Donaldson, A. D. LoBue, J. L. Cannon, D.-P. Zheng, J. Vinje, and R. S. Baric.** 2008. Mechanisms of GII.4 norovirus persistence in human populations. *PLOS Med.* **5**:269-290.
82. **Loisy, F., R. L. Atmar, P. Guillon, P. L. Cann, M. Pommepeuy, and F. S. L. Guyader.** 2005. Real-time RT-PCR for norovirus screening in shellfish. *J. Virol. Methods* **123**:1-7.
83. **Lopman, B. A., H. Vennema, E. Kohli, P. Pothier, A. Sanchez, A. Negredo, J. Buesa, E. Schreier, M. Reacher, D. W. Brown, J. Gray, M. Iturriza, C. Gallimore, B. Bottiger, K. O. Hedlund, M. Torven, C. H. von Bonsdorff, L. Maunula, M. Poljsak-Prijatelj, J. Zimsek, G. Reuter, G. Szucs, B. Meleg, L. Svensson, Y. van Duinhoven, and M. Kijpmans.** 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. *Lancet* **363**:682-688.
84. **Maguire, A. J., J. Green, D. W. Brown, U. Desselberger, and J. Gray.** 1999. Molecular epidemiology of outbreaks of gastroenteritis associated with small round structured viruses in East Anglia, United Kingdom, during the 1996-1997 season. *J. Clin Microbiol.* **37**:81-89.
85. **Maunula, L., I. T. Miettinen, and C.-H. von Bonsdorff.** 2005. Norovirus outbreaks from drinking water. *11* **11**.
86. **McDonnell, S., K. B. Kirkland, W. G. Hardy, C. Aristeguita, R. S. Hopkins, S. S. Monroe, and R. I. Glass.** 1997. Failure of cooking to prevent shellfish-associated viral gastroenteritis. *157*:111-116.
87. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**:607-625.

88. **Medici, M. C., M. Martinelli, L. A. Abelli, F. M. Ruggeri, I. D. Bartolo, M. C. Arcangeletti, F. Pinardi, F. de Conto, G. Izzi, S. Bernasconi, C. Chezzi, and G. Dettori.** 2006. Molecular epidemiology of norovirus infections in sporadic cases of viral gastroenteritis among children in Northern Italy. *J. Med. Virol.* **78**:1486-1492.
89. **Miller, I., R. Gunson, and W. F. Carmen.** 2002. Norwalk like virus by light cyclor PCR. *J. Clin Microbiol.* **25**.
90. **Moe, C., J. Gentsch, T. Ando, G. S. Grohmann, S. Monroe, X. Jiang, J. Wang, M. K. Estes, Y. Seto, C. Humphrey, S. Stine, and R. I. Glass.** 1994. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. *J. Clin Microbiol.* **32**:642-648.
91. **Mullendore, J. L., M. D. Sobsey, and Y.-S. C. Shieh.** 2001. Improved method for the recovery of hepatitis A virus from oysters. *J. Virol. Methods* **123**:1-7.
92. **Myrmel, M., E. M. M. Berg, E. Rinstad, and B. Grinde.** 2004. Detection of enteric viruses in shellfish from the Norwegian coast. *Appl. Environ. Microbiol.* **70**:2678-2684.
93. **Nishida, T., H. Kimura, M. Saiton, M. Shinohara, M. Kato, S. Fukuda, T. Munenura, T. Mikami, A. Kawamoto, M. Akiyama, Y. Kato, K. Nishi, K. Kozawa, and O. Nishio.** 2003. Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. *Appl. Environ. Microbiol.* **69**:5782-5786.
94. **Noel, J. S., T. Ando, J. P. Leite, K. Y. Green, K. E. Dingle, M. K. Estes, Y. Seto, S. S. Monroe, and R. I. Glass.** 1997. Correlation of patient immune responses with genetically characterized Small Round-Structured Viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990-1995. *J Med Virol* **53**:372-383.

95. **Noel, J. S., R.L. Fankhauser, T. Ando, S.S. Monroe, and R.I. Glass.** 1999. Identification of a distinct common strain of "Norwalk-like viruses" having a global distribution. *J. Infect. Dis.* **179**:1334-1344.
96. **O'Neill, H. J., C. McCaughey, D. E. Wyatt, F. Mitchell, and P. V. Coyle.** 2001. Gastroenteritis outbreaks associated with Norwalk-like viruses and their investigation by nested RT-PCR. *BMC Microbiol.* **1**:14.
97. **Ohyama, T., S. Yoshizumi, H. Sawada, Y. Uchiyama, Y. Katoh, N. Hamaoka, and E. Utagawa.** 1999. Detection and nucleotide sequence analysis of human caliciviruses (HuCVs) from samples in non-bacterial gastroenteritis outbreaks in Hokkaido, Japan. *Microbiol. Immunol.* **43**:543-550.
98. **Parshionikar, S. U., S. Willian-True, G. S. Fout, D. E. Robbins, S. A. Seys, J. D. Cassady, and R. Harris.** 2003. Waterborne outbreak of gastroenteritis associated with a norovirus. *Appl. Environ. Microbiol.* **69**:5263-5268.
99. **Phan, T. G., T. Kuroiwa, K. Kaneshi, Y. Ueda, S. Nakaya, S. Nishimura, A. Yamamoto, K. Sugita, T. Nishimura, F. Yagyu, S. Okitsu, W. E. G. Muller, N. Maneekarn, and H. Ushijima.** 2006. Changing distribution of norovirus genotypes and genetic analysis of recombinant GIIB among infants and children with diarrhea in Japan. *J. Med. Virol.* **78**:971-978.
100. **Pina, S., M. Puig, F. Lucena, J. Jofre, and R. Girones.** 1998. Viral pollution in the environment and in shellfish; human adenovirus detection by PCR as an index of human viruses. *Appl. Environ. Microbiol.* **64**:3376-3382.
101. **Potasman, I., A. Paz, and M. Odeh.** 2002. Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clin. Infect. Dis.* **35**:921-928.

102. **Richards, G. P., D. Goldmintz, D. L. Green, and J. A. Babinchak.** 1982. Rapid methods for extraction and concentration of poliovirus from oyster tissues. *J. Virol. Methods* **5**:285-291.
103. **Saito, K., N. Sato, A. Takahashi, R. Tsutsumi, and S. Sato.** 2006. Study on pollutant pathway of norovirus contamination in oysters. *Kansenshogaku Zasshi* **80**:399-404.
104. **Saitoh, T., K. Yamashita, and N. Okabe.** 2003. Recent trend of the detection of SRSV. *IASR* **24**:321-322.
105. **Schultz, A. C., P. Saadbye, J. Hoorfar, and B. Norrung.** 2007. Comparison of methods for detection of norovirus in oysters. *Intl J Food Microbiol* **114**:352-356.
106. **Schwab, K. J., F. H. Neill, M. K. Estes, T. G. Metcalf, and R. L. Atmar.** 1998. Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. *J. Food Prot.* **61**:1674-1680.
107. **Seah, E. L., I. C. Gunsekere, J. A. Marshall, and P. J. Wright.** 1999. Variation in ORF3 of genogroup 2 Norwalk-like viruses. *Arch. Virol.* **144**:1007-1014.
108. **Shieh, Y.-S. C., S. S. Monroe, R. L. Fankhauser, G. W. Langlois, W. B. III, and R. S. Baric.** 2000. Detection of Norwalk-like virus in shellfish implicated in illness. *J. Infec. Dis.* **181**:S360-S366.
109. **Shieh, Y. C., R.C. Baric, J.W. Woods, and K.R. Calci.** 2003. Molecular surveillance of enterovirus and norwalk-like virus in oysters relocated to a municipal-sewage-impacted gulf estuary. *Appl. Environ. Microbiol.* **69**:7130-7136.
110. **Siebenga, S., A. Kroneman, H. Vennema, E. Duizer, and M. Koopmans.** 2008. Food-borne viruses in Europe network report: the norovirus GII.4 2006b (for US named

- Minerva-like, for Japan Kobe034-like, for UK V6) variant now dominant in early seasonal surveillance. *Euro. Surveill.* **13**.
111. **Sobsey, M. D., C. Wallis, and J. L. Melnick.** 1975. Development of a simple method for concentrating enteroviruses from oysters. *Appl. Microbiol.* **29**:21-26.
112. **Straub, T. M., K. Honer zu Bentrup, P. Orosz-Coghlan, A. Dohnalkova, B. K. Mayer, R. A. Bartholomew, C. O. Valdez, C. J. Bruckner-Lea, C. P. Gerba, M. Abbaszadegan, and C. A. Nickerson.** 2007. In vitro cell culture infectivity assay for human noroviruses. *Emerg. Infect. Dis.* **13**:396-403.
113. **Sugieda, M., K. Nakajima, and N. S.** 1996. Outbreaks of Norwalk-like virus associated gastroenteritis traced to shellfish: coexistence of two genotypes in one specimen. *Epidemiology and Infection* **116**:339-346.
114. **Ueki, Y., D. Sano, T. Watanabe, K. Akiyama, and T. Omura.** 2005. Norovirus pathway in water environment estimated by genetic analysis of strains from patients of gastroenteritis, sewage, treated wastewater, river water and oysters. *Water Research* **39**:4271-4280.
115. **Ueki, Y., M. Shoji, A. Suto, T. Tanabe, Y. Okimuro, Y. Kikuchi, N. Saito, D. Sano, and T. Omura.** 2007. Persistence of caliciviruses in artificially contaminated oysters during depuration. *Appl. Environ. Microbiol.* **73**:5698-5701.
116. **Vinje, J.** 2008. Unpublished.
117. **Vinje, J., S. Altena, and M. Koopmans.** 1997. The incidence and genetic variability of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. *J. Infect. Dis.* **176**:1374-1378.

118. **Vinje, J., R. A. Hamidjaja, and M. D. Sobsey.** 2004. Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. *J. Virol. Methods* **116**:109-117.
119. **Vinje, J., and M. P. G. Koopmans.** 1996. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in The Netherlands. *J. Infect. Dis.* **173**:610-615.
120. **Vinje, J., and M. P. G. Koopmans.** 2000. Simultaneous detection and genotyping of Norwalk-like viruses by oligonucleotide array in a reverse line blot hybridization format. *J. Clin Microbiol.* **38**:2595-2601.
121. **Wang, J., X. Jiang, H. P. Madore, J. Gray, U. Desselberger, T. Ando, Y. Seto, I. Oishi, J. F. Lew, and K. Y. Green.** 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. *J. Virol.* **68**:5982-5990.
122. **Wang, Q.-H., M. G. Han, S. Cheetham, M. Souza, J. A. Funk, and L. J. Saif.** 2005. Porcine norovirus related to human norovirus. *Emerg. Infect. Dis.* **11**:1874-1881.
123. **Widdowson, M.-A., A. Sulka, S. N. Bulens, R. S. Beard, S. S. Chaves, R. Hammond, E. D. P. Salehi, E. Swanson, J. Totaro, R. Woron, P. S. Mead, J. S. Bresee, S. S. Monroe, and R. I. Glass.** 2005. Norovirus and foodborne disease, United States, 1991-2000. *Emerg. Infect. Dis.* **11**.
124. **Wolf, S., W. M. Williamson, J. Hewitt, M. Rivera-Aban, S. Lin, A. Ball, P. Scholes, and G. E. Greening.** 2007. Sensitive multiplex real-time reverse transcription-PCR assay for the detection of human and animal noroviruses in clinical and environmental samples. *Appl. Environ. Microbiol.* **73**:5464-5470.

125. **Wright, P. J., I. C. Gunesekere, J. C. Doultree, and J. A. Marshall.** 1998. Small round-structured (Norwalk-like) viruses and classical human caliciviruses in southeastern Australia 1980-1996. *J Med Virol* **55**:312-320.
126. **Yan, H., F. Yagy, S. Okitsu, O. Nishio, and H. Ushijima.** 2003. Detection of norovirus (GI, GII), sapovirus, and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. *J. Virol. Methods* **14**:37-44.

CHAPTER 3

Rapid and Efficient Quantification of Norovirus Genogroups I and II from Oyster and Plankton¹

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ABSTRACT

The human health risk associated with the consumption of molluscan shellfish grown in sewage-contaminated waters is well established. Noroviruses are the principal agent of shellfish-related illness. We describe the evaluation of two silica-based viral RNA extraction protocols as well as two recently published norovirus real time TaqMan RT-PCR assays for the detection of norovirus in shellfish and adapted for use in plankton. Using a GII fecal sample, the Qiagen RNeasy method was able to detect 8, 5.2×10^3 , and 8.6×10^3 copies in the oyster, small plankton, and large plankton samples, respectively whereas the silica binding method was able to detect 38 copies in the oyster samples. The detection limit of two published TaqMan RT-PCR assays (A and B), evaluated with three RNA run-off transcripts GI.4, GI.3b, and GII.4, established assay B was more sensitive for detecting the GI.4 and the GII.4 run-off transcripts whereas assay A was more sensitive for detecting low copies of GI.3 RNA and both NoV genogroups in naturally contaminated oysters. Both assays were evaluated on naturally contaminated samples which demonstrated that the use of the RNeasy method was superior for detecting norovirus RNA, but both assay A and B were equally sensitive. In conclusion, the combination of a rapid RNA extraction method followed by TaqMan RT-PCR assay A offers significant advantages for development of routine assays for the detection of noroviruses in bivalve shellfish and plankton.

Keywords: norovirus, shellfish, plankton, RT-PCR

INTRODUCTION

Human norovirus (NoV), a genus of the family *Caliciviridae*, is estimated to cause two-thirds of non-bacterial foodborne illness and 30-50% of all foodborne outbreaks of acute gastroenteritis in the U.S. (30). NoV infection due to contaminated oysters has been investigated extensively, and bivalve molluscan shellfish have been implicated in many outbreaks of acute gastroenteritis (10, 23). Hence, monitoring of NoVs in shellfish and the surrounding water environment is critical to reducing shellfish-related norovirus illness; however, methods to detect NoVs were originally developed for use in stool samples, whereas environmental samples tend to have higher concentrations of inhibitors (e.g., polysaccharides) and viral detection methods must be adapted accordingly (8).

With no simple method to detect infectious NoV available yet, methods focus on detection of viral particles or their nucleic acids, both of which have limitations. Detection by electron microscopy requires at least 1×10^6 virions ml^{-1} (15, 21). Although the detection limit for conventional RT-PCR is lower, this method requires examination by gel electrophoresis followed by sequencing or hybridization (28). Recently, rapid and sensitive real-time RT-PCR assays have been developed following the successful sequencing of complete norovirus genomes (2, 14, 24); however, many laboratories analyzing environmental samples continue to use laborious RNA extraction methods of varying efficiencies (15), which counteracts the goal of a rapid detection system.

Common methods used for the extraction of enteric viruses and viral RNA from environmental samples include acid adsorption-elution (19, 38), direct glycine buffer elution (26), silica bead extraction (7, 26), virus precipitation using Cat-Floc (34) or polyethylene glycol

(16, 19), and solvent extraction using chloroform (5, 31) or chloroform/butanol (4). The drawback to most of these methods is the time required for extraction and the loss of viral genomes during successive steps (15). To date, the most reliable and straightforward method for purifying viral nucleic acids from shellfish and water samples is the silica bead extraction method, on which most commercial kits are now based (15). These extraction methods can be followed by variants of the RT-PCR assay such as nested RT-PCR, semi nested RT-PCR, RT-PCR followed by hybridization, and real-time RT-PCR, which, when paired with RNA run-off transcripts, can be used to quantify the viruses present.

Recently, a novel virus extraction method including concentration of viruses using Zirconia beads and extraction of viral RNA using the Qiagen RNeasy Mini kit following the Plant and Fungi Method was reported (8). In the present study, we build upon that method using an alternate concentration step with PBS + 100 $\mu\text{g ml}^{-1}$ proteinase K and quantitative detection of norovirus by real time RT-PCR using RNA run-off transcripts. We also extrapolated the protocol for use on other environmental samples subject to high concentrations of inhibitors (i.e., plankton). Efficient detection and enumeration of norovirus in estuarine sample types will allow for a better overall understanding of NoV presence in the water environment and its transmission to shellfish. The present study describes an optimized protocol for the rapid and efficient quantification of norovirus genogroups I and II from both oyster and plankton samples.

MATERIALS AND METHODS

Controls.

Positive controls. Three norovirus-positive fecal samples, representing genotypes GI.4, GI.3b, and GII.4 Minerva, were provided by the Centers for Disease Control and Prevention

(CDC). Stool samples were diluted in phosphate-buffered saline (PBS) to obtain a 20% suspension, vortexed, and centrifuged at 15,700 x g for 2 min to remove the solids and the supernatant was stored in 1 ml aliquots at -70°C.

Viral RNA extraction from stool. For stool samples, viral RNA was extracted from the clarified PBS extracts using the MagMAX-96 Viral Isolation Kit (Ambion, Austin, TX) and the KingFisher Instrument (Thermo Electron Corporation, Waltham, MA). Purified RNA was eluted into 55 µl of elution buffer provided by the kit.

RNA Transcript Standards. To obtain positive control standards and to enable quantification in real-time RT-PCR, RNA run-off transcripts for genotypes GI.4, GI.3, and GII.4 were obtained from the CDC. The concentration for each of the RNA transcripts was previously determined to be 1×10^6 copies μl^{-1} . A 10-fold serial dilution was made for each transcript and analyzed by real time RT-PCR to create the standard curves as described below.

Shellfish and plankton samples.

The shellfish used in this study were *Crassostrea gigas* oysters, frozen on the half-shell, imported from South Korea and implicated in a norovirus outbreak in the western United States in December 2006. The Food and Drug Administration (FDA) confirmed norovirus in oysters from this lot on December 8, 2006 and issued a multi-state recall (1). The USDA provided several hundred oysters, which were shipped frozen by overnight courier for this study.

The plankton samples used in this study were obtained from Sapelo Sound off the coast of McIntosh County, Georgia in September 2006 and March 2007. Two plankton size fractions were collected using a 63-µm plankton net and a 200-µm plankton net, which were towed horizontally for 5 minutes. Each fraction was collected in pre-sterilized 1-liter polypropylene bottles. The samples were held at ambient temperatures (22°C- 25°C) and transported to the

laboratory within 6 hours of collection and processed. The plankton was re-filtered through 63- and 200- μm nets to attain exact size fractions, 63-200 μm and $>200 \mu\text{m}$, and homogenized for 5 minutes using a Pro Scientific Series Pro 200 homogenizer (Oxford, CT).

NoV Concentration from Oysters and Plankton. Four oysters were removed from their shell and the peripheral flesh and organs were cut away from the hepatopancreas (18). The combined oyster hepatopancreas material was finely chopped with a sterile razor and approximately 5 grams were added to an equal volume of PBS plus $100\text{-}\mu\text{g ml}^{-1}$ proteinase K (5 ml) to aid in the degradation of the shellfish tissue and release the virions into suspension (Fig. 3.1). A plankton suspension was made in a method similar to that for the oysters. Briefly, approximately 300 μg of homogenized plankton was separated into a microcentrifuge tube. An equal volume-to-weight of PBS + $100 \mu\text{g ml}^{-1}$ proteinase K was added to the tube (Fig. 3.1).

For the concentration of norovirus, both oyster and plankton suspensions were incubated at 37°C for 1 hour with shaking at 320 rpm on a C24 Incubator Shaker (Edison, NJ), vortexed, and further incubated at 65°C for 15 min to inactivate the enzyme, and vortexed again. The suspensions were centrifuged at $3,000 \times g$ for 5 min and the soluble portion (approximately 8 ml) was stored at -80°C in 1-ml aliquots. The aliquots were used for the extraction assays and seeding experiments below.

Viral RNA extraction. Viral RNA from the oyster and plankton suspensions was extracted using a method modified from Boom et al. (7) and Lees et al. (26) as described in Jothikumar et al. (20). Buffers L6 (Guanidine thiocyanate, Tris-HCl, Triton X-100, EDTA), L2 (Guanidine thiocyanate, Tris-HCl), and silica were prepared as described previously (7). Briefly, for each sample, 133- μl aliquots of shellfish or plankton homogenate were separated into 1.5-ml microcentrifuge tubes containing 10 μl of a silica powder suspension (glassmilk: Science Stuff,

Inc., Austin, TX). PBS was added to all tubes for a final volume of 420 μ l. Two positive controls were included: 50 μ l of either a GI or GII norovirus-positive fecal sample plus 5 μ l of a silica powder suspension was added to PBS for a final volume of 405 μ l. One negative control was included: 400 μ l of PBS plus 5 μ l of a silica powder suspension. Viral RNA was extracted by binding to the size-fractionated silica after treatment with 900 μ l buffer L6, 1 ml buffer L2 twice, 1 ml 70% ethanol, and 1 ml ice-cold acetone. After elution with 52.5 μ l Tris-EDTA buffer, RNA was precipitated in 5 μ l 3M sodium acetate (pH 5.2) and 110- μ l ice cold 100% ethanol and resuspended in 50- μ l nuclease-free water.

For the Qiagen RNeasy Mini kit, 150 μ l of oyster or plankton homogenate was used for RNA extraction, following the plant and fungi procedure. The kit was utilized for its acidic polysaccharide-removing properties, as asserted by de Roda Husman et al. (8), who reported the Qiagen RNeasy Mini kit to be the most time- and recovery-efficient norovirus RNA extraction protocol for shellfish tissue (8). Briefly, 150 μ l of the oyster or plankton concentrate was added to 450 μ l of Buffer RLT (a guanidine thiocyanate solution containing 45 μ l β -mercaptoethanol) and vortexed vigorously. Three controls were included with the samples and processed similarly: 150 μ l of either a GI or GII norovirus-positive fecal sample suspension as positive controls and 150 μ l of PBS as a negative control. Remaining cell debris in the suspensions was removed using the Qiashredder spin columns. The flow-through from the Qiashredder column was added to 0.5 volumes (~250 μ l) 100% ethanol and loaded into the RNeasy spin columns where bound RNA was washed with guanidine salts and ethanol and finally eluted into 50 μ l nuclease-free water.

Quantification of inhibition in oyster and plankton samples. The level of inhibition in both oyster and plankton samples was compared to that of sterile water by seeding both

plankton and oyster homogenates (140 µl) with 10 µl of a GII.4 fecal suspension and comparing this to 140 µl sterile water spiked with 10 µl of a GII.4 fecal suspension. RNA from oyster and plankton homogenates and spiked-sterile water was extracted using the two extraction methods listed above and subjected to real time RT-PCR for NoV GII.

Comparison of viral RNA extraction methods using seeded oysters and plankton samples. To compare the silica-binding extraction method with the Qiagen kit, the oyster and plankton homogenates were split and spiked with a norovirus GII RNA run-off transcript or a norovirus GII-positive fecal suspension. Only norovirus genogroup II was used for spiking experiments because it is the most frequently detected genogroup identified in outbreaks (12).

Oysters and plankton were seeded with a GII RNA run-off transcript (2×10^6 copies of GII.4 RNA) and viral RNA was extracted and purified to determine the efficiency of each extraction method. For the silica-binding extraction method, 2 µl of RNA transcript (2×10^6 copies) was added and bound to the size-fractionated silica after the oyster or plankton suspension was treated with 900 µl buffer L6. For the Qiagen kit, the flow-through from the Qiashtredder column was added to a new 1.5-ml tube and spiked with 2 µl of the GII RNA run-off transcript containing 2×10^6 copies of GII.4 RNA.

Oyster and plankton suspensions were also compared for extraction efficiency when seeded with a GII-positive fecal sample. In the silica-binding extraction method, 123 µl of the suspension was spiked with 10 µl of a fecal suspension before extraction following the above procedures. For the Qiagen extraction kit, 140 µl of the suspension was spiked with 10 µl of a fecal suspension before extraction

Detection and quantification of norovirus RNA. Two established detection assays for norovirus real time RT-PCR that have been tested for use in naturally contaminated shellfish

were compared (Table 3.1). Assay A targets an 84-bp fragment (Genogroup I) and a 97-bp fragment (Genogroup II) of the conserved region at the ORF1-ORF2 junction of the norovirus genome (21) and assay B targets a 96-bp and an 89-bp fragment, respectively, of the same area of the genome (20).

The RT-PCR reaction mixture for both primer sets contained 2 µl of template, each primer at a final concentration of 400 nM, each probe mixture at a final concentration of 120 nM, 12.5 µl of 2X RT-PCR buffer, 1 µl of 25X RT-PCR enzyme mix, 1.67 µl of detection enhancer, and nuclease-free water for a total reaction mixture of 25 µL (Ambion AgPath-ID One-Step RT-PCR kit). The reaction mixture was subjected to a one-step assay on an ABI 7900 (Applied Biosystems, Foster City, CA), an ABI StepOne (Applied Biosystems, Foster City, CA), or an Eppendorf Mastercycler ep realplex using the following conditions: (i) RT for 10 min at 45°C, (ii) 10 min at 95°C, (iii) 45 cycles of 10 s at 95°C, 30 s at 55°C, and 15 s at 72°C.

All amplification reactions were carried out in duplicate. Samples that gave a positive result in either or both of the duplicate reactions were re-tested. If the sample gave a second positive result, it was counted as an overall positive.

Statistics. SAS (Cary, NC) software for windows was used to perform Student's T-tests to compare assays (A and B) and extraction methods. Linear regressions were performed to describe the relationship between RNA run-off transcript concentrations and Ct values. All tests had the significance level set to $p=0.05$.

RESULTS

Comparison of NoV Taqman realtime RT-PCR assays using RNA transcripts. A GI.3, a GI.4, and a GII.4 RNA run-off transcript were examined by real-time RT-PCR using

assay A and assay B. Figure 3.2 gives the standard curves for all three RNA transcripts. Assay A was more sensitive than assay B for the GI.3 RNA transcript over each dilution. Additionally, assay A had a detection limit of 20 copies of GI.3 norovirus μl^{-1} while assay B had a detection limit of 200 copies μl^{-1} (Fig 3.2A). However, for the GI.4 RNA run-off transcript, assay B was more sensitive than assay A. Crossing point values were an average of 5.7 cycles lower (95% CI= -6.2, -5.3) for assay B; however, both assays had a detection limit of 20 copies μl^{-1} for the GI.4 RNA transcript (Fig 3.2B). Finally, assay B was more sensitive for the GII.4 RNA transcript by an average of 1.7 cycles (95% CI= -2.6, -0.8), but both assays had a detection limit of 2,000 copies of GII.4 norovirus μl^{-1} (Fig 3.2C). The high detection limit for GII was not due to a lack of amplification, as amplification for both 200 and 20 copies of GII norovirus was visible, but the background noise prohibited the computer from accurately determining the crossing point value for the lower copy numbers (Figure 3.3). Based on these observations, our study continued with both sets of primers and probes with a preference to assay B when spiking with genogroup II.

Quantification of inhibition in oyster and plankton samples. The level of inhibition in oyster and plankton RNA extracts (using both extraction methods) was compared to the level of inhibition in sterile water. Plankton and oyster homogenates (140 μl) were seeded with 10 μl of a GII.4 fecal sample and compared to the Ct values of 140 μl sterile water spiked with 10 μl of a GII.4 fecal sample. The average Ct difference between ten-fold dilutions is given for each sample type and extraction method (Table 3.2). A Ct difference of 3.3 is equivalent to a ten-fold difference in DNA. For sterile water, an average Ct difference of 3.5 and 3.3 cycles between ten-fold dilutions was given for the silica-binding extraction method and Qiagen RNeasy kit, respectively. For oyster homogenates, a Ct difference of 6.5 and 4.0 cycles between ten-fold

dilutions was given for the silica-binding extraction method and Qiagen RNeasy kit, respectively. For plankton homogenates, a Ct difference of 3.2 cycles between ten-fold dilutions was given for the Qiagen RNeasy kit while no positives were detected using the silica-binding method. Table 3.2 also gives the average Ct difference between seeded sample and sterile water over ten-fold dilutions. Dilutions of seeded oyster samples extracted with the silica-binding method produced an average Ct 9.4 cycles higher than sterile water dilutions while seeded oyster samples extracted with the Qiagen RNeasy method yielded an average Ct 1.1 cycles higher than sterile water dilutions. Seeded plankton sample dilutions were an average 1.0 cycles higher than seeded sterile water dilutions using the Qiagen RNeasy method while no norovirus positive samples were detected with the silica-binding method.

Comparison of the silica-binding extraction method with the Qiagen RNeasy Mini kit for seeded oysters. Two RNA extraction methods were compared with regard to their speed and efficiency of norovirus RNA recovery. It took approximately 30 min. to obtain norovirus RNA from 4 oysters with the Qiagen RNeasy kit while it took more than 5 hours for the same 4 oysters with the silica-binding extraction method.

The recovery efficiency of each method was compared by spiking the oyster suspension with a GII RNA transcript after the lysis step in both extraction methods. The silica-binding extraction method estimated the number of copies of RNA to be 3.5×10^3 , significantly lower ($p=0.0013$) than the spiked value of 2.0×10^6 copies. The Qiagen extraction kit estimated the number of copies of RNA to be 1.6×10^6 copies, which was not significantly different from the actual number of RNA spiked into solution, 2.0×10^6 copies (Figure 3.4A).

The recovery efficiency of each method was also compared by spiking the oyster suspension with a GII-positive fecal sample and extracting RNA using both methods. Endpoint-

dilution real-time RT-PCR using assay B identified the greatest sensitivity with the RNeasy Mini kit (Table 3.3). The silica-binding method was positive only to a dilution of 1.0×10^0 , corresponding to an average of 38 RNA copies at a Ct value between 36.6 and 43.9. The Qiagen RNeasy Mini kit was positive to a dilution of 1.0×10^{-3} , corresponding to approximately 8 RNA copies at a Ct value of 40.0 (Table 3.3).

Evaluation of protocols on naturally contaminated oysters. The entire protocol for detection of norovirus genogroups I and II was evaluated on naturally-contaminated oysters with all alternatives, including extraction with the silica-binding method and Qiagen RNeasy Mini Kit, and TaqMan realtime RT-PCR with both assays (Table 3.4). Extraction with the silica-binding method resulted in no positive norovirus samples. Using the two sample t-test, there was no evidence that either assay was more sensitive for norovirus amplification from naturally contaminated oysters ($p=0.6171$); however, assay A was able to detect both genogroups I and II while assay B only detected genogroup I in the naturally contaminated oysters.

Comparison of the silica-binding extraction method with the Qiagen RNeasy Mini kit for seeded plankton. The two RNA extraction methods were compared with regard to their speed and efficiency of norovirus RNA recovery from two plankton samples, 63-200 μm and $>200 \mu\text{m}$, in addition to shellfish samples. The time for norovirus extraction using both methods was the same as for shellfish samples. It took approximately 30 min to obtain norovirus RNA from 6 plankton samples (either size fraction) with the Qiagen RNeasy Mini kit while it took more than 5 hours for the same 6 samples with the silica-binding extraction method.

The recovery efficiency of each method was compared by spiking four 150- μl plankton suspensions (two 63-200 μm plankton and two $>200 \mu\text{m}$ plankton) with a GII RNA transcript after the lysis step in both extraction methods. The silica-binding extraction method estimated

the number of copies of RNA in the seeded 63-200 μm plankton samples between 52-125 (<0.001%), significantly lower ($p < 0.0001$) than the spiked value (2.0×10^6 copies) using the two sample t-test, while the Qiagen extraction kit estimated the number of copies of RNA to be 3.7×10^4 (0.01%), which was also significantly lower than the actual number of RNA spiked into solution ($p < 0.0001$) but much closer to the initial seeded value (2.0×10^6 copies) (Figure 3.4B). The silica-binding extraction method estimated the number of copies of RNA in the seeded >200 μm plankton samples to be 11.1 (<0.001%), significantly lower ($p < 0.0001$) than the spiked value, while the Qiagen RNeasy Mini kit estimated the number of copies of RNA to be 2.8×10^3 copies (0.001%), which was also significantly lower than the actual number of RNA spiked into solution ($p < 0.0001$) but much closer to the initial seeded value (2.0×10^6 copies).

The recovery efficiency of each method was also compared by spiking the plankton suspensions with a GII-positive fecal sample and extracting RNA using both methods. Endpoint-dilution real-time RT-PCR using assay B identified the greatest sensitivity with the RNeasy Mini kit for the 63-200 μm plankton and >200 μm plankton samples (Table 3.5). The silica-binding method did not detect norovirus in either sample at any dilution. The Qiagen RNeasy kit was positive to a dilution of 1.0×10^{-1} for both plankton size fractions, corresponding to approximately 5.2×10^3 NoV genomes at a Ct value of 36.0 for 63-200 μm plankton and 8.6×10^3 NoV genomes at a Ct value of 35.0 for >200 μm plankton.

Evaluation of protocol on naturally contaminated plankton. The entire protocol for detection of norovirus genogroups I and II was evaluated on naturally-contaminated plankton collected from Sapelo Sound, GA in March 2007. Viral RNA was concentrated with PBS + 100 $\mu\text{g ml}^{-1}$ proteinase K, extracted with the silica-binding method and Qiagen RNeasy Kit, and subjected to real-time RT-PCR with assays A and B (Table 3.6). Extraction with the silica-

binding method resulted in no positive norovirus samples. Using the Qiagen RNeasy Mini kit, assay A only detected NoV genogroup I in the >200 μm plankton with Ct means of 4-11 (N=2), while assay B only detected NoV genogroup I in the 63-200 μm plankton with Ct means of 30-35 (N=2).

Based on these results, we determined an optimal detection protocol: concentrating noroviruses in oyster and plankton suspensions with PBS + 100 $\mu\text{g ml}^{-1}$ proteinase K, norovirus extraction with the Qiagen RNeasy method, and real-time RT-PCR amplification with assay A is a rapid and sensitive method for naturally contaminated plankton in addition to naturally contaminated shellfish (Figure 3.5).

DISCUSSION

Many different viral RNA extraction methods for shellfish are in use today (3, 9, 13, 17, 25, 29, 36, 37). Only a handful of them take less than twenty-four hours to perform (22, 36). The protocol presented here was successfully used on both contaminated oysters and plankton to rapidly and efficiently extract norovirus-spiked or naturally accumulated RNA. The total time from raw environmental sample to RT-PCR results was less than six hours. In general, our results were consistent with the findings of de Roda Husman et al. (8) that the Qiagen RNeasy Mini kit had a higher recovery efficiency than the silica-binding extraction method for shellfish and we found that it is more efficient for plankton samples as well.

One disadvantage of the Qiagen RNeasy Mini kit is that generally in-house methods are cheaper than commercial kits, enhancing the appeal of the silica-binding extraction method. However, a standard method is necessary in order to objectively compare results from different

experiments and laboratories and the silica-binding method was less sensitive by several orders of magnitude.

Assay B produced a lower Ct value for each copy number of the GII.4 RNA transcript indicating that these primers and probes are highly sensitive as previously reported (20). While assay B was more sensitive for the GI.4 RNA run-off transcript, assay A had a lower detection limit for the GI.3b RNA transcript, which represents one of the most prevalent genogroup I genotypes (39). Assay A and B were equally sensitive for naturally contaminated oysters but only assay A was able to detect NoV in the naturally contaminated >200 µm plankton and genogroup I and II noroviruses in the naturally contaminated oyster samples. Based on these results, we recommend A for detection of NoV from environmental samples.

To compare the relative levels of inhibition in our two sample types, oyster and plankton, we seeded both with a GII.4 norovirus-positive fecal suspension. We detected a greater level of inhibition in plankton than oysters and found that the Qiagen RNeasy Mini kit removes more inhibition from both sample types than the silica-binding method. Similar results were obtained when comparing extraction methods for oysters and plankton. Whereas De Roda Husman et al. (8) asserted that pooling oysters would dilute norovirus RNA and concentrate inhibitors, we produced a positive norovirus signal from not only pooled oysters, but also pooled plankton samples as well. Inhibition was not observed for the artificially contaminated oysters (as determined by observation of a dilution effect) but significant inhibition was observed for the estuarine plankton samples. However, while both extraction methods were subject to inhibition for NoV extraction from plankton, as detected by use of RNA transcripts, the Qiagen RNeasy Mini kit outperformed the silica-binding method. Similarly, when seeded with a NoV-contaminated fecal sample, the Qiagen RNeasy Mini Kit was able to detect norovirus to a

dilution of 1.0×10^{-1} while the silica-binding extraction method was unable to detect NoV genomes at any dilution of NoV RNA sample from plankton. The Qiagen RNeasy Mini kit proved to be the superior viral RNA extraction method in all seeded experiments and was the critical factor in detecting NoV in samples subject to high concentrations of inhibitors.

Viral gastroenteritis outbreaks related to shellfish have been increasing around the world over the past decade (11, 21, 32, 33, 35). Norovirus has been suspected in many of them but it has been difficult to link shellfish consumption with the outbreak using molecular techniques (6, 11) because it is often difficult to collect and analyze the specific food items implicated. Low levels of contamination of shellfish, such as those seen in the naturally contaminated oysters in our study ($3 \times 10^4 - 2 \times 10^8$ genomes g^{-1}), yielding threshold cycle values of 38 to 42, can generally only be observed by the use of real-time TaqMan RT-PCR (20, 28). Since the infectious dose of norovirus is estimated between 1-10 viral particles (27) these low environmental levels can, when infectious, be enough to lead to infection and disease but norovirus levels in shellfish are rarely quantified. Additionally, norovirus has not yet been examined for its association with estuarine plankton, but as seen in this study, human norovirus can be detected at quantifiable levels in such samples. A protocol as presented in this paper that is rapid and sensitive enough to detect and quantify norovirus from multiple samples in a suspected waste-contaminated shellfish bed would be important for shellfish monitoring and prevention of shellfish-borne gastroenteritis.

REFERENCES

1. **U.S. Food and Drug Administration.** 2006. FDA Investigating Norovirus Outbreak Linked to Oysters. Internet Press Release.
2. **Ando, T., S. S. Monroe, J. R. Gentsch, Q. Jin, D. C. Lewis, and R. I. Glass.** 1995. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and Southern hybridization. *J. Clin. Microbiol.* **35**:64-71.
3. **Arnal, C., V. Ferre-Aubineau, B. Besse, B. Mignotte, L. Schwartzbrod, and S. Billaudel.** 1999. Comparison of seven RNA extraction methods on stool and shellfish samples prior to hepatitis A virus amplification. *J. Virol. Methods* **77**:17-26.
4. **Atmar, R. L., F.H. Neill, J.L. Romalde, F. LeGuyader, C.M. Woodley, T.G. Metcalf, and M.K. Estes.** 1995. Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. *Appl. Environ. Microbiol.* **61**:3014-3018.
5. **Atmar, R. L., T. G. Metcalf, F. H. Neill, and M. K. Estes.** 1993. Detection of enteric viruses in oysters by using the polymerase chain reaction. *Appl. Environ. Microbiol.* **59**:631-635.
6. **Berg, D. E., M. A. Kohn, T. A. Farley, and L. M. McFarland.** 2000. Multi-state outbreaks of acute gastroenteritis traced to fecal-contaminated oysters harvested in Louisiana. *J. Infec. Dis.* **181**:381-386.
7. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. W.-v. Dillen, and J. v. d. Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**:495-503.

8. **de Roda Husman, A. M., F. Lodder-Verschoor, H. H. J. L. V. d. Berg, F. S. L. Guyader, H. V. Pelt, W. H. M. V. d. Poel, and S. A. Rutjes.** 2007. Rapid virus detection procedure for molecular tracing of shellfish associated with disease outbreaks. *J. Food Prot.* **70**:967-974.
9. **Dix, A. B., and L. A. Jaykus.** 1998. Virion concentration method for the detection of human enteric viruses in extracts of hard-shelled clams. *J. Food Prot.* **61**:458-465.
10. **Dowell, S. F., C. Groves, K. B. Kirkland, H. G. Cicirello, T. Ando, Q. Jin, J. R. Gentsch, S. S. Monroe, C. D. Humphrey, C. Slemp, D. M. Dwyer, R. A. Meriwether, and R. I. Glass.** 1995. A multistate outbreak of oyster-associated gastroenteritis: implications for interstate tracing of contaminated shellfish. *J. Infec. Dis.* **171**:1497-1503.
11. **Doyle, A., D. Barataud, A. Gallay, J. M. Thiolet, S. Le Guyader, E. Kohli, and V. Vaillant.** 2004. Norovirus foodborne outbreaks associated with the consumption of oysters from the Etang de Thau, France, December 2002. *Euro. Surveill.* **9**:24-26.
12. **Fankhauser, R. L., S. S. Monroe, J. S. Noel, C. D. Humphrey, J. S. Bresee, U. D. Parashar, T. Ando, and R. I. Glass.** 2002. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. *J. Infec. Dis.* **186**:1-7.
13. **Gouvea, V., N. Santos, M. d. C. Timenetsky, and M. K. Estes.** 1994. Identification of Norwalk virus in artificially seeded shellfish and selected foods. *J. Virol. Methods* **48**:177-187.
14. **Green, J., J. P. Norcott, D. Lewis, C. Arnold, and D. W. Brown.** 1993. Norwalk-like viruses: demonstration of genomic diversity by polymerase chain reaction. *J. Clin. Microbiol.* **31**:3007-3012.

15. **Griffin, D. W., K. A. Donaldson, J. H. Paul, and J. B. Rose.** 2003. Pathogenic Human Viruses in Coastal Waters. *Clin. Microbiol. Rev.* **16**:129-143.
16. **Guillard, R. R., and J. H. Ryther.** 1962. Studies of marine planktonic diatoms. *Can. J. Microbiol.* **8**:229-239.
17. **Hafliger, D., M. Gilgen, J. Luthy, and P. Hubner.** 1997. Semi-nested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood. *Intl. J. Food Microbiol.* **37**:27-36.
18. **Henshilwood, K., W. J. Dore, S. Anderson, and D. N. Lees.** 2003. Investigation of Norwalk like virus elimination during depuration using a real time quantitative PCR. *In* J. L. R. A. Villalba, B. Reura, and R. Beiras (ed.), *Molluscan shellfish safety*. Conselleria de Pesca e Asuntos Maritimos da Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Galicia, Spain.
19. **Jaykus, L. A., R. DeLeon, and M. D. Sobsey.** 1996. A virion concentration method for detection of human enteric viruses in oysters by PCR and oligoprobe hybridiation. *Appl. Environ. Microbiol.* **62**:2074-2080.
20. **Jothikumar, N., J. A. Lowther, K. Henshilwood, D. N. Lees, V. R. Hill, and J. Vinje.** 2005. Rapid and sensitive detection of norovirus by using TaqMan-based one-step reverse-transcription-PCR assays and application to naturally contaminated shellfish samples. *Appl. Environ. Microbiol.* **71**:1870-1875.
21. **Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama.** 2003. Broadly reactive and highly sensitive assay for norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J. Clin. Microbiol.* **41**:1548-1557.

22. **Kingsley, D. H., and G. P. Richards.** 2001. Rapid and Efficient Extraction Method for Reverse Transcription-PCR Detection of Hepatitis A and Norwalk-Like Viruses in Shellfish. *Appl. Environ. Microbiol.* **67**:4152-4157.
23. **Kohn, M. A., T. A. Furley, T. Ando, M. Curtis, S. A. Wilson, Q. Jin, S. S. Monroe, R. C. Baron, L. M. MacFarland, and R. I. Glass.** 1995. An outbreak of Norwalk virus gastroenteritis associated with eating raw oysters. Implications for maintaining safe oyster beds. *J. Am. Med. Assoc.* **273**:466-471.
24. **Lambden, P. R., E. O. Caul, C. R. Ashley, and I. N. Clarke.** 1993. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. *Science* **259**:516-519.
25. **Le Guyader, F., F. H. Neill, M. K. Estes, S. S. Monroe, T. Ando, and R. L. Atmar.** 1996. Detection and analysis of a small round-structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. *Appl. Environ. Microbiol.* **62**:4268-4272.
26. **Lees, D. N., K. Henshilwood, and W. J. Dore.** 1994. Development of a method for detection of enteroviruses in shellfish by PCR with poliovirus as a model. *Appl. Environ. Microbiol.* **61**:2999-3005.
27. **Lindsmith, L., C. Moe, S. Marionneau, N. Ruvoen, X. Jiang, L. Lindblad, P. Stewart, J. Lependu, and R. Baric.** 2003. Human susceptibility and resistance to Norwalk virus infection. *Nat. Med.* **9**:548-553.
28. **Loisy, F., R. L. Atmar, P. Guillon, P. L. Cann, M. Pommepuy, and F. S. L. Guyader.** 2005. Real-time RT-PCR for norovirus screening in shellfish. *J. Virol. Methods* **123**:1-7.

29. **Lopez-Sabater, E. I., M. Y. Deng, and D. O. Cliver.** 1997. Magnetic Immunoseparation PCR assay (MIPA) for detection of hepatitis A virus (HAV) in American oyster (*Crassostrea virginica*). *Lett. Appl. Microbiol.* **24**:101-104.
30. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. *Emerg. Infec. Dis.* **5**:607-625.
31. **Mullendore, J. L., M. D. Sobsey, and Y.-S. C. Shieh.** 2001. Improved method for the recovery of hepatitis A virus from oysters. *J. Virol. Methods* **123**:1-7.
32. **Potasman, I., A. Paz, and M. Odeh.** 2002. Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clin. Infec. Dis.* **35**:921-928.
33. **Prato, R., P. L. Lopalco, M. Chironna, G. Barbutti, C. Germinario, and M. Quarto.** 2004. Norovirus gastroenteritis general outbreak associated with raw shellfish consumption in south Italy. *BMC Infec. Dis.* **4**:37.
34. **Richards, G. P., D. Goldmintz, D. L. Green, and J. A. Babinchak.** 1982. Rapid methods for extraction and concentration of poliovirus from oyster tissues. *J. Virol. Methods* **5**:285-291.
35. **Sanchez, G., M. Pinto, H. Vanaclocha, and A. Bosch.** 2002. Molecular characterization of hepatitis A virus isolates from a transcontinental shellfish-borne outbreak. *J. Clin. Microbiol.* **40**:4148-4155.
36. **Schwab, K. J., F.H. Neill, F.F. Le Guyader, M.K. Estes, and R.L. Atmar.** 2001. Development of a reverse transcription-PCR-DNA enzyme immunoassay for detection of Norwalk-like viruses and hepatitis A virus in stool and shellfish. *Appl. Environ. Microbiol.* **67**:742-749.

37. **Shieh, Y. C., K. R. Calci, and R. S. Baric.** 1999. A method to detect low levels of enteric viruses in contaminated oysters. *Appl. Environ. Microbiol.* **65**:4709-4714.
38. **Sobsey, M. D., C. Wallis, and J. L. Melnick.** 1975. Development of a simple method for concentrating enteroviruses from oysters. *Appl. Microbiol.* **29**:21-26.
39. **Vinje, J.** 2008. Unpublished.

TABLES

Table 3.1. Oligonucleotide primer and probe sequences for norovirus real-time RT-PCR used in this study.

Assay	Genogroup	Oligonucleotide	Sequence (5'-3') ^a	Location	Reference
A	GI	COGIF	CGY TGG ATG CGN TTY CAT GA	5291-5310 ^e	Kageyama 2003
		COGIR	CTT AGA CGC CAT CAT CAT TYA C	5375-5358 ^e	
		Ring1a	FAM^b-AGA TYG CGA TCY CCT GTC CA-BHQ^c	5340-5359 ^e	
		Ring1b	FAM-AGA TCG CGG TCT CCT GTC CA-BHQ	5340-5321 ^e	
B	GI	JJVIF	GCC ATG TTC CGI TGG ATG	5282-5299 ^d	Jothikumar 2005
		JJVIR	TCC TTA GAC GCC ATC ATC AT	5377-5358 ^d	
		JJVIP	FAM-TGT GGA CAG GAG ATC GCA ATC TC-BHQ	5319-5341 ^d	
		Ring1b	FAM-AGA TCG CGG TCT CCT GTC CA-BHQ	5340-5321 ^e	
A	GII	COG2F	CAR GAR BCN ATG TTY AGR TGG ATG AG	5003-5023 ^f	Kageyama 2003
		COG2R	TCG ACG CCA TCT TCA TTC ACA	5100-5080 ^f	
		Ring2	FAM-TGG GAG GGC GAT CGC AAT CT-BHQ	5048-5067 ^f	
B	GII	JJV2F	CAA GAG TCA ATG TTT AGG TGG ATG AG	5003-5028 ^d	Jothikumar 2005
		COG2R	TCG ACG CCA TCT TCA TTC ACA	5100-5080 ^f	
		Ring2	FAM-TGG GAG GGC GAT CGC AAT CT-BHQ	5048-5067 ^f	

^aMixed bases in degenerate primers and probes are as follows: Y, C or T; R, A or G; B, not A; N, any.

^b6-carboxyfluorescein (FAM)

^cblack hole quencher (BHQ)

^d Nucleotide positions based on the Norwalk (GI) (accession no. M87661) and Lordsdale (GII) (accession no. X86557) sequences.

^e Corresponding nucleotide position of Norwalk1/68 virus (accession no. M87661) of the 5' end.

^f Corresponding nucleotide position of Camberwell virus (accession no. AF145896) of the 5' end.

Table 3.2. Quantification of inhibition in oyster and plankton samples by comparing NoV-seeded samples to NoV-seeded water samples (N=42).

Sample Type	Extraction Method	Ct Difference between cycles	Ct Difference from Sterile Water
Water	Silica-binding	3.5	
	Qiagen	3.3	
Oyster	Silica-binding	6.5	9.4
	Qiagen	4.0	1.1
Plankton	Silica-binding	ND ^a	ND
	Qiagen	3.2	1.0

^aND not detected

Table 3.3. Comparison of extraction methods using endpoint dilution real-time RT-PCR using assay B from oyster suspensions seeded with a GII norovirus-positive fecal sample (N=16).

Extraction Method	Dilution	Mean Ct	Mean Estimated Copies
Qiagen RNeasy	1 x 10 ⁰	28.1	8.9 x 10 ³
	1 x 10 ⁻¹	28.6	7.0 x 10 ³
	1 x 10 ⁻²	31.45	1.1 x 10 ²
	1 x 10 ⁻³	39.85	7.9 x 10 ⁰
	1 x 10 ⁻⁴	ND ^a	ND
Silica-binding	1 x 10 ⁰	40.25	3.8 x 10 ¹
	1 x 10 ⁻¹	ND	ND
	1 x 10 ⁻²	ND	ND
	1 x 10 ⁻³	ND	ND
	1 x 10 ⁻⁴	ND	ND

^aND not detected

Table 3.4. Crossing-point values of amplification of genogroup I and II norovirus from naturally contaminated oysters extracted from oyster suspensions using Qiagen RNeasy kit and amplified with both assays A and B (silica-binding extraction resulted in no positive amplifications) (N=16).

Assay	Genogroup	Mean Ct	Estimated copies oyster⁻¹
A	GI	42.5	2×10^5
	GI	28.6	2.4×10^{13}
	GII	27.6	1.5×10^8
	GII	34.9	3.3×10^6
B	GI	ND ^a	ND
	GI	40.9	3.3×10^7
	GII	ND	ND
	GII	ND	ND

^aND not detected

Table 3.5. Comparison of extraction methods using endpoint dilution real-time RT-PCR using assay B from plankton suspensions seeded with a GII norovirus-positive fecal sample (N=24).

Plankton Sample	Extraction Method	Dilution	Mean Ct	Mean Estimated Copies
63-200um	Qiagen	1.0 x 10 ⁰	36.2	4.5 x 10 ²
	RNeasy	1.0 x 10 ⁻¹	36.0	5.2 x 10 ³
		1.0 x 10 ⁻²	ND ^a	ND
	Silica-binding	1.0 x 10 ⁰	ND	ND
		1.0 x 10 ⁻¹	ND	ND
>200um	Qiagen	1.0 x 10 ⁻²	ND	ND
		1.0 x 10 ⁰	32.9	2.8 x 10 ³
	RNeasy	1.0 x 10 ⁻¹	35	8.6 x 10 ³
		1.0 x 10 ⁻²	ND	ND
	Silica-binding	1.0 x 10 ⁰	ND	ND
		1.0 x 10 ⁻¹	ND	ND
		1.0 x 10 ⁻²	ND	ND

^aND not detected

Table 3.6. Crossing-point values of amplification of genogroup I and II norovirus from naturally contaminated 63-200 μm and $>200 \mu\text{m}$ plankton samples from Sapelo Sound, GA using Qiagen RNeasy Mini kit and amplified with both assay A and assay B (silica-binding extraction resulted in no positive amplifications) (N=48).

Assay	Genogroup	Fraction	Mean Ct	Estimated copies g⁻¹
A	GI	>200	11.1	9.0×10^{12}
B	GI	63-200	33	1.6×10^7
A	GI	>200	5	4.5×10^{14}
B	GI	63-200	30.2	2.7×10^4

FIGURE LEGEND

FIG. 3.1. Flow chart for evaluation of norovirus detection protocols in raw oyster and plankton samples.

FIG. 3.2. Examination of RNA run-off transcripts: GI.3 (A), GI.4 (B), and GII.4 (C) using assays A (Kageyama 2003) and B (Jothikumar 2003).

FIG. 3.3. Real-time RT-PCR graph of reaction vs. cycle for GII.4 RNA run-off transcript displaying amplification of 200 and 20 copies μl^{-1} concentrations within background noise.

FIG. 3.4. Comparison of extraction methods using endpoint dilution real-time RT-PCR using Assay B from oyster and plankton suspensions seeded with a GII norovirus RNA run-off transcript.

FIG 3.5. Flow chart of final norovirus detection protocol for oyster and plankton samples.

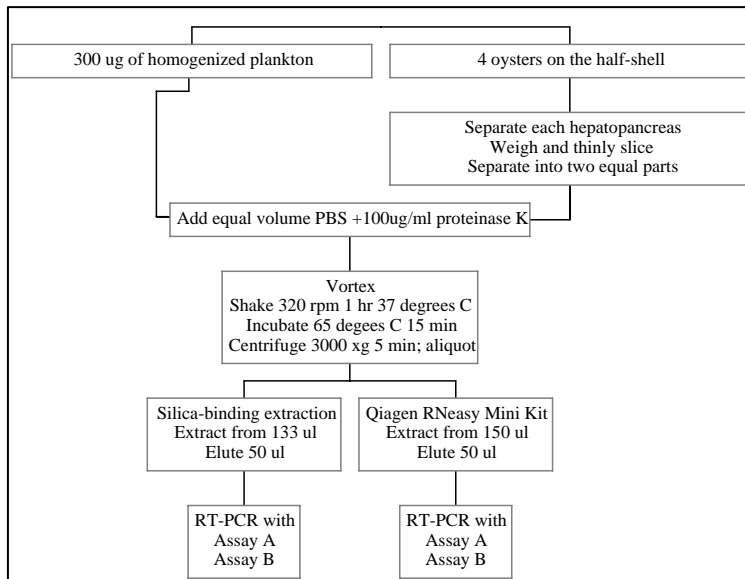


FIG. 3.1

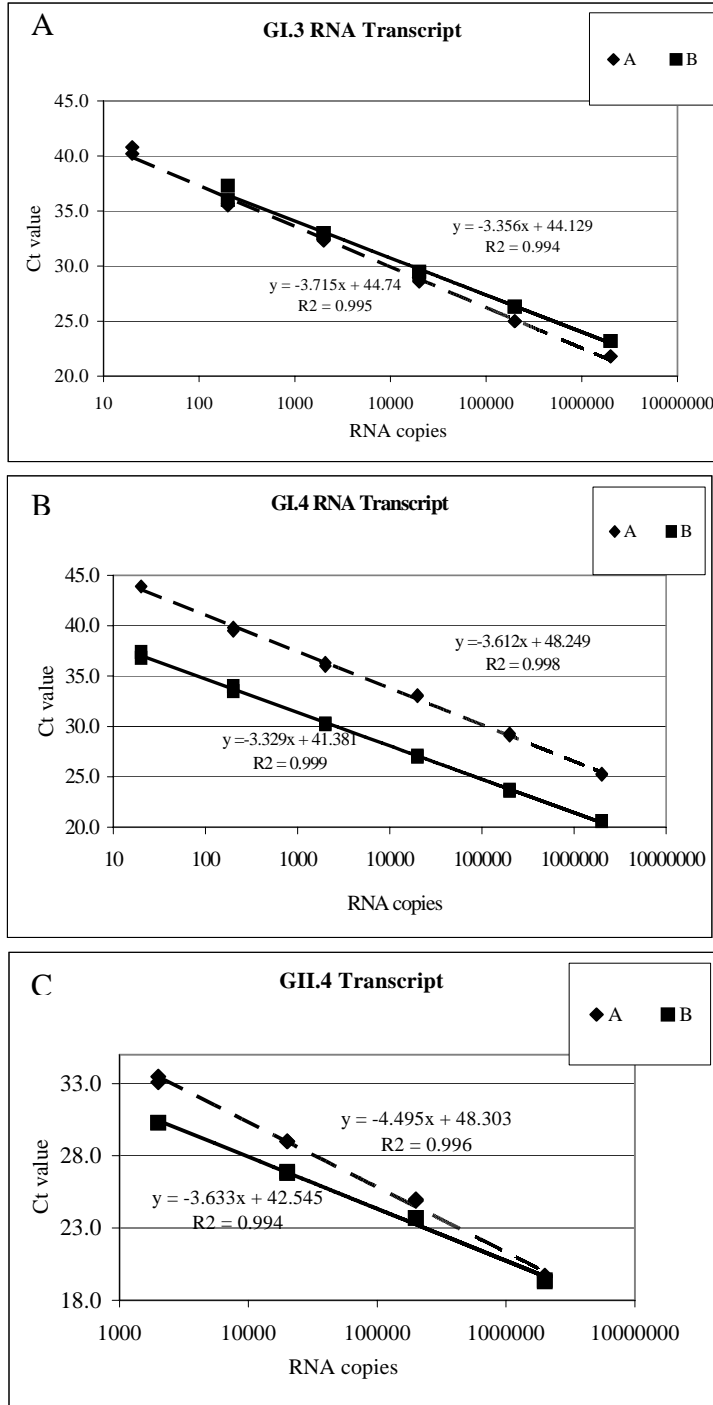


FIG. 3.2

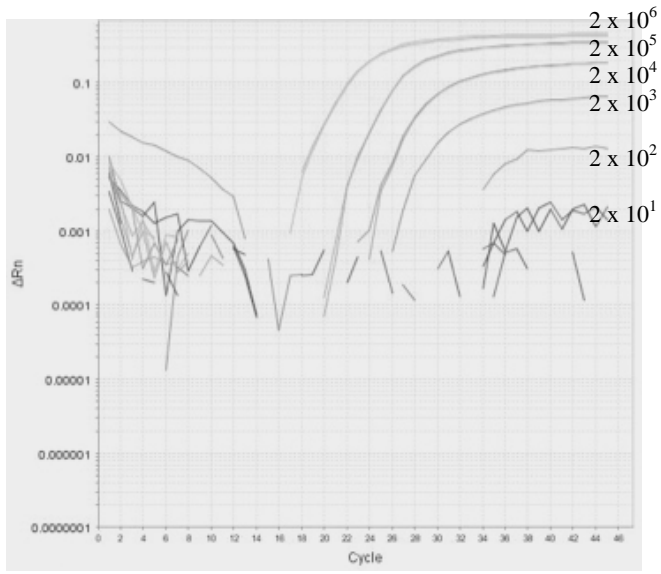


FIG. 3.3

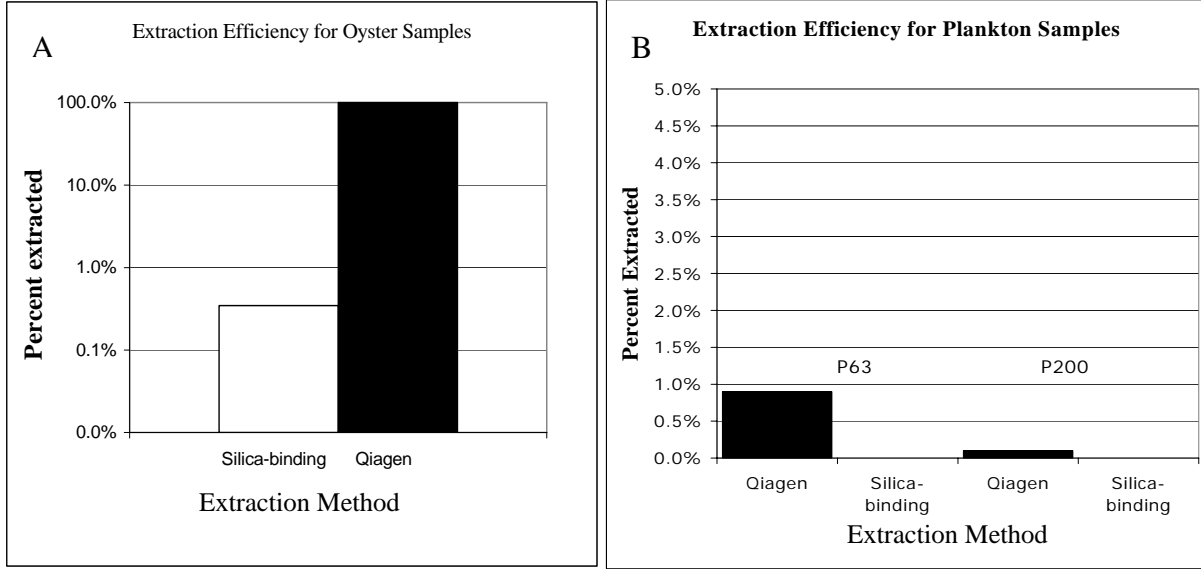


FIG. 3.4

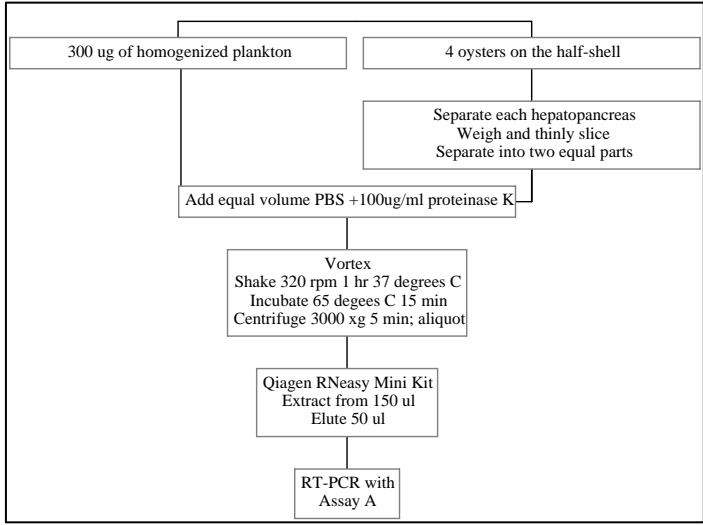


FIG. 3.5

CHAPTER 4

Quantification and Distribution of Human Norovirus from Oyster, Water, and Plankton Samples Over a One-Year Period from two Georgia Estuaries.

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ABSTRACT

Human norovirus (NoV) has been studied extensively as an important cause of gastroenteritis outbreaks worldwide. While oysters are a primary vehicle for infection, only a fraction of NoV studies have examined the virus' distribution in the estuarine environment. This study addressed this issue by examining two active shellfish harvesting areas in Georgia (U.S.A.) for prevalence, genotype diversity, and concentrations in a variety of estuarine sample types. In total, 225 samples (9 oyster, 72 water, 72 63-200 μm plankton, 72 $>200 \mu\text{m}$ plankton) were analyzed. Of those, 21 samples (9.3%) were positive for norovirus. By sample type, 55.0% (5/9) of all oyster samples collected, 8.3% (6/72) of all water samples, 11.1% (8/72) of all 63-200 μm plankton samples, and 2.8% (2/72) of all $>200 \mu\text{m}$ plankton samples were positive for human norovirus. The $>200 \mu\text{m}$ plankton, which contained mainly zooplankton, included only two NoV-positive samples, but those samples had the highest quantity of NoV genomes of any sample with 9.0×10^{12} and 4.5×10^{14} genomes g^{-1} . The majority, 90.5%, (19/21) of the positive samples were from genogroup I; only 9.5% of the positive samples were from genogroup II (2/21). The high concentrations of NoV in plankton samples compared to water and oyster samples was unexpected and provides clues about the presence and distribution of human NoV in the water environment.

Keywords: water environment, norovirus, shellfish

INTRODUCTION

Human norovirus (NoV) is the leading cause of nonbacterial gastroenteritis worldwide (2). The Centers for Disease Control and Prevention (CDC) estimate that 23 million cases of acute gastroenteritis due to norovirus occur each year, with symptoms including acute-onset vomiting, watery non-bloody diarrhea with abdominal cramps, and nausea (38). Norovirus outbreaks are pervasive for many reasons, but particularly because the virus is highly contagious and environmentally hardy (8). Infection occurs through the fecal-oral route, through consumption of contaminated food or water or contact with an infected person. Once infection occurs, individuals can excrete millions of viral particles in feces, allowing for large numbers in sewage (19). Without proper removal or inactivation during wastewater treatment, the viruses can be released into recreational and shellfish harvesting water bodies. Complete inactivation of norovirus during sewage treatment is rare and even in areas with proper wastewater treatment, contamination of oyster beds has been reported (5, 19, 20, 35, 41, 43). Because bivalve molluscan shellfish are believed to act as filters for viruses and other microbes and because norovirus is extremely infectious (as few as 10 viral particles are required for disease) the disease risk for consumption of raw oysters is high (36).

Human norovirus genogroups I (GI) and II (GII) have been detected in oyster samples harvested from bays and estuaries worldwide (5, 11, 23). Ueki et al. (45) detected NoV in both shellfish and the surrounding river water in Japan and concluded that NoV contamination was most likely due to sewage and treated wastewater input into the river; however, no study has yet been able to characterize how NoV may be naturally distributed in an estuarine system, including in water, adhered to particles (including plankton), and in shellfish (45). Limitations are due in

part to lack of adequate detection methods specifically adapted for different environmental sample types (9).

Human NoV infection due to contaminated oysters has been investigated extensively, but the reverse of this, contamination of shellfish as well as their surrounding estuarine environment, has not. The goal of this study was to explore the wider distribution of NoV in an estuarine system. In particular, we aimed to evaluate estuarine biota as reservoirs for this virus.

MATERIALS AND METHODS

Controls.

Norovirus Positive controls. Three NoV-positive fecal samples, representing genotypes GI.4, GI.3b, and GII.4 Minerva, were provided as controls for this study by the CDC. Stool samples were diluted to obtain a 20% suspension in phosphate-buffered saline (PBS), vortexed, and centrifuged at 15,700 x g for 2 min.

Viral RNA extraction from stool. For stool samples, viral RNA was extracted from the clarified PBS extracts using the MagMAX-96 Viral Isolation Kit (Ambion, Austin, TX) and the KingFisher Instrument (Thermo Electron Corporation, Waltham, MA) that automatically purifies viral RNA. Purified RNA was eluted into 55- μ l elution buffer, provided by the kit.

RNA Transcript Standards. To obtain positive control standards and to enable quantification in real-time RT-PCR, RNA transcripts for genotypes GI and GII were obtained from the CDC. The concentrations of the RNA transcripts were previously determined to be 1×10^6 copies μ l⁻¹. A 10-fold serial dilution was made (in duplicate) and used to create the standard curves for quantification.

Environmental Samples.

Oyster, plankton, and water samples used in this study were obtained from 12 stations representing 2 estuaries off the coast of Georgia. Six stations (B1-B6) were located in Sapelo Sound off the coast of McIntosh County and six (D1-D6) were located in Wassaw Sound off the coast of Chatham County, near Savannah (Figure 4.1). As is typical of the Georgia Coast, these estuaries have large tidal ranges (1.7-2.1 m) but are somewhat unique in having minimal fresh water influence from rivers (27). Each estuary was sampled bimonthly between September 2006 and September 2007 (no samples were collected in August 2007). At each sampling, temperature, salinity, dissolved oxygen content, and pH were measured from the surface water at each station using a YSI 556 MPS (Yellow Springs, OH). All samples were collected on a falling spring tide.

Oyster samples. At each sample collection, between 20 and 40 intact oysters were collected from one public shellfish-harvesting site in each estuary. In Wassaw Sound, oysters were collected from Station D2 each sampling collection. From Sapelo Sound, oysters were collected from Station B5 from Sept 06- Jan 07 and from Station B3 from March 07- July 07. The oysters were kept on ice during transport and were frozen at -20°C before processing.

Plankton samples. Two plankton size fractions were collected using a 63- μm plankton net and a 200- μm plankton net that were towed horizontally for 5 minutes. Each fraction was collected in pre-sterilized 1-liter polypropylene bottles. The samples were held at ambient temperatures (22°C- 25°C) and transported to the laboratory within 6 hours of collection and processed. Each raw plankton collection was re-filtered through 63- and 200- μm nets to attain exact size fractions, 63-200 μm and >200 μm , and homogenized for 5 minutes using a Pro Scientific Series Pro 200 homogenizer (Oxford, CT.). The 63-200 μm fraction primarily included phytoplankton and some juvenile zooplankton, while the >200 μm fraction contained

zooplankton (40). Equivalent wet weights were determined for each ml of sample for each fraction.

Water samples. Two liters of water were taken just below the surface at each site in pre-sterilized polypropylene bottles off the side of the boat. The samples were held at ambient temperatures (22°C- 25°C) and transported to the laboratory within 6 hours of collection and processed. Temperatures during transport were monitored with a min/max recording thermometer.

Oyster dissection and viral RNA extraction. Norovirus RNA was extracted from oyster hepatopancreas tissue as described previously (24). Briefly, the hepatopancreas of between four and eleven oysters, equaling at least 5 grams, was removed from the shell and peripheral flesh and finely chopped with a sterile razor (26). Approximately 5 grams were added to an equal volume of PBS plus 100- $\mu\text{g ml}^{-1}$ proteinase K to degrade the shellfish tissue and release the virions into suspension. The suspension was incubated at 37°C for 1 hour with shaking at 320 rpm on a C24 Incubator Shaker (Edison, NJ) and vortexed. It was then incubated at 65°C for 15 min to inactivate the enzyme, vortexed, and centrifuged at 3,000 xg for 5 min. The soluble portion (approximately 8 ml) was aliquoted into cryovials and stored at -80°C.

One hundred and fifty microliters of oyster homogenate was used for RNA extraction using the Qiagen RNeasy Mini Kit, following the plant and fungi procedure (15). Briefly, 150 μl of the oyster concentrate was added to 450 μl of a guanidine thiocyanate solution (including 45 μl β -mercaptoethanol) and vortexed vigorously. Any remaining cell debris in this suspension was removed using the Qias shredder spin columns. The flow-through was then added to 0.5 volumes (~250 μl) 100% ethanol and loaded into the RNeasy Mini columns where bound RNA was washed with guanidine salts and ethanol and finally eluted into 50 μl nuclease-free water.

Viral RNA concentration and extraction from plankton samples. A plankton suspension was made, and RNA extracted, in a method similar to that for the oysters (24). Briefly, approximately 300 μg of homogenized plankton was separated into a microcentrifuge tube. An equal volume-to-weight of PBS + 100 $\mu\text{g ml}^{-1}$ proteinase K was added to the tube and the solution was vortexed and shaken at 320 rpm for one hour at 37°C in a C24 Incubator Shaker (Edison, NJ). The tube was then heated to 65°C for 15 minutes to deactivate the Proteinase K and centrifuged at 3,000 x g for 5 minutes. The supernatant (approximately 6.5 ml) was carefully removed, aliquoted, and stored at -80°C. The Qiagen RNeasy Mini kit, following the plant and fungi procedure, was used to extract RNA from 150 μl of the plankton concentrate and eluted into 50- μl nuclease-free water (Qiagen, Valencia, CA).

Concentration and extraction of norovirus RNA from water. The adsorption-elution method described by Katayama et al. (30) and modified by Fong et al. (18) was used to concentrate viruses from water samples. One liter of water was adjusted to pH ~4.0 using a 1 N solution of acetic acid. This was passed through a 90 mm, 0.45 μm pore size HA membrane filter (Millipore MF Membrane Filters, Billerica, MA) using a sterile filter housing. The filter was rinsed with 100 ml 0.5 M sulfuric acid (pH 3.0). Viruses were eluted from the membrane with 10 ml 1 mM sodium hydroxide (pH 10.5-10.8). Eluent was added to 100 μl of 50 mM sulfuric acid (pH 3.0) and 0.1 ml of 100X Tris EDTA (pH 8.0) in a sterile 15 ml polypropylene tube. The eluent was further purified and concentrated using Centriprep YM-50 concentrator columns (Millipore, Billerica, MA) to a final volume of 2 ml. Concentrates were saved at -80°C. RNA was extracted from 200 μl of concentrate using the Qiagen RNeasy Mini kit to elute a final concentrated virus sample in 50- μl nuclease-free sterile water (Qiagen, Valenica, CA).

Detection and quantification of norovirus RNA. The two most sensitive and broadly

reactive real-time norovirus RT-PCR primer and probe sets currently available were used to detect and enumerate noroviruses (Table 4.1). Degenerate primer sets COG1F/COG1R and COG2F/COG2R, specifically target an 84-bp fragment from GI and a 97-bp fragment from GII, respectively, of the conserved region at the ORF1-ORF2 junction of the norovirus genome (29). A second set of primers and probes without degeneracies, JJV1F/JJV1R and JJV2F/COG2R, for genogroup I and II specifically target a 96-bp and an 89-bp fragment, respectively, of the same area of the genome (28).

The RT-PCR reaction mixture for both primer sets contained 2 µl of sample, each primer at a concentration of 400nM, each probe mixture at a concentration of 120nM, 12.5 µl of 2X RT-PCR buffer, 1 µl of 25X RT-PCR enzyme mix, 1.67 µl of detection enhancer, and nuclease-free water for a total reaction mixture of 25 µL (Ambion AgPath-ID One-Step RT-PCR kit). The reaction mixture was subjected to a one-step assay on an ABI 7900 (Applied Biosystems, Foster City, CA), an ABI StepOne (Applied Biosystems, Foster City, CA), or an Eppendorf Mastercycler ep realplex using the following conditions: (i) RT for 10 min at 45°C, (ii) 10 min at 95°C, (iii) 45 cycles of 10 s at 95°C, 30 s at 55°C, and 15 s at 72°C.

All amplification reactions were carried out in duplicate. Samples that gave a positive result in either or both of the duplicate reactions were amplified by RT-PCR again. Only after a sample gave a second positive result was it counted as an overall positive.

Statistical Analyses. Data were analyzed using SAS (Cary, NC) and Minitab (State College, PA) software for Windows. Spearman's Rank correlations were performed to compare NoV concentrations with environmental parameters including rainfall and temperature. Kruskal-Wallis Tests were used to compare norovirus concentrations between sample types (e.g., oysters, water, and plankton). Pearson Chi-Square Analyses were used to compare frequency of

norovirus detection between season, location, and sample type. In all cases, the significance was declared at $p=0.05$.

RESULTS

Only 10 oyster samples were collected during the year due to high water levels, preventing the collection of oysters during March and April of 2007. Additionally, the oysters collected in October 2006 were depleted before an effective extraction method was implemented.

In all, 225 samples (9 oyster, 72 water, 72 63-200 μm plankton, 72 >200 μm plankton) were analyzed and of those, 21 samples (9.3%) were positive for norovirus (Table 4.2). Concentrations for all NoV-positive samples ranged from 1.2×10^0 genomes g^{-1} to 4.5×10^{14} genomes g^{-1} . Concentrations ranged from 2.7×10^4 to 1.7×10^8 genomes g^{-1} in oysters, from 1.2×10^0 to 1.8×10^6 genomes ml^{-1} in water, from 1.7×10^2 to 1.6×10^7 genomes g^{-1} in 63-200 μm plankton, and from 9.0×10^{12} to 4.5×10^{14} genomes g^{-1} in >200 μm plankton.

Comparison by sample location. In Wassaw Sound, of 112 total samples analyzed, 11 (10%) were positive for NoV. In Sapelo Sound, of 113 total samples analyzed, 10 (9%) were positive for NoV. The percent of NoV-positive samples detected in each sound was not significantly different. The average positive concentrations for Sapelo Sound were 7.0×10^6 genomes g^{-1} for oyster samples, 2.2×10^2 genomes ml^{-1} for water samples, 3.3×10^6 genomes g^{-1} for 63-200 μm plankton, and 2.3×10^{14} genomes g^{-1} for >200 μm plankton. Similarly, the average positive concentrations for Wassaw Sound were 4.3×10^7 genomes g^{-1} for oyster samples, 3.6×10^5 genomes ml^{-1} for water samples, and 2.9×10^5 genomes g^{-1} for 63-200 μm plankton (NoV was not detected in the >200 μm plankton). In Wassaw Sound, 50% (5/10) of positive samples (and 25% of total positive samples, including both Wassaw and Sapelo Sounds)

were detected at station D2, which was significantly greater than other Wassaw Sound stations ($P= 0.02$). In Sapelo Sound, there was no station that had a statistically greater percentage of positive samples, although 75% of NoV-positive samples were detected in Stations B3 and B5.

Comparison of prevalence by date and season. Norovirus-positive samples were found throughout the year, with the exception of September, October, and November 2006 and April and July 2007 (Table 4.2 and Figure 4.4). The sample with the greatest concentration of NoV was a $>200 \mu\text{m}$ plankton sample (4.5×10^{14} genomes g^{-1}) collected in March 2007 when the highest concentration in the 63-200 μm fraction (1.6×10^7 genomes g^{-1}) was also detected. However, the largest concentration in water (1.8×10^6 genomes ml^{-1}) occurred in September 2007 and the highest concentration in oysters (1.7×10^8 genomes g^{-1}) occurred in February 2007. When analyzed by season, 2 of 57(4%) were positive for NoV in fall (Oct-Dec), 9 of 57(16%) were positive in winter (Jan-Mar), 5 of 56(9%) were positive in spring (Apr-June), and 5 of 56(9%) were positive in summer (July–Sept), but none were significantly different. Among norovirus positive samples, forty-two percent occurred in winter, 23.8% occurred in the spring, 23.8% occurred in the summer, and 9.5% occurred in the fall (Figure 4.2).

The potential relationship of NoV levels to environmental parameters were also examined, and while there was a negative association between both norovirus concentration and rainfall and norovirus concentration and temperature, there was no statistically significant relationship.

Comparison by fraction. Norovirus was detected in every sample type analyzed. By sample type, 55.0% (5/9) of all oyster samples collected, 8.3% (6/72) of all water samples, 11.1% (8/72) of all 63-200 μm plankton samples, and 2.8% (2/72) of all $>200 \mu\text{m}$ plankton samples were positive for human norovirus. Using Pearson Chi-Square analysis, the detection

frequency in oyster samples was significantly higher than the other sample types and the detection frequency in the >200 μm plankton samples was significantly lower than the other sample types ($P < 0.0001$). Among all NoV-positive samples, 23.8% (5/21) were oyster samples, 28.6% (6/21) were water samples, 31.0% (8/21) were 63-200 μm plankton samples, and 9.5% (2/21) were >200 μm plankton samples (Figure 4.3).

Average NoV concentrations among the sample type varied from 2.5×10^4 genomes ml^{-1} in water (N=72), 2.8×10^7 genomes g^{-1} in oysters (N=9), 2.4×10^5 genomes g^{-1} in 63-200 μm plankton (N=72), and 6.3×10^{12} genomes g^{-1} in >200 μm plankton (N=72). Kruskal-Wallis showed no significant difference in NoV concentration between the sample types; however, this reflects the few number of positive samples detected.

When only norovirus positive samples were compared, concentrations ranged from a low of 1.2×10^0 genomes ml^{-1} (water, September 2007) to a high of 4.5×10^{14} genomes g^{-1} (>200 μm plankton, March 2007) (Figure 4.3). Positive water samples averaged 3.0×10^5 genomes ml^{-1} (N=6). For oysters, the mean concentration among positive samples was 5.0×10^7 genomes g^{-1} (N=5). Among the 63-200 μm plankton samples, the mean concentration was 2.2×10^6 genomes g^{-1} (N=8) and the mean concentration for the >200 μm plankton samples was 2.3×10^{14} genomes g^{-1} (N=2) (Figure 4.3).

Positives in concurrently collected samples occurred five times during the study period, in December 2006 and February, March, May and June 2007 (Figure 4.4). In February 2007, oyster, water, and 63-200 μm plankton samples were all positive for NoV and in March 2007, water, 63-200 μm plankton, and >200 μm plankton were all positive for NoV.

Comparison by genogroups. Genogroup I and II represented 90.5% (19/21) and 9.5% (2/21) of the positive samples identified, respectively. The number of GI positive samples were

significantly greater than the number of GII positive samples ($P < 0.0001$). Genogroup II samples were only recovered from a 63-200 μm plankton sample from Sapelo Sound in May 2007 and in a water sample from Wassaw Sound in September 2007. These two samples contained the lowest quantity of human norovirus detected in any sample throughout the study period (average 8.7×10^1 genomes g^{-1}) (Table 4.2).

DISCUSSION

Human norovirus is the most significant viral pathogen associated with food- and water-borne outbreaks of acute gastroenteritis (2). Outbreaks due to contaminated water and oysters pose a serious risk in the U.S. and abroad. This study investigated norovirus in shellfish harvesting waters, a key component in the circulation of human NoV between contaminated water, food and humans. RNA from oyster, water, and plankton samples was detected by qRT-PCR with two sets of primers and probes in the ORF1/ORF2 region, the most conserved region of the genome. Two sets of primers and probes were used to limit the number of false negatives, a common problem in identifying NoV in environmental samples (28, 29).

Positive NoV samples were evenly distributed between the two estuaries studied; however, the samples with the highest quantity of NoV genomes, 9.0×10^{12} and 4.5×10^{14} genomes g^{-1} (in the $>200 \mu\text{m}$ plankton) were collected in March 2007 from Sapelo Sound. This was surprising given that McIntosh County, adjacent to Sapelo Sound, is sparsely populated (11,248 citizens) compared to Chatham County (241, 411 citizens), adjacent to Wassaw Sound, which includes Savannah (7); however, data from Walker et al. (46) show that McIntosh County has a high concentration of septic tanks very near the shore of Sapelo Sound as well as the surrounding creeks and rivers. The study mapped 1, 056 septic systems and discovered 53 (5%)

were obviously dysfunctional, 100 (10%) were within one foot of a water body, and an additional 11 (1%) were within 1 to 25 feet of a marsh or water body. Since a drain field of 25 feet is required for septic tank drainage (16), combined with the fact that there is a low water table and sandy soil in this area (46), there is little doubt that the septic tanks are releasing fecal pollutants into the surrounding rivers, creeks, and estuaries (46). Comparing the GIS maps of septic tanks provided by Walker et al. (46) to the sampling locations used in this study, Stations B2, B3, B5, and B6 coordinated with a dense area of septic systems. Additionally, Stations B3 and B5 are close to faulty septic systems reported in Walker et al. (46). Stations B3 and B5 are public-picking areas for shellfish (25) and contained 9/12 (75%) of the NoV positive samples from this sound. The data indicate that (faulty and incorrectly placed) septic systems are probably a major contributor to NoV contamination of Sapelo Sound shellfish harvesting areas.

Despite the trends in clinical data which describe NoV as a winter disease (39), NoV detection showed no significant seasonality in this study. This may reflect an important distinction between clinical outcome and environmental prevalence. Whereas norovirus displays a seasonal distribution in restricted environments such as hospitals, nursing homes, schools, and the military, the seasonal variation is much less pronounced in the larger community (37). Although studies of waterborne NoV seasonality are limited, results of this study and others (47) suggest that norovirus may be more persistent in the estuarine system than previously thought, and as such, the estuarine distribution of the virus may be an important factor in the occurrence of disease cases and outbreaks.

Oyster samples had the highest percentage, 55%, of norovirus positive samples of any sample type. This is consistent with previous work showing norovirus prevalence in oysters as high as 44% (13). Our higher detection rate may be related to improved detection efficiency

(24). Detection rates of 8% in water from this study is also consistent with results of La Rosa et al. (31) that showed a lower overall detection in natural water samples.

A novel component of this study was the examination of plankton for NoV. Here, we found the largest percentage of NoV-positive samples, 31.0% (8/21), among the 63-200 μm plankton samples (primarily phytoplankton). Additionally, the $>200 \mu\text{m}$ (primarily zooplankton) plankton had the highest concentrations of NoV genomes of any sample, 9.0×10^{12} and 4.5×10^{14} genomes g^{-1} (Table 4.2). Although we are treating the diverse composition of each plankton fraction as a “black box,” we speculate NoV may be adsorbed to plankton particles in general via electrostatic interactions, similar to viral adsorption to sediment and other particles (32). Most enteric viruses are negatively charged under neutral and alkaline pH conditions (1). While no work has yet characterized the charge on zooplankton particles, Bayne et al. (4) found that among phytoplankton in the 6-100 μm size fraction, the majority of Chlorophyta, Cyanophyta, and Euglenophyta were neutral to positively-charged (4). While there is no definitive evidence for the mechanism promoting virus association with plankton, it may be a significant area for future research.

NoV can survive in water and oyster samples for extended periods (42); however, the full distribution of NoV in the environment is as yet unknown. The association between viruses and particles prolongs the virus’ survival. For example, laboratory studies show that poliovirus1 is protected from microorganisms, heat, and salts when associated with marine sediments (33). Additionally, if plankton, or other organic particulates also act as a reservoir, oysters’ ability to selectively filter feed in the environment may indicate an important link for promoting virus concentration in shellfish (hepatopancreas) tissues.

Selective filter feeding involves three mechanisms: particle retention, pre-ingestive selection, and differential absorption (12) and is affected by the particle's size, seston load, and relative organic composition (6). Oysters use particle retention to distinguish between particle sizes; their gills can retain a wide range of particles, from 10-200 μm , which includes phytoplankton, zooplankton, and protists (ciliates and flagellates) (17). Pre-ingestive selection on the gills and labial palps distinguishes between organic and inorganic particles and differential absorption in the gut distinguishes between microalgal species (12). Viral adsorption to plankton particles (from sewage-contaminated water) would allow for viral particles to be retained in the oyster and ingested as opposed to adsorption to sediment particles or presence in water, which would be eliminated as pseudofeces rather than ingested in the gut. Thus, adsorption to plankton would be a more likely model for transmission to bivalve molluscan tissues and, in turn, transmission to humans.

Another unexpected result of this study was that the majority of positive samples were from GI; only 9.5% of the positive samples were from GII (2 of 21 samples). The GII-positive samples also contained the lowest quantity of human norovirus detected in any sample throughout the study period. While both GI and GII noroviruses are commonly found in sewage samples, outbreaks are more frequently attributed to genogroup GII (22, 31, 34, 44, 45). The prevalence of NoV GI strains in this study may be attributed to the use of two improved real-time assays (24), reducing the number of NoV GI false negatives and possibly giving a more accurate view of NoV GI presence in the environment. Also, recent evidence suggests that NoV GI may be more pervasive than previously thought. For example, GI strains detected in NoV outbreaks increased from 4% between 1996-1997 to 25% between 1997-2000 according to one U.S. study (21). Additionally, studies in France, Mexico, and Guatemala have reported a high

NoV GI presence in cases and outbreaks (14) or even greater concentrations of NoV GI than GII (10, 40). They (14) also found that NoV GI and GII were present in 43 and 88% of sewage influent samples, respectively, but in 24 and 14% of effluent samples, respectively, suggesting that sewage treatment is less effective for treating NoV GI than GII. Additionally, da Silva et al. (14) observed that NoV GI presence was more variable, had higher peaks, and had higher average positive influent concentrations than NoV GII. This suggests that there are differences in environmental persistence between the genogroups. Genogroups detected in sewage and in the environment may more accurately reflect the true NoV circulation in the population, rather than reported cases, which is a small proportion of the total cases (14).

Previous studies have investigated the presence of norovirus in environmental oyster samples, but few studies have examined the presence of NoV in oysters over a full year and even fewer have examined NoV presence in water environments surrounding shellfish harvesting areas. This is the first study to examine the association between norovirus and oysters, water, and plankton. We discovered that NoV is associated differentially with plankton size fractions and that the $>200 \mu\text{m}$ fraction can harbor concentrations greater than 10^{10} genomes g^{-1} . This fraction may serve as an environmental reservoir for the virus. Additionally, this study found a greater prevalence of NoV GI than GII, which suggests that NoV GI is either more environmentally hardy than GII in the estuarine environment or that this genotype represents strains circulating widely in the population. All of these dynamics are significant findings that point to the need for more studies of human norovirus in the water environment.

REFERENCES

1. **Asano, T. (ed.).** 1998. Wastewater Reclamation and Reuse, vol. 10. CRC Press.
2. **Atmar, R. L., and M.K. Estes.** 2006. The epidemiologic and clinical importance of norovirus infection. *Gastroenterol. Clin. N. Am.* **35**:275-290.
3. **Barille, L., J. Prou, M. Heral, and S. Bougrier.** 1993. No influence of food quality, but ration-dependent retention efficiencies in the Japanese oyster *Crassostrea gigas*. *J. Exp. Mar. Bio. Ecol.* **171**:91-106.
4. **Bayne, D. R., and J. M. Lawrence.** 1972. Separating constituents of natural phytoplankton populations by continuous particle electrophoresis. **17**:481-489.
5. **Beuret, C., A. Baumgartner, and J. Schluep.** 2003. Virus-contaminated oysters: a three-month monitoring of oysters imported to Switzerland. *Appl. Environ. Microbiol.* **69**:2292-2297.
6. **Bougrier, S., A. J. S. Hawkins, and M. Heral.** 1997. Preingestive selection of different microalgal mixtures in *Crassostrea gigas* and *Mytilus edulis*, analysed by flow cytometry. *Aquaculture* **150**:123-134.
7. **US Census Bureau.** 2008. US Census 2000.
8. **CDC.** 2006. Norovirus in Healthcare Facilities Fact Sheet.
http://www.cdc.gov/ncidod/dhqp/id_norovirusFS.html.
9. **CDC.** 2008. Norovirus: Technical Fact Sheet.
<http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus-factsheet.htm>.
10. **Chapin, A. R., C. M. Carpenter, W. C. Dudley, L. C. Gibson, R. Pratdesaba, O. Torres, D. Sanchez, J. Belkind-Gerson, I. Nyquist, A. Karnell, B. Gustafsson, J. L. Halpern, A. L. Bourgeois, and K. J. Schwab.** 2005. Prevalance of norovirus among

visitors from the United States to Mexico and Guatemala who experience traveler's diarrhea.

11. **Cheng, P. K. C., D. K. K. Wong, T. W. H. Chung, and W. W. L. Lim.** 2005. Norovirus contamination found in oysters worldwide. *J Med Virol* **76**:593-597.
12. **Cognie, B., L. Barille, and Y. Rince.** 2001. Selective feeding of the oyster *Crassostrea gigas* fed on natural microphytobenthos assemblage. *Estuaries and Coasts* **24**:126-134.
13. **Constanini, V., F. Loisy, L. Joens, F. S. Le Guyader, and L. J. Saif.** 2006. Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. *Appl. Environ. Microbiol.* **72**:1800-1809.
14. **da Silva, A. K., J.-C. L. Saux, S. Parnaudeau, M. Pommepuy, M. Elimelech, and F. S. L. Guyader.** 2007. Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: Different behaviors of genogroups. *Appl. Environ. Microbiol.* **73**:7891-7897.
15. **de Roda Husman, A. M., F. Lodder-Verschoor, H. H. J. L. V. d. Berg, F. S. L. Guyader, H. V. Pelt, W. H. M. V. d. Poel, and S. A. Rutjes.** 2007. Rapid virus detection procedure for molecular tracing of shellfish associated with disease outbreaks. *J. Food Prot.* **70**:967-974.
16. **Georgia Department of Human Resources.** Rules and Regulations On-site Sewage Management Systems Chapter 290-5-26.
17. **Dupuy, C., S. L. Gall, H. J. Hartmann, and M. Breret.** 1999. Retention of ciliates and flagellates by the oyster *Crassostrea gigas* in French Atlantic coastal ponds: protists as a trophic link between bacterioplankton and benthic suspension-feeders. *Mar. Ecol. Prog. Ser.* **177**:165-175.

18. **Fong, T.-T., and E. K. Lipp.** 2005. Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools. *Microbiol. Mol. Biol. Rev.* **69**:357-371.
19. **Formiga-Cruz, M., A.K. Allard, A.C. Conden-Hansson, K. Henshilwood, B.E. Hernroth, J. Jofre, D.N. Lees, F. Lucena, M. Papapetropoulou, R.E. Rangdale, A. Tsibouxi, A. Vantarakis, and R. Girones.** 2003. Evaluation of potential indicators of viral contamination in shellfish and their applicability to diverse geographical areas. *Appl. Environ. Microbiol.* **69**:1556-1563.
20. **Formiga-Cruz, M., G. Tofino-Quesada, S. Bofill-Mas, D. N. Lees, K. Henshilwood, A. L. Allard, A. C. Conden-Hansson, B. E. Hernroth, A. Vantarakis, A. Tsibouxi, M. Papapetropoulou, M. D. Furones, and R. Girones.** 2002. Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden and the United Kingdom. *Appl. Environ. Microbiol.* **68**:5990-5998.
21. **Frankhauser, R. L., S.S. Monroe, J.S. Noel, C.D. Humphrey, J.S. Bresee, U.D. Parashar, T. Ando, and R.I. Glass.** 2002. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. *J. Infect. Dis.* **186**:1-7.
22. **Gallimore, C. I., M. A. Barreiros, D. W. Brown, J. P. Nascimento, and J. P. Leite.** 2004. Noroviruses associated with acute gastroenteritis in a children's day care facility in Rio de Janeiro, Brazil. *Braz. J. Med. Biol. Res.* **37**:321-326.
23. **Gallmore, C. I., J. S. Cheesbrough, K. Lamden, C. Bingham, and J. J. Gray.** 2005. Multiple norovirus genotypes characterised from an oyster-associated outbreak of gastroenteritis. *Intl J Food Microbiol* **103**:323-330.

24. **Gentry, J. B., J. Vinje, and E. K. Lipp.** 2008. Rapid and Efficient Quantification of Norovirus Genogroups I and II from Oyster and Plankton (Unpublished).
25. **Guadagnoli, D.** 2007. Georgia DNR Coastal Resources Division. (personal communication).
26. **Henshilwood, K., W. J. Dore, S. Anderson, and D. N. Lees.** 2003. Investigation of Norwalk like virus elimination during depuration using a real time quantitative PCR. *In* J. L. R. A. Villalba, B. Reura, and R. Beiras (ed.), Molluscan shellfish safety. Conselleria de Pesca e Asuntos Maritimos da Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Galicia, Spain.
27. **National Park Service.** 1970. An Ecological Survey of the Coastal Region of Georgia.
28. **Jothikumar, N., J. A. Lowther, K. Henshilwood, D. N. Lees, V. R. Hill, and J. Vinje.** 2005. Rapid and sensitive detection of norovirus by using TaqMan-based one-step reverse-transcription-PCR assays and application to naturally contaminated shellfish samples. *Appl. Environ. Microbiol.* **71**:1870-1875.
29. **Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama.** 2003. Broadly reactive and highly sensitive assay for norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J. Clin. Microbiol.* **41**:1548-1557.
30. **Katayama, K., A. Shimasaki, and S. Ohgaki.** 2002. Development of a virus concentration method and its application to detection of enterovirus and Norwalk virus from coastal seawater. *Appl. Environ. Microbiol.* **68**:1033-1039.
31. **La Rosa, G., S. Fontana, A. Di Grazia, M. Iaconelli, M. Pourshaban, and M. Muscillo.** 2007. Molecular identification and genetic analysis of norovirus genogroups I

- and II in water environments: comparative analysis of different reverse transcription-PCR assays. *Appl. Environ. Microbiol.* **73**:4152-4161.
32. **Labelle, R., and C. P. Gerba.** 1980. Influence of estuarine sediment on virus survival under field conditions. *Appl. Environ. Microbiol.* **39**:749-755.
 33. **Labelle, R., and C. P. Gerba.** 1982. Investigations into the protective effect of estuarine sediment on virus survival. *Water Research* **16**:469-478.
 34. **Le Guyader, F., F. Bon, D. DeMedici, S. Parnaudeau, A. Bertone, S. Crudeli, A. Doyle, M. Zidane, E. Suffredini, E. Kohli, F. Maddalo, M. Monini, A. Gallay, M. Pommepuy, P. Pothier, and F. M. Ruggeri.** 2006. Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. *J. Clin Microbiol.* **44**:3878-3882.
 35. **Le Guyader, F., L. Haugarreau, L. Miossec, E. Dubois, and M. Pommepuy.** 2000. Three-year study to assess human enteric viruses in shellfish. *Appl. Environ. Microbiol.* **66**:3241-3248.
 36. **Le Guyader, F., F. Loisy, R. L. Atmar, A. M. Hutson, M. K. Estes, N. Ruvoen-Clouet, M. Pommepuy, and J. Le Pendu.** 2006. Norwalk virus-specific binding to oyster digestive tissues. *Emerg. Infect. Dis.* **12**:931-936.
 37. **Lopman, B. A., G. K. Adak, M. Reacher, and D. W. Brown.** 2003. Two Epidemiologic Patterns of Norovirus Outbreaks: Surveillance in England and Wales, 1992–2000. *Emerg. Infect. Dis.* **9**:71-77.
 38. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**:607-625.

39. **Mounts, A. W., T. Ando, M. Koopmans, J. S. Bresee, J. Noel, and R. I. Glass.** 2000. Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses. *J. Infect. Dis.* **181**:S284-S287.
40. **Omori, M., and T. Ikeda.** 1992. *Methods in marine zooplankton ecology.* Krieger Publishing Company, Malabar, USA.
41. **Pina, S., M. Puig, F. Lucena, J. Jofre, and R. Girones.** 1998. Viral pollution in the environment and in shellfish; human adenovirus detection by PCR as an index of human viruses. *Appl. Environ. Microbiol.* **64**:3376-3382.
42. **Schwab, K. J., F. H. Neill, M. K. Estes, T. G. Metcalf, and R. L. Atmar.** 1998. Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. *J. Food Prot.* **61**:1674-1680.
43. **Shieh, Y. C., R.C. Baric, J.W. Woods, and K.R. Calci.** 2003. Molecular surveillance of enterovirus and norwalk-like virus in oysters relocated to a municipal-sewage-impacted gulf estuary. *Appl. Environ. Microbiol.* **69**:7130-7136.
44. **Subekti, D. S., P. Tjaniadi, M. Lesmana, C. Simanjuntak, S. Komalarini, H. Digdowirogo, B. Setiawan, A. L. Corwin, J. R. Campbell, K. R. Porter, and B. A. Oyoyo.** 2002. Characterization of Norwalk-like virus associated with gastroenteritis in Indonesia. *J. Med. Virol.* **67**:253-258.
45. **Ueki, Y., D. Sano, T. Watanabe, K. Akiyama, and T. Omura.** 2005. Norovirus pathway in water environment estimated by genetic analysis of strains from patients of gastroenteritis, sewage, treated wastewater, river water and oysters. *Water Research* **39**:4271-4280.

46. **Walker, R. L., C. F. Cotton, and K. A. Payne.** 2003. A GIS inventory of on-site septic systems adjacent to the coastal waters of McIntosh County, Georgia.1-44.
47. **Westrell, T., P. Teunis, H. v. d. Berg, W. Lodder, H. Ketelaars, T. A. Stenstrom, and A. M. de Roda Husman.** 2006. Short- and long-term variations of norovirus concentrations in the Meuse river during a 2-year study period. *Water Research* **40**:2613-2620.

TABLES

Table 4.1. Primer and Probe sequences for real time RT-PCR

Genogroup	Oligonucleotide	Sequence (5'-3') ^a	Genome Location	Reference
GI	COGIF	CGY TGG ATG CGN TTY CAT GA	5291-5310 ^e	29
	COGIR	CTT AGA CGC CAT CAT CAT TYA C	5375-5358 ^e	
	Ring1a	FAM ^b -AGA TYG CGA TCY CCT GTC CA- BHQ ^c	5340-5359 ^e	
	Ring1b	FAM -AGA TCG CGG TCT CCT GTC CA- BHQ	5340-5321 ^e	
GI	JJVIF	GCC ATG TTC CGI TGG ATG	5282-5299 ^d	28
	JJVIR	TCC TTA GAC GCC ATC ATC AT	5377-5358 ^d	
	JJVIP	FAM -TGT GGA CAG GAG ATC GCA ATC TC- BHQ	5319-5341 ^d	
	Ring1b	FAM -AGA TCG CGG TCT CCT GTC CA- BHQ	5340-5321 ^e	
GII	COG2F	CAR GAR BCN ATG TTY AGR TGG ATG AG	5003-5023 ^f	29
	COG2R	TCG ACG CCA TCT TCA TTC ACA	5100-5080 ^f	
	Ring2	FAM -TGG GAG GGC GAT CGC AAT CT- BHQ	5048-5067 ^f	
GII	JJV2F	CAA GAG TCA ATG TTT AGG TGG ATG AG	5003-5028 ^d	28
	COG2R	TCG ACG CCA TCT TCA TTC ACA	5100-5080 ^f	
	Ring2	FAM -TGG GAG GGC GAT CGC AAT CT- BHQ	5048-5067 ^f	

^a Mixed bases in degenerate primers and probes are as follows: Y, C or T; R, A or G; B, not A; N, any.

^b 6-carboxyfluorescein (FAM)

^c black hole quencher (BHQ)

^d Nucleotide positions based on the Norwalk (GI) (accession no. M87661) and Lordsdale (GII) (accession no. X86557) sequences.

^e Corresponding nucleotide position of Norwalk1/68 virus (accession no. M87661) of the 5' end.

^f Corresponding nucleotide position of Camberwell virus (accession no. AF145896) of the 5' end.

Table 4.2. Positive Norovirus Samples among sample types.

Source	Genogroup	Estuary	Station	Date	Viral Genomes g ⁻¹ or ml ⁻¹
Oyster	GI	Wassaw	D2	Dec-06	2.7 x 10 ⁴
	GI	Sapelo	B5	Jan-07	2.7 x 10 ⁴
	GI	Wassaw	D2	Feb-07	1.7 x 10 ⁸
	GI	Sapelo	B3	May-07	1.4 x 10 ⁷
	GI	Wassaw	D2	Jun-07	6.4 x 10 ⁷
Water	GI	Wassaw	D4	Dec-06	2.2 x 10 ²
	GI	Wassaw	D2	Feb-07	4.3 x 10 ²
	GI	Sapelo	B2	Mar-07	2.2 x 10 ²
	GI	Wassaw	D2	Sep-07	1.8 x 10 ⁶
	GII	Wassaw	D3	Sep-07	1.2 x 10 ⁰
	GI	Wassaw	D4	Sep-07	4.0 x 10 ⁰
	GI	Wassaw	D3	Feb-07	6.9 x 10 ³
Plankton 63-200 μm	GI	Sapelo	B3	Mar-07	1.6 x 10 ⁷
	GI	Sapelo	B5	Mar-07	2.7 x 10 ⁴
	GI	Sapelo	B2	May-07	2.7 x 10 ³
	GII	Sapelo	B2	May-07	1.7 x 10 ²
	GI	Sapelo	B5	May-07	2.7 x 10 ⁴
	GI	Wassaw	D3	Sep-07	8.1 x 10 ³
	GI	Wassaw	D5	Sep-07	8.5 x 10 ⁵
Plankton >200 μm	GI	Sapelo	B3	Mar-07	9.0 x 10 ¹²
	GI	Sapelo	B5	Mar-07	4.5 x 10 ¹⁴

FIGURE LEGEND

FIG. 4.1. Map of sampling stations in Wassaw and Sapelo Sounds, Georgia (USA).

FIG. 4.2. Distribution of NoV by season (fall: Sept, Oct, Nov; winter: Dec, Jan, Feb; spring: Mar, Apr, May; summer: June, July, Aug)

FIG. 4.3. Distribution of NoV by sample type (oyster, water, 63-200 μm , and $>200 \mu\text{m}$) and mean NoV concentration among each sample (calculated for positive samples only).

FIG. 4.4. Total of NoV load by month showing contribution from each sample type (no samples were collected in Aug 2007).

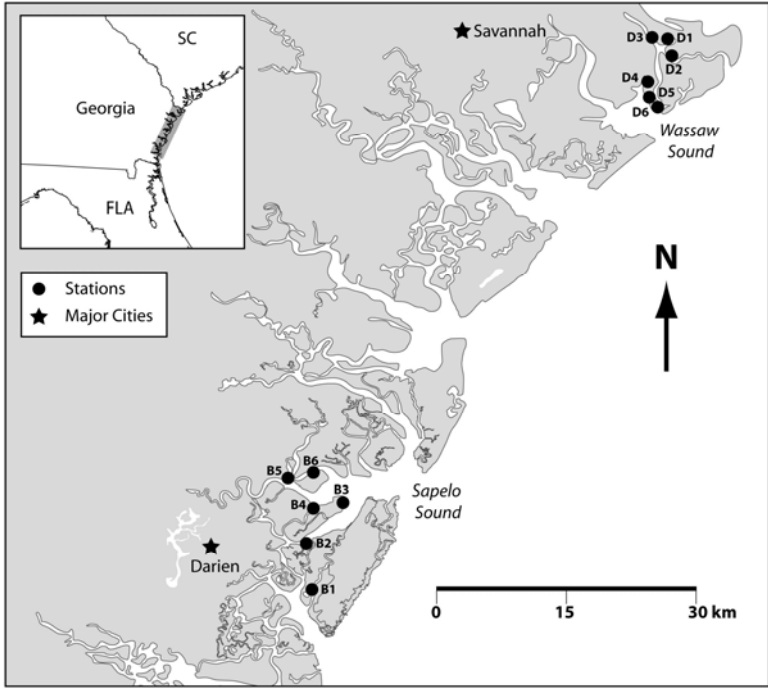


FIG. 4.1

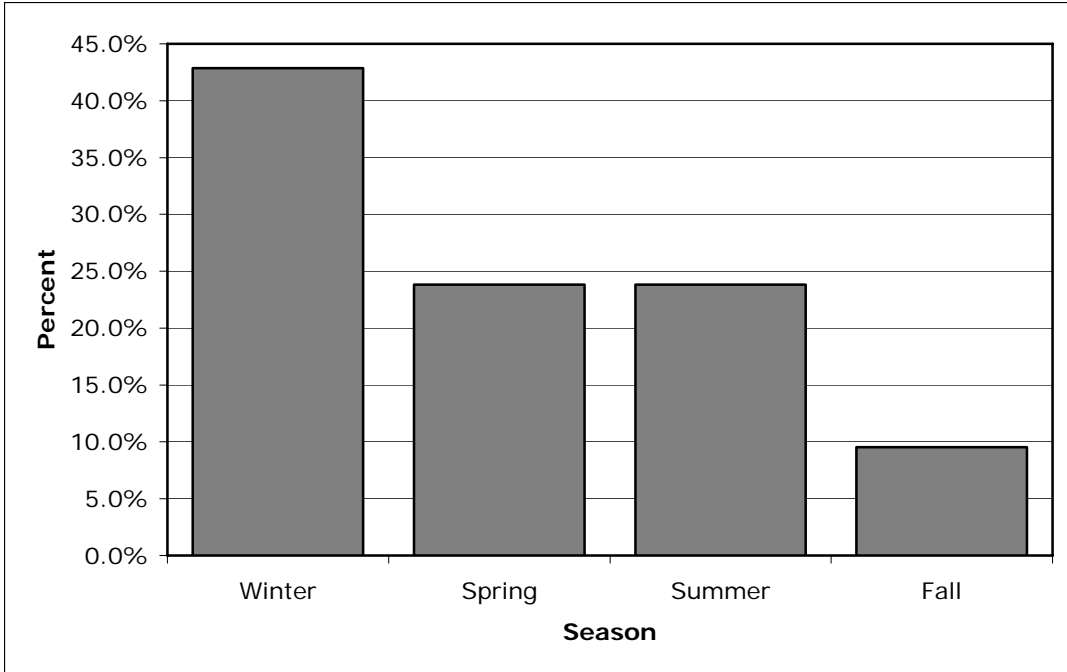


FIG. 4.2

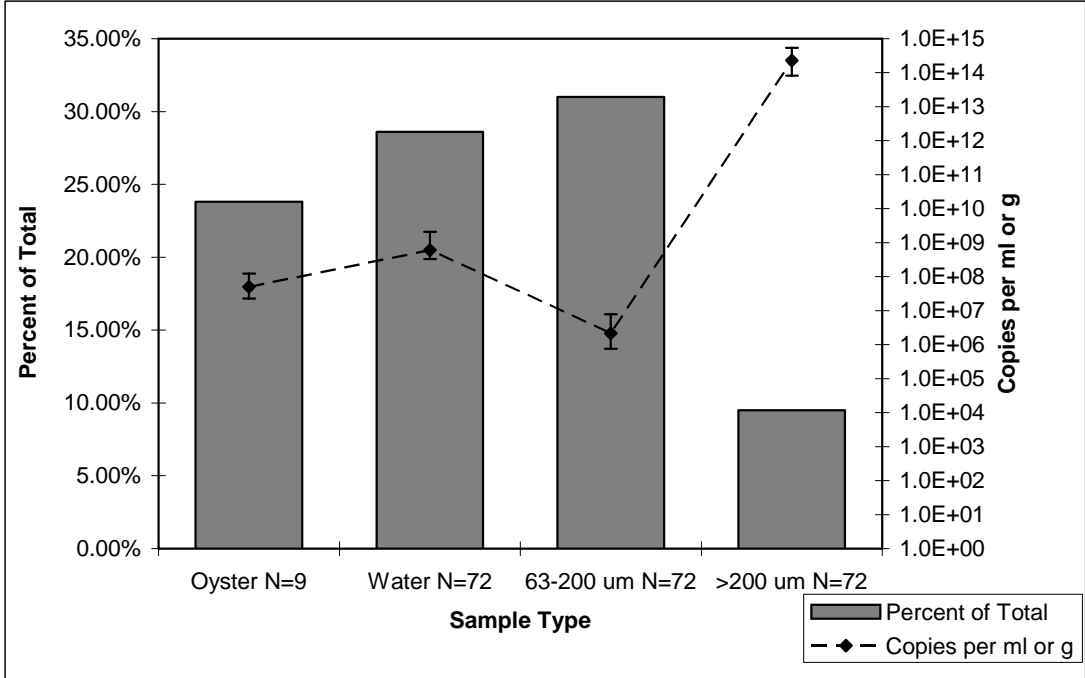


FIG. 4.3

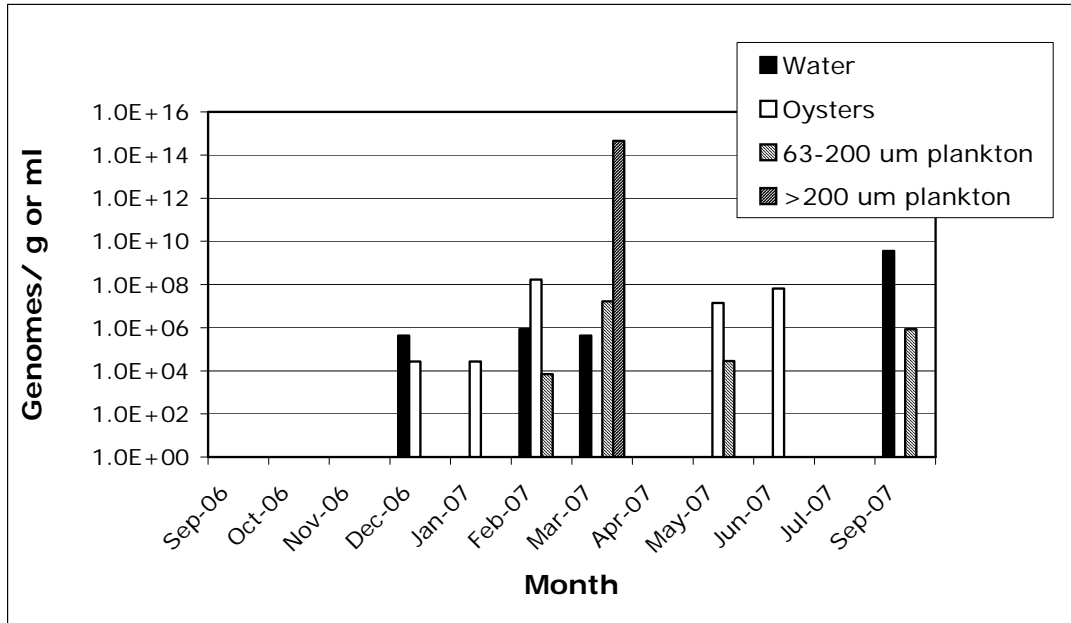


FIG. 4.4

CHAPTER 5

CONCLUSIONS

With the inception of shellfish monitoring using fecal coliforms, the level of bacterial gastroenteritis (from shellfish) dropped dramatically. The prevailing pathogens related to shellfish-borne gastroenteritis are now viruses, especially norovirus (NoV). NoV causes a range of gastrointestinal disorders and, as it can be accumulated in the mid-gut of oysters, poses a serious health risk with the consumption of raw oysters. While the characteristics and pathogenicity of NoVs have been studied extensively, only a fraction of studies have examined the presence of this virus in the environment. To reduce the incidence of NoV disease, improved detection methods must be developed and shellfish must be rigorously monitored.

We focused first on the development of a rapid, sensitive, and quantitative protocol for the detection of NoVs in estuarine samples and evaluated two published assays (A and B). We compared the detection limit for genogroup I and II NoV using RNA transcripts of a known concentration. Assay A had a detection limit of 20 copies for GI.3 and GI.4 norovirus μl^{-1} and 2,000 copies for GII.4 norovirus μl^{-1} . Assay B had a detection limit of 200 copies μl^{-1} for GI.3, 20 copies μl^{-1} for GI.4, and 2,000 copies μl^{-1} for GII.4. While assay B was more sensitive for the GI.4 RNA run-off transcript, assay A had a lower detection limit for the GI.3b RNA transcript and was able to detect genogroup II noroviruses in naturally contaminated oyster samples (assay B did not). Based on these results, we recommended assay A for the detection of NoV from environmental samples.

Two well-described viral RNA extraction protocols were evaluated for shellfish and plankton samples. The Qiagen RNeasy Mini kit had a higher recovery efficiency than the silica-binding extraction method for both artificially- and naturally-contaminated shellfish and plankton samples. The RNeasy extraction method was able to remove a majority of the inhibitors from artificially contaminated oysters (as determined by observation of a dilution effect) but significant inhibition was still observed for estuarine plankton samples. While detection was subject to inhibition for both NoV extraction methods, the Qiagen RNeasy Mini kit outperformed the silica-binding method when both RNA transcripts and NoV-contaminated fecal samples were used. Use of the Qiagen RNeasy Mini kit proved to be the critical step in NoV detection from environmental samples and allowed for a final protocol, which combined a rapid extraction step and real-time TaqMan RT-PCR detection in less than six hours.

This assay was then employed to screen oyster, environmental water, and plankton samples from two estuaries over a one-year period. In all, 225 samples (9 oyster, 72 water, 72 P63-size fraction plankton, 72 P200-size fraction plankton) were analyzed and of those, 21 samples (9.3%) were positive for norovirus. By sample type, 55% (5/9) of all oyster samples, 8.3% (6/72) of all water samples, 11.1% (8/72) of all P63 plankton samples, and 2.8% (2/72) of all P200 plankton samples were positive for human norovirus. Among all NoV-positive samples, 23.8% (5/21) were oyster samples, 28.6% (6/21) were water samples, 31.0% (8/21) were P63 plankton samples, and 9.5% (2/21) were P200 plankton samples.

The majority of positive samples were from genogroup I; only 9.5% of the positive samples tested positive for genogroup II (2 of 21 samples). The genogroup II-positive samples also contained the lowest quantity of human norovirus detected in any sample throughout the study period. This was unexpected, because while both GI and GII noroviruses are commonly

found in sewage samples, most outbreaks are attributed to genogroup GII viruses. The high prevalence of GI strains in this study may be attributed to the sensitive real-time assay, the geographic region sampled, or GI may survive longer in the environment than previously thought. One published study suggests that NoV GI may be more prevalent in the human population but that only a few cases caused by GI viruses are seen by physicians, possibly due to less severe symptoms. Our data support this hypothesis but more studies testing both environmental samples and clinical specimens are needed.

No plankton samples have ever been examined for NoV, but we found that 31% (8/21) of the 63-200 μm plankton samples tested positive for NoV. Additionally, the $>200 \mu\text{m}$ plankton samples had the highest quantity of NoV genomes of any sample, with 9.0×10^{12} and 4.5×10^{14} genomes g^{-1} . It is possible that NoV is adsorbed to plankton particles as some studies have characterized a positive electric charge on phytoplankton genera. No information on the charge on zooplankton particles has been reported as far as we know. Thus, while detection and identification of viruses associated with planktonic sediment may be an important part of the distribution of norovirus in the estuarine environment, more studies are needed to confirm our findings that plankton can serve as a NoV reservoir.

No statistically significant seasonal pattern was found for positive NoV samples. Norovirus may be more persistent in the estuarine system than previously thought, and as such, the estuarine distribution of the virus may be an important factor in the occurrence of disease outbreaks.

Previous studies have investigated the presence of norovirus in environmental oyster samples, but few studies have examined the presence of NoV in oysters over the period of one year and even fewer have examined NoV presence in water environments surrounding shellfish

harvesting areas. This is the first study to examine the association between norovirus and oysters, water, and plankton. We discovered that NoV is associated differentially with plankton size fractions and that the greater than 200 μm fraction can harbor concentrations greater than 10^{10} genomes g^{-1} . This fraction may serve as an environmental reservoir for the virus. Additionally, we found a greater persistence of NoV GI than GII. This suggests that NoV GI is either more environmentally hardy than GII in the estuarine environment or that this genotype represents strains circulating widely in the population. Both of these dynamics are significant findings that point to the need for more studies of human norovirus in the water environment in order to prevent NoV-related gastrointestinal disease.